Manual for the Use of Stable Isotopes in Entomology

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Nuclear Techniques in Food and Agriculture

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FOREWORD

This publication was originally intended as an update to the Laboratory Training Manual on the Use of Nuclear Techniques in Insect Research and Control (Third Edition) which was published in 1992. It focused mainly on the use of radionuclides in entomological research and the application of gamma irradiation to entomological problems, in particular for Sterile Insect Technique (SIT) programmes.

Since the publication of the third edition of this manual the global scientific environment has changed dramatically, in part due to technological advances, evident from the widespread use of molecular techniques and the ubiquitous use of computing. There have also been significant changes in the external political environment which have had an impact on the day to day scientific work. From an environmental perspective it is no longer acceptable to release radionuclides into the field, and it has become increasingly expensive to use them in the laboratory due to essential safety considerations. From a social perspective the perceived risk associated with using radionuclides is deemed far greater than it may have been in the past. So, as with many technologies, it is the changes in the external political and social environment which has led to a shift in usage. The decline in the use of radionuclides in entomological research is a result of pressures from the external environment rather than a reflection of their scientific usefulness.

Considering these aspects, many of the methodologies described in the 1992 manual are now deemed outdated or obsolete, with the exception of the use of the SIT. Given the increasing global importance of SIT as an effective insect pest control method that is integrated as part of an area-wide approach, the Insect Pest Control subprogramme of the Joint FAO/IAEA Division published a definitive handbook on SIT in 2005, Sterile Insect Technique. Principles and Practice in Area-wide Integrated Pest Management. Editors: V.A. Dyck, J. Hendrichs and A.S. Robinson, which covered in detail many aspects of SIT which were touched upon in the previous laboratory manual.

Scientific boundaries are extended as a result of problem driven inquisitiveness and technological advances, and are framed by the social and political environment. Although the external environment may mould the technological path, a technology will only become obsolete if there are viable substitution products or methods. Stable isotope methods are a substitute for many radionuclide methods. The progress made in stable isotope science over the past twenty years is a direct result of the interplay of the above factors. Stable isotopes are omnipresent in the environment and pose no health or environmental risks. Advances in isotope ratio mass spectrometry in terms of detection, accuracy and automation have broadened experimental possibilities immensely over the past twenty years.

It was recognised that there was significant potential for answering many of the entomologist's biological and ecological questions using stable isotopes, an expertise the Soil Science Unit of the FAO/IAEA Agriculture and Biotechnology Laboratory in Seibersdorf had long fostered; therefore collaboration with the Entomology Unit at the same Laboratory was established. A number of collaborative experiments were carried and subsequently published. It was soon recognised that stable isotopes have tremendous potential in entomological research and although there were numerous studies using stable isotopes in ecology, their use in entomology per se was limited. Thus it was felt that a publication was required to make stable isotope techniques more widely known among entomologists. This manual will attempt to provide an introduction to the use of stable isotopes in entomological research. It will strive to communicate the basic principles and techniques of stable isotope science and provide a springboard for further interest and research in this area.

The IAEA officer responsible for this publication was R. Hood-Nowotny.

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GENERAL INTRODUCTION

This publication is aimed at entomologists and ecologists who have not previously used stable isotopes in their research but have a good general scientific understanding.

Stable isotopes are non-radioactive, do not decay, do not emit radiation and occur naturally in the environment. Stable isotopes are completely safe to handle and therefore there are few safety considerations to be addressed. Specialised equipment and building regulations are not required and personnel can be assured that they face no adverse health risks when handling them. Stable isotopes pose no health risks to the insects being studied and it is possible to safely release labelled insects into the environment. These factors all help to reduce costs and facilitate their use. Stable isotopes are present in the biosphere and natural processes lead to distinctive isotopic signals. These isotopic landscapes or isoscapes can be extremely useful in tracing insect movement, feeding patterns, and answering specific questions about resource usage etc. It is the comparative advantages of stable isotope techniques and the advances of online sample preparation systems which have led to the virtual substitution of radioactive techniques in many areas of ecological and biological research.

One of the main disadvantages of using stable isotopes is the capital cost of isotope ratio mass spectrometers required; these can cost in excess of 100,000 USD. Additionally the equipment requires a temperature controlled environment and highly skilled personnel to maintain and service the sensitive instrumentation. These problems may be overcome either by working in collaboration with an institution that has access to such equipment or by contracting-out isotope analysis to a commercial analysis laboratory. There are now many laboratories which offer isotope analysis on a pay per sample basis. Analysis costs are reasonable, depending on the isotope and the matrix ranging from 5-100 USD per sample. It is simple, safe and inexpensive to ship stable isotope samples across the world and easy to pick up analysed data via e-mail or web pages in real-time. Out-sourcing offers the advantage that researchers can focus their funds and minds on research, whilst the mass spectrometer analyst gets on with sample analysis. The disadvantages, of lack of immediacy and flexibility may be outweighed by the significantly reduced total costs, when personnel, laboratory and equipment costs, have been taken into consideration.

