5. *RESEARCH PROGRAMME*

The overall objective of this initiative is to create a national centre of expertise and facilities within which the natural abundance stable isotope approach can permeate and integrate all of the life sciences at every level of organisation and every scale of study. It is envisaged that a well–founded nucleus of expertise and facilities organised around a well–chosen research theme, will enable the original researchers to attract further external funding for related research as well as attract talented scholars and students from all over the world. Especially we seek to attract the collaborative efforts of two groups: (1) our growing body of European research partners and (2) the American, Australian and Japanese researchers already active, separately, in various facets of stable isotope work.

We have shown, in previous sections, our serious commitment to this work, as well as the need to put it on a solid footing if it is to grow and thrive. For growth to occur we must overcome, the lack of job security for key personnel, the lack of key analytical instruments and sample preparation facilities, as well as the present lack of coherent integration of research around a central, expandable, theme. We must be seen to be on the cutting edge of this research, while still founding a base of intelligently collected descriptive work. The SCRI should become the collector and the repository of basic work needed to establish a baseline, as well as a culture medium from which useful questions and fruitful hypotheses will grow. With adequate funding, the SCRI can quite properly give the foundation of this research the continuity, expertise, and planning it needs. In order to facilitate this, we will establish a **Management Group**, organised around and including, the key personnel to be funded by this proposal (ecologist, metabolic physiologist and research chemist) but also including other important researchers and organisations.

The central theme of this proposal is the functional community ecology of plants. We want to learn more about how plant/soil systems use and transport inorganic nutrients (e.g., N and C) and water. This theme is further focused on plant nutrition and growth, because in both agricultural systems and unmanaged ecosystems, plants serve as a central mediator and transducer of environmental information; plants integrate environmental signals in dynamic ways and physically integrate the pools of available nutrients and water as they acquire resources for their own nutrition and growth. Plants record much of their history (and the history of their environment) in the form of natural abundance levels of stable isotope signatures. Learning to read these stable isotope signals will reveal much new information about plant ecophysiology (i.e., how plants regulate their uptake, distribution and utilisation of water and inorganic nutrients). This will be especially true of plant responses to sub–optimal conditions. Under marginal conditions the responses to environmental signals will be magnified. It is clear from the current literature of plant ecophysiology that natural abundances of stable isotopes are the major way forward in this field.

Although there is a large body of broad–bush information about plant/soil systems, most of the details are still hidden from us. Fortunately, detailed processes have left records of their existence and their importance. These records occur as natural abundance levels of stable isotope ratios. It remains for us to learn to read these records; their language is subtle and still largely undeciphered. To learn the natural language of stable isotopes will require integrated studies involving all of the major sciences, including physics, chemistry, mathematics, microbiology, soils science, plant biology, geochemistry, aquatic sciences, etc.

as problems require.

Further, to advance our understanding of the mechanisms and processes which recorded these signals we must adopt an iterative approach involving repeated cycles of observation and experimentation. Field observation will teach us what signals exist under what environmental conditions; subsequent laboratory experimentation (hypothesis testing) will assist in interpreting these signals; the interpretations gained from the laboratory will aid us in understanding the natural systems under study. Studies discussed in earlier portions of this document clearly demonstrate that this iterative approach has already yielded enormously valuable new information.

A **Model System** is proposed: Savannah* defined by us as woody plants with an undergrowth of Gramineae: grass, wheat or barley. Geographically and economically this system is found from diversified farms in Scotland (e.g., nutrient depleted heather moors) and Scandinavia, to mid-latitudinal Europe (dehesas, usually including oak trees in Spain, France, and Portugal), to the tropics where the term, savanna, is used. The model, thus, embraces most of our major geographic areas of international collaboration. As a research model, it provides opportunities for work in all of the major processes, at all of the levels and scales which we wish to encompass in this initiative and leaves great scope for rational expansion. It potentially includes research into woody plant species (N_2-fixing and non-N_2-fixing), herbaceous understorey plant species, well-documented cultivars, soils and soil nutrient cycling, plant-soil-water interactions, carbon and nitrogen cycling and animals (grazers, soil fauna, pathogens) interactions, as well as plant-microbial interactions. It provides, as a research focus, enormous potential for collaboration and additional external funding. Additionally, this relates closely to funded and proposed research on nitrogen metabolism and the water cost of growth in potatoes and barley.

