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Compound-specific amino acid $^{15}$N stable isotope probing of nitrogen assimilation by the soil microbial biomass using gas chromatography/combustion/isotope ratio mass spectrometry

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RATIONALITY: Organic nitrogen (N) greatly exceeds inorganic N in soils, but the complexity and heterogeneity of this important soil N pool make investigations into the fate of N-containing additions and soil organic N cycling challenging. This paper details a novel approach to investigate the fate of applied N in soils, generating quantitative measures of microbial assimilation and of newly synthesized soil protein.

METHODS: Laboratory incubation experiments applying $^{15}$N-ammonium, $^{15}$N-nitrate and $^{15}$N-glutamate were carried out and the high sensitivity and selectivity of gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) exploited for compound-specific $^{15}$N stable isotope probing ($^{15}$N-SIP) of extracted incubation soil amino acids (AAs; as N-acetyl, O-isopropyl derivatives). We then describe the interpretation of these data to obtain a measure of the assimilation of the applied $^{15}$N-labelled substrate by the soil microbial biomass and an estimate of newly synthesized soil protein.

RESULTS: The cycling of agriculturally relevant N additions is undetectable via bulk soil N content and $\delta^{15}$N values and AA concentrations. The assimilation pathways of the three substrates were revealed via patterns in AA $\delta^{15}$N values with time, reflecting known biosynthetic pathways (e.g. ammonium uptake occurs first via glutamate) and these data were used to expose differences in the rates and fluxes of the applied N substrates into the soil protein pool (glutamate > ammonium > nitrate).

CONCLUSIONS: Our compound-specific $^{15}$N-SIP approach using GC/C/IRMS offers a number of insights, inaccessible via existing techniques, into the fate of applied $^{15}$N in soils and is potentially widely applicable to the study of N cycling in any soil, or indeed, in any complex ecosystem. © 2016 The Authors. Rapid Communications in Mass Spectrometry Published by John Wiley & Sons Ltd.

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) remain poorly understood (Fig. 1$^{[1–5]}$). A particular problem has been resolving more resistant 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.$^{[4]}$) 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.$^{[6]}$); however, the complexity and heterogeneity of soil organic N have 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.$^{[7]}$), 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.$^{[8,9]}$). A shortcoming of both of these methods is that they only provide an estimate of the bulk N isotopic composition of what is an extremely complex and non-uniformly $^{15}$N-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$^{[10]}$ and subsequent isotopic analysis to determine the N isotopic 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. However, this extraction method can only generate estimates of bulk soil microbial biomass N.

A more recently developed technique that interrogates the active microbial community in more detail is 15N stable isotope probing (15N-SIP) of nucleic acids (deoxyribonucleic acid (DNA) or ribonucleic acid (RNA)). This culture-independent method employs isopycnic centrifugation to separate out 15N-enriched nucleic acids, which, following fingerprinting, may enable the taxonomic identity of actively assimilating microorganisms to be established. The technique is limited by: the high final nucleic acid 15N enrichments required (>50%); the potential for cross feeding and trophic cascades under highly 15N-labelled substrates and longer incubation times; and the complexity of matching terminal restriction fragments (TRFs; which may be shared by multiple organisms) with sequences from clone libraries. In addition, although potentially valuable in identifying active microorganisms, nucleic acid 15N-SIP does not afford insights into the N cycling of the soil community as a whole and the fate of applied 15N.

A technique which can offer this capability is compound-specific 15N-SIP of soil organic N using mass spectrometry. Many studies have used conventional gas chromatography/mass spectrometry (GC/MS) in compound-specific 15N-SIP studies (e.g. for 15N-enriched amino sugars; investigating microbial amino acid (AA) utilisation; investigating barley leaf proteins with high turnover rates; but this approach can generally only be used precisely (±0.01 atom %) with highly 15N-enriched compounds where the 15N enrichment is easily detectable above natural background values. Only a handful of workers, however, have exploited the far higher potential precision (0.5–2.0 ‰; 0.0002–0.0008 atom %) of gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) in 15N-SIP studies. Almost half of these relate to mammalian physiology (e.g. ) and the remainder consist of a few reports in several different research areas: N uptake in aquatic systems (e.g. ); plant N uptake (e.g. ); plant-microbe associations (e.g. ); microbial cultures; and soil N partitioning (e.g. ).

