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The use of stable isotopes in fungal ecology

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Many an undergraduate biologist will remember the excitement/fear of using radio-labelled substrates (usually ^{14}C) during carefully supervised laboratory practicals. They will also remember the precautions taken to avoid contact with the radioactive source and to account for all the materials used. The use of radiolabelled compounds (mainly ^{3}H, ^{14}C, ^{32}P or ^{35}S) is commonplace though carefully controlled, and the utilisation of such compounds for field experiments is severely restricted. A further limitation in ecological research is that the radioactive isotope of nitrogen (^{13}N) has a half life of only a few minutes.

However, many elements have stable isotopes with a higher atomic weight (the same number of protons/electrons but one or two more neutrons in the atomic nucleus). These do not undergo radioactive decay and can thus be used without risk to health. The best known of the stable isotopes is deuterium (^{2}H and its oxide, heavy water [D_2O]) but in ecological research it is the stable isotopes of nitrogen (^{15}N) and carbon (^{13}C) that are most widely used. Compounds containing these isotopes are for the most part chemically indistinguishable from the commoner, lighter isotope though they do have some unusual properties (for instance, heavy water freezes at +3.8°C).

Pulse labelling (stable isotope probing)

Despite lacking the advantage of radioisotopes in terms of easy detection with X-ray film or scintillation counting, stable isotope levels can now be routinely measured by isotope ratio mass spectrometry (IRMS). Commercial analytical costs fall within the region of £5 - £30 per sample. Thus a substrate can be labelled with a ^{15}N-enriched tracer compound and its fate determined by measuring elevated ^{15}N levels along meta/catabolic pathways. As is usually the case, such technology is often developed for use in biomedical research (stable isotope analysis has been used for decades in studies of human nutrition (e.g. Gaebler *et al.*, 1966), and only later feeds down to less-well-funded disciplines like ecology or mycology (Boschker & Middelburg, 2002). However, by now their use is commonplace in animal ecology where the technique is particularly informative in studies of trophic interactions, feeding preference and study of migration and dispersal patterns (Coleman & Fry, 1991; Knowles & Blackburn ,1997; Olive *et al.*, 2003). For instance, stable isotope analyses of prey animals (fed on ^{15}N enriched substrates) are used to identify predators in a way that is more informative than examination of gut contents, since the latter provide only a snapshot of the predator's diet. Both techniques can be used in conjunction; however, where it is not possible to analyse gut contents, the stable isotope approach can provide a method of determining both long term and short term dietary variations. Briers *et al.* (2004) dosed an upland stream with ^{15}N-labelled ammonium chloride and were able to follow dispersal of ^{15}N-labelled stoneflies for distances of >1 km. Similarly, in plant ecology $^{15}NH_4Cl$ was injected at different depths into soil by Jumpponen *et al.* (2002) to quantify N uptake by different plant species, whilst Ostle *et al.* (2000) used a $^{13}CO_2$ pulsing system to monitor CO_2 assimilation *in situ* in grasslands (Fig 1). In labelling experiments where high levels of ^{13}C or ^{15}N are being measured by IRMS, the values are commonly expressed as atom %. This expresses the number of atoms of a particular isotope of an element in the sample as a fraction of the total number of atoms of that element present.

Fig 1 Dr. Nick Ostle of the CEH Lancaster Environment Centre and his $^{13}CO_2$ pulsing system in action at the NERC Soil Biodiversity field site at Sourhope. Inset a close-up view of one of the six pulsing chambers that can be used simultaneously. The cost of $^{13}CO_2$ used for single pulsing experiments can be very high (ca. £30,000).

