SOIL Discuss., 2, 1135–1160, 2015
www.soil-discuss.net/2/1135/2015/
doi:10.5194/soild-2-1135-2015

SOIL Discussions Open Access EGU

Compound-specific ^{15}N stable isotope probing of N assimilation by the soil microbial biomass: a new methodological paradigm in soil N cycling

A. F. Charteris[1], T. D. J. Knowles[1], K. Michaelides[2], and R. P. Evershed[1]

[1]Organic Geochemistry Unit, School of Chemistry, University of Bristol, Cantock's Close, Bristol, BS8 1TS, UK
[2]School of Geographical Sciences, University of Bristol, University Road, Bristol, BS8 1SS, UK

Received: 25 September 2015 – Accepted: 6 October 2015 – Published: 30 October 2015

Correspondence to: R. P. Evershed (r.p.evershed@bristol.ac.uk)

Published by Copernicus Publications on behalf of the European Geosciences Union.

Abstract

A compound-specific nitrogen-15 stable isotope probing (^{15}N-SIP) technique is described which allows investigation of the fate of inorganic- or organic-N amendments to soils. The technique uses gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) to determine the δ^{15}N values of individual amino acids (AAs; determined as *N*-acetyl, *O*-isopropyl derivatives) as proxies of biomass protein production. The δ^{15}N values are used together with AA concentrations to quantify N assimilation of ^{15}N-labelled substrates by the soil microbial biomass. The utility of the approach is demonstrated through incubation experiments using inorganic ^{15}N-labelled substrates ammonium ($^{15}NH_4^+$) and nitrate ($^{15}NO_3^-$) and an organic ^{15}N-labelled substrate, glutamic acid (^{15}N-Glu). Assimilation of all the applied substrates was undetectable based on bulk soil properties, i.e. % total N (% TN), bulk soil N isotope composition and AA concentrations, all of which remained relatively constant throughout the incubation experiments. In contrast, compound-specific AA δ^{15}N values were highly sensitive to N assimilation, providing qualitative and quantitative insights into the cycling and fate of the applied ^{15}N-labelled substrates. The utility of this ^{15}N-AA-SIP technique is considered in relation to other currently available methods for investigating the microbially-mediated assimilation of nitrogenous substrates into the soil organic N pool. This approach will be generally applicable to the study of N cycling in any soil, or indeed, in any complex ecosystem.

1 Introduction

Organic nitrogen (N) concentrations far exceed those of inorganic N in most soils and despite much investigation, the composition and cycling of this complex pool of soil organic matter (SOM) remains poorly understood (Stevenson, 1982; Schulten and Schnitzer, 1998; Friedel and Scheller, 2002; Jones and Kielland, 2012; Michaelides et al., 2012; van Groenigen et al., 2015). A particular problem has been resolving more

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recalcitrant soil organic N from that actively cycling through the soil system; an important consideration in soil N cycling studies, especially those focusing on nutrient supply. Studies monitoring the concentrations of added substrates and potential products (e.g. Jones and Kielland, 2012) are useful because the concentrations of actively cycled components will fluctuate, providing indications of "reactivity" in the soil. However, such approaches do not allow elucidation of the pathways of N transformation and are rather a blunt tool for interrogating soil-based transformations critical to the global N cycle.

The use of ^{15}N-labelled substrates as stable isotope tracers has contributed much to our understanding of N cycling in the soil system (e.g. Barraclough, 1995), however, the complexity and heterogeneity of soil organic N has, up until now, prevented interrogation of the *biomolecular* fate of applied N in any detail. As a result, a considerable proportion of previous work has either assumed that since the majority of soil N is organic, all of the ^{15}N retained in the soil is organic N (e.g. Sebilo et al., 2013), or has derived estimates for the N isotopic composition of organic N by extracting/subtracting ^{15}N-labelled inorganic compounds from bulk soils/values (e.g. Ladd and Amato, 1986; Pilbeam and Hutchison, 1998). A shortcoming of both of these methods is that they *only* provide an estimate of the bulk N isotope composition of what is an extremely complex and non-uniformly labelled organic N pool. Critically, in these methods the ^{15}N substrates/amendments mostly serve as a physical tracer rather than a true *biochemical* tracer. A more refined approach has been to use microbial biomass N extraction (Brookes et al., 1985) and subsequent isotopic analysis to determine the N isotope composition of biomass N, representing the fraction of ^{15}N assimilated by microorganisms or the ^{15}N cycling through the "living", "active" or "available" portion of soil organic N (e.g. Nannipieri et al., 1999; Zhou et al., 2011; Liang et al., 2013). However, this extraction method can only generate estimates of bulk soil microbial biomass N (Dalal, 1998; Haubensak et al., 2002; Müller et al., 2003; Jenkinson et al., 2004).

Here we describe a novel approach to investigate the fate of N in soils by obtaining a measure of the assimilation of an applied ^{15}N-labelled substrate by the soil microbial biomass. In addition, we derive a quantitative estimate of newly synthesized soil

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protein, which is representative of the functioning of the soil microbial biomass and biomass protein production using the applied substrate. This approach builds on our previous work (Knowles et al., 2010) but now we demonstrate the utility of the technique for the study of any N-containing soil amendment. The technique uses ^{15}N stable isotope tracers and compound-specific ^{15}N analysis, but unlike previous ^{15}N stable isotope tracer work, uses amino acids (AAs; representing biochemical products) rather than ammonium (NH_4^+) and nitrate (NO_3^-) to directly investigate the flow of N into the biologically active soil organic N pool. Our ^{15}N compound-specific stable isotope probing (^{15}N-SIP) approach exploits the high selectivity and sensitivity of gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS; Meier-Augenstein, 1999; Evershed et al., 2006; Maxfield et al., 2012; Petzke and Metges, 2012) ensuring highly "diluted" metabolic products of the ^{15}N tracer are readily detectable in the incubated soils. Furthermore, by using only "low" tracer doses perturbations to native soil conditions are minimised. AAs are the target analytes as these are major "building blocks" of all life, forming the proteins which regulate essential biochemical reactions. Proteinaceous matter (proteins, peptides and AAs) generally comprises 20–50 % of total soil N and is ubiquitous in living organisms, so is a major "organic product" of microbial activity/assimilation (Stevenson, 1982; Schulten and Schnitzer, 1998; Friedel and Scheller, 2002; Roberts and Jones, 2008). Indeed, microbial N assimilation (of NH_4^+) itself occurs via pathways involving AAs – either via the reductive amination of α-ketoglutarate to L-glutamate (L-Glu) catalysed by glutamate dehydrogenase (GDH) or via the glutamine synthetase (GS), glutamate synthase (glutamine oxoglutarate aminotransferase; GOGAT) pathway (Tempest et al., 1970; Meers et al., 1970; Santero et al., 2012). Since AAs represent major organic nitrogenous products in soil they provide a highly sensitive integrating tool across the many thousands of proteins present in soil, revealing important general features of the dynamics and pathways of assimilation of N-containing substrates into the organic N pool. Critically, the percentage of applied ^{15}N detectable in the total hydrolysable AA pool offers a measure of (or "proxy" for) the assimilation of applied ^{15}N-labelled substrate by the soil microbial biomass and an

