A Dundee contribution to science: high-throughput mass spectrometry and new biological insights

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Recalcitrant problems in biology, agriculture, conservation, and environmental management need a continual input of the best new interpretations and technologies. A relatively recent innovation, which has produced much useful new information, is continuous-flow isotope ratio mass spectrometry (CF-IRMS). The CF-IRMS instrument provides a fast, reliable way of measuring the isotope ratios of elements (e.g. C, N, O, H, and S) in different kinds of samples. Much has already been learned and new phenomena have been identified by observing deviations from the expected values of isotope ratios. Much of this new science began, and is still being led, by research in Tayside.

As UK government policy turns increasingly to environmental concerns, and to agricultural conservation and sustainability of farming and biodiversity issues, this powerful new ability to study the processes of nature requires pride-of-place in research planning, environmental assessment and conservation. This is especially so in Scotland where the science was born, and where it continues to evolve new ways of resolving old and new problems.

Innovation of the technology and the chemistry

CF-IRMS was invented between 1977 and 1984 by Dr Tom Preston and Professor Nick Owens, two former graduate students of the then Professor W.D.P. Stewart of the Department of Biological Sciences, Dundee University. Dr Preston realised, while laboriously analysing hundreds of samples of lake sediments and plants for their nitrogen isotope abundances, that the analyses could be automated if an instrument called an elemental analyser was coupled to an isotope ratio mass spectrometer which had multiple signal collectors. In 1981, Dr Preston, by then a researcher at the Scottish Universities Research and Reactor Centre, was finally able to develop this concept in collaboration with Dr Owens, by then at Plymouth Marine Laboratory. The new instrument[1,2,3] so impressed two employees of a major mass spectrometer manufacturer that they quit their jobs and formed a new company (Europa Scientific Ltd, now PDZ Europa), which has twice won the Queen's Award for Industry.

Designed originally to measure ^{15}N enrichments for tracer studies, CF-IRMS was rapidly developed to measure isotope compositions at natural abundance levels: $\delta^{13}C$, $\delta^{18}O$, and $\delta^{15}N$. Over the last decade, the CF-IRMS approach has been extended to other elements (^{2}H and ^{34}S) and other sample types (^{18}O in organics and nitrate). Dundee has continued to be a major centre of activity in CF-IRMS development, with collaborative projects between SCRI and Europa Scientific Ltd on ^{2}H and ^{34}S and ^{18}O analysis. All of the wide range of CF-IRMS instruments in use around the world are descendants of this fundamentally Scottish invention. CF-IRMS was thus conceived in Dundee, proven in Plymouth and is now, globally, an essential research tool.

The simple (in hindsight) coupling of an elemental analyser to an IRMS to produce the CF-IRMS opened up a whole new range of applications and possibilities. For $\delta^{15}N$ and $\delta^{13}C$, the chief impact was sample throughput. Handling large sample sets for statistically sound interpretation of ecological and genetic problems became a realistic possibility. Once the potential for ^{13}C and ^{15}N was demonstrated, there was widespread interest in extending this productive analytical system to other elements, particularly H, O and S. The elemental analyser sample converter was ideal for bulk plant and soil samples, but there was also growing interest in the isotopic composition of individual compounds. This drove a further level of instrument integration, where a gas-chromatograph, separating complex mixtures, precedes a micro-combustion interface to the IRMS.

SCRI has continued the development of novel systems for both bulk sample and compound-specific stable isotope analysis of H, O and S, working closely with instrument manufacturers and international collaborators. Along with other developments in the field, the result is that instruments are now available to tackle a wide range of samples and elements. The pace of instrument development has been rapid and has sometimes run ahead of the production of adequate calibration materials. Models for interpreting the natural variations in isotope composition have still to catch up with the instrument developments. There is consequently a unique opportunity for timely studies of isotope natural history, not possible until now, which promise powerful tools to complement $\delta^{13}C$ and $\delta^{15}N$ in environmental and other fields.