The latter studies have begun to hint at the potential of 15N-SIP using GC/C/IRMS as an extremely powerful tool for tracing the fate of 15N in soils. The critical advantage is that this technique offers the sensitivity required to follow 15N substrates applied at environmentally relevant concentrations and appropriately low enrichments, through a variety of ecosystems and into a range of N-containing products. Given this, it is surprising that this approach remains largely unexploited and, hence, the full range of applications and insights is yet to be realised. This may be partly due to the challenges associated with compound-specific 15N analyses via GC/C/IRMS, as compared with those of carbon-13 (13C; N is generally much less abundant than C in organic molecules; two N atoms are required to produce each N2 molecule for analysis; additional reduction chemistry is required to successfully convert a N-containing molecule into N2 for analysis; the ionisation efficiency of N2 is only 70% that of carbon dioxide (CO2); small leaks can be detrimental due to the high abundance of N2 in air; and there is potential for interfering ionic species, such as [CO3]–, at m/z 28, 29 and 30, which can make the technique somewhat temperamental, but may also be due to a lack of awareness regarding the potential of compound-specific 15N-SIP using GC/C/IRMS to investigate soil N cycling.

Herein we describe the advantages of compound-specific AA 15N-SIP using GC/C/IRMS to investigate the fate of N in soils and obtain a measure of the assimilation of an applied 15N-labelled substrate by the soil microbial biomass. We demonstrate the utility of the approach for the study of any N-containing soil amendment via the results of laboratory incubations applying inorganic (15N-ammonium; 15NH4+), 15N-nitrate; (15NO3)– and organic (15N-glutamate; 15N-Glu) substrates. We derive quantitative estimates of newly synthesised soil protein, which is representative of the functioning of the soil microbial biomass and biomass protein production using the applied substrate. The high selectivity and sensitivity of GC/C/IRMS enable the use of environmentally relevant 15N-tracer doses that minimise perturbations to native soil conditions and ensure the highly ‘diluted’ metabolic products of the 15N tracer are readily

Figure 1. Conceptual diagram of the soil N cycle emphasising the relative contributions of organic and inorganic N to soil N (ca 90:10%) and the actively cycling soil organic N pool which is currently difficult to assess, but which may be estimated via the approach discussed in this paper. Inputs of the type applied in this study correspond to ‘Fertilisation’ and ‘Animals and plants’.

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detectable in the incubated soils. The power of the approach lies in the analysis of AAs 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.[1–3,49] Since AAs represent major organic nitrogenous products in soil they provide a highly sensitive integrating tool across the many thousands of proteins present, 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 15N detectable in the total hydrolysable AA pool offers a measure of (or ‘proxy’ for) the assimilation of applied 15N-labelled substrate by the soil microbial biomass and an estimate of newly synthesised soil protein. We discuss the range of potential insights and highlight the wider applicability of the approach in the investigation of complex N cycling ecosystems.

EXPERIMENTAL

Incubations

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.[41] The soil is classified as a clayey non-calcareous Podsolnagloey 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).[30] Sampled soils were homogenised, air-dried to allow sieving (2 mm) and then adjusted to 50% water holding capacity (WHC) 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 15N-labelled ammonium chloride ([15NH4Cl, 10 atom %, 400 μg in 200 μL DDW; Sigma-Aldrich, St. Louis, MO, USA], 15N-labelled potassium nitrate (K15NO3, 10 atom %, 400 μg in 200 μL DDW; Sigma-Aldrich), 15N-labelled glutamic acid ([15N-Glu, 98 atom %, 2 mg in 200 μL 0.1 M hydrochloric acid; HCl, 15N-Glu from Spectra Stable Isotopes, Columbia, MD, USA and HCl was reagent grade from Fisher Scientific, Loughborough, UK) 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 an optimal distribution. The incubation experiments were halted by a n d 1 2 ha n d 1 , 2 , 4 , 8 , 1 6 a n d 3 2 d a y s i n t h e d a r k and stored at 400 mesh ion-exchange resin (Acros Organics, Morris Plains, NJ, USA).[54] This was followed by conversion into their N-acetyl, O-isopropyl derivatives for analysis.[51,55] Derivatising agents were supplied by Sigma-Aldrich (Steinheim, Germany); acetyl chloride was puriss. p.a. grade; trimethylamine had ≥99.50% purity; and acetic anhydride was ReagentPlus® grade. All solvents were of HPLC grade and were supplied by Rathburn Chemicals Ltd. (Walkerburn, UK). DDW was produced using a Bibby Aquatron still. Where not applied to living soil, DDW was extracted with dichloromethane prior to use in order to remove dissolved organic compounds.