In future, the cost of stable isotope analysis may come down further due to advances in optical laser based technologies. Laser based equipment is available which can measure isotopic ratios of carbon dioxide at ambient concentrations and natural abundance levels. It is the current lack of commercially available sample preparation units linked to these systems, which limits their analytical and experimental capability. If there is sufficient interest in these products they will become commercially available. The advantages of the laser systems are their relative lack of complexity and therefore inherent robustness which leads to reduced capital costs of the instrument and ultimately to reduced personnel costs for running and servicing the equipment.

One of the great advantages of using and learning the art of stable isotope science is its potential scope. Stable isotopes are now being routinely used in a wide range of disciplines including hydrology, geology, medicine, archaeology, forensics and physiology etc. An understanding of the principles and an ability to transfer that knowledge and experience into another scientific discipline means that a training in isotope science is an excellent grounding for many areas of science today.

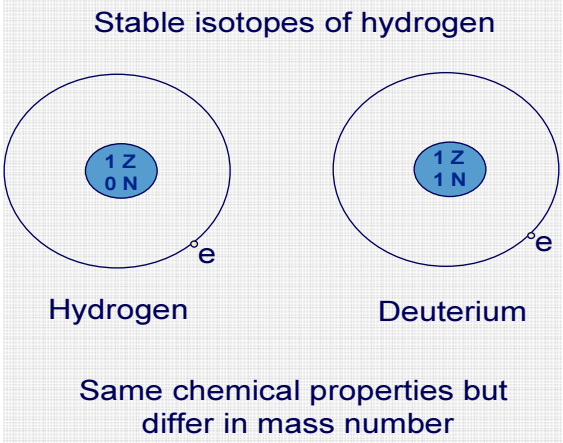
1. STABLE ISOTOPES

Stable isotopes are naturally abundant in the environment, safe and non-radioactive and therefore do not decay, which make them useful natural tracers. For example approximately 1% of the carbon in our diet is ^{13}C the rest being ^{12}C and about 0.4% of the nitrogen in our diet is ^{15}N with the rest being ^{14}N.

Many of the processes and reactions which have been investigated using radioactive tracers can be undertaken using stable isotopes, with the distinct advantage that there are no environmental or bio-safety issues. It is also possible to release stable-isotope labelled insects into the environment.

Stable isotopes are particularly useful in ecophysiology, the science of how whole populations and communities behave in relation to environmental constraints, as they provide a means to follow pathways with minimal disturbance or impact to the system.

Figure 1. Simplification of an isotope.



1.2 Isotope basics.

An isotope of an element has the same atomic number but a different number of neutrons and thus a different atomic weight; therefore stable isotopes react almost chemically identically to the more common isotope. Different rates of reaction due to isotope mass differences at an enzymatic level can result in slight variations in isotopic composition in nature and these natural signatures can be used in ecological studies to trace food web structure, migration patterns, feeding preferences etc. Studies of variations in naturally occurring isotopic signatures are referred to as natural abundance studies. Whereas studies in which enriched isotopes are added to the system are known as enrichment studies.

Natural abundance studies rely on the differences in isotope signatures between compounds, organisms or across the biome or isoscape, and therefore are constrained to situations where isotopic differentiation occurs.

Enrichment studies generally assume that the isotopically labelled compound acts identically to its lighter isotope, therefore isotopes can be used as tracers to follow the fate of compounds in complex systems. This is an extremely powerful approach but may be limited by the availability and cost of the labelled isotope.

1.1 Stable isotopes routinely used.

Hydrogen	^{1}H, ^{2}H
Carbon	^{12}C, ^{13}C
Nitrogen	^{14}N, ^{15}N
Oxygen	^{16}O, ^{17}O, ^{18}O
Sulphur	^{32}S, ^{33}S, ^{34}S, ^{36}S

Definitions:

- Mass number (A) = neutrons (N) + protons (Z)
- Nuclide: A species of atom as characterized by the number of protons and neutrons in the nucleus.
- Isotope: Atom of an element which has a different number of neutrons, (N), but the same number of protons (Z), i.e. a nuclide having the same atomic number but with a different mass number.

2. ISOTOPE ANALYSIS

Analysis is traditionally undertaken using isotope ratio mass spectrometry (IRMS). There are other successful spectroscopic techniques to measure stable isotopes in gases, many of which are laser based. These techniques are likely to expand the scope and affordability of isotope measurement capabilities still further, as they facilitate rapid analysis at pico-mole concentrations and are generally simpler than mass spectrometry.

Sample preparation will depend on the physical form of the sample and the element to be measured. The main stable isotopes used in entomology are ^{13}C, ^{15}N, ^{18}O, ^{2}H, ^{34}S.