Our design of new analytical systems has centred on two parts of the CF-IRMS, the sample converter and the mass-spectrometer. The conventional mass-spectrometer design is satisfactory for C, N, O and S analysis, but not for H; the helium carrier, which sweeps the analyte gas from the sample converter to the IRMS, cannot be separated fully from hydrogen in the conventional analyser. The helium is present in vast excess and is only one mass unit heavier than the very low intensity Hydrogen-Deuterium (HD) ion. Precise quantification of the HD ion is essential for natural abundance measurements, and this was achieved by using a specially designed analyser which produced a much greater separation of the ion beams[4]. Once measurement of hydrogen gas was possible, sample conversion of water and organic compounds was developed using pyrolysis and reduction on carbon[5,6]. Pyrolysis not only converts sample hydrogen to hydrogen gas, but also converts any oxygen to carbon monoxide which is suitable for measuring oxygen isotopes. Measurements of hydrogen and oxygen isotopes can now be made on very small volumes of water and organic compounds, bulk samples and individual components of complex mixtures. Appropriate standards, however, do not exist for organic compounds; we see a research opportunity for SCRI in such development and subsequent use of appropriate standards.

The CF-IRMS system for sulfur analysis is substantially the same as for C and N, but uses modified elemental analyser chemistry to achieve complete conversion of sample sulfur to sulfur dioxide. Using conventional off-line methods, sulfur has always been a difficult element to handle for isotope analysis; in a dedicated CF-IRMS, high throughput of plant samples of low sulfur content is possible. Enriched sulfur isotopes are not readily available and this has restricted the application of stable isotope methods to plant metabolism. However, using naturally distinct sulfate sources, we have studied uptake and re-location of sulfur in wheat over a growing season[7].

Biological problem solving

Since 1991, Dr Linda Handley (SCRI) has specialised in understanding the biological bases for the patterns of $\delta^{15}N$ values found in nature. These patterns are important because they lead to an understanding of natural processes which cannot be studied by any other means. After a substantial, critical review of the existing literature and a 3-year study of $\delta^{15}N$ patterns in complex vegetation and soils[8], Dr Handley called for a complete revision of this area of study and pointed out to an international audience[9] that $\delta^{15}N$ could not be used to trace, directly, sources of nitrogen, as almost all previously reports assumed, but chiefly revealed the occurrence and extent of biological and physical processes which transform nitrogen chemically and physically (e.g. nitrification, assimilation and loss by organisms and gaseous nitrogen loss).

The $\delta^{15}N$ research at SCRI has developed into two main areas, both of which are directly useful for UK environmental studies, conservation, sustainable farming and for providing the scientific bases for policy and regulation. The first main area concerns the chemical transformations and fates of nitrogen in soils and waters, including the role of plants. The second major area concerns functional plant biodiversity: its protection in nature, the assessment of biodiversity in crop plants for potential breeding, and whole plant performance of genetically modified crop plants. Both

of these lines of research have commercial potential.

Transformations and Fates of Nitrogen Soil nitrogen is changed naturally into nitrate-nitrogen, which leaks into drinking water, streams and ultimately into nearshore marine waters[10]; nitrogen arising from fish farm waste is suspected to have a role in the recently reported amnesiac shellfish poisoning[11] found in Scottish west coast waters, and atmospheric nitrogen pollution has been identified, globally, as a threat to the biodiversity of naturally nutrient-poor ecosystems[12], such as native moorlands and grasslands. It has been asserted for several decades that excess nitrate in drinking water caused the so-called blue baby disease in infants and has been suspected, on theoretical grounds, of causing stomach cancer. Today, the evidence is mixed and being revised[13], with some positive benefits apparently accruing to human health and some new concerns arising as well. It is established that nitrogen from land sources causes major changes in the nearshore marine environment[10,14] and nitrates from agriculture and urban sources are suspected of playing a role in causing nuisance and toxic algal blooms in estuaries and freshwaters, to the extent that this presumption forms one of the main bases for an EU Directive requiring designation of Nitrate Vulnerable Zones.

There has been no way to study the chemical transformations and fates of nitrogen on a long-term, large-scale basis such as whole fields or catchments. Some progress has been made in the last decade in studying the variations of nitrogen forms and amounts in receiving waters (surface and ground waters). However, the majority of nitrogen cycling events, including the generation of nitrate, occur in soils, and the lack of appropriate methods has prevented studying these processes in soils until recently. Most of the information that we have about the transformations and fates of nitrogen was determined by adding isotopically heavy (^{15}N-enriched) nitrogen tracers to either small-scale field plots or to laboratory experiments. This approach has limited use because nitrogen transformations are highly variable in space and time, and unpredictable on the larger scales which are relevant to environmental stewardship. To the extent that such methods are limited to point measurements in space and time, they are also unreliable. Additionally, most research conclusions are based on site-dependent case studies, which cannot be generalised to other sites. Hence, scientific underpinning is weak or absent for much of the management and regulation of nitrogen in the environment, and a substantial niche exists for basic and strategic research, were more suitable methods and technologies available.

