Developing an interpretation for $\delta^{15}N$ in plants

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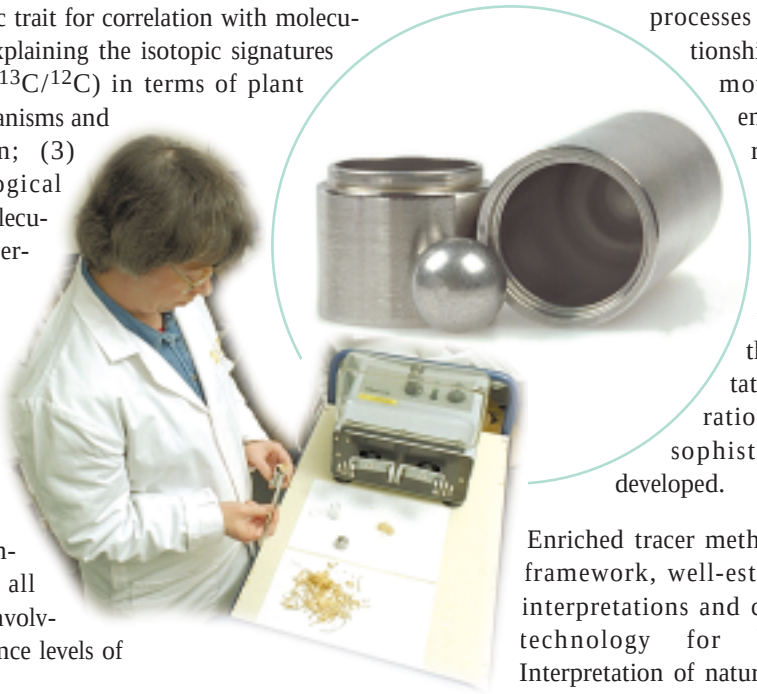
The Unit for Stable Isotope Studies in Biology uses all levels of isotope enrichment as research requires, but specifically investigates the conceptual, methodological and instrumentation approaches to complex problems using the naturally-occurring levels of the biologically important stable isotopes (i.e. $^{15}N/^{14}N$, $^{13}C/^{12}C$, $^{18}O/^{16}O$, $^{2}H/^{1}H$ and $^{34}S/^{32}S$). Here we report mainly on research into the interpretation of isotopic compositions of C ($\delta^{13}C$) and N ($\delta^{15}N$) in plants and plant N sources. This research lies in four major areas of plant research: (1) the use of $\delta^{15}N$ as a genetic trait for correlation with molecular markers; (2) explaining the isotopic signatures of $^{15}N/^{14}N$ (and $^{13}C/^{12}C$) in terms of plant physiological mechanisms and plant N nutrition; (3) linking physiological mechanisms to molecular markers *via* interpretation of the isotope values; and (4) the continuing development of new chemical preparation methods and instruments which is fundamental to progress in all areas of research involving natural abundance levels of stable isotopes.



Our biological isotope research is done in a variety of environments, e.g. microbial cultures, highly controlled glasshouse studies and designed sampling of the natural environment in terrestrial and aquatic systems.

Two major questions underpin all of our research on $\delta^{15}N$: (1) what are the extent and causes of $\delta^{15}N$ variations in plants and plant N sources; and (2) how can their mechanisms be quantitatively modelled?

Elements artificially enriched in their heavier stable isotope have been used for many years as tracers in biological systems. These can be used to describe the amounts of an element which move from a source to a sink. The naturally-occurring levels of stable isotopes (identified by the δ notation), and especially those of N, C, O, H, and S, can be used to investigate processes within complex biological systems. These processes can be opaque to more traditional techniques, such as measuring changes in mass amounts, and difficult to study using heavy-isotope-enriched tracers. If enough information is known about the system under investigation, the naturally-occurring levels of stable isotopes can be used with a so-called 'fractionation' model to provide a combined description of the processes and source-sink relationships of the element. In moving from the use of enriched tracers to natural abundance levels, the conceptual framework changes dramatically, and the limits to interpretation are different. Additionally, the required instrumentation and sample preparations are, at once, more sophisticated and less well developed.