Usually samples are collected, dried, finely ground and weighed into a small tin or silver cup (approximately 5 by 8 mm) for C, N and S analysis or O analysis, respectively. However in insect studies it is often possible to analyse a whole insect or insect part, by loading it in to the metal cup and drying it directly thus substantially reducing sample preparation time.

The sample preparation systems on the front end of the isotope ratio mass spectrometers convert liquid or solid samples into a gaseous form and then isolate the sample gas of interest prior to introduction into the mass spectrometer.

Figure 2. Isotope ratio mass spectrometer.

Figure 3. Laser based system for breath test analysis.

2.1 Analysis of organic nitrogen and carbon compounds.

Dried samples are pre-weighed into tin cups. Samples are automatically dropped into a furnace at 1000°C and the flash combustion of the tin in an atmosphere of oxygen raises the temperature to around 1700°C. Following complete oxidation, the samples are carried in a stream of helium through a series of scrubbers to remove sulphurous impurities and residual water, and over hot copper (600° C) to reduce oxides of nitrogen to elemental nitrogen (N_2). CO_2 and N_2 peaks are separated on a gas chromatography column and bled into the IRMS. Under vacuum the gases are ionized on a hot filament, accelerated and separated by a magnetic field based on their mass to charge ratio (m/z). The separated ions are collected in Faraday cups where the ratios of the isotopes of 28, 29, 30 peaks (N_2) and 44, 45 and 46 (CO_2) are determined.

The output of the IRMS is a ratio which can be converted to an atom % value or a delta value (see later section), depending on the standards used and the experimental requirements. Current fully automated IRMS systems are capable of accurately determining isotope ratios in approximately 10 µg of carbon and 5 µg of nitrogen and this usually takes 5-10 minutes per sample.

2.2 Analysis of oxygen and hydrogen in organic compounds.

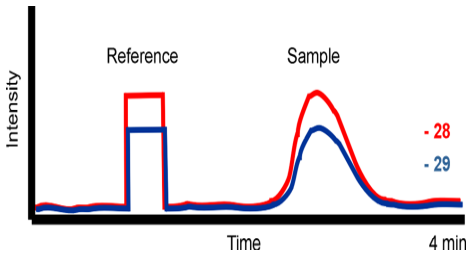
Oxygen and hydrogen isotopes of organic compounds are determined by pyrolysis, i.e. the high temperature conversion of organic matter to carbon monoxide and hydrogen in the presence of glassy carbon. . H_2 and CO are separated on a gas chromatograph; the separate peaks are bled into the ion source of the IRMS where they are ionised and accelerated, ions are then separated by a magnetic field based on their mass to charge ratio (m/z). The separated ions are collected in a Faraday cup universal collector array where for CO, masses 28, 29 and 30 are monitored, while for H_2 masses 2 and 3 are monitored. Analysis can be performed on as little as 75 µg of organic matter.

Using this technique it is also possible to analyse oxygen and hydrogen isotopes in liquid samples, however the precision is less that the standard equilibration method, but less sample is required (around 0.5-10 µL). It is also possible to analyse oxygen isotopes in nitrates, phosphates and sulphates using this method.

2.3 Analysis of organic sulphur.

Organic sulphur is initially converted to pure SO_2. Samples are dropped into a furnace held at 1080ºC and the flash combustion of the tin in an atmosphere of excess oxygen causes the temperature to rise to 1,700ºC. The sample is carried in a stream of helium through tungstic oxide and zirconium oxide catalysts, then over high purity coppe and finally water is removed. Gaseous SO_2 is separated on a gas chromatography column and bled into the mass spectrometer. Under vacuum the gases are ionized on a hot filament, accelerated and separated by a magnetic field based on their mass to charge ratio (m/z). The separated ions are measured by a Faraday cup universal collector array where the ratios of the isotopes of masses 64, and 66 are determined.

Figure 4. A typical continuous flow sample run spectrum obtained on IRMS and tin cups and isotope sample storage in micro titre plates.



2.4 Analysis of oxygen using the equilibration method.

Determinations of ^{18}O in water have been traditionally performed using an equilibration technique. After temperature controlled equilibration of water with CO_2 gas, the isotopic composition of the CO_2 is measured. As there is ready exchange between the oxygen of the water and the CO_2, the isotopic ratio of the water can easily be determined from the gas measurement using dual inlet mass spectrometry. Sample size is typically 0.5-10 ml which is one disadvantage of this technique.

2.5 Analysis of hydrogen in water.

It is possible to determine deuterium in water by equilibration with hydrogen gas and a platinum catalyst or off-line zinc reduction of water to hydrogen gas (Kendall and Coplen 1985). However on-line reduction of water, using a hot (1000°C) chromium reactor is becoming increasingly popular as sample size and run times are minimal (Schoeller *et al.* 2000).

Figure 5. Schematic of the C/N analyser system linked to an isotope ratio mass spectrometer set up for C and N.