The **Central Question** of this proposal, as explained in our model Savannah, is: what mechanisms determine the patterns in nature of the stable isotope values of $^{15/14}N$ and $^{13/12}C$? To answer this question, a great many smaller questions must be answered; other isotopes such as $^{34/32}S$, $^{18/16}0$ and $^{2/1}H$ must be used; descriptive data documenting these patterns must be accumulated in a focused, informed way; and it must be done systematically by a stable nucleus of expert researchers, so that institutional memory is retained, expanded, and exploited. It must embrace the use of simpler model systems or organisms to explore detailed sub-questions, contributing to the whole.

The needs for research are both great and varied. However, this proposal provides the structural framework for this important work. We have divided the following more detailed research plans into two categories: (1) research to be accomplished by the personnel funded in this proposal, along with an approximate time frame for the work (2) research which closely integrates with these projects, but to be tendered for additional external funding. The scope of the initial research will reflect the level of funding, which should include enough travel (both European and non-EC) to raise the profile of this work and attract other workers.

*We have used the historical spelling (e.g., Savannah, Georgia) to distinguish our concepts from the more restrictive usage of tropical savannah. We thank Dr John Proctor of Stirling University for suggesting this usage.

We have requested only a bare-bones budget for the minimum staffing and facilitation of a viable investment in this important new research area.

5.1 *Research Plan*

Draft ROAME documentation will be provided on request.

5.1.1 *Core Research*

Research at the core of this proposal (to be done by personnel funded in this proposal). We propose three closely integrated and mutually supportive research plans; all three are directed toward our central question and formulated to be expandable. We propose to implement the project incrementally, initially consolidating our existing investments. They will also be complementary to additional externally funded projects and to much research which is on-going, pending or under discussion at SCRI with Dundee University researchers and others (see also Section 2). Our central research brings together ecological/whole organism research, plants metabolic physiology and analytical/experimental methods developments. Additional technical support will be sought from external funding.

Research Plan 1: Patterns of $\delta^{15}N$ and $\delta^{13}C$ in Plants as a function of N-source.
In this instance, N-source has a broad meaning: NO_3^--N, NH_4^+-N, N_2-fixation, sources of N having undergone different amounts of mineralisation, mediated or not by a symbiont (e.g., mycorrhizas), as well as taxon "preferences" for reduced *versus* oxidised N. In addition, plant phenology and changing soil sources through time or seasons will be considered. This work will describe patterns of occurrence under laboratory and field conditions, while Research Plan 2 investigates the plant metabolic physiology giving rise to the observed patterns. Both research plans will be supported by Plan 3, the analytical chemistry development.

Several workers (e.g., Handley, unpubl; Virginia & Delwiche, 1982, 1982) have noticed large variations of $\delta^{15}N$ in non-N_2-fixing plants; both between and among individuals and taxa at the same sites. The observed variations record past events in N-cycling including acquisition, assimilation, translocation, and return to the environment. We expect that descriptive studies will reveal multi-factorial relationships, as, for example, hypothesised in Handley and Raven (1992). Additionally, the $\delta^{13}C$ value of plants, recently linked to the water costs of plant growth, has also been linked by Raven and Farquhar (1990) to plant pH regulation and organic acid synthesis. These are, in large part, determined by N-source.

1.A. Whole Ecosystems: Pattern analysis of Savannah at SCRI.