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estimate of newly synthesized soil protein. In this paper we describe the technique and demonstrate its utility by examining the results from laboratory incubations applying inorganic (NH_4^+, NO_3^-) and organic (Glu) substrates.

2 Materials and methods

2.1 Experimental design

Soil was sampled randomly from Rowden Moor experimental site (plot six) at North Wyke Research Station near Okehampton, Devon, UK; the same site used by Knowles et al. (2010). The soil is classified as a clayey non-calcareous Pelostagnogley of the Hallsworth series (British Classification), a Stagni-vertic cambisol under the Food and Agriculture Organisation of the United Nations (FAO) scheme or a Typic haplaquept by the United States Department of Agriculture (USDA; Harrod and Hogan, 2008). Sampled soils were homogenised, air-dried to allow sieving (2 mm) and then adjusted to 50 % water holding capacity by the addition of double distilled water (DDW). Incubations were carried out in small glass tubes (10 cm height × 2 cm diameter) containing 10 g soil under aerobic conditions and maintained by weight at 50 % WHC. After a 4 day pre-incubation period to allow for equilibration to the new conditions, the microcosms received treatments of either ^{15}N-labelled ammonium chloride ($^{15}NH_4Cl$, 10 at. %, 400 µg in 200 µL DDW), ^{15}N-labelled potassium nitrate ($K^{15}NO_3$, 10 at. %, 400 µg in 200 µL DDW), ^{15}N-labelled glutamic acid (^{15}N-Glu, 98 at. %, 2 mg in 200 µL 0.1 M hydrochloric acid (HCl)) or for the control samples, DDW (200 µL). Substrates were introduced by injection and the needle was drawn up through the soil as the plunger was depressed in order to achieve a good distribution. The incubation experiments were halted by immersion in liquid nitrogen (N_2) after periods of 1.5, 3, 6 and 12 h and 1, 2, 4, 8, 16 and 32 days in the dark and stored at −20 °C until freeze-drying. All incubations were carried out in triplicate so there were three tubes for each time point of each treatment (see also Knowles et al. 2010). The 10 at. % enrichment of $^{15}NH_4^+$

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and $^{15}NO_3^-$ was chosen based on recent research highlighting changes in ^{15}N discrimination and isotopic fractionation in biological mechanisms at very high enrichments (Mathieu et al., 2007; Tang and Maggi, 2012). Enrichments of 10 at. % were considered low enough for these effects to be negligible. The ^{15}N-Glu incubation experiments were carried out earlier, hence the undesirably high enrichment of the applied Glu.

2.2 Extraction, isolation and derivatisation of hydrolysable AAs

Finely ground, freeze-dried incubation soil samples (100 mg) were weighed into culture tubes and 100 µL of norleucine (Nle; 400 µg mL^{-1} in 0.1 M HCl) was added as an internal standard. Hydrolysis with 5 mL 6 M HCl was carried out at 100 °C for 24 h under an atmosphere of N_2. Acid hydrolysis extracts both free and proteinaceous AAs as well as catalysing the breakdown of living microbial biomass (Roberts and Jones, 2008). The relatively harsh conditions are necessary for the cleavage of peptide bonds between hydrophobic residues (e.g. isoleucine (Ile), leucine (Leu) and valine (Val)), but also result in the deamination of asparagine (Asn) to aspartate (Asp) and glutamine (Gln) to Glu and the complete destruction of cysteine (Cys) and tryptophan (Trp; Fountoulakis and Lahm, 1998; Roberts and Jones, 2008). The technique may also partially destroy serine (Ser; ca. 10 % loss), threonine (Thr; ca. 5 % loss) and tyrosine (Tyr; loss depends on level of trace impurities in hydrolysis agent; Fountoulakis and Lahm, 1998) and has the potential to hydrolyse AA chains from non-proteinaceous sources, such as peptidoglycan, resulting in an overestimation of some AAs, mostly alanine (Ala), Glu, lysine (Lys) and glycine (Gly; Roberts and Jones, 2008). The technique is, however, considered the most reliable method for determining the total protein content of soils (Roberts and Jones, 2008) and as such, we equate total hydrolysable AA concentrations to the size of the soil protein pool. The hydrolysis is performed under N_2 as the presence of oxygen (O_2) can induce the thermal breakdown of hydroxyl- and sulfur-containing AAs (e.g. methionine (Met), Ser, Thr and Tyr; Roberts and Jones, 2008).

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Hydrolysates were collected by centrifugation, dried under a stream of N_2 at 60 °C and stored at −20 °C under 1 mL 0.1 M HCl. AAs were isolated from hydrolysates by cation exchange column chromatography using acidified Dowex 50WX8 200-400 mesh ion exchange resin (Metges and Petzke, 1997). This was followed by conversion to their *N*-acetyl, *O*-isopropyl derivatives for analysis (Corr et al., 2007; see also Knowles et al., 2010).