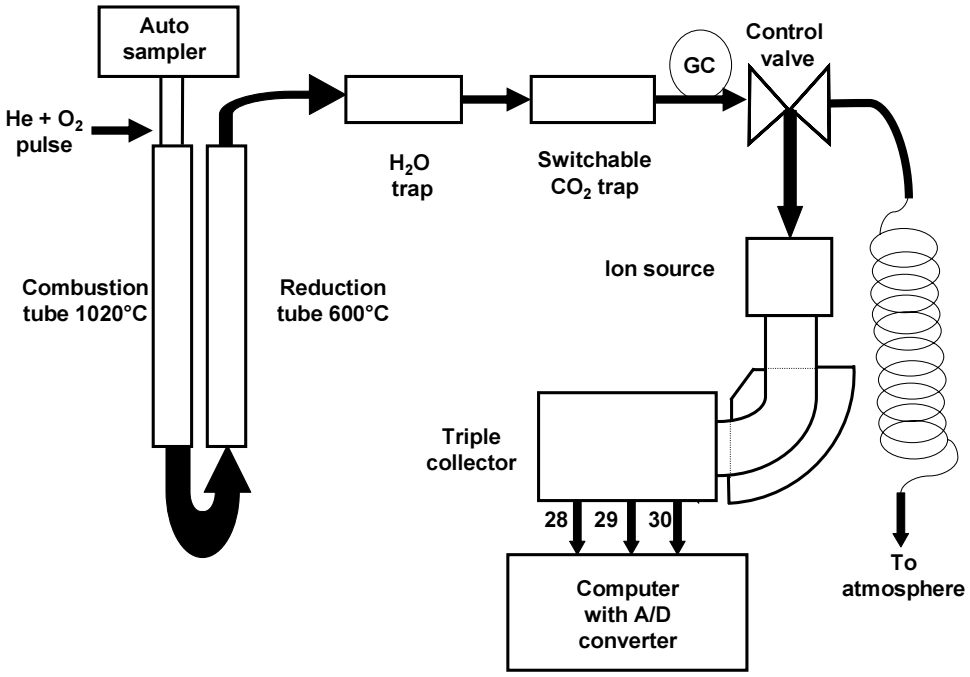
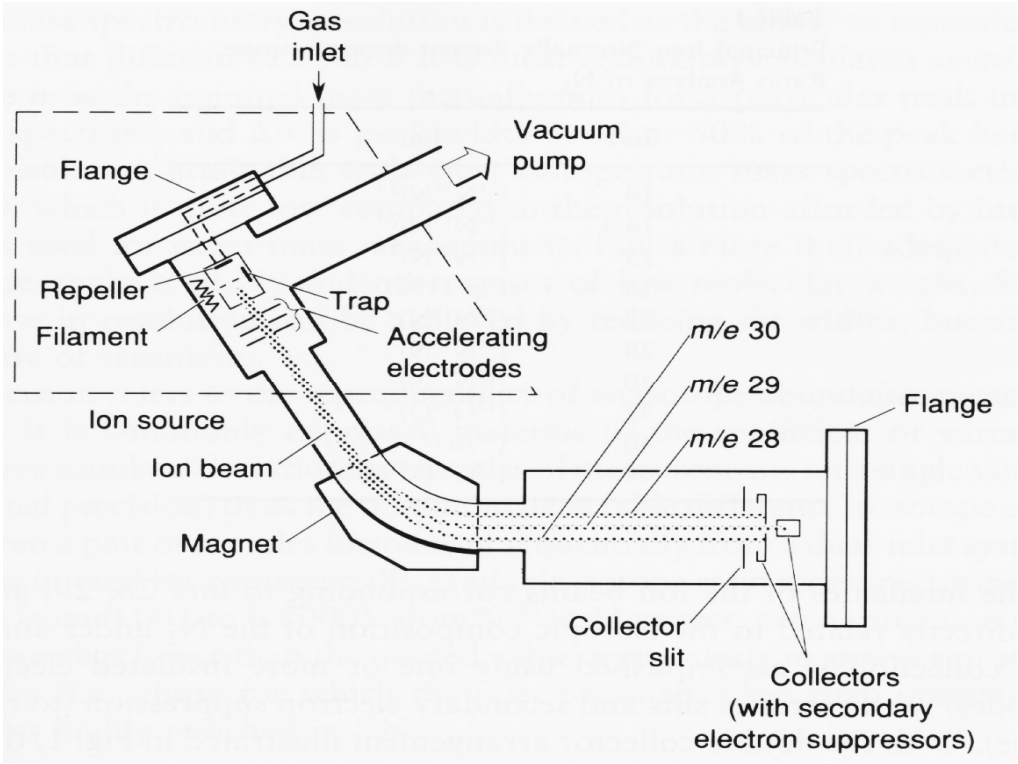


Figure 6. Schematic of the isotope ratio mass spectrometer.