Enriched tracer methods have a conceptual framework, well-established mechanistic interpretations and comprise off-the-shelf technology for biological studies. Interpretation of natural abundance level isotopes has no such firm basis yet, and constitutes a new area of basic research, whose results will provide a new means of understanding some of the more subtle biological processes.

Controlled experiments

$\delta^{15}N$ of barley as influenced by genotype and abiotic stresses Wild and cultivated barley genotypes were ranked by their $\delta^{15}N$ values after growth in highly controlled hydroponics on NO_3^--N as the sole N source. This work[1,2] showed that barley shoots expressed large differences in $\delta^{15}N$ which were correlated with genotype, with salt stress and with genotype interacting with stress. Whole plant $\delta^{15}N$ for barley genotypes subjected to the stresses of drought and N

deficiency, also expressed a large range of values, and a variety of intra-plant partitioning patterns of $\delta^{15}N$, which were correlated with genotype, stress and the interaction of these. While $\delta^{13}C$ has been used as an index of stress tolerance (water loss *versus* carbon gained) in a great many studies, this was the first systematic demonstration that plant $\delta^{15}N$ is genetically and environmentally determined, rather than being directly and solely a function of the $\delta^{15}N$ of the plants' external N source.

As a first step in explaining the mechanisms underlying the plant $\delta^{15}N$ values obtained from these experiments, Robinson *et al.*[3] developed a theoretical model explaining the patterns of intra-plant $\delta^{15}N$ resulting from primary assimilation of NO_3^--N. This model is based on three simple rules: (1) when an N pool divides without transformation, there is no change in $\delta^{15}N$ values of pools (this reflects that little fractionation is associated with transport processes); (2) when an N pool divides by enzyme-catalysed transformation (e.g. during NO_3^- reduction catalysed by nitrate reductase), the resulting $\delta^{15}N$ values depend on $^{15}N/^{14}N$ fractionations as approximated by a Rayleigh-type equation; and (3) when two N pools mix (e.g. when reduced N transported from shoots to roots mixes with that already present in roots), the resulting $\delta^{15}N$ value is a mass-weighted average of $\delta^{15}N$ values of the component pools.

Despite the simplicity of these rules (and the restriction to plant growth on NO_3^--N), this initial $^{15}N/^{14}N$ fractionation theory for use of a single chemical type and isotopic source of N (NO_3^--N of a known and constant $\delta^{15}N$) is more complex than that for e.g. plant $\delta^{13}C$ which can be largely explained by a single, simple equation[4]. The new model for $\delta^{15}N$ requires 30 sets of equations, cannot be condensed into a single expression, and must be solved numerically. The processes it describes include: NO_3^- reduction in roots and shoots; transport from roots to shoots of unreduced and reduced N; transport in the opposite direction of reduced N; and the efflux of reduced and unreduced N from roots, the only plausible mechanism for a net $^{15}N/^{14}N$ discrimination between the plant and its N source. It provides estimates of these processes which match the calculated $\delta^{15}N$ values of certain N pools (e.g. shoot and root total N) to those which have been measured in a particular experiment. In reality (even in potted plants in glasshouses), the available N pool is much more complex, commonly comprising NO_3^--N, NH_4^+-N and various types of organic N, as well as influences on assimilation by other organisms such as N_2-fixing microbes, various pathogens, mycorrhiza-forming fungi (see below) and abiotic stresses. This means, *inter alia*, that the net plant response in terms of $\delta^{15}N$ is composed of a time-and-mass-averaged, net $\delta^{15}N$ value related to the assimilatory processes of all of the types of N used by the plant, and as moderated by environmental and biotic influences. This is presently uninterpretable in the field. Understanding the mechanisms related to NH_4^+-N nutrition is the next goal, to be followed by modelling the assimilation of mixed N sources (chemical and isotopically mixed) and then relating these models to perennial as well as annual plants. In the meantime, controlled experiments have been done to understand better biotic influences on plant $\delta^{15}N$.