2.3 Instrumental analyses

Bulk soil N analyses were carried out on a Eurovector EA3000 Elemental Analyser (EA). A weighed sample was sealed in a tin capsule with a combustion aid and introduced into a combustion tube at 1016 °C containing pure O_2. Helium (He) carrier gas then carried the combustion products over heated copper (Cu) wire to remove excess O_2 and reduce any N oxides. The resulting N, carbon dioxide (CO_2) and water (H_2O) was passed through a separation column and then measured using a thermal conductivity detector. Soil for bulk $\delta^{15}N$ analysis was weighed into tin capsules and combusted using a Eurovector EA. The N_2 resulting from the reduction of combustion products was then used to determine $\delta^{15}N$ values using a Micromass Isoprime IRMS.

A Hewlett Packard 5890 Series II GC fitted with a *VF-23*ms column (60 m × 0.32 mm i.d., 0.15 µm phase thickness; Varian, Inc.) and flame ionisation detector (FID) was used for quantification of individual AAs as their *N*-acetyl, *O*-isopropyl derivatives by comparison with the internal standard, Nle. The *N*-acetyl, *O*-isopropyl AAs were identified by their known elution order (Corr et al., 2007) and by comparison with standards. The carrier gas was hydrogen (H_2), at a flow rate of 3 mL min^{-1}. The temperature programme utilised was: 40 °C (1 min) to 120 °C at 15 °C min^{-1}, then to 190 °C at 3 °C min^{-1} and finally to 260 °C (12 min) at 5 °C min^{-1}. Data were acquired and analysed using Clarity chromatographic station for Windows by DataApex (Prague).

The $\delta^{15}N$ values of individual AAs as their *N*-acetyl, *O*-isopropyl derivatives were determined using a ThermoFinnigan Trace 2000 GC coupled with a ThermoFinni-

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gan Delta^{Plus} XP IRMS via a ThermoFinnigan Combustion III Interface (Thermo Electron Corporation). Samples were introduced using a CTC Analytics GC Pal autosampler and via a programmable temperature vaporisation (PTV) inlet. The carrier gas was He at a flow rate of 1.4 mL min^{-1} and the GC was fitted with a DB-35 column (30 m × 0.32 mm i.d. × 0.5 µm stationary phase thickness; Agilent Technologies). The temperature programme utilised was: 40 °C (5 min) to 120 °C at 15 °C min^{-1}, to 180 °C at 3 °C min^{-1}, then to 210 °C at 1.5 °C min^{-1} and finally to 270 °C at 5 °C min^{-1}. The copper oxide, nickel oxide and platinum (CuO/NiO/Pt) wire oxidation reactor was maintained at 980 °C and the Cu wire reduction reactor at 650 °C. Four pulses of a reference N_2 gas of known isotopic composition, previously calibrated against the AIR international isotope standard, were introduced directly into the ion source via an open split at the beginning and end of each run in order to calibrate sample δ^{15}N values. Samples were run in duplicate and an AA standard mixture of known N isotopic composition was run between each sample as a quality control (QC) standard to monitor instrument performance. Data were acquired and analysed using Isodat NT 3.0 (ThermoFinnigan). Figure 1 shows a typical sample chromatogram including the ion current signals for each m/z ratio recorded.

2.4 Calculations

If the total hydrolysable AA pool is taken to be representative of the soil protein pool, then any ^{15}N enrichment (E) in hydrolysable AAs can be summed to represent newly synthesised soil protein in the soil at that time:

$$\text{Newly synthesised soil protein} \approx \sum E \text{ in hydrolysable AAs} \quad (1)$$

The E of an AA may be expressed as the number of moles of ^{15}N derived from the applied substrate that are present in that AA in the soil:

$$E = n \times \text{AFE} \quad (2)$$

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where n is the number of moles of N in the AA (i.e. if the molecular structure of the AA contains only one N atom, n is the same as the number of moles of the AA in the soil, but twice this if the AA structure consists of two N atoms and so on) and AFE is the atom fraction excess of the AA after incubation compared with the control:

$$\mathrm{AFE} = \mathrm{AF}_{\mathrm{Sample}} - \mathrm{AF}_{\mathrm{Control}} \tag{3}$$

AF is the atom fraction of ^{15}N in the AA, i.e.:

$$\frac{\mathrm{Number}\,^{15}\mathrm{N}\,\mathrm{atoms}}{(^{14}\mathrm{N}+^{15}\mathrm{N})\,\mathrm{atoms}}. \tag{4}$$

This can be calculated from the AA's δ^{15}N value as in Knowles et al. (2010):

$$\mathrm{AF} = \frac{R_{\mathrm{Std}}(\delta^{15}\mathrm{N}/1000 + 1)}{1 + (\delta^{15}\mathrm{N}/1000 + 1)} \tag{5}$$

where R_{Std} is the $^{15}\mathrm{N}\,/\,^{14}\mathrm{N}$ ratio of air, the international isotopic standard for N. The data may also be expressed in terms of the percentage of the applied ^{15}N incorporated into each AA, as in Knowles et al. (2010):

$$\%\,\mathrm{Incorporation} = \left(\frac{E}{N}\right) \times 100 \tag{6}$$

where N is the number of moles of ^{15}N applied. These values reflect both the concentration and enrichment (δ^{15}N value) of the AA (i.e. how much was incorporated if the AA at x concentration was enriched by x ‰) and the percentage of applied ^{15}N incorporated into newly synthesised soil protein is determined by summing these results for individual AAs.

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3 Results and discussion

The results generated using our compound-specific AA ^{15}N-SIP approach provide hitherto unattainable insights into N cycling from any (inorganic or organic) N-containing substrate (≡ amendments). The utility of the method is discussed in terms of: (i) enhanced detection of ^{15}N substrate assimilation by soil microorganisms; (ii) pathways of assimilation of different N-containing substrates; (iii) revealing differences in rates and fluxes of N between applied substrates; and (iv) interpretations of ^{15}N-SIP determinations in relation to complex N dynamics.