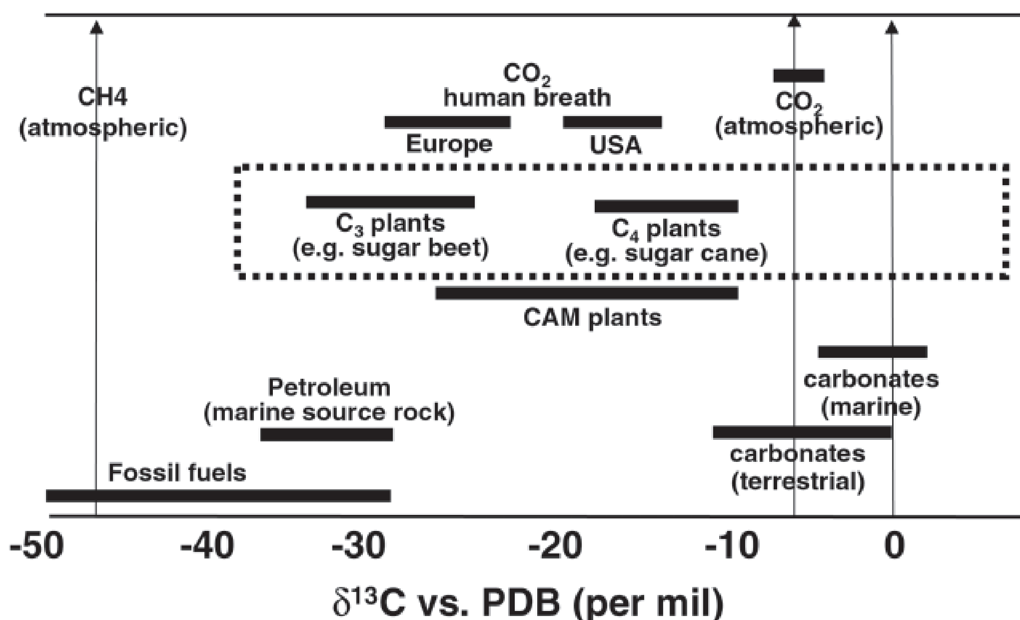

Fig 3 Natural variations in the abundance of $\partial^{13}C$ values (drawn by Dr. Andrew Stott, NERC Stable Isotope Facility; raw data taken from Boutton [1991]).

Natural abundance studies

Stable isotopes occur in nature with atmospheric N_2 and CO_2 consisting of 0.37 atom % ^{15}N and 1.1 atom % ^{13}C respectively (i.e. 0.37% of the atoms for N, rather than weight; deuterium [^2H] is present at 0.02 atom % in water). A characteristic which has proved very useful in ecological studies is that enzymatic reactions fractionate to a greater or lesser extent against these heavier isotopes and consequently significant differences in the natural abundance of these stable isotopes are found between different biological samples (Hoering, 1955). The relative enrichment or depletion of ^{15}N vs ^{14}N or ^{13}C vs ^{12}C is usually quoted in comparison to reference materials, atmospheric N_2 in the case of ^{15}N and the Cretaceous limestone (PeeDee Belemnite [PDB]) for ^{13}C. Thus in natural abundance studies stable isotopes ratios are often expressed as the delta notation (∂), for example $\partial^{15}N_{AIR}$(‰) or $\partial^{13}C_{PBD}$(‰) (note that ‰ [per mil] is per thousand) to quantify enrichment or depletion relative to these reference standards (calculated as ^{13}C ‰ = [($^{13}C/^{12}C$sample - $^{13}C/^{12}C$ reference standard) / ($^{13}C/^{12}C$ reference standard)] x 1000).

An example of the utility of these differences in stable isotope natural abundance (as distinct from