2.6 Inductively coupled plasma mass spectrometry, (ICPMS).

Although ICPMS is not routinely used for stable isotope determination, the ability to determine concentrations in the part per trillion region and the regular coupling of ICPMS with laser ablation preparation systems may make them useful tools in entomological research. It allows isotopic determination of specific micro-tissues or micro-symbionts. ICPMS is also widely used in the analysis of heavier stable isotopes of ecological importance such as strontium.

The laser ablation preparation system consists of a laser which is focused on the material and vaporises it, this forms a plume of ablated material which can then be introduced into the plasma. The spatial resolution of this laser ablation is dependent on the beam focus; but can get down to a 12 µm spot or pit diameter, this is a comparable spatial resolution to secondary ionisation mass spectrometry (SIMS). The ablated material is introduced into an extremely hot plasma of 10,000ºC, where it is broken down into atoms and ionised. The ions from the plasma pass through a series of cones and are introduced into the mass spectrometer (usually a quadrupole) where they are separated dependent on their mass-charge ratio.

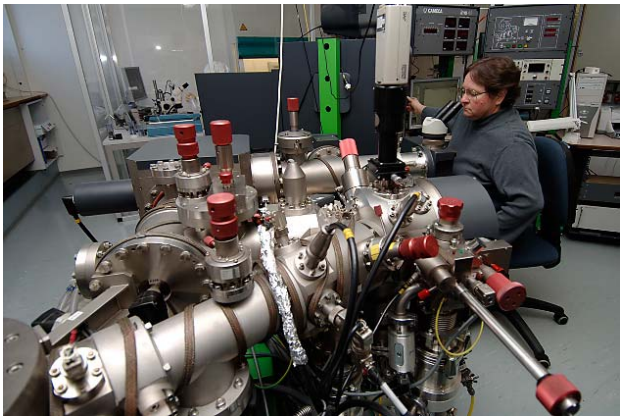
Figure 7. Laser ablation system.

2.7 Secondary ionisation mass spectrometry, (SIMS).

The advantage of laser ablation ICPMS over SIMS is the simpler sample preparation and availability of instruments globally which in turn leads to lower costs for ICPMS analysis.

In SIMS the sample is loaded onto a small slide or plate. Under vacuum the sample is bombarded by a thin beam (µm) of primary ions. This results in a spluttering of atoms some of which are ionised. The ions are then accelerated through a split into the mass spectrometer where they are separated depending on their mass-charge ratio.

Figure 8. A SIMS machine.



SIMS can perform elemental analysis of sub-ppm concentrations and can achieve per mil precision in ^{18}O analysis. The big advantage of the SIMS system is the spatial resolution both horizontally and with sample depth. This is obviously dependent on the model but resolutions of 0.12 µm are achievable.

Although SIMS has been used to look at nano-scale stable isotope differentiations in soils and their associated bacteria it appears the technique has yet to be used in an entomological context. This may be due to lack of useful entomological applications or due to the relative rarity of SIMS machines.

3. COMPOUND SPECIFIC ISOTOPE ANALYSIS

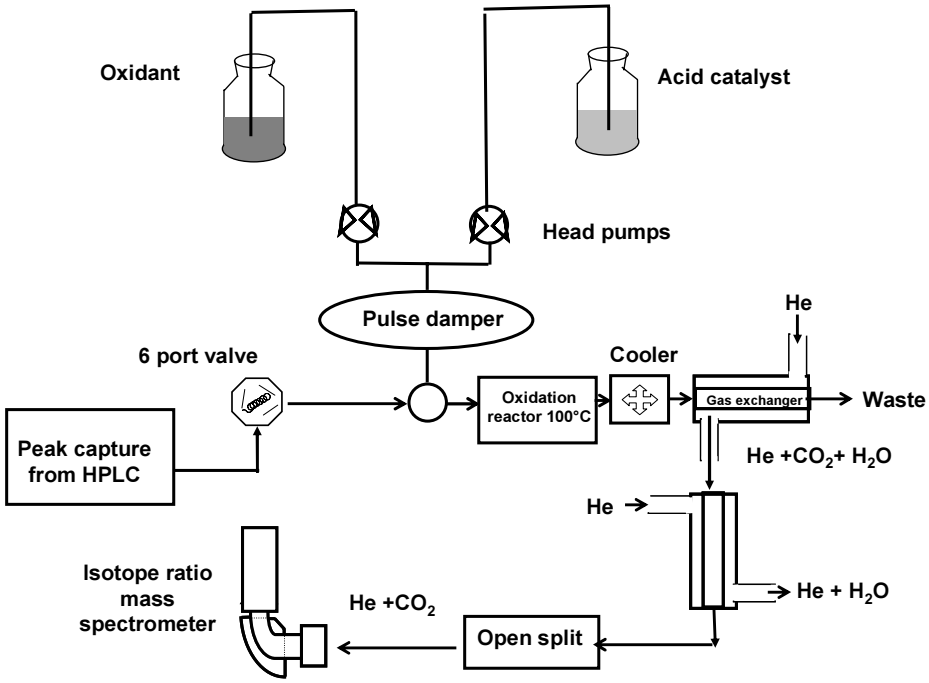
3.1 Isotope ratio monitoring liquid mass spectrometry (IRM-LC-MS).