Instrumental analyses

Bulk soil percentage total N (% TN) and δ15N analyses were carried out using a Eurovector elemental analyser (Milan, Italy) coupled to a Micromass Isoprime isotope ratio mass spectrometer (Stockport, UK) at the Lancaster node of the Natural Environment Research Council Life Sciences Mass Spectrometry Facility (NERC LSMSF; UK). Soil (ca 10 mg) was weighed into tin capsules, combusted in an overestimation of some AAs, mostly alanine (Ala), Glu, lysine (Lys) and glycine (Gly).[49] The technique is, however, considered the most reliable method for determining the total protein content of soils[40] and, as such, we equate total hydrolysable AA concentrations to the size of the soil protein pool. The hydrolysis is performed under N2 as the presence of oxygen (O2) can induce the thermal breakdown of hydroxyl- and sulfur-containing AAs (e.g. methionine; Met, Ser, Thr and Tyr).[49]

Hydrolysates were collected by centrifugation, dried under a stream of N2 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 (Acros Organics, Morris Plains, NJ, USA).[54] This was followed by conversion into their N-acetyl, O-isopropyl derivatives for analysis.[51,55] Derivatising agents were supplied by Sigma-Aldrich (Steinheim, Germany); acetyl chloride was puriss. p.a. grade; trimethylamine had ≥99.50% purity; and acetic anhydride was ReagentPlus® grade. All solvents were of HPLC grade and were supplied by Rathburn Chemicals Ltd. (Walkerburn, UK). DDW was produced using a Bibby Aquatron still. Where not applied to living soil, DDW was extracted with dichloromethane prior to use in order to remove dissolved organic compounds.
passed into the isotope ratio mass spectrometer for determination of % TN contents and δ15N values.

A model 5890 Series II gas chromatograph (Hewlett Packard, Wilmington, DE, USA) fitted with a VF-23ms column (60 m × 0.32 mm i.d., 0.15 μm phase thickness; Varian Inc., Palo Alto, CA, USA) and a flame ionisation detector (FID; Hewlett Packard) 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\(^{[85]}\) and by comparison with AA standards (TLC grade, 98%; Sigma-Aldrich). The carrier gas was hydrogen (H\(_2\); The BOC Group plc, Guildford, UK), at a flow rate of 3 mL min\(^{-1}\). The temperature programme utilised was: 40 °C (1 min) to 120 °C at 1 °C min\(^{-1}\), then to 190 °C at 3 °C min\(^{-1}\) and finally to 270 °C (12 min) at 5 °C min\(^{-1}\). Data were acquired and analysed using Clarity chromatographic station for Windows by DataApex (Prague, Czech Republic).

The δ15N values of individual AAs as their N-acetyl, O-isopropyl derivatives were determined using a ThermoFinnigan Trace 2000 gas chromatograph coupled with a ThermoFinnigan DeltaPlus XP isotope ratio mass spectrometer via a ThermoFinnigan Combustion III Interface (Thermo Electron Corporation, Waltham, MA, USA). Samples were introduced using a GC Pal autosampler (CTC Analytics, Zwingen, Switzerland) and via a programmable temperature vapourisation (PTV) inlet (Thermo Electron Corporation). The carrier gas was helium (He; The BOC Group plc) at a flow rate of 1.4 mL min\(^{-1}\) and the gas chromatograph was fitted with a DB-35 column (30 m × 0.32 mm i.d. × 0.5 μm stationary phase thickness; Agilent Technologies, Santa Clara, USA). The temperature programme utilised was: 40 °C (5 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 Isodat NT 3.0 (Thermo Electron Corporation). Figure 2 shows a typical sample chromatogram including the ion current signals recorded by 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 15N/14N isotope ratios.