3.1 Enhanced detection of ^{15}N substrate assimilation by soil microorganisms

The addition of an agriculturally relevant, but sufficiently low N concentration to prevent alteration of the soil's N status (and thereby limit perturbation), almost by definition results in no notable changes in the % total N (% TN) of the soil over the course of the experiment. Tables 1, 2 and 3 confirm this – there is no observable trend in the % TN of the incubation microcosms and the standard deviations (SDs) and standard errors (SEs) of the means of the % TNs for all incubation microcosms is small. Thus, the application of ^{15}N-enriched amendments is clearly valuable in allowing added N to be differentiated from native soil N. However, following addition of all three substrates, bulk soil δ^{15}N values, although showing an initial rise, remained relatively constant throughout the rest of the incubation experiment (Table 4). The elevated δ^{15}N values compared to $t = 0$ values confirm the continued presence of the ^{15}N tracer in the soil, but no insights can be gained about the form or internal processing of the amendments within the soil, i.e. are they still present as $^{15}NH_4^+$, $^{15}NO_3^-$ or ^{15}N-Glu or have they been assimilated by the soil microbial biomass?

As essential biomolecules, AAs are likely products of amendment assimilation, however, the concentrations of individual AAs show little change over the course of the incubation and there is no observable increase in concentration with incubation duration, as might be expected from the synthesis of new AAs using the applied NH_4^+, NO_3^-

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or Glu (Tables 1, 2 and 3). This does not imply that the supplied NH_4^+, NO_3^- or N-Glu has not been used in the synthesis of AAs, but rather that the concentration of NH_4^+, NO_3^- or N-Glu added has not stimulated protein biosynthesis above that present prior to the additions. Accordingly, the total hydrolysable AA N content of the soil is on average 23.9 % (SD: 2.84; SE: 0.480), 23.7 % (SD: 3.33; SE: 0.554) and 48.4 % (SD: 14.3; SE: 2.71) of total soil N throughout the $^{15}NH_4^+$, $^{15}NO_3^-$ or ^{15}N-Glu experiments, respectively (Tables 1, 2 and 3). These concentrations fall within the range (20–50 %) generally reported for total hydrolysable soil AAs (Stevenson, 1982; Schulten and Schnitzer, 1998; Friedel and Scheller, 2002; Roberts and Jones, 2008) and are equated to the concentration of the soil protein pool (Roberts and Jones, 2008). We note that acid hydrolysis does not extract all proteinaceous AAs and extracts some non-proteinaceous AAs (Friedel and Scheller, 2002; Roberts and Jones, 2008), however, the fraction of soil AAs recovered is constant and combined with the use of ^{15}N-labelled substrates, allows newly biosynthesised AAs (mostly new protein) to be identified and critically, the dynamics of the assimilation of an N-containing amendment into the soil organic N pool to be traced.

3.2 Pathways of assimilation of different N-containing substrates

The use of GC-C-IRMS allows determination of the δ^{15}N values of individual hydrolysable soil AAs. Figure 2 shows the trends in hydrolysable AA δ^{15}N values over the course of the incubation experiments applying $^{15}NH_4^+$, $^{15}NO_3^-$ or ^{15}N-Glu. The advantages of the compound-specific approach are immediately apparent, with readily detectable changes being seen in the δ^{15}N values of all AAs in all experiments (Fig. 2). It should be noted that this is the first time the assimilation of NH_4^+ and NO_3^- by the soil microbial biomass has been measured in this way and the results clearly emphasise the importance of investigating N cycling in different N-containing soil amendments.

Incorporation of $^{15}NH_4^+$ (Fig. 2a) occurs in two phases for all AAs except Glx – fast over the first 2–4 days, then more slowly for the remainder of the experiment. Assim-

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ilation into Glx occurs more quickly over the first 2 days than into any other AA and the degree of ^{15}N-labelling is 2- to 5-fold greater, before declining during the rest of the experiment. These differences in the patterns of ^{15}N incorporation relate to the fundamental biosynthetic pathways most microorganisms use for the assimilation of NH_4^+, i.e. via the reductive amination of α-ketoglutarate to L-Glu catalysed by GDH or via the GS-GOGAT pathway (Tempest et al., 1970; Meers et al., 1970; Santero et al., 2012). Glu is of central importance to the biosynthesis of new proteins as other AAs are synthesised from Glu, using it as a substrate for the amination of appropriate α-ketoacid carbon (C) skeletons. The fast rise in the δ^{15}N values of Glu reflects the initial incorporation of $^{15}NH_4^+$, with the subsequent decline after 2 days reflecting redistribution of the ^{15}N into newly synthesised AAs, hence their δ^{15}N values rise.

The results for the $^{15}NO_3^-$ experiment are extremely interesting, further emphasising the importance of this approach (Fig. 2b). Broadly, most AAs initially, and somewhat surprisingly, show lower δ^{15}N values before rising slightly over the rest of the incubation. The initial dip indicates that at the start of the incubation more AAs with a lower N isotopic composition are present than at $t = 0$. The reason for this is unknown, but one possibility is that contact with initial high NO_3^- concentrations causes cell lysis providing non-proteinaceous substrates of a lower N isotopic composition for AA biosynthesis. The subsequent smaller and more irregular rise in δ^{15}N values for all AAs compared to the $^{15}NH_4^+$ experiment is likely because NO_3^- requires reduction prior to incorporation into AAs (Puri and Ashman, 1999).

Comparison with the ^{15}N-Glu incubation carried out by Knowles et al. (2010; data represented for this discussion in Fig. 2c and d) exposes a more complex situation as the applied substrate is itself an AA in the total hydrolysable AA pool. As might be expected, the δ^{15}N value of Glu falls with time, whilst those of the other AAs rise (Fig. 2c and d). Interestingly, a comparable pattern to that of $^{15}NH_4^+$ incorporation emerges for the transfer of ^{15}N-Glu into other hydrolysable AAs, but in this case all non-substrate AAs, except Asx, exhibit two-phase incorporation. As Knowles et al. (2010) concluded, this is again likely due to the fundamental biosynthetic pathways that oper-

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ate in most microorganisms; Asp is produced by the transamination of oxaloacetate using an amino group from Glu (Gelfand and Steinberg, 1977), the remaining C-skeleton of which is α-ketoglutarate, which is used in the tricarboxylic acid (TCA) cycle, an essential metabolic process that generates energy in aerobic respiration. Decarboxylation of α-ketoglutarate as part of the cycle then generates another molecule of oxaloacetate. Interpreting the rate data alongside this known biochemistry, Knowles et al. (2010) concluded that the patterns of isotope incorporation are consistent with Asp being the AA closest in biosynthetic proximity to Glu.