In a separate experiment, Robinson & Conroy[5] found that the plant itself influences the $\delta^{15}N$ of its own rhizosphere during growth and does this in proportion to the adequacy of available soil water.

Biotic influences on plant $\delta^{15}N$ One of the applications of $\delta^{15}N$ in terrestrial plant-soil studies was to use the $\delta^{15}N$ of a potentially N_2-fixing plant to calculate how much of its N came from N_2-fixation by symbiotic microbes. This, and other uses of $\delta^{15}N$ as a 'pseudo-tracer' of N sources, has come under periodic criticism from many researchers. We conducted a glasshouse experiment[6], comparing estimates of the amount of N fixed using three enrichment levels of N (natural abundance, 0.5 atom % and 5 atom %) with a *Rhizobium*-clover symbiosis. Even in closely controlled glasshouse studies, $\delta^{15}N$ was unacceptably variable and inaccurate as a 'pseudo-tracer'.

It has long been assumed that N_2-fixation incurs no isotopic fractionation and that the $\delta^{15}N$ of atmospheric N_2 fixed by microbes associated with vascular plants is nil. We have shown recently[7] that N_2-fixation can be accompanied by $\delta^{15}N$ values ranging from 0‰ to -4.4‰ and that the net $\delta^{15}N$ value is correlated with the type of N_2-fixing enzyme (nitrogenase) which is most active. This new information will have a considerable impact on perceptions of the influence which N_2-fixation has in determining net $\delta^{15}N$ in plants, ecosystems and palaeo-studies.

Following a field report by Högberg[8], that foliar $\delta^{15}N$ at an African site correlated with type of ectomycorrhiza, we conducted two controlled experiments[9,10] using arbuscular mycorrhizas (AM). These showed that infection by AM-forming fungi could influence the whole plant $\delta^{15}N$ by as much as 3‰ and that the

largest influences on plant $\delta^{15}N$ (studied in these experiments) were species of fungus and external N concentration. Amount of fungal infection had no effect on plant $\delta^{15}N$. New experiments will test the extent of $\delta^{15}N$ variation induced by drought and fungal infection on plant $\delta^{15}N$.

$\delta^{15}N$ (and $\delta^{13}C$) were used to assess the effects of biotic stress caused by pathogens, where *Petunia hybrida* was infected with a systemic virus, nematodes and a combination of virus and nematodes. Pathogen-induced effects were not confined to the sites of virus infection. All treatments resulted in a depletion of shoot and root ^{15}N compared with uninfected controls, and pathogen loading altered the $\delta^{15}N$ of whole plants, shoots and roots. In the double infection treatment, $\delta^{15}N$ patterns suggested that N nutrition was impaired.

Controlled experimentation has, thus, established minimum ranges of $\delta^{15}N$ variation and some of their proximate causes. This work underscores the futility of using $\delta^{15}N$ as a 'pseudo-tracer' of N sources in plant, soil and animal studies and emphasises the process-based nature of $\delta^{15}N$.

Field-based studies

The overall approach of the Stable Isotopes Unit is iterative, from field observation to controlled experiments (designed on the basis of field results), and then field verification of experimental results. Our first large field study was done over three full years, examining the spatial and seasonal $\delta^{15}N$ of the vegetation of a field in the first stages of primary succession[11]. The plants comprised legume-rhizobia symbioses and non-N_2-fixers, woody components and a herbaceous sward, xerophytes and mesophytes. This study established that the type of chemical N source used by the different plant species and life-forms could not be determined on the basis of $\delta^{15}N$ from field samples. It also confirmed the findings of an earlier study in Africa[12] that $\delta^{15}N$ could not be used to calculate the amounts of plant N derived from N_2-fixation. In contradiction to reports from another European laboratory, clear seasonal changes in leaf $\delta^{15}N$ were documented for all of the plants studied.