We will establish a *Hippophae* + *Triticum* + perennial ryegrass model system for spatial and temporal pattern analysis of $\delta^{15}N$ and $\delta^{13}C$. This site will serve as a historically documented research model for many years. Mathematical analyses will be done by both traditional statistical methods (in consultation with Scottish Agricultural Statistics Service) and by consultation with our own Group for Non-linear Mathematics.

To determine spatial and temporal sampling frequencies, as well as to test some elementary concepts about $\delta^{15}N$ patterns of occurrence, a nearby secondary succession community has

been systematically sampled over the last year. The samples are archived, already for analysis and will be analysed over the next few months.

Year 1 and 2 Analyse archived samples (about 600) for $\delta^{15}N$. Do baseline site preparation and soils sampling of model Savannah site. Establish model system. Determine sampling protocol.

Year 3, 4 and 5 Continue sampling, analysis and pattern analysis of model site.

Amounts of N_2-fixation will be estimated in the *Hippophae* as well as $\delta^{15}N$ values across the site. $\delta^{13}C$ of the trees and under plantings will be tested seasonally. This will also be compared, for the trees, with age in years. δD analysis will reveal changes in major water, and hence, dissolved nutrient sources, as well as estimating rooting depth. Harvested grass, as well as less frequent soil sampling, will be used to estimate the N productivity of the site and $\delta^{15}N$ values.

1.B. Whole Plant Controlled Experiments

Dr Handley has begun (in consultation with Professor David Read of Sheffield University and Dr Melvin Daft of Dundee University) experiments attempting to explain Högberg's (1990) field observations. He found, in Tanzania, that the $\delta^{15}N$ value of trees was empirically correlated with the type of associated mycorrhizas. A paper is in preparation on this first experiment which shows significant new results and implicates mycorrhizas as altering δ-values in several ways for more than one isotope pair. The underlying mechanisms will also be related in further work to the type of N-source being mediated by the fungus. Dr Handley will do the following experiments in controlled, glasshouse conditions: Year 1. studies of the isotope effects of mycorrhizas (ecto- *versus* VA) on woody species capable of associating with both types. Year 2-3. further mycorrhizal studies on the same plant species using different N-sources, including amino acids. Year 4-5 further controlled glasshouse work as indicated by the results of years 1, 2 and 3. This work will be done in close consultation with Professor Read (Sheffield University) and Dr C T Wheeler (Glasgow University).

Research Plan 2: Improving the carbon stable isotope technique for estimating the water costs of growth: corrections for carbon dioxide incorporation by *anaplerotic* pathways. Metabolic Physiology

Background. The use of carbon stable isotope measurements to estimate the water cost of growth for plants (for example, in breeding programmes) is subject to a number of constraints. One such constraint relates to the extent to which variations in carbon assimilation by quantitatively minor carboxylases which discriminate much less between carbon isotopes than the predominant carboxylase contribute to total plant carbon. The main such carboxylase (phosphoenolpyruvate carboxylase) is involved in the production of carbon skeletons for assimilation of nitrogen and in making organic acids for acid base regulation during nitrate assimilation. Raven and Farquhar (1990) show that variations in the nitrogen source, the site of assimilation within the plant, and the nitrogen content could yield changes in plant carbon isotope ratio as great as those associated with large variations in the water cost of growth. Melzer and O'Leary (1991) show how analysis of within-molecule distribution of carbon isotopes can yield valuable information on this question, while studies

on transgenic plants expressing another plant's phosphoenolpyruvate carboxylase as well as its own have great potential (Hudspeth *et al.,* 1992).

Programme. Years 1–3: The *initial objective* is to work up techniques for analysis of carbon isotope ratios of specific carbon atoms in key metabolites (aspartate, malate) and to determine the spatial pattern within the plant of these ratios for *Triticum* grown in a controlled environment on nitrate (years one–two).

Years 2–3: The *second objective* is to examine how variations in the contribution of carbon from anaplerotic carbon fixation, and the site of its assimilation, influence whole–plant carbon isotope ratios, by altering the nitrogen source and concentration supplied to *Triticum* (years two–three).