Isotope ratio monitoring liquid chromatography mass spectrometry (IRM-LC/MS) has now developed to the extent that the measurement of isotope ratios of specific compounds is possible. Preparation procedures vary; many rely on the post LC separation of the liquid phase prior to isotope analysis, such as the moving wire technique, where the solvent is evaporated followed by combustion of the sample (Brand and Dobberstein 1996). Reproducibility of 0.2‰ for samples down to 400 ng sample on a column have been reported using this method (Krummen *et al.* 2004). However, more recent developments rely on conversion of organic carbon to CO_2 in the liquid phase and on-line capture of CO_2 in a helium stream through a gas-exchange membrane.

The oxidation reagent used is ammonium peroxide disulphate and an acid catalyst of phosphoric acid and silver nitrate, once mixed with the post HPLC sample peak, flows through a capillary oxidation reactor at 100°C. The liquid stream is then cooled and the individual CO_2 peaks are separated from the liquid phase by a gas exchange membrane. Following drying of the sample in the He carrier stream, it enters the isotope ratio mass spectrometer via an open split.

Figure 9. An isotope ratio monitoring liquid chromatography mass spectrometer system.

Figure 10. Schematic isotope ratio monitoring liquid chromatography mass spectrometer system (IRM-LC/MS).



3.2 Gas chromatograph/combustion/ isotope ratio mass spectrometry, GC/C/IRMS.

GC/C/IRMS systems differ from the elemental analyser systems in as much as there is an initial separation of compounds on the GC followed by individual combustion or pyrolosis of the peaks as they come off the GC column. This allows for compound specific isotope analysis (CSIA).

This technique is useful for compounds that can be easily vaporized. The sample is injected into the system and immediately vaporizes. A carrier gas, usually helium, sweeps the sample along the column. The compounds are separated on the column by differential interaction. The compounds that have the greatest interaction move the slowest.

The interactions can be through absorption or any type of chemical interaction depending on the packing material and configuration of the column, making these very versatile systems.

The peaks coming off the GC can be either individually combusted or pyrolysed to form gaseous forms that can then be bled into the mass spectrometer for isotopic analysis of C, N, H or O. Many compounds cannot be analysed directly but require chemical manipulation to yield GC amenable components. Phospholipids are saponified and methylated to generate fatty acid methyl esters (FAMEs). Solvent un-extractable bio-polymers such as carbohydrates, proteins, aliphatic biomacromolecules e.g. chitin must be chemically or pyrolytically cleaved prior to GC/C/IRMS. Reactions must convert all substrate to product as incomplete reactions result in kinetic isotope effects (see Table 1 for common derivatisation procedures). Care must be taken to account for isotopic contamination due to derivatisation procedures.

Figure 11. Gas chromatograph combustion isotope ratio mass spectrometer (GC/C/IRMS).

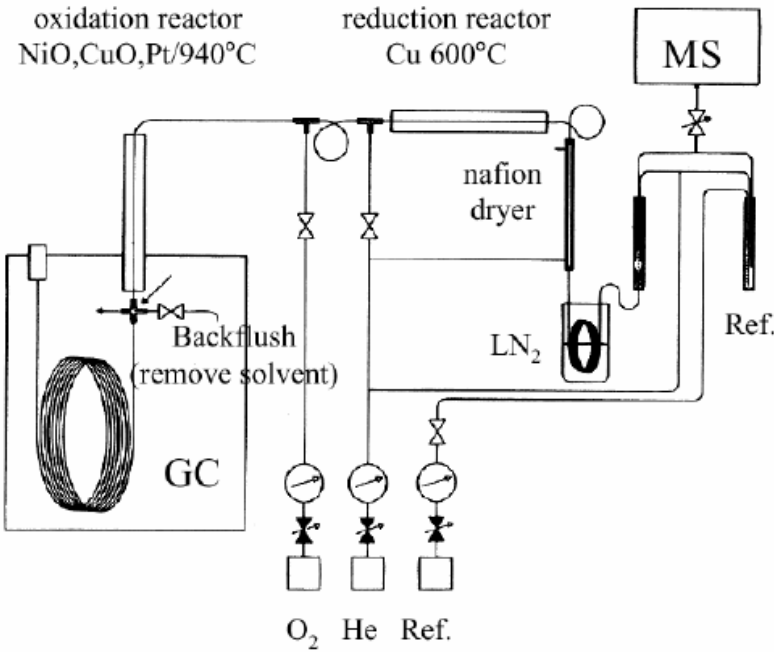


Figure 12. Schematic of preparation procedures for GC/C/IRMS.