In a field study of the native juniper tree, *Juniperus communis*[13], we found that foliage $\delta^{15}N$ co-varied with both tree gender and soil moisture content. This suggests that juniper may vary its success in different soil water regimes and that the different soil-moistures are reflected in the foliar $\delta^{15}N$.

Soils and soil invertebrate communities The tradition of isotope research has been (and still is, to some extent) based on single samples or at least very little replication. With the advent of the new automated mass spectrometers, it is now possible to analyse >100 samples overnight, making statistically-designed field experiments feasible. However, no work had been done to determine optimum sampling patterns for natural abundance level stable isotopes. We quantified the spatial variability of three soil properties (total N, total C and pH) and two stable isotopes ($\delta^{13}C$ and $\delta^{15}N$ of whole soil), using geostatistical techniques in upland Scottish pastures given contrasting management regimes (grazed, fertilised and ungrazed, unfertilised)[14]. The results suggested that to obtain statistically independent samples, a sampling distance of ≥13.5 m is required for $\delta^{15}N$ of total soil N.

Following the geostatistical results, $\delta^{15}N$ (and $\delta^{13}C$) analyses of soil, plant and invertebrate samples showed that the land-use treatments greatly affected the trophic interactions of soil invertebrates and primary producers[15]. These data also showed that the total soil $\delta^{15}N$ changed seasonally and was not, therefore, a constant background value, as previously assumed. Seasonal declines of $\delta^{13}C$ and $\delta^{15}N$ were detected in earthworms and slugs and may reflect previously unsuspected invertebrate behaviour.

$\delta^{15}N$ fractionation modelling for aquatic studies The concentration of nitrate in the River Ythan catchment, north-eastern Scotland, is above 10 mg NO_3^--N l^{-1}. Information is sought on the source of the nitrate and the extent to which the nuisance blooms of the green seaweed, *Enteromorpha* sp., are dependent on the high levels of nitrate entering the estuary. We are using stable isotopes to investigate the movements of nitrogen through soil N pools, streams and the river into the estuary. Theoretically, as the nitrate moves towards the estuary, its $^{15}N/^{14}N$ isotopic composition should change. By characterising the isotopic composition of the nitrogen in each major pool and the growth and isotopic composition of aquatic plants, the movement of inorganic nitrogen will be modelled.

In parallel with this study is the characterisation of the isotopic composition of the oxygen atom in the nitrate. Naturally derived and industrially produced nitrate have distinct $\delta^{18}O$ values. The addition of inorganic N fertilisers to a number of test fields located close to targeted burns will allow us to track the movement of nitrate by the ^{18}O composition. This

approach is further strengthened by the characterisation of the isotopic composition of the inorganic carbon in the river and estuary water. Carbon dioxide is the primary substrate for photosynthesis. By tracking the seasonal changes in the isotopic composition of organic carbon in the seaweeds, we will be able to define the major growing season of the algae and relate this to the acquisition and assimilation of inorganic nitrogen.

Chemistry

The foregoing projects exploiting stable isotopes depend on our ability to measure stable isotope levels in a range of samples. For the large number of plant and invertebrate samples studied in our laboratory (up to 25,000 per year), we use continuous flow systems with elemental analyser sample converters (see analytical facilities entry). This approach allows us to make most of the measurements we need, but there is a clear need for isotope data which can not be met with existing methods. This is addressed by an active research effort aimed at developing novel instrumentation and sample preparation methods, to meet both immediate and future needs.

We have successfully completed an EU-funded project to extend continuous flow methods to hydrogen and oxygen isotopes. Full exploitation of this methodology may be some time in the future, but it has already proved robust in collaborative research on the energy expenditure of foraging bumble bees, using stable isotope labelled water. The sample conversion methods developed in this project will also find application in compound specific analysis of soil N species. This is a difficult problem, to which solutions will be welcomed in our laboratories and in the wider community of stable isotope users.

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