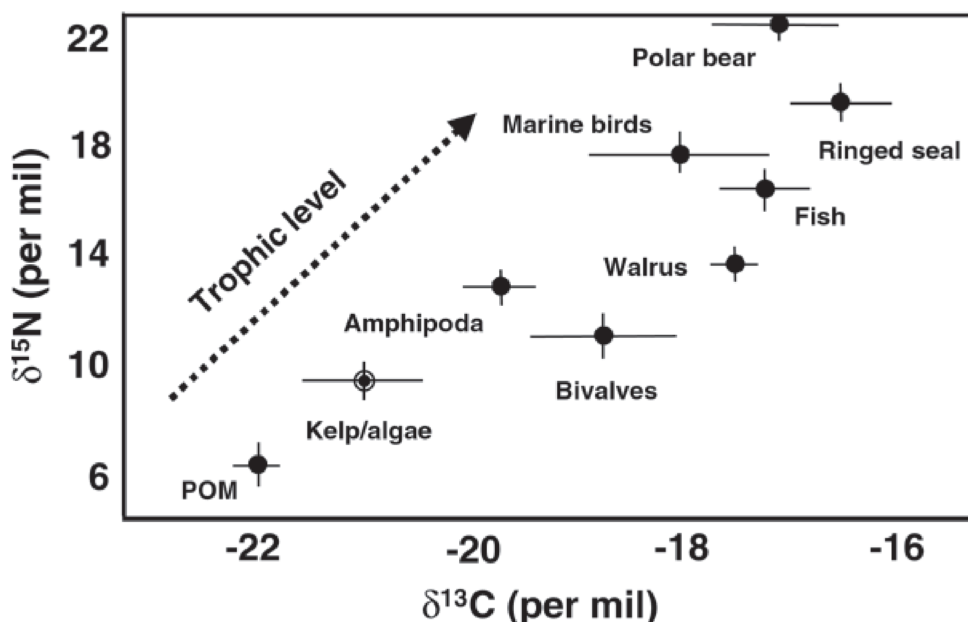

Fig 2 Relationship of $\partial^{13}C$ and $\partial^{15}N$ values in a marine food web. Redrawn from (Hobson & Welch, 1992).

pulsing with enriched substrates) in ecology is in determining the trophic structure of animal food chains. Due to fractionation against ^{15}N during assimilation and biosynthesis (e.g. urine is depleted in ^{15}N), the bulk tissues of animals higher up a food chain are found to have progressively enriched $\partial^{15}N_{AIR}$ values (3-5‰ increase at each trophic level; Fig 2).

Thus the hair keratin of carnivores is more enriched in ^{15}N than that of herbivores, reflecting intake of animal protein by the former group — as the saying goes 'you are what you eat' or more appropriately what you 'assimilate'. Indeed this distinct separation can distinguish between humans consuming 'high meat' diets and those consuming vegetarian or vegan diets. Under certain burial conditions, hair of human corpses is very well preserved, and hair keratin isotopic analysis in conjunction with bone collagen $\partial^{15}N$ analysis has become popular with isotope archaeologists to show dietary shifts in ancient populations, for example providing information about the past social status of the dead individual (i.e. more enriched for people of higher status) (Macko *et al.*, 1999).

It is generally considered that carbon stable isotope ratios tend to change little with trophic level in animal food webs but are informative with regard to diet origin. Many tropical/subtropical plants (such as maize, sugarcane etc) fix CO_2 by a different metabolic pathway (the C_4 pathway) than temperate C_3 plants which are more widespread in temperate regions. These metabolic differences result in differential discrimination of ^{13}C, so that $\partial^{13}C_{PBD}$ for C_4 plants is ca. 10‰ enriched relative to C_3 plants. These distinct isotopic variations are thus reflected in the bulk tissues and specific biomolecules (e.g. lipids) of herbivores consuming this plant material (Fig 3). Thus, the $\partial^{13}C$ values in the exhaled breath of Americans (who eat more maize) are lower than for wheat-eating Europeans (Wagenmakers *et al.*, 1993). Such assays also have medical applications, for example for detecting changes in exhaled breath CO_2 due to infection by the ulcer-causing bacterium *Helicobacter pylori* (Riepl *et al.*, 2000) and also by archaeologists who have analysed ^{13}C levels in hair to map the spread of maize cultivation in pre-Columbian America (Katzenberg *et al.*, 1993).

Other stable isotopes are also fractionated by biotic and abiotic processes and variations in the natural abundance of sulphur in mosses has been used to map different pollutant sources (Nriagu & Glooschenko, 1992), whilst variations in natural abundance of deuterium and ^{18}O in water from different sources is used (alongside other stable isotopes) to map long-distance migratory patterns in birds (Farmer *et al.*, 2003; Hobson *et al.*, 2003). Natural abundance of deuterium and ^{18}O are also being used in the fight against bioterrorism to identify the geographic origin of *Bacillus* spores based on the water used for the fermentation process (Kreuzer-Martin *et al.*, 2003).