3.3 Revealing differences in rates and fluxes of N between applied substrates

Quantifying the fate of N-containing substrates (inorganic or organic) in different soils is essential to understanding the N cycle in natural or semi-natural ecosystems but is especially important in agricultural systems where managing fertiliser applications has ecological and economic relevance. The new insights gained into N cycling through this novel approach offer potential to enhance fundamental understanding in this area. Using Eqs. (3)–(6), increases in AA δ^{15}N values can be used to determine the percentage of the applied ^{15}N incorporated into each AA and by summation, the percentage incorporated into the total hydrolysable AA or soil protein pool and cycling through the "living", "active" or "available" portion of soil organic N at that time (Fig. 3). These calculations are straightforward where the applied substrate is not a hydrolysable AA (e.g. $^{15}NH_4^+$ and $^{15}NO_3^-$) as any ^{15}N enrichment in the hydrolysable AA pool must be derived from the applied substrate via microbial processing during the experiment. The assessment is more complicated however when the applied substrate is a hydrolysable AA (e.g. ^{15}N-Glu) as this must be accounted for in the analytical approach (Knowles et al., 2010) and calculations (Fig. 3).

The use of several different treatments applied separately to the same soil allows comparison of their relative 'availabilities' to the soil microbial biomass – in the case of NH_4^+, NO_3^- and Glu here, clear differences in the assimilation of these substrates into newly synthesised hydrolysable soil AAs are revealed. Alternatively, the technique

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can also be used to compare the fate of particular N amendments in different soils to provide hitherto unattainable estimates of the relative "activity" of the microbial biomass of the soils under selected incubation conditions. In both cases, a measure of newly synthesised protein can be obtained by summing the ^{15}N enrichments of all the AAs at each time point for each treatment (Eqs. 1 and 2) to give the moles of ^{15}N in the soil protein pool at that time. Note however, that new protein will also be biosynthesised from non-labelled sources during the experiment, e.g. following cell lysis or concomitant organic matter mineralisation.

3.4 Interpretations of ^{15}N-SIP determinations in relation to complex N dynamics

Due to the dynamic nature of the soil system any estimates of ^{15}N in the soil protein pool represent the balance of assimilation into/loss from the pool at a given point in time. ^{15}N incorporated into the soil protein pool does not simply accumulate with time, but is turned over as native soil N turns over, e.g. via catabolic mineralisation. Insights into the dynamics of this aspect of the N cycle in soil can now be gained for the first time. In these experiments, applied labile substrates (NH_4^+ and Glu) are initially assimilated rapidly, with the amount assimilated increasingly considerably between each time point until a transient equilibrium with slower soil N turnover/loss develops (Figs. 2a, d and 3). For NO_3^- (an energy demanding substrate), on the other hand, the dynamics are more complex and the rate of assimilation is always closer to that of turnover (Figs. 2b and 3). In natural systems these assimilation-turnover dynamics would be subject to external forcings (e.g. rainfall event, soil type, etc.). Time-course incubations of this type allow the overall assimilation-turnover dynamics of the substrate with time, and other environmental variables, to be investigated and provide a measure of substrate availability/lability and value (via rate of incorporation and flux). Although this approaches cannot currently generate absolute values for the assimilation of an applied ^{15}N substrate by the soil microbial biomass or the amount of newly synthesized soil protein, it does provide enhanced insights compared to other currently available

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methods. It is reassuring that the percentages of applied ^{15}N recovered in soil microbial biomass N studies (e.g. Nannipieri et al., 1999; Liang et al., 2013) are comparable (0.8–15.3 % across these two studies) to those obtained herein.

4 Conclusions

The compound-specific ^{15}N-SIP technique described herein to investigate the fate of N amendments in soils offers a number of advantages over existing approaches to reveal a range of new insights, in particular:

1. The method provides a sensitive and relatively selective means of assessing microbial assimilation of ^{15}N-labelled substrates/amendments applied at appropriately low concentrations to minimise perturbations. Substrate-product concentration monitoring and bulk N isotope analysis cannot provide such insights.

2. Valuable insights into microbial biochemical assimilation pathways can be gained and differences are readily revealed in the microbial processing of N-containing amendments of differing chemical/biochemical natures, e.g. inorganic versus organic or different types of inorganic or organic amendment.

3. Estimates are provided for newly synthesized soil protein, which are inaccessible based on currently available methods.

4. Detailed quantitative insights can be gained into the dynamics of N cycling from an applied substrate through the soil protein pool.

5. Scope exists for using this new approach to probe soil N cycling in relation to a wide range of soil biota, ecosystem variables and anthropogenic management regimes. Opportunities for further refinement of the method are exemplified by our recent paper (Redmile-Gordon et al., 2015), wherein additional insights were gained by considering different soil protein fractions.

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6. The method is potentially adaptable to investigate N cycling into other N-containing biochemical pools, e.g. amino sugars.

Acknowledgements. This work was carried out in part during T. D. J. Knowles's PhD studentship funded by the Biotechnology and Biological Sciences Research Council and supported by the UK Natural Environment Research Council Life Sciences Mass Spectrometry Facility and in part during A. F. Charteris's UK Natural Environment Research Council (NERC) Open CASE PhD studentship between the University of Bristol and Wessex Water. We thank NERC for partial funding of the mass spectrometry facilities at Bristol (contract no. R8/H10/63; www.lsmsf.co.uk) and H. Grant of the UK NERC Life Sciences Mass Spectrometry Facility (Lancaster node) for stable isotopic characterisation of reference standards and derivatising agents and bulk soil N isotope analysis.