Research at SCRI, using the natural background levels of the nitrogen isotopes (δ^{15}N) of soil and water, are changing this situation by innovating new methods and new interpretations. Early results show that mathematical modelling approaches can be used with direct sampling and analyses of δ^{15}N of soils or waters can be used to describe the chemical transformations and fates of environmental nitrogen. When fully developed, this approach will allow us to identify the polluter. It will be possible to assess whether nitrogen pollution regulations are working properly, long before the ultimate results can be detected in receiving waters, to identify trouble spots quickly and suggest improvements. It will also provide a rapid assay of soil nitrogen nutritional status by quickly assessing the relative importances of nitrification and denitrification in releasing and/or depleting the soil nitrogen bound in organic matter. This will enable increased precision of timing for fertilisation and provide an index to optimum fertilisation amounts by filling in the long-missing part of the puzzle - how much is lost as nitrogen gases.

Others have recently attempted to model soil nitrogen cycling using δ^{15}N of nitrogen pools, but have been forced to gloss over the lack of reliable chemical preparation methods for the isotopic analysis of these pools. Hence, the real work remains to be done. At SCRI, we have innovated the only method, so far, which is capable of measuring the δ^{15}N of nitrate-nitrogen in complex samples such as soil solutions and highly eutrophic waters[15]. A new and reliable method for determining the δ^{15}N of ammonium-nitrogen from soil solutions and eutrophic waters will be announced shortly from our laboratory.

Meanwhile, we have completed one full calendar year of measuring, fortnightly, the concentrations and δ^{15}N values of nitrate-nitrogen in the soil solution of a barley field in the Ythan River catchment, Aberdeenshire, and in its field drains. We have also measured the concentrations and δ^{15}N of nitrate in the Ythan River and its tributary burns. This is the first, accurate description of the spatial and seasonal, farming-related changes of the δ^{15}N of nitrate in parallel with nitrate concentrations in soils and surface waters and hence of the transformations of nitrogen in the soil which lead to the formation of nitrate. The δ^{15}N of the nitrate in soil solution also reveals the extent to which the sample comprises newly nitrified nitrogen available to the crop or for leaching with per-

colating waters *versus* the extent to which the analysed nitrogen is that remaining after excess nitrogen has been lost gaseously.

In the current climate of nitrate regulation and related environmental concerns, such as ozone depletion and atmospheric nitrogen pollution, it is increasingly important to be able to assess the transformations and losses of nitrogen from soil and waters. Researchers using conventional water chemistry[e.g.16,17] and now-acknowledged, inadequate methodologies for $\delta^{15}N$ of nitrate[18,19], have produced data suggesting that some wetlands routinely achieve very high (up to 60%) removal of nitrate as denitrification, mostly as N_2 gas, but that the process from soil may incur denitrification *via* a larger proportion of ozone-depleting N_2O gas[17]. Martin[20] reported wetlands reducing incoming dissolved nitrate to as low as 2 mg l^{-1}, while Sidle *et al.*[21] found that a constructed and managed wetland in Indiana, USA was less efficient at nitrate removal than an adjacent natural riparian marsh. Using existing expertise, experience and unique methodologies, SCRI is poised to further our understanding and ability to assess important aspects of nitrogen cycling, particularly in relation to nitrogen pollution and denitrification losses. We are especially well prepared to deliver innovative research on the role of wetlands in attenuating nitrogen loads.

Recently, hydrologists in Germany, Canada and the US have been able to distinguish empirically, in some cases, between nitrate arising from agriculture and nitrate in waters arising from animal sources, including human waste[22]. This has been done by simultaneously analysing the $\delta^{15}N$ and $\delta^{18}O$ of nitrate in waters. The underlying biological mechanisms are not understood, and we see this as a fruitful line of inquiry for the expertise existing at SCRI, building on, *inter alia*, the innovative work and accumulated experience in ^{18}O analysis at SCRI.