Application to fungal ecology

In fungal ecology, use of stable isotope analysis has largely been restricted to the macrofungi which form tissue masses greater than 10-20 mg fresh weight (corresponding to ca. 100 µg N), although EA (elemental analysis)-IRMS measurements are often made on sample masses containing between 10-100 µg N. Since many of these macrofungi are often difficult to grow or examine on agar media or in laboratory microcosms, stable isotope analysis provides a very useful alternative to more conventional physiological approaches. For instance, it might be expected that ectomycorrhizal and saprotrophic fungi would differ in their $\partial^{13}C$/ $\partial^{15}N$ profiles, since the former receive carbon from and also supply N to adjacent plants by a more direct route than the latter. One note of caution is that habitats or ecosystems with different nutrient inputs and plant communities can show large differences in overall $\partial^{13}C$ and $\partial^{15}N$ values (Stapp *et al.*, 1999), so it is always essential to obtain background information about natural abundance levels in soil, vegetation and litter.

Gebauer & Taylor (1999) found different patterns of ^{15}N enrichment in ectomycorrhizal and saprotrophic fungi, which they interpreted as reflecting differences in N uptake (from inorganic N, organic soil N or organic N in litter). Consistent differences in ^{15}N and ^{13}C patterns between mycorrhizal and saprotrophic taxa in several woodland habitats were shown by (Hobbie *et al.*, 1999; Kohzu *et al.*, 1999) with ectomycorrhizal fungi showing greater enrichment for ^{15}N and greater depletion for ^{13}C than saprotrophic fungi from the same habitats. However, Henn & Chapela (2001) also found that an 'EM-SAP Divide' could be observed when comparing data from a single site but did not agree that this approach could be used as a diagnostic tool since data from different habitats were strongly dependent on substrate and potentially different degrees of isotope fractionation by different fungi.

Henn & Chapela (2000) have also provided evidence that uptake of sucrose originating from C_3 plants such as sugar beet (but not C_4 plants such as maize which fix CO_2 by a different mechanism) by three basidiomycete species show taxon-specific patterns of fractionation in pure culture. Thus the assumption made by many plant and animal ecologists that $\partial^{13}C$ patterns are primarily