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Table 1. Soil % total nitrogen (% TN) and composition and concentrations of soil hydrolysable AAs for the $^{15}NH_4^+$ SIP experiment. THAA N, total hydrolysable amino acid nitrogen; SD, standard deviation; SE, standard error. Values for % TN and AA concentrations are shown to 2 decimal places with means, SDs and SEs shown to the appropriate number of significant figures. Values for THAA N and % THAA N of N are shown to 3 significant figures throughout.

		Time/days													
		0	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	Mean	SD	SE
Mean concentration/mg g^{-1}	% TN	0.72	0.74	0.79	0.73	0.77	0.76	0.76	0.73	0.75	0.74	0.74	0.74	0.036	0.0060
	Alanine	2.38	1.92	1.79	2.04	1.91	1.87	1.93	1.90	2.02	2.11	2.09	2.03	0.267	0.0453
	Aspartate	1.61	2.21	1.80	1.96	2.01	1.94	2.10	2.00	2.11	2.08	2.03	1.95	0.303	0.0512
	Glutamate	1.59	2.02	1.81	1.87	1.88	1.81	1.89	1.83	1.92	1.89	1.89	1.83	0.260	0.0440
	Glycine	1.83	1.26	1.14	1.48	1.34	1.33	1.52	1.37	1.49	1.49	1.44	1.46	0.257	0.0435
	Hydroxyproline	0.12	0.12	0.11	0.13	0.12	0.11	0.12	0.11	0.13	0.13	0.13	0.12	0.014	0.0024
	Isoleucine	0.38	0.61	0.54	0.49	0.46	0.52	0.55	0.45	0.48	0.48	0.47	0.48	0.083	0.014
	Leucine	1.02	1.16	1.15	1.10	1.07	1.05	1.09	1.03	1.09	1.13	1.12	1.09	0.0703	0.0119
	Lysine	0.48	0.41	0.27	0.23	0.25	0.34	0.48	0.40	0.52	0.59	0.55	0.42	0.17	0.029
	Methionine	0.07	0.12	0.12	0.12	0.14	0.10	0.08	0.13	0.12	0.11	0.13	0.1	0.03	0.005
	Phenyalanine	0.48	0.55	0.58	0.46	0.46	0.46	0.48	0.54	0.61	0.59	0.64	0.53	0.079	0.014
	Proline	1.23	1.14	1.10	1.24	1.14	1.09	1.14	1.07	1.14	1.18	1.20	1.16	0.117	0.0197
	Serine	0.89	1.02	0.85	0.89	0.94	0.92	1.04	0.95	1.07	1.12	1.05	0.97	0.14	0.023
	Threonine	0.73	1.07	0.89	0.84	0.90	0.86	0.96	0.92	0.99	1.01	0.93	0.91	0.15	0.025
	Tyrosine	0.22	0.29	0.29	0.23	0.24	0.24	0.26	0.30	0.36	0.35	0.35	0.28	0.059	0.0099
	Valine	0.75	0.95	0.80	0.76	0.72	0.83	0.90	0.78	0.82	0.83	0.78	0.81	0.13	0.022
	THAA N	13.8	14.9	13.2	13.8	13.6	13.5	14.5	13.8	14.8	15.1	14.8	14.1	1.17	0.197
	% THAA N of TN	24.8	24.7	20.7	24.1	22.0	22.3	24.0	23.7	25.1	25.8	25.4	23.9	2.84	0.480

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Table 2. Soil % total nitrogen (% TN) and composition and concentrations of soil hydrolysable AAs for the $^{15}NO_3^-$ SIP experiment. THAA N, total hydrolysable amino acid nitrogen; SD, standard deviation; SE, standard error. Values for % TN and AA concentrations are shown to 2 decimal places with means, SDs and SEs shown to the appropriate number of significant figures. Values for THAA N and % THAA N of N are shown to 3 significant figures throughout.

		Time/days													
		0	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	Mean	SD	SE
Mean concentration/mg g^{-1}	% TN	0.72	0.72	0.70	0.73	0.70	0.72	0.70	0.70	0.71	0.76	0.75	0.72	0.020	0.0059
	Alanine	2.38	1.86	1.93	1.97	1.93	2.11	1.97	2.18	1.81	1.85	2.65	2.06	0.330	0.0550
	Aspartate	1.61	1.92	1.99	2.02	2.01	1.83	2.19	1.82	1.55	1.56	1.23	1.77	0.390	0.0650
	Glutamate	1.59	1.78	1.85	1.73	1.81	1.83	1.92	1.80	1.51	1.57	1.20	1.68	0.326	0.0544
	Glycine	1.83	1.27	1.39	1.38	1.36	1.53	1.42	1.42	1.20	1.47	2.09	1.49	0.298	0.0496
	Hydroxyproline	0.12	0.12	0.12	0.12	0.12	0.14	0.12	0.13	0.11	0.10	0.14	0.12	0.017	0.0028
	Isoleucine	0.38	0.49	0.49	0.40	0.35	0.25	0.34	0.39	0.34	0.26	0.41	0.37	0.086	0.014
	Leucine	1.02	1.07	1.11	1.02	0.98	0.95	0.95	1.07	0.85	0.91	0.97	0.98	0.010	0.017
	Lysine	0.48	0.34	0.41	0.57	0.46	0.39	0.40	0.46	0.53	0.39	0.69	0.44	0.15	0.025
	Methionine	0.07	0.13	0.12	0.12	0.10	0.09	0.10	0.07	0.06	0.08	0.07	0.09	0.03	0.004
	Phenylalanine	0.48	0.58	0.59	0.59	0.54	0.56	0.43	0.51	0.37	0.49	0.46	0.50	0.091	0.015
	Proline	1.23	1.11	1.14	1.08	1.10	1.23	1.12	1.22	1.01	0.97	1.40	1.14	0.154	0.0257
	Serine	0.89	0.89	0.95	1.12	1.05	0.90	1.00	0.98	0.87	0.82	0.95	0.93	0.15	0.026
	Threonine	0.73	0.82	0.90	0.97	0.88	0.68	0.87	0.86	0.77	0.67	0.70	0.80	0.16	0.027
	Tyrosine	0.22	0.34	0.31	0.37	0.34	0.34	0.23	0.26	0.19	0.25	0.24	0.27	0.069	0.0120
	Valine	0.75	0.72	0.77	0.73	0.63	0.44	0.63	0.76	0.67	0.52	0.67	0.67	0.15	0.025
	THAA N	13.8	13.4	14.1	14.2	13.6	13.3	13.7	13.9	11.7	11.9	13.9	13.3	1.45	0.241
	% THAA N of TN	24.7	23.2	25.3	24.5	24.5	23.6	24.7	25.3	20.8	20.3	22.5	23.7	3.33	0.554