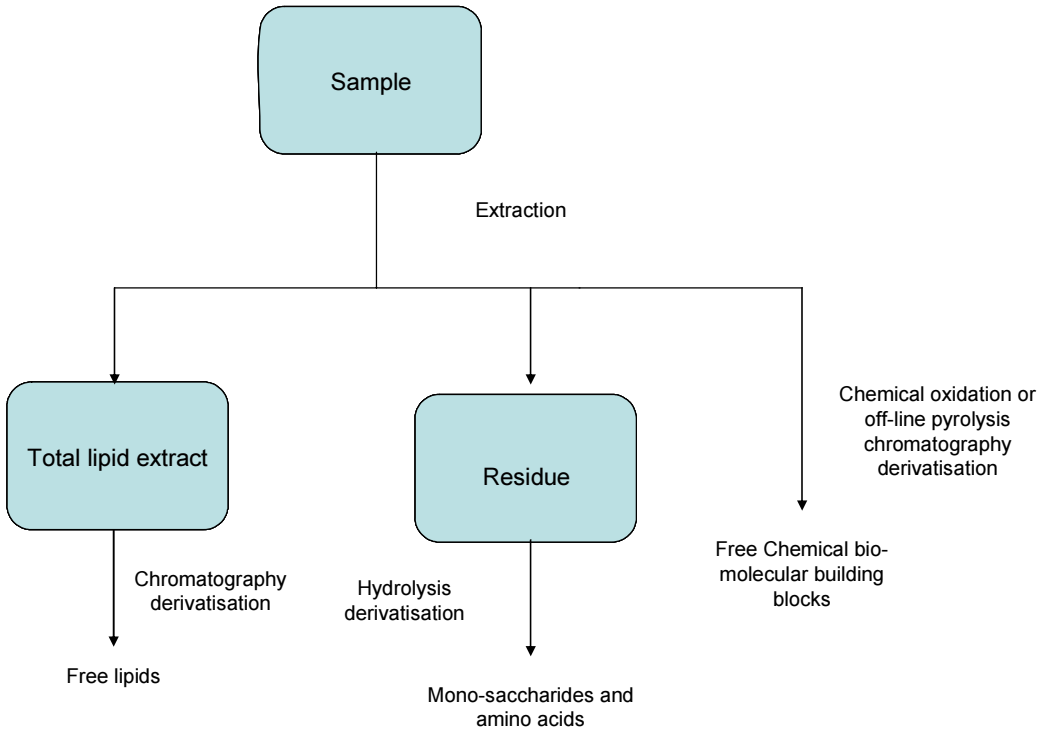


Table 1. Derivatisation procedures for GC/C/IRMS.

Procedure	Initial compound	Reagent	Reference
Silylation	Sterols, alcohols	Ntertbutydimethysilyl-*N*-methyl-trifluoroacetamide. (MSBSTFA)	Lockheart (1997) Stott and Evershed (1996) Derrien *et al,* (2003)
	Amino acids		
	Monosaccharides	*N,O*- bis (trimethylsilyl) trifluoro-acetamide (BSTFA)	
	Lignin, Polyphenol		
Esterification	Fatty acids	BF_3/ methanol acetyl chloride/methanol	Howland *et al*. (2003) Doherty *et al*. (2001)
	Amino acids		
Acylation	Amino acids	Acetic anhydride Trifluoroacetic-anhydride Pivaloyl chloride	Demmelmair and Schmidt (1993) Doherty *et al*. (2001) Metges and Danenzer (2000)
	Monosaccharides		
Methylboronatrion	Monosaccharides	Methane boronic acid (MBA)	Van Dongen *et al*. (2001)

4. ENRICHMENT STUDIES

It is possible to purchase a wide variety of isotopically enriched compounds that have higher concentrations of the rarer isotope than the natural background (or natural abundance). For example in 99 atom% ^{15}N labelled potassium nitrate, 99% of the atoms are ^{15}N atoms compared to ~ 0.4% in "normal" potassium nitrate. Stable isotope labelled compounds can be easily integrated into feeding regimes of insects and used in capture/recapture, feeding preference and resource allocation studies.

Labelling studies are usually referred to as enrichment studies, although different rates of reaction can result in slight variations in isotopic composition and are important in natural abundance studies; in enrichment studies this discrimination is generally irrelevant. However, natural abundance and enrichment studies are subject to different assumptions, terminologies and caveats. There is a wealth of knowledge of enriched tracer techniques in agriculture, especially plant nutrition, which can be drawn on by entomologists.

Given the advances in mass spectrometry it is now possible to undertake micro-enrichment studies. In these studies minute amounts of label are added to a system to create isotopic differences in the target organism in the isotopic range of natural abundance differences. The advantage of these techniques is that the system is minimally disturbed however there is enough signal to detect differences. In these studies care must be taken to account for isotope discrimination effects. Micro-enrichment techniques offer a wealth of possibilities for entomological research as

4.1 Costs of isotopes and analysis.

Over the past 20 years the cost of stable isotope analysis and enriched compounds has decreased dramatically, the cost of highly labelled ^{13}C glucose for example is approximately 100-160 USD per gm. Samples are easy and safe to dispatch, as a consequence there are now a number of laboratories offering isotope analysis commercially and at affordable prices ranging from 5-100 USD per sample, depending on the isotope to be analyzed. These developments have widened the accessibility and scope of stable isotope science, and have led to an increase in use in an eclectic mix of disciplines, from archeology, nutrition, geology, physiology through to forensics.