The basic technologies and concepts, largely initiated in Scotland, have started a cascade of isotope research centred on nitrate and on the $\delta^{15}N$ and $\delta^{18}O$ of nitrate and the $\delta^{13}C$ and $\delta^{18}O$ of dissolved organic carbon compounds in ground and surface waters, tracking pollution and studying water quality. For use with ground and surface waters (of relatively low organic nitrogen content and therefore susceptible to methods which are unsuitable for soil solutions analyses), scientists at the United States Geological Survey have developed new methods[23,24] for measuring the $\delta^{15}N$ and $\delta^{18}O$ of dissolved nitrate, a technology which found immediate acceptance among hydrologists and geochemists[22,25]; this same group is funded by the US Government to examine how nitrate concentrations increase under forest soils in a large catchment. The intensity of interest in the $\delta^{15}N$ and $\delta^{18}O$ of nitrate for use in hydrological studies is such that new methods are beginning to appear from several laboratories[e.g.23,24,26], although most of these laboratories still use older manual preparation methods instead of relying on the newer continuous-flow technology for determining $\delta^{18}O$. As the United States carries out a large programme of assessing water quality, several major research projects, on the scale of major continental river catchments, are using $\delta^{15}N$ and $\delta^{18}O$ to study the age and source of nitrates in surface and ground waters[27] . The US Environmental Protection Agency is beginning new research using $\delta^{15}N$ and $\delta^{18}O$, together with conventional water chemistry and hydrological modelling, to study the impacts of landforms and land use, interacting with vegetation, on surface and ground water quality[28] , an approach we have long argued is necessary for Scotland.

The method developed at SCRI by Johnston *et al.*[15] is the only suitable one to date for measuring the $\delta^{15}N$ of nitrate in soil solutions. This method, and other expertise at SCRI, could fill a widely recognised need[20,29] in soil nutrient cycling research, for systems of measuring nitrification, denitrification and soil respiration at depth in the soil profile.

SCRI researchers published the first evidence that previously unsuspected processes in the top layer of the soil caused sufficient changes in total soil nitrogen to change the $\delta^{15}N$ of whole soil substantially in the course of a growing season. The initial work was done on an abandoned agricultural field in primary succession[8], then in Scottish pastures in collaboration with Dr Carol Marriott of the Macaulay Land Use Research Institute[30,31]. Similar findings have now been reported by Butler *et al.*[32] for arable (maize) and grassland in Spain and in southern England. Our work was done for the upper 10 cm of the soil profile. Butler *et al.*[32] showed the same patterns occurring to 30 cm depth. We attempted to explain these new observations in terms of known processes and rates for nitrogen mineralisation and loss; such calculations did not account for the observations, demonstrating serious deficiencies in our information base (*inter alia* interpretations underpinning regulations) and highlighting the need for new research using new isotope techniques.

Current laboratory and field experiments at SCRI examine the nature of the nitrogen transformations which cause such isotopic patterns in soils and percolate waters. In an early study of Kenyan savannah vegetation[33] (done collaboratively with Professor Janet Sprent (Dundee University) and Dr David Odee (KEFRI, Kenya), then a student of Dundee University), an important link was demonstrated between plant $\delta^{15}N$ and soil moisture status. Soil moisture, intermittent or consistent, dominated the $\delta^{15}N$ patterns of the local vegetation, regardless of whether the potential source of plant nitrogen was atmospheric nitrogen, fixation by legumes, or soil nitrogen, and regardless of type of plants examined. Later work[34] with another student at Dundee University (Paul Hill) on the functional diversity of native *Juniperus communis* trees in Scotland, established similar relationships for foliar $\delta^{15}N$ and soil water relations. Most recently, a global survey of $\delta^{15}N$ values of plants and soils[35] documented that rainfall and temperature (as surrogate measures of soil moisture) so dominated ecosystem nitrogen cycling that it was reflected consistently in the nitrogen isotope values of plants and soils. The findings of this study were consistent with those of Austin and Vitousek[36], who found a trend for foliar $\delta^{15}N$ along a rainfall gradient in Hawaii.

Collaborative work in Spain[37] uses $\delta^{15}N$ and ^{15}N-enriched tracers to reveal the processes of nitrogen cycling in soils and plants following periodic fires in that region. In a collaborative study with a Canadian researcher[38], $\delta^{15}N$ values enabled the solution of a problem which years of research had been unable to resolve otherwise: how prevalent management of western red cedar forestry (including post-harvest burning) leads to a serious weed infestation which debilitates newly planted replacement forests.