**CALCULATIONS**

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

\[
\text{ Newly synthesised soil protein } \approx \sum \text{E in hydrolysable AAs} \tag{1}
\]

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

\[
E = n_N \times \text{AFE} \tag{2}
\]

where \(n_N\) is the number of moles of N in the AA (i.e. if the molecular structure of the AA contains only one N atom, \(n_N\) 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:

\[
\text{AFE} = \text{AFE}_{\text{Sample}} - \text{AFE}_{\text{Control}} \tag{3}
\]

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

\[
\frac{15\text{N atoms}}{14\text{N} + 15\text{N} \text{ atoms}} \tag{4}
\]
This can be calculated from the AA’s \( \delta^{15}N \) value as in Knowles et al.:\(^{41}\)

\[
AF = \frac{R_{\text{std}}(\delta^{15}N/1000 + 1)}{1 + (\delta^{15}N/1000 + 1)} \tag{5}
\]

where \( R_{\text{std}} \) is the \( ^{15}N/^{14}N \) 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.:\(^{41}\)

\[
\% \text{ Incorporation} = \left( \frac{E}{N} \right) \times 100 \tag{6}
\]

where \( N \) is the number of moles of excess \( ^{15}N \) applied (above natural abundance). Percentage incorporations reflect both the concentration and \( ^{15}N \) enrichment (\( \delta^{15}N \) value) of the AA (i.e. how much was incorporated if the AA at \( x \) concentration was \( ^{15}N \)-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. Note that these percentage incorporation data will be affected by the conservation of applied \( ^{15}N \) in the system; thus, if \( ^{15}N \) is lost from the system (e.g. over time), AA percentage incorporations may become skewed as there is less \( ^{15}N \) available for incorporation than expected. The incubation experiment design described aims to limit any \( ^{15}N \) losses from the system in order to obviate this issue, but it is equally possible to calculate percentage incorporations at time, \( t \), based on the moles of \( ^{15}N \) retained (\( N_{\text{r}} \); above natural abundance/control soil values) in the system at time, \( t \) (Eqn. (7)), if bulk recovery of the applied \( ^{15}N \) is low or decreases with time.

\[
N_{\text{r}} = AFE \left( \frac{\% \text{TN}}{1400} \right) \tag{7}
\]

where AFE is the atom fraction excess of \( ^{15}N \) in the bulk soil (calculated from bulk and control soil \( \delta^{15}N \) values using Eqns. (3) and (5)) and \( \% \text{TN} \) is the percentage total N content of the soil. Percentage retention of applied \( ^{15}N \) was calculated as follows:

\[
\% \text{ Retention} ^{15}N = \left( \frac{N_{\text{r}}}{N} \right) \times 100 \tag{8}
\]

It can also be argued, however, that loss from the system is just another process competing against AA biosynthesis for N, so should not be discounted in this way. All of these approaches are valid and the most appropriate one will depend on the specifics of the experimental design and desired outcomes.

RESULTS AND DISCUSSION

Compound-specific AA \( ^{15}N \)-SIP using GC/C/IRMS has the potential to provide hitherto unattainable insights into soil N cycling (Fig. 1) from any (inorganic or organic) N-containing substrate (= amendments). The utility of the method is discussed in terms of: (i) limitations of bulk N and AA concentrations to detect appropriate N additions/cycling in soils; (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 in soils.

Limitations of bulk N and AA concentrations to detect appropriate N additions/cycling in soils

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 \( \% \text{TN} \) of the soil over the course of the experiment. Tables 1, 2 and 3 confirm this – there is no observable trend in the \( \% \text{TN} \) of the incubation microcosms and the standard errors of the means (SEs) of the \( \% \text{TN} \) contents for all incubation microcosms are small. Thus, the application of \( ^{15}N \)-labelled amendments is clearly valuable in allowing added N to be differentiated from native soil N. However, following addition of all three substrates, bulk soil \( \delta^{15}N \) values, following the initial rise, remained relatively constant throughout the rest of the incubation experiment (i.e. overall percentage retentions of \( ^{15}N \) in the system were high and close to 100%; Table 4). The elevated \( \delta^{15}N \) 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. is the \( ^{15}N \) still present as \( ^{15}\text{[NH}_4\text{]}^+ \), \( ^{15}\text{[NO}_3\text{]} \) or \( ^{15}\text{N-Glu} \) or has it been assimilated by the soil microbial biomass?