Years 3–5: The *third objective* is to determine the water cost of growth of *Triticum* under the various nitrogen supply conditions, to determine the balance of diffusion and carboxylation limitations, and to correlate these findings with the carbon isotope data (years three–four). The *final objective* is to extend the findings to a range of *Triticum* genotypes, and to *Hordeum*.

Intellectual Benefits. The research will benefit our understanding of the synthesis, turnover and transport of compounds derived *via* phosphoenolpyruvate carboxylase. It will interface, at the metabolic level, with larger scale work on $\delta^{15}N$, N–sources, and $\delta^{13}C$, as well as providing a mechanistic basis for interpreting work on soil organic turnover and transfer.

Research Plan 3: Methods Developments Supporting Other Research and Expanding Capabilities. Research Chemistry.

New or improved analytical development is needed throughout natural abundances research. Much critical research awaits appropriate techniques development. $\delta^{13}C$ work, now becoming central to ecology and crop selection research, could not have proceeded until automated high–precision, analyses of whole plant tissues was routine. $\delta^{15}N$, as a research tool, is still held back by the general lack of expertise available to use an ANCA at natural abundance levels. Only a few of these major hurdles have been overcome, by a relatively few workers two of whom are listed as key personnel in this proposal. Additionally, new experimental approaches (see, for example, Research Plan 2) will constantly require new methods and new methods development. We have identified the following as both necessary and possible in the next five years. These methods will underpin our own research and answer global needs of stable isotope research in general.

Year 1–2 – Isolation and $\delta^{15}N$ measurement of nitrate and ammonia nitrogen from dilute water and soil sources.

There is a clearly identified need for reliable $\delta^{15}N$ measurements for nitrate and ammonia in dilute water and samples. These two sources result in much the same problem, since soluble nitrogen can be washed from soil samples, but results in dilute solutions requiring as much as 50 ml to give sufficient nitrogen for IRMS analysis. The pressing need is to develop an isolation and analysis method that is reliable, convenient and sufficiently cheap in equipment

and consumables to permit a high sample throughput. The existing methods rely on the isolation of ammonia (with or without prior conversion of nitrate to ammonia) and range from Kjeldahl distillation and trappping of ammonia in acid solution, to diffusion methods where the ammonia is trapped on acidified glass fibre filters or acid washed zeolite (see A Barrie, in Stable Isotopes in Plant Nutrition, Soil Fertility and Environmental Studies, IAEA, 1991).

The proposed method development will look at these existing methods to determine which are most suitable for the sample material available and particularly at their suitability for natural abundance analysis. The most promising method will then be refined into a standard procedure capable of high sample throughout.

Year 1 – δD measurement in water contained in woody plant material and soil samples.

Useful information about plant water sources and water economy is available from measurement of δD for possible source waters and plant sap. Most hydrogen preparation methods for $^{2/1}H$ ratio measurements (uranium or zinc reduction in batch or on–line systems) require relatively pure water samples or involve vacuum distillation of the water as part of the procedure. Woody plant material and soil samples contain relatively little free water and vacuum distillation of this water is tedious and difficult. Failure to distil over **all** the water results in serious fractionation errors.

An alternative approach for δD analysis is the use of a platinum catalyst to bring about equilibration of deuterium between water and hydrogen gas. The hydrogen can then be analysed directly and used as an index of the composition of the water. This equilibration method was originally used for geochemical studies of brines, using a porous polymer supported catalyst floating on the water surface. This method has recently been modified to allow the use of readily available platinum/alumina catalyst with plasma and urine (Scrimgeour unpublished), by avoiding direct contact with the impure water phase. The equilibration is slow (days rather than hours) but excellent results have been obtained in low level tracer studies. Use of this method for plant and soil samples should be possible, as the prolonged equilibration period will allow diffusive mixing of all the available liquid water with the vapour phase which is equilibrated with the hydrogen gas. The proposed development of this method will involve standardisation of the method for plant and soil samples, using different amounts of reference water samples to allow normalisation with respect to the variable water content of the samples. The method will be validated using water of known composition added to dried soil, and woody material and comparison with existing literature data.