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Table 3. Soil % total nitrogen (% TN) and composition and concentrations of soil hydrolysable AAs for the ^{15}N-Glu SIP experiment. THAA N, total hydrolysable amino acid nitrogen; SD, standard deviation; SE, standard error. Values for % TN and AA concentrations are shown to 2 decimal places with means, SDs and SEs shown to the appropriate number of significant figures. Values for THAA N and % THAA N of N are shown to 3 significant figures throughout.

		Time/days												
		0	0.125	0.25	0.5	1	2	4	8	16	32	Mean	SD	SE
Mean concentration/mg g^{-1}	% TN	0.76	0.76	0.78	0.77	0.79	0.77	0.76	0.77	0.78	0.77	0.77	0.019	0.0033
	Alanine	5.41	7.87	7.35	7.44	5.73	4.12	4.38	5.46	3.45	4.47	5.58	2.05	0.387
	Aspartate	3.60	4.22	3.75	3.17	3.56	3.11	3.61	3.26	2.21	2.38	3.26	1.04	0.189
	Glutamate	2.93	3.88	3.60	3.08	2.83	2.54	3.01	2.97	1.91	2.04	2.88	0.919	0.168
	Glycine	4.71	6.38	6.02	6.17	4.82	3.67	3.54	5.19	3.19	3.76	4.75	1.63	0.297
	Isoleucine	1.25	1.44	1.40	1.32	1.15	0.94	0.97	2.34	1.01	0.72	1.3	0.65	0.12
	Leucine	0.69	0.97	0.90	0.78	0.70	0.53	0.84	0.60	0.33	0.48	0.68	0.25	0.047
	Lysine	0.64	0.21	0.14	0.32	0.15	0.92	0.46	1.30	0.98	0.28	0.53	0.43	0.078
	Methionine	0.17	0.23	0.23	0.20	0.17	0.17	0.24	0.14	0.07	0.10	0.2	0.07	0.01
	Phenylalanine	0.41	0.40	0.41	0.40	0.36	0.35	0.36	0.49	0.28	0.26	0.37	0.11	0.020
	Proline	2.51	3.57	3.79	3.66	2.69	1.81	2.23	3.01	1.64	2.26	2.73	1.08	0.197
	Serine	2.75	3.69	3.33	3.03	2.53	2.17	2.50	2.59	1.79	1.77	2.61	0.871	0.159
	Threonine	2.28	2.24	1.91	1.68	1.95	1.38	1.88	1.81	1.17	1.08	1.70	0.579	0.106
	Valine	1.29	1.39	1.39	1.05	1.07	0.51	1.00	0.75	0.46	0.48	0.91	0.43	0.079
	THAA N	28.6	36.5	34.2	32.3	27.7	22.2	25.0	29.9	18.5	20.1	27.4	7.99	1.46
	% THAA N of TN	50.6	65.6	60.0	57.7	47.2	39.1	43.6	52.9	32.5	35.9	48.4	14.33	2.71

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Table 4. Bulk soil δ^{15}N values for the $^{15}NH_4^+$, $^{15}NO_3^-$ and ^{15}N-Glu incubation experiments. SD, standard deviation; SE, standard error. Values are shown to 3 significant figures.

	$t = 0$			$t = 3$ h			Overall incubation mean		
	Mean δ^{15}N value	SD	SE	Mean δ^{15}N value	SD	SE	Mean δ^{15}N value	SD	SE
$^{15}NH_4^+$	4.47	0.106	0.043	87.3	6.98	4.03	85.4	6.82	1.25
$^{15}NO_3^-$	4.47	0.106	0.043	35.8	2.92	1.69	36.8	5.95	3.43
^{15}N-Glu	7.16	1.89	0.773	1050	116	67.0	1070	72.8	14.0

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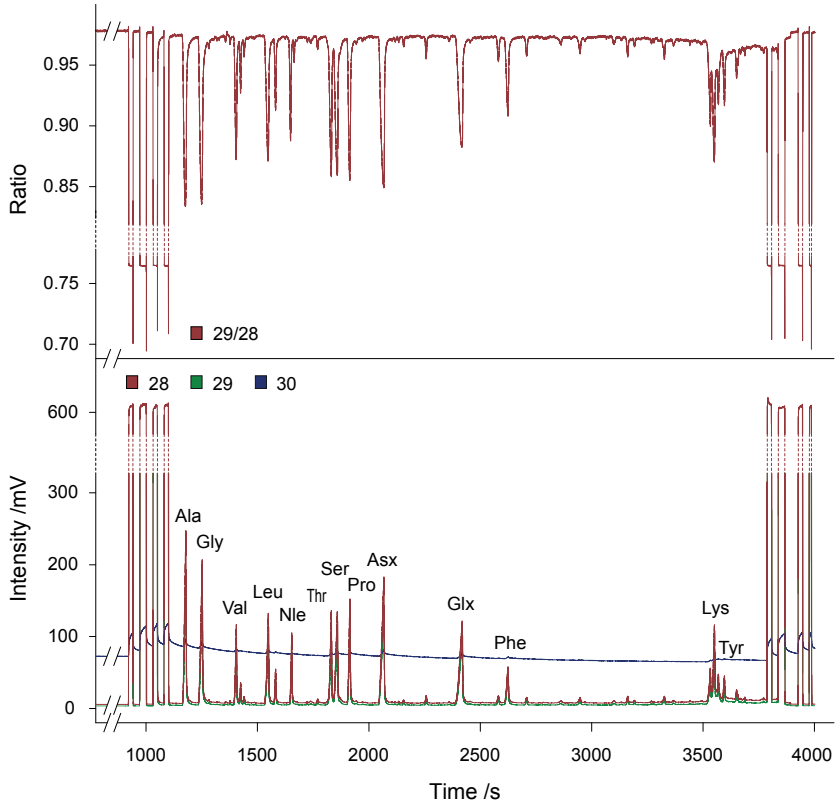

Figure 1. Typical GC-C-IRMS chromatogram of *N*-acetyl, *O*-isopropyl derivatised hydrolysable soil AAs showing the ion current signals recorded by the GC-C-IRMS operating for N_2 (m/z 28, 29 and 30) and the ratio of m/z 28 to m/z 29 which is used to generate $^{15}N/^{14}N$ isotope ratios.