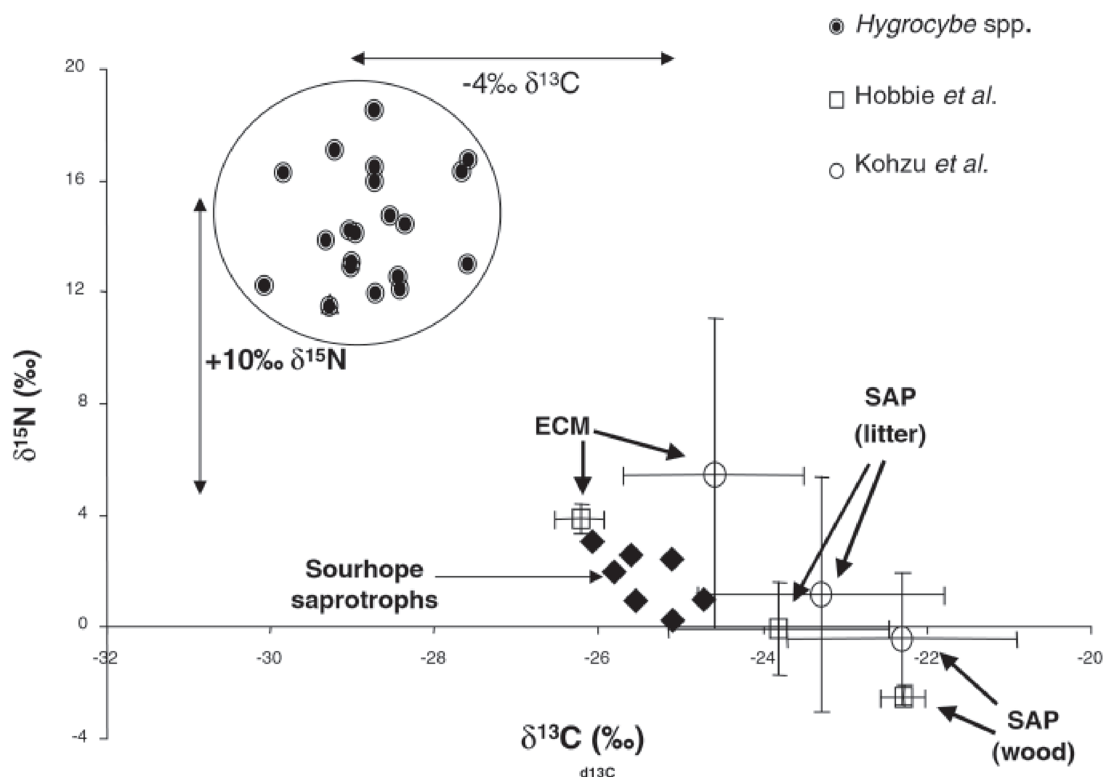

Fig 4 Patterns of ^{13}C and ^{15}N in macromycete fungi from Sourhope, modified from Griffith *et al.*, (2002). Mean data (and standard error bars) for ectomycorrhizal (ECM) fungi, litter saprotrophs (SAP[litter]) and wood saprotrophs (SAP[wood]) from earlier studies by Hobbie *et al.* (1999) and Kohzu *et al.* (1999) are also shown. Data for *Hygrocybe* spp. from Sourhope and other field sites are shown as solid circles and saprotrophs as solid diamonds. The ca 4‰ difference in $\partial^{13}C$ values and ca. 10‰ difference in $\partial^{15}N$ values between data for *Hygrocybe* spp. and other fungi is highlighted.

indicative of photosynthesis-determined isotope discrimination must be treated with care when studying fungi. The suggestion that significant ^{13}C fractionation can be mediated by decomposer fungi is likely to prompt a re-evaluation of models of ^{13}C partitioning. Although fractionation of ^{15}N is known to occur (e.g. in animals $\partial^{15}N$ of urine is ca. 3‰ lower than body tissues; Fig 2), Emmerton *et al.* (2001) found that patterns of isotopic fractionation in three mycorrhizal fungi were strongly influenced by the N source used (glycine/glutamate vs. ammonium or nitrate), leading to $\partial^{15}N$ values in mycelia which differed by 5-10‰.

The theoretical models that underlie natural abundance levels are complex (Olive *et al.*, 2003) including differential fractionation of different compounds and the effects of varying substrate concentrations. Given the diverse range of (often undefined) substrates that fungi can utilize in nature, crude interpretation of stable isotope data using the same assumptions as those made by animal or plant ecologists can be risky. However, when used in conjunction with other ecological data, natural abundance patterns can be informative. Hogberg *et al.*, (1999) found that the higher levels of ^{13}C depletion for some ectomycorrhizal species was related to their host-specificity. Thus, the basidiocarps of those species specifically associated with *Betula* had lower $\partial^{13}C$ values than those specific to conifers. In another study which aimed to discover the reason for huge numbers of *Suillus luteus* basidiocarps found by Hedger (1986) in conifer plantations in the Andean Paramo, Chapela *et al.* (2001) used a combination of stable isotope data, radiocarbon analysis and basidiocarp surveys to show *S. luteus* populations were making a significant contribution to the depletion of soil carbon that has been observed in these plantations.

As part of a project within the NERC Soil Biodiversity Programme (http://soilbio.nerc.ac.uk/) aimed at elucidating the role played by *Hygrocybe* spp (waxcaps) in soil nutrient cycling, we have studied $^{13}C/^{15}N$ natural abundance levels in macromycete fruit

bodies at a grassland site in Scotland (Sourhope, nr. Kelso). Isotopic analyses were performed at the NERC Life Sciences Mass Spectrometry Facility (http://www.nerc.ac.uk/funding/services/15nsif.shtml) who specialise in ecological and environmental isotopic analyses for the UK research community. The basidiocarps of several typical litter saprotrophs (e.g. *Cystoderma, Mycena, Panaeolus, Psilocybe*) showed $^{13}C/^{15}N$ levels similar to saprotrophic fungi from other habitats (Fig 4). However, basidicoarps of. *Hygrocybe* spp. exhibited very unusual patterns of ^{15}N enrichment and ^{13}C depletion when compared to other fungi from this site and also to data from various earlier studies mentioned above (Fig 4; ca. 4‰ more depleted in ^{13}C and >10‰ more enriched in ^{15}N than saprotrophs) (Griffith *et al.*, 2002). In some cases $\partial^{15}N$ values exceeding +20‰ were found, a level only found in animal food chains in top level carnivores (Fig 2) .