As essential biomolecules, proteinaceous 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 \( \text{[NH}_4\text{]}^+ \), \( \text{[NO}_3\text{]}^\text{-} \) or Glu (Tables 1, 2 and 3). This could imply that the supplied \( \text{[NH}_4\text{]}^+ \), \( \text{[NO}_3\text{]}^\text{-} \) or N-Glu has not been used in the synthesis of AAs, but it could also be that the concentration of \( \text{[NH}_4\text{]}^+ \), \( \text{[NO}_3\text{]}^\text{-} \) 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 28.5% (SE: 0.555), 25.9% (SE: 0.523) and 48.4% (SE: 2.71) of total soil N throughout the \( ^{15}\text{[NH}_4\text{]}^+ \), \( ^{15}\text{[NO}_3\text{]} \) or \( ^{15}\text{N-Glu} \) experiments, respectively (Tables 1, 2 and 3). These concentrations fall within the range (20–50%) generally reported for total hydrolysable soil AAs\(^{1–3,49}\) and are equated to the concentration of the soil protein pool.\(^{49}\) We note that acid hydrolysis does not extract all proteinaceous AAs and extracts some non-proteinaceous AAs;\(^{3,49}\) however, the fraction of soil AAs recovered is constant. These results emphasise the need to undertake compound-specific N isotope analysis using GC/C/IRMS of the newly biosynthesised AAs (mostly new protein) to gain detailed insights into the dynamics of the assimilation of a \( ^{15}N \)-containing amendment into the soil organic N pool.

Pathways of assimilation of different N-containing substrates

The use of GC/C/IRMS allows precise (0.5–2.0 %; 0.0002–0.0008 atom %) determination of the \( \delta^{15}N \) values of
individual hydrolysable soil AAs. The advantages of the compound-specific approach are immediately apparent, with readily detectable changes being seen in the $\delta^{15}$N values of all AAs in all experiments (Fig. 3). It should be noted that this is the first time the assimilation of $\text{NH}_4^+$ and $\text{NO}_3^-$ by the soil microbial biomass has been measured in this way and the results clearly emphasise the importance of investigating N cycling from different N-containing soil amendments. Incorporation of $\text{NH}_4^+$ (Fig. 3(a)) occurs in two phases for all AAs except Glx – fast over the first 2 to 4 days, then more slowly for the remainder of the experiment. Assimilation into Glx occurs more quickly over the first 2 days than into any other AA and the degree of $\text{N}$ enrichment is 2- to 5-fold.
Table 3. Soil % TN and composition and concentrations of soil hydrolysable AAs for the 15N-Glu-SIP experiment

| Time / days | 0 | 0.125 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | Mean | SE |
|-------------|---|-------|------|-----|---|---|---|---|----|----|------|----|
| Mean concentration /mg g⁻¹ | % TN | 0.76 | 0.76 | 0.76 | 0.77 | 0.79 | 0.77 | 0.76 | 0.77 | 0.78 | 0.77 | 0.77 | 0.0033 |
| Ala | 5.41 | 7.87 | 7.35 | 7.44 | 5.73 | 4.12 | 4.38 | 5.46 | 3.45 | 4.47 | 5.58 | 0.387 |
| Asx | 3.60 | 4.22 | 3.75 | 3.17 | 3.56 | 3.11 | 3.61 | 3.26 | 2.21 | 2.38 | 3.26 | 0.189 |
| Glx | 2.93 | 3.88 | 3.60 | 3.08 | 2.83 | 2.54 | 3.01 | 2.97 | 1.91 | 2.04 | 2.88 | 0.168 |
| Gly | 4.71 | 6.38 | 6.02 | 6.17 | 4.82 | 3.67 | 3.54 | 5.19 | 3.19 | 3.76 | 4.75 | 0.297 |
| Ile | 1.25 | 1.44 | 1.40 | 1.32 | 1.15 | 0.94 | 0.97 | 2.34 | 1.01 | 0.72 | 1.3 | 0.12 |
| Leu | 0.69 | 0.97 | 0.90 | 0.78 | 0.70 | 0.53 | 0.84 | 0.60 | 0.33 | 0.48 | 0.68 | 0.047 |
| Lys | 0.64 | 0.21 | 0.14 | 0.32 | 0.15 | 0.92 | 0.46 | 1.30 | 0.98 | 0.28 | 0.53 | 0.078 |
| Met | 0.17 | 0.23 | 0.23 | 0.20 | 0.17 | 0.17 | 0.24 | 0.14 | 0.07 | 0.10 | 0.2 | 0.01 |
| Phe | 0.41 | 0.40 | 0.41 | 0.40 | 0.36 | 0.35 | 0.36 | 0.49 | 0.28 | 0.26 | 0.37 | 0.020 |
| Pro | 2.51 | 3.57 | 3.79 | 3.66 | 2.69 | 1.81 | 2.23 | 3.01 | 1.64 | 2.26 | 2.73 | 0.197 |
| Ser | 2.75 | 3.69 | 3.33 | 3.03 | 2.53 | 2.17 | 2.50 | 2.59 | 1.79 | 1.77 | 2.61 | 0.159 |
| Thr | 2.28 | 2.24 | 1.91 | 1.85 | 1.77 | 1.18 | 1.88 | 1.81 | 1.17 | 1.08 | 1.70 | 0.106 |
| Val | 1.29 | 1.39 | 1.39 | 1.05 | 1.07 | 0.51 | 1.00 | 0.75 | 0.46 | 0.48 | 0.91 | 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 | 1.46 |
| % THAA N of TN | 50.6 | 65.6 | 60.0 | 57.7 | 47.2 | 39.1 | 43.6 | 52.9 | 35.2 | 35.9 | 48.4 | 2.71 |