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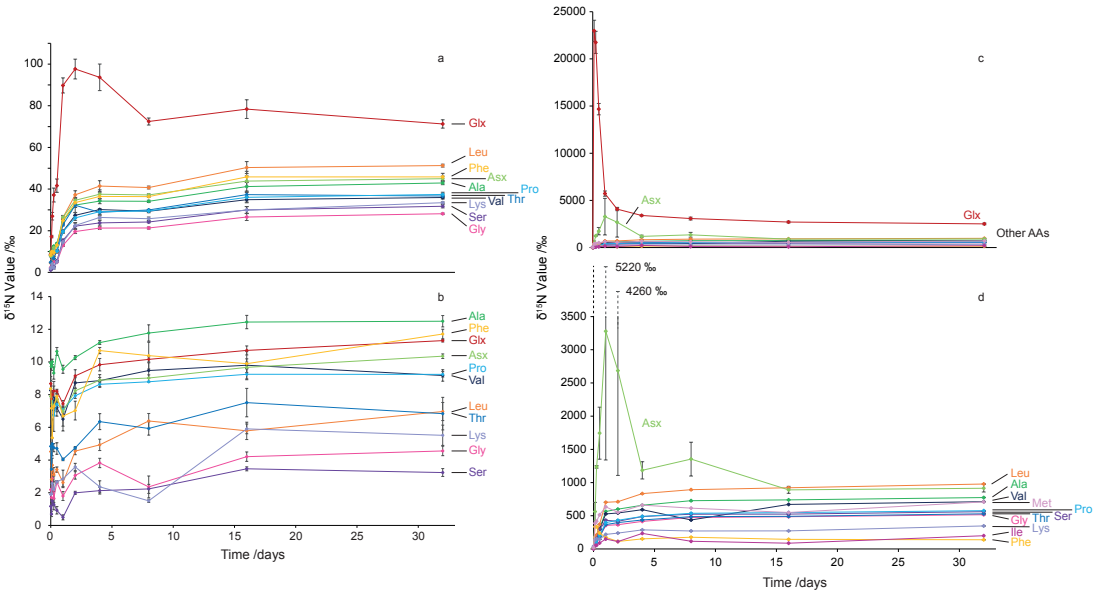

Figure 2. δ^{15}N values of individual AAs over the course of a 32 day incubation experiment: **(a)** $^{15}NH_4^+$ incubation, **(b)** $^{15}NO_3^-$ incubation, **(c)** ^{15}N-Glu incubation, including the applied ^{15}N-Glu and **(d)** ^{15}N-Glu incubation, excluding the applied ^{15}N-Glu.

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A new methodological paradigm in soil N cycling

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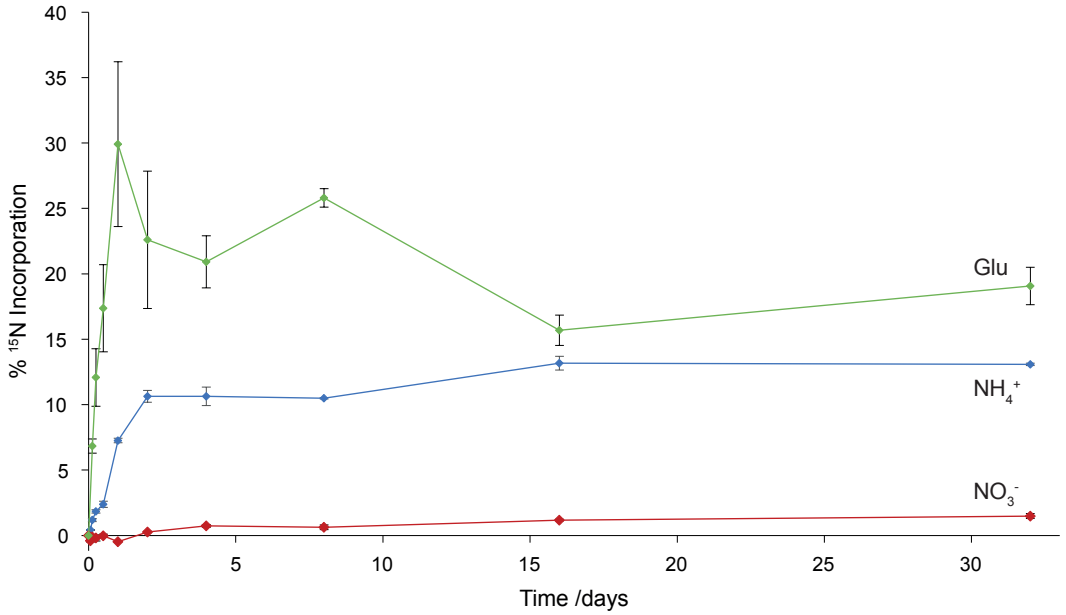

Figure 3. Percentage of applied $^{15}NH_4^+$, $^{15}NO_3^-$ and ^{15}N-Glu incorporated into the total hydrolysable AA pool or soil protein pool. Calculations for $^{15}NH_4^+$ and $^{15}NO_3^-$ are straightforward summations of the percentage of the applied ^{15}N incorporated into each AA, while results for ^{15}N-Glu incubation were, in this case, calculated excluding the ^{15}N residing in Glu as a relatively high level of enrichment remains at the apparent equilibrium compared with the enrichment of the other AAs (Fig. 2c) indicating considerable intact use of the applied ^{15}N in preference to de novo AA biosynthesis.

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