Table 2. Approximate cost of useful enriched compounds.

Compound	Approximate enrichment	Approximate cost per gram in USD
^{13}C mixed fatty acids	98	200
Amino acids		
Glycine ^{13}C	98	95
Aspartic acid ^{13}C	98	300
Leucine ^{13}C	98	200
Valine ^{13}C	98	275
Glycine $^{13}C/^{15}N$	98	900
$^{13}C_6$ D glucose	98	160
^{13}C-UL-fructose		
Water		
$^{2}H_2O$	98	0.3
$H_2{}^{18}O$	97	195
$^{15}NH_4Cl$	99	40
$K^{15}NO_3$	99	25

5. NATURAL ABUNDANCE STUDIES

Natural abundance studies are based on the small differences in isotopic composition brought about by fractionation or discrimination, for or against the heavier isotopes due to kinetic and equilibrium isotope effects. Although stable isotope forms of the same element are chemically identical, as determined by the number of protons in the nucleus, the rates of reaction can be very slightly different for the heavier atoms. These differences are in the order of parts per thousand or more commonly referred to as per mil (‰). In nature values are typically in the -100 to +100‰ range.

These small differences lead to distinctive isotopic landscapes as there are dominant processes in the biosphere which have specific fractionation factors.

The main cycles of N, C, S, H and O have characteristic fractionation factors associated with the dominant transformation processes. The resultant isotopic differences can be used to study the ecology and biology of insects.

In natural abundance studies, values are reported as ratios referenced against international standards in delta (δ) unit parts per thousand ‰. There are a number of conventional reference standards ranging from air to limestone (Vienna PeeDee Belemnite) that are listed below in Table 3.

Generally it is always best to calculate back to atom % or atom fraction when doing mixing equations, especially when using micro-enrichment techniques. Additionally it is also important to convert back when doing statistics as atom % and fractions are absolute values rather than ratios.

Table 3. Ratios for the reference materials used as origins for the delta scales.

Element	R isotopic ratio of the reference materials used for the delta scales	Reference materials used for the delta scales.
Carbon $^{13}C/^{12}C$	0.0112372	PeeDee Belemnite (PDB)
Carbon $^{13}C/^{12}C$	0.01224	ViennaPee Dee Belemnite (PDB)
Nitrogen $^{15}N/^{14}N$	0.0036765	Air (regular atmospheric nitrogen)
Hydrogen $^{2}H/^{1}H$	0.00015576	Vienna Standard Mean Ocean Water (VSMOW)
Oxygen $^{18}O/^{16}O$	0.0020052	VSMOW
Sulphur $^{34}S/^{32}S$	0.0450045	Canyon Diablo Troilite (CDT)
Sulphur $^{34}S/^{32}S$	0.0441626	Vienna Canyon Diablo Troilite (VCDT)

Gröning (2004). *PDB and VPDB are considered equivalent and CDT and VCDT are considered equivalent.

Note: These are advanced concepts not necessary for the isotope beginner, but are the basis of isotopic effects.

5.1 Kinetic effects.

Light isotope molecules usually react faster than heavier isotope molecules. As the rates of reaction are slightly different if a reaction proceeds from substrate to product there will be a continual selection against the heavier molecule, causing the product to be depleted in the heavier isotope and the substrate to be enriched in the heavier isotope. Nearly all biological reactions are subject to kinetic isotope effects which are generally not temperature dependent.

5.2 Equilibrium isotope effects.

Equilibrium isotope effects happen when the chemical reaction is in equilibrium. When the whole of the substrate does not get converted to product, the concentration of the substrate and product is constant, however there is some flux between substrate and product due to reversible reactions. The equilibrium isotope effect is the sum of the two opposing kinetic isotope effects. In these reactions the heavier isotope generally concentrates where it is more strongly bound. Equilibrium isotope effects are generally temperature dependent.

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

A good example of an isotope equilibrium effect is the dissolution of CO_2 in water. CO_2 reacts with water to give carbonic acid, it then disassociates to bicarbonate, both are reversible exchange reactions. CO_2 is only free in the water and not strongly bound so it concentrates in the bicarbonate fraction. This results in the bicarbonate being about 9‰ heavier than the dissolved CO_2 at 15°C. On a global scale this means ocean bicarbonate has a value of around +1‰ and the CO2 in the atmosphere having a value of -8‰.

5.3 Open verses closed systems.

In an open system the substrate is continually replenished and residual substrate leaves the system. In this situation if there is fractionation it will result in a lighter product and a consequently heavier substrate.