THAA N; total hydrolysable amino acid nitrogen. (Note that soil used in the 15N-Glu-SIP incubation experiment was sampled at a different time from soil for the 15[15NH4]⁺ and 15[15NO3]⁻-SIP incubation experiments and this has resulted in the higher concentrations of hydrolysable AAs observed throughout.)

Table 4. Bulk soil δ¹⁵N values for the 15[15NH4]⁺, 15[15NO3]⁻ and 15N-Glu incubation experiments

| t = 0 | t = 3 h | Overall incubation mean |
|-------|---------|------------------------|
| Mean | δ¹⁵N value | SE | Mean | δ¹⁵N value | SE | Mean | δ¹⁵N value | SE | % Retention | 15N | SE |
| 15NH₄⁺ | 4.47 | 0.0432 | 87.3 | 4.03 | 85.4 | 1.09 | 88 | 3.1 | 2.07 |
| 15NO₃⁻ | 4.47 | 0.0432 | 35.8 | 1.69 | 35.1 | 0.89 | 88 | 3.1 | 2.07 |
| 15N-Glu | 7.16 | 0.773 | 1050 | 67.0 | 1070 | 14.0 | 100 | 1.32 |

Greater, before declining during the rest of the experiment. These differences in the patterns of ¹⁵N incorporation relate to the fundamental biosynthetic pathways most microorganisms use for the assimilation of [15NH₄]⁺, i.e. via the reductive amination of α-ketoglutarate to L-Glu catalysed by glutamate dehydrogenase (GDH) or via the glutamine synthetase (GS), glutamate synthase (glutamine oxoglutarate aminotransferase; GOGAT) pathway.][60] 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 α-ketoacid C skeletons. The fast rise in the δ¹⁵N values of Glu reflects the initial incorporation of 15[15NH₄]⁺, with the subsequent decline after 2 days reflecting redistribution of the 15N into newly synthesised AAs, hence their δ¹⁵N values rise.

The results for the 15[15NO₃]⁻ experiment are extremely interesting, further emphasising the importance of this approach (Fig. 3(b)). Broadly, most AAs initially, and somewhat surprisingly, show lower δ¹⁵N values before rising slightly over the rest of the incubation. The initial dip indicates that at the start of the incubation AAs are 15N-depleted compared with at t = 0. The reason for this is unknown, but one possibility is that contact with initial high [NO₃]⁻ concentrations causes cell lysis providing non-proteinaceous substrates with low δ¹⁵N values for AA biosynthesis. The subsequent smaller and more irregular rise in δ¹⁵N values for all AAs compared to the 15[15NH₄]⁺ experiment is likely because [NO₃]⁻ requires reduction prior to incorporation into AAs.][60]