In collaboration with Dr Chris Wheeler of Glasgow University[39], we were able to demonstrate, via $\delta^{15}N$ measurements, that most of the plant-available soil nitrogen in a west coast Scottish mire originated from atmospheric nitrogen fixation by free-living soil microorganisms, and was not contributed by the more conspicuous leguminous plants. A separate study provided new information about the nitrogen status of important forestry trees which fix atmospheric N_2 in association with the actinomycete, *Frankia*[40].

Functional Plant Biodiversity The UK is bound by international treaties and by EU Directives[41] to address the problems of conserving biodiversity, and there is a special biodiversity group for Scotland[42]. However, little is known about the existing whole-organism, functional biodiversity of plants. Additionally, it has been impossible to assess, within a reasonable length of time, whether environmental factors, such as air pollution, are affecting the functional biodiversity of plants. Research at SCRI, relying heavily on the stable isotopes of carbon and nitrogen, is providing a novel way to assess whole-plant functional biodiversity in interaction with environmental conditions.

The publication of a model explaining the central mechanism determining whole leaf[43] $\delta^{13}C$, opened a large new area of research on the environmental and population-based components of photosynthesis and water loss in plants using the C_3 pathway of photosynthesis[e.g.44,45]. A significant few of these papers related $\delta^{13}C$ to inter- and intra population genetic variations[e.g.44,46], especially in wild plants. As far as we are aware, SCRI's isotope group is the only laboratory, so far, to follow through extensively on the genetic component of plant $\delta^{13}C$ and relate $\delta^{13}C$ to molecular markers of identified genotypes; we were the first laboratory[47] to establish a genetic component for plant $\delta^{15}N$. This lead is now being taken up by others, such as Professor Richard Guy at the University of British Columbia[48]. Our approach has been unique from the beginning in taking advantage of the newly emerging techniques of molecular biology to establish at the outset that experimental material for physiological studies consists of molecularly-identified different genotypes. The next step is to understand the mechanisms behind the observed isotopic differences among genotypes and life forms. We continue to study the functional biodiversity of native British plants as expressed in the whole organism by $\delta^{13}C$ and $\delta^{15}N$ [49,50,51].

From the first field studies of foliar $\delta^{13}C$ and $\delta^{15}N$, it was recognised that there were inter-species, and intra-population variations around mean values, which could not be explained easily by a central mechanism. For $\delta^{13}C$, it was widely assumed that these variations represented small differences in water availability or leaf temperature, until Comstock and Ehleringer pointed out the genetic component to these variations[46]. For plant $\delta^{15}N$, the common, but untested, assumption was that variations of plant $\delta^{15}N$ represented small differences in the type and soil depth of the nitrogen which these plants used, e.g.[52]. In a series of papers[53,54,55,56,57,58,59,60,61,62,63,64,65,66],

researchers at SCRI described highly controlled hydroponics experiments, and one soil-based glasshouse experiment, which showed that genotypes of wild and cultivated barley varied in their shoot and whole plant $\delta^{15}N$ and $\delta^{13}C$ against a common isotope background for nitrogen and carbon. Further data[51] have now shown the same genotype-dependent variations of these isotopes for wild plants (a sedge, grass and dicotyledon) growing in a nutrient-poor soil, for native Scots pine in a common garden and in sites-of-origin[49], and for the important upland pasture grass, *Agrostis capillaris*, grown in a controlled environment chamber in sand culture[50].

Analyses of plant $\delta^{13}C$ and $\delta^{15}N$ are fast and reliable, and the results allow us to make some reasonable hypotheses about their causes, i.e. what is functionally different about genotypes having different isotopic values and, therefore, what genetic variations in a population of plants allow that population to survive the vicissitudes of competition and environmental changes. The hypotheses which arise from initial isotope screening can then be tested on individual genotypes which have shown extreme isotopic values, thus reducing the number of test organisms to a manageable level and increasing the certainty with which experimentation is designed.

Variations among plant genotypes of each isotope pair direct further investigations toward specific areas of research. Variations in $\delta^{13}C$ tell us that we should investigate genotypic differences in plant anatomy[67,68,69] and physiology[34,70,71] related to photosynthesis and post-photosynthetic carbon metabolism as well as differences in water use. $\delta^{15}N$ variations tell us that we should examine variations in nitrogen assimilation, internal allocation and losses of nitrogen for describing the biodiversity of plant strategies.