Given the caution with which stable isotope data from fungi should be treated, it would be unwise to infer a particular nutritional mode but these data suggest that the waxcaps either have a very unusual mechanism of nutrient uptake leading to strong fractionation in favour of ^{15}N or that their main source of N from soil is highly enriched for ^{15}N. These data are consistent with suggestions that these fungi are obtaining nitrogen from deeper soil horizons, since recalcitrant compounds in lower soil horizons tend to be enriched for ^{15}N (Gebauer & Taylor, 1999). It is interesting to note that members of the genus *Cortinarius* showed significant levels of ^{15}N enrichment (up to +15.4‰) (Taylor *et al.*, 1997). These fungi are also considered to be adapted to N-poor environments and thus particularly sensitive to anthropogenic N enrichment by atmospheric deposition (Arnolds, 1991) which can alter pathways of N cycling in soil. Taken together with other knowledge of the biology of waxcap fungi, such as their reluctance to grow on agar media and susceptibility to nitrogen fertilizers, stable isotope analyses are useful in creating a clearer picture of their nutritional biology.

RNA-based stable isotope probing and future developments

One fascinating development within the NERC Soil Biodiversity Programme has been the use of pulses of $^{13}CO_2$ at Sourhope as a tool to monitor nutrient flows within the soil ecosystem (Ostle *et al.*, 2000). This has allowed monitoring of the fate of $^{13}CO_2$ pulsed *in situ* in the field as carbon flows from the plants into the soil microbes. For instance, Johnson *et al.* (2002) found that over 6% of the ^{13}C fixed by plants in the pulsing chamber was released within 21 hours from the mycelia of arbuscular mycorrhizal fungi. Another recent and exciting stable isotope methodology is the development of methods for compound-specific stable isotope techniques, whereby individual compounds (e.g. lipids) are separated using gas chromatography prior to measurement of isotope ratios (GC-C-IRMS) (Boschker & Middelburg, 2002). Copley *et al.* (2003) analysed fatty deposits on archaeological remains, using this approach to identify specific fatty acids with $\partial^{13}C$ values characteristic of ruminant milk, in order to demonstrate that Neolithic farmers in Britain were involved in dairying at least 7000 years ago. Different microbial taxa also have distinctive phospholipid fatty acids (PFLA), and by combining *in situ* $^{13}CO_2$ stable isotope probing techniques at Sourhope with compound specific GC-C-IRMS, it was possible to identify microbial groups actively involved in the assimilation of root-derived carbon in limed grassland soils (Treonis *et al.*, 2004).

The fact that molecules containing ^{13}C or ^{15}N are slightly heavier can also be exploited to identify organisms able to degrade particular substrates. Addition of labelled ^{13}C substrates leads to incorporation of ^{13}C into the ribosomal RNA of organisms able to use these substrates. These 'heavy' RNA molecules can be separated from the RNA of non-utilizers by isopycnic (density gradient) centrifugation. Once isolated, the heavy RNA is subjected to reverse transcriptase PCR to create a clone library containing sequence information relating to those organisms involved in the particular degradative process. The stable isotope probing (SIP) method has been used to examine phenol (Manefield *et al.*, 2002) and methanol (Lueders *et al.*, 2004) degradation. The former study identified that previously uncultured bacteria of the genus *Thauera* were important in the degradation of phenol in industrial bioreactors while the latter study, conducted in microcosms to mimic paddy field soil, revealed the previously unsuspected role of certain fungi (related to *Aspergillus* and *Fusarium* spp.), probably decomposing methylotroph bacteria. These novel approaches have yet to make an impact on fungal ecology but they illustrate the intriguing possibility of linking up utilisation of particular substrates to genetic information which allows identification of the degrading microbe. Increasingly sensitive mass spectrometers, combined with techniques such as laser ablation (e.g. Bruneau *et al.*, 2002) will eventually make it possible to analyse isotope signature at the microscopic scale.

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Useful Websites

http://www.iso-analytical.com/page21.html (Beginners guide to stable isotopes)

http://www.uga.edu/~sisbl/stable.html (Overview of Stable Isotope Research)

http://www.chelt.ac.uk/gdn/origins/life/carbon.htm (Further information about carbon isotopes)

http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/C/C4plants.html (basics of photosynthesis in C3 and C4 plants)

http://gcte-focus1.org/basin.html (Biosphere - Atmosphere Stable Isotope Network [BASIN] website)

http://www.nerc.ac.uk/funding/services/15nsif.html (for research groups requiring SI analyses)

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