Comparison with the 15N-Glu incubation carried out by Knowles et al.[41] (data represented for this discussion in Figs. 3(c) and 3(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 δ¹⁵N value of Glu falls with time, whilst those of the other AAs rise (Figs. 3(c) and 3(d)). Interestingly, a comparable pattern to that of 15[15NH₄]⁺ incorporation emerges for the transfer of 15N-Glu into other hydrolysable AAs, but in this case all non-substrate AAs, except Asx, exhibit two-phase incorporation. As Knowles et al.[41] concluded, this is again likely due to the fundamental biosynthetic pathways that operate in most microorganisms; Asp is produced by the transamination of oxaloacetate using an amino group from Glu, 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. [41] concluded that the patterns of isotope incorporation are consistent with Asp being the AA closest in biosynthetic proximity to Glu.

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 Eqns. (3)–(6), increases in AA δ15N values can be used to determine the percentage of the applied 15N 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. 4). These calculations are straightforward where the applied substrate is not a hydrolysable AA (e.g. 15[NH4]+ and 15[NO3]–) as any 15N 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. 15N-Glu) as this must be accounted for in the analytical approach [41] and calculations (Fig. 4).

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 [NH4]+, [NO3]– and Glu here, clear differences in the assimilation of these substrates into newly synthesised hydrolysable soil AAs are revealed. Alternatively, the technique 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 15N enrichments of all the AAs at each time point for each treatment (Eqns. (1) and (2)) to give the moles of 15N in the soil protein pool at that time. Note, however, that new protein will also be

Figure 3. δ15N values of individual AAs over the course of a 32-day incubation experiment: (a) 15[NH4]+ incubation, (b) 15[NO3]– incubation, (c) 15N-Glu incubation, including the applied 15N-Glu and (d) 15N-Glu incubation, excluding the applied 15N-Glu. Error bars are ± SE (n = 3).

Figure 4. Percentage of applied 15[NH4]+, 15[NO3]– and 15N-Glu incorporated into the total hydrolysable AA pool or soil protein pool. Error bars are ± SE (n = 3). Calculations for 15[NH4]+ and 15[NO3]– are straightforward summations of the percentage of the applied 15N incorporated into each AA, while results for 15N-Glu incubation were, in this case, calculated excluding the 15N residing in Glu as a relatively high level of enrichment remains at the apparent equilibrium compared with the enrichment of the other AAs (Fig. 2(c)) indicating considerable intact use of the applied 15N in preference to de novo AA biosynthesis.
biosynthesised from non-labelled sources during the experiment, e.g. following cell lysis or concomitant organic matter mineralisation.

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

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 at the AA level. In these experiments, applied labile substrates ([NH$_4$]$^+$ and Glu) are initially assimilated rapidly, with the amount assimilated increasing considerably between each time point until a transient equilibrium with slower soil N turnover/loss develops (Figs. 3(a), 3(d) and 4). 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. 3(b) and 4). In natural systems these assimilation-turnover dynamics would be subject to external forcings (e.g. rainfall events, 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 approach 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 methods. It is reassuring that the percentages of applied $^{15}$N recovered in soil microbial biomass N studies (e.g.$^{[11,13]}$) are comparable (0.8–15.3% across these two studies) to those obtained herein.

CONCLUSIONS

The novel compound-specific $^{15}$N-SIP approach using GC/C/IRMS described herein to investigate the fate of N amendments (e.g. via fertilisation; Fig. 1) in soils offers a number of advantages that existing techniques cannot to reveal a range of new insights, in particular:

(i) The method provides a sensitive and relatively selective means of assessing microbial assimilation of $^{15}$N-labelled substrates/amendments applied at environmentally relevant concentrations and appropriately low $^{15}$N enrichments to minimise perturbations and $^{15}$N discrimination/isotopic fractionation, respectively. Bulk N isotope analysis cannot provide such insights and GC/MS $^{15}$N-SIP studies require highly $^{15}$N-labelled substrates for sufficient product AA $^{15}$N enrichment, making such studies more susceptible to $^{15}$N discrimination/isotopic fractionation effects and much more expensive.

(ii) 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.

(iii) Estimates are provided for newly synthesised soil protein, which are inaccessible based on currently available methods.

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

(v) 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,$^{[13]}$ wherein additional insights were gained by considering different soil protein fractions.

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

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