Much less is known about plant $\delta^{15}N$ than is known about $\delta^{13}C$; there is no central mechanism described which chiefly determines plant $\delta^{15}N$. However, progress is being made. Handley and Scrimgeour[8,9] have been major forces in causing a complete re-assessment of the way this isotope is regarded. $\delta^{15}N$ was initially regarded as a tracer, a naturally occurring form of ^{15}N-enriched compounds which had been used successfully to trace the fates of added nitrogen and estimate the amounts of nitrogen in systems. Especially after CF-IRMS instruments became widely available, a host of papers appeared purporting to use foliar $\delta^{15}N$ to trace the 'source of plant nitrogen.' Shearer and Kohl[72] claimed for many years (pre-CF-IRMS, when few could analyse $\delta^{15}N$) that they could quantify the amount of plant nitrogen which was derived from biological N_2-fixation and do it under field conditions. Although the educated isotope community never accepted this claim, Handley and Scrimgeour[8] were the first to argue strongly and overtly, with supporting data, that plant $\delta^{15}N$ is chiefly determined by isotopically fractionating processes and not by the isotopic value of the source nitrogen; they argued, further, that plants have an active and dynamic role in their own nitrogen metabolism and are not passive acceptors of an external nitrogen source. Handley and Scrimgeour's rejection of $\delta^{15}N$ as a direct measure of amounts of nitrogen fixed from the atmosphere (or from any other source) was based on theory, field observation and the results of a rigorously controlled glasshouse study[73]. Theirs was also the first clear rejection of $\delta^{15}N$ as a tracer of nitrogen in soils since the early 1970's[e.g. 74]. The view that $\delta^{15}N$ integrates processes, rather than traces sources, is now widely accepted.

In one of a series of experiments with genotypes of wild barley[63], we were able to show that the $\delta^{15}N$ of the plants was related to how much nitrogen was retained in the plants *versus* that lost through the roots, under environmental stress. This series of experiments[e.g. 63,47] documented that $\delta^{15}N$ of plants was variable according to the kind of environmental stress to which the plants were subjected (salinity, drought and nitrogen deficiency). These were the first reports that plant $\delta^{15}N$ was related to genotype and could be modified by stress. These experiments, and subsequent work, are unravelling the relationship between this easily measured variable and the biodiversity of plant responses to stress insofar as the nutritionally important element, nitrogen, is concerned. SCRI researchers described this new approach in a series of papers presented to a conference on linking plant physiology with molecular genetic approaches[58,59]. In another molecular based study[75], $\delta^{15}N$ helped to describe genetically based variations in the nitrogen and carbon metabolism of pea.

Most plants form fungal associations known as mycorrhizas, the most common type of mycorrhiza are, arguably, arbuscular mycorrhizas (AM). To understand plant $\delta^{15}N$, and how it varies in response to environmental stresses, the role of mycorrhizas must be understood. It is known that AM fungi assist their

plant associates in obtaining soil phosphorus[76]; no special role in nitrogen nutrition was known. In collaboration with Dr Melvin Daft of Dundee University and later with Dr Charo Azcón of Granada, Spain, Dr Chris Wheeler of Glasgow University and Dr Henrique Fonseca of Portugal, we showed that the presence or absence of AM[77,78,79,40], the type of AM and the interaction of these factors with drought or nitrogen deficiency, have a large effect on the nitrogen relations of herbaceous plants. In a study of Canadian western red cedar forests, the use of $\delta^{15}N$ enabled Chang and Handley[38] to link type of mycorrhizal association with changes in post-fire nitrogen and phosphorus cycling and determine the cause of a serious weed infestation problem. This study also demonstrated how the biodiversity of organisms in the plant-soil partnership can respond differently to contrasting climates under physical stress.

Our research group has made a unique contribution to the knowledge of biological nitrogen-fixation: it has been undisputed, general wisdom for decades that biological nitrogen-fixation does not discriminate between the heavy and light isotopes of atmospheric nitrogen. We have shown, in collaboration with Dr Peter Rowell, Dundee University, that biological N_2-fixation can incur substantial fractionations relative to source nitrogen, especially in organisms which are able to live independently of symbioses with higher plants[80]. We have also shown that the amount of this fractionation is correlated with the chemical type of nitrogen-fixing enzyme, nitrogenase, which is present. Exploitation of these results could lead to new and useful information about microbial nitrogen-fixation.