Year 2–3 – ^{15}N natural abundance variation of amino acid nitrogen in proteins

The natural variation of ^{15}N from whole plant material grown under different conditions results from a complex interaction of many factors. A little documented factor is the often significant differences in the ^{15}N content of different chemical species present and changes in the relative amounts of these compounds. For example, the ^{15}N content of different amino acids shows considerable variation (Scrimgeour unpublished), and changes in protein composition would alter the relative amounts of different amino acids present. The significance of such composition changes can only be assessed if the isotopic signatures of the different amino acids are known. This data will be obtained by isolating amino acids

from different plant protein extracts and measuring $\delta^{15}N$ for each, enabling an extensive catalogue to build up. This study will also provide a useful system for testing and refining isolation methods such as HPLC for use in natural abundance studies.

Years 1–5. Isolation and analysis of specific carbon atoms in key plant metabolites, to support Research Plan 2.

Years 2–3. Work with instrument manufacturers to bring on–line an automated machine for $^{34/32}S$ analyses.

5.1.2 *Closely related research for near–term external tender*

Resource partitioning in perennial plant communities

This project consists of two parallel studies: (1) to measure the C and N transport between plants via common mycorrhizal networks and (2) and to use stable isotopes of C to distinguish dead from living roots and estimate the amounts of respired soil C derived from living roots. Post–doctoral and technical assistance is required along with glasshouse/header house/custom growth chamber facilities and travel for collaboration and field work. Dr David Robinson

Volatile N losses from soil: associated stable isotope fractionations under controlled conditions.

Soils containing pure and mixed cultures of known nitrifying and denitrifying organisms, will be incubated under a sequence of defined conditions. $\delta^{15}N$ values found in the laboratory will be used to define the ranges of processes sought in the field. Additional technical assistance is required. Mr R E Wheatley

The use of stable isotopes to determine the type of prey eaten by the New Zealand flatworm.

$\delta^{13}C$ and $\delta^{34}S$ will be used to describe feeding patterns across species of soil fauna and through the seasons to determine whether the recently invasive New Zealand flatworm will affect other soil animals (and hence associated plants) than just the earthworm. Additional technical assistance and an automated $\delta^{34}S$ mass spectrometer is required. Dr Brian Boag

Energy forestry in Sweden

$\delta^{13}C$, and to lesser extents, $\delta^{15}N$ and δD will be used to select clonal material for *Salix* species used in biomass production forestry. Contracts have been signed with Energy Forestry Project in Uppsala to do initial feasibility experiments. Additional technical assistance and travel are required. Dr L Handley

Selection of vegetable crop cultivars

High cost vegetable crops, especially in England, have been subject to increasing drought stress. Economic viability is threatened by both water shortage and cost of irrigation. Preliminary feasibility experiments have begun with one large grower in England, leading to a larger scale research project, possibly involving a large horticultural firm and, perhaps, DTI. Glasshouse/header house facilities and growth chambers are required. Dr L Handley

Real-time experiments on photosynthesis and gas exchange

Wheat or barley will be grown in controlled atmosphere microcosms to explore the effects of environmental variables and cultivars on these processes. Mutants will be used to explore metabolic pathways. A growth chamber dedicated mass spectrometer in a glasshouse/header house is required. Prof J A Raven and Dr L Handley

The use of carbon isotope ratios to study metabolic regulation in transgenic plants

$\delta^{13}C$ will be used to study modifications of metabolic fluxes induced by genetic manipulation in potato plants. The approach will address 1) the mechanisms of sink-source relationships at the whole plant level and 2) the fate of carbon at the cellular level (changes in isotope ratios within metabolite pools). Additional technical assistance is required. Dr R Viola.

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