Soil Invertebrate Studies and Food Webs Food webs (who eats whom and what) have been notoriously difficult to study. Many organisms, because of their migrations, their small size, or because they live in soil or other largely inaccessible locations, cannot be observed directly, and their eating habits must be inferred from indirect evidence. Environmentalists have always been interested in the feeding habits of animals, but it is also crucial to agriculture to know what soil-dwelling organisms eat and what effect plant-eating pathogens have on agricultural crops.

Deniro and Epstein[81] showed that the $\delta^{13}C$ of a variety of organisms could be used to determine what they actually assimilated (as opposed to ingested) and that, on average[82], there was a 3.3 ‰ increase in whole animal $\delta^{15}N$ with each increase in trophic level (i.e., this work predicts that tigers would be 3.3 ‰ more isotopically enriched than the cows they ate, and cows would be 3.3 ‰ more enriched than the plants they ate). The relationship breaks down between primary producers (plants and algae) and their nitrogen sources.

The expected $\delta^{15}N$ increase with height in the food chain is not absolute, and deviations from this expected value have revealed new information about animal life cycles and feeding habits. Using isotopic techniques, Scrimgeour *et al.*[83] found that beetles, which are pests on local raspberry crops, remain in the soil, between crop rotations, much longer than formerly suspected.

To study the below-ground feeding habits of soil-dwelling invertebrates in Scottish pastures, a multidisciplinary team (soil zoologists, plant biologists and ecophysiologists) was assembled, including scientists from the Macaulay Land Use Research Institute. This was the first, statistically designed spatial and seasonal study of the isotopic signatures of soil invertebrates, and one of the first such studies of soils and plants[30,31]. We found, *inter alia*, that grazing intensity altered below-ground feeding habits.

With continually improving technology at SCRI, and consequently better ability to analyse very small samples, the natural abundance isotope research was extended into plant-pathogen interactions, i.e. animal parasites, nematodes and the effects of nematodes and viruses on plants. During these studies, Dr Brian Boag (SCRI) published[84] the first $\delta^{13}C$ and $\delta^{15}N$ study of the internal parasitic feeding habits within an animal host, which also showed that the rabbit embryo behaves (nutritionally and isotopically) as a parasite on the mother. Using $\delta^{13}C$ and $\delta^{15}N$, Drs Neilson and Brown[85] showed that combined pathogen infection (virus application and nematode feeding) produced a greater physiological effect in *Petunia hybrida* seedlings than that recorded with only a single pathogen infection. Related work[86] established that some nematodes could alter their feeding preferences for plants.

The research at SCRI is closely allied with that of Professor John A. Raven FRS, Boyd Baxter Professor of Biology at the University of Dundee, and with his associates. Professor Raven's research is of a fundamental nature; he has specialised in plant physiology and has used marine algae and higher plants as model organisms. His use of enrichment and natural abundance levels of stable isotopes has revealed much new

and fundamental information about plant physiology and biochemistry. Although he began using natural abundance level stable isotopes to understand plant physiology while still at Cambridge University[87], the first isotope-based research he did in Dundee was enrichment-level studies of $^{18}O_2$ uptake in algae in the light to investigate the Mehler reaction and the (then still controversial) oxygenase activity of RuBISCO *in vivo*[88,89]. This work was followed by an investigation of the pathway(s) of oxalic acid synthesis in higher plants using enrichment studies with $^{18}O_2$ and $^{13}CO_2$ and natural abundance $^{13}C/^{12}C$ ($\delta^{13}C$) measurements[90]. The suggestion that the $\delta^{13}C$ of C_3 plant parasites (which are photosynthetically competent and living on C_4 hosts) can be used to assess the fraction of parasite C obtained from the host[91], has been widely followed up by subsequent studies elsewhere. This higher plant work led to the use of $\delta^{13}C$ data to interpret variations in the water-use efficiency of plants as a function of nitrogen source (ammonium, nitrate, nitrogen gas, ammonia gas)[92,93,94]. Food chain reconstruction from fossil (Cretaceous) data has also been attempted[95].

Using both aquatic plants and algae, Professor Raven's laboratory has used primarily $\delta^{13}C$ to make fundamental discoveries about photosynthesis. The innovative *Chlorella* (alga) work involved the use of carbon dioxide and nitrogen availability in regulating expression of the carbon dioxide concentrating mechanism, again with interpretative aid from stable isotopes[96]. Research on freshwater plants related $\delta^{13}C$ to the inorganic carbon source as determined by other, physiological measurements; these measurements, in combination, led to estimates of the diffusion pathlength for carbon dioxide in the freshwater alga, *Lemanea*, which does not use bicarbonate ions[97,98].

The freshwater algal work led to investigations on the ecophysiology of *Lemanea* with several lines of evidence (including carbon isotope natural abundance), corroborating the diffusion pathlength deduced earlier[99,100], and subsequent work on $\delta^{13}C$ of plants from freshwater habitats[101,102].

The other major theme in Professor Raven's algal work is the ecophysiology of marine micro- and macroalgae. As primary producers, these organisms are fundamental to the entire marine ecology. As is the case for freshwater organisms, the capacity for obtaining carbon from bicarbonate and, apparently, carbon dioxide concentrating mechanisms in general, involves less change of $\delta^{13}C$ relative to source than does diffusive entry of carbon dioxide[98,103,104]. The complication of atmospheric carbon dioxide as well as dissolved inorganic carbon for intertidal algae and plants was also studied[104].

A key event in algal studies was research on a range of marine macroalgae from the east of Scotland, which confirmed the correlation of diffusive entry of carbon dioxide, these organisms having very negative $\delta^{13}C$ values[105,106]. This work also showed that the *in situ* growth rate and $\delta^{13}C$ can, as for the freshwater *Lemanea*, be used to estimate the length of the carbon dioxide diffusion pathway in marine algae such as *Delesseria*. Further work on these algae investigated effects of variations in light supply[107,108], and of variations of inorganic carbon and oxygen supply[109,110], on photosynthetic and growth rates and on $\delta^{13}C$. The taxonomic and geographical spread of the data set subsequently has been extended[111,112] and currently is being analysed prior to publication.

A continuing theme in the work on aquatic macrophytes has been the use of $\delta^{13}C$ for interpreting symbiosis. This work followed from research on the role of the N_2-fixing cyanobacterium, *Anabaena*, in supplying carbon to its symbiont, the (freshwater) fern *Azolla*[98]. Work on the marine lichen, *Lichina*, has helped to define the mechanism of inorganic carbon supply to the cyanobacterial symbiont, *Calothrix*[113]. Raven *et al.*[114] investigated the metabolic relationship between the brown Australasian macroalga, *Hormosira*, and *Notheia*, a brown alga which only grows on *Hormosira* or the closely related *Xiphophora*. This work found, from gas exchange and $\delta^{13}C$ measurements, that the epiphyte could satisfy all of its energy and carbon requirements from its own photosynthesis and was not parasitic. Because *Notheia* contains the sugar alcohol altritol, which only occurs in a few other brown algae, including both *Hormosira* and *Xiphophora* which are relatively distantly related to *Notheia*, it was thought that the altritol in *Notheia* could be derived from *Hormosira* or *Xiphophora*[115,116]. This possibility has now been dismissed using mass spectrometric and NMR analyses of $\delta^{13}C$ and ^{13}C enrichment studies; hence, we now know that altritol synthesis occurs in both *Notheia* and its relatively distant relatives *Hormosira* and *Xiphophora* (and *Bifurcariopsis* and *Himanthalia*)[115].

Kleptoplasty by sacoglossan molluscs living on green (and red) macroalgae is a further example of symbiosis involving marine macroalgae. Here, some of the chloroplasts ingested by the molluscs during feeding

are retained by the sacoglossan in which they continue to photosynthesise for days to weeks (and occasionally months). Raven *et al.*[117] have used $\delta^{13}C$ to estimate the fraction of animal carbon which is derived from kleptoplastic photosynthesis rather than from direct feeding on the host algae in Western Australia.

Finally, work on microalgal cultures led by Dr Andrew Johnston (now at SCRI) has yielded important data on the relationship among the inorganic carbon supply to marine microalgae, their growth rate, and algal $\delta^{13}C$ relative to source inorganic carbon[118,119]. These, and other data, cast doubt on some recent attempts to use $\delta^{13}C$ in marine sediments to 'hindcast' sea-surface, and hence atmospheric, carbon dioxide levels in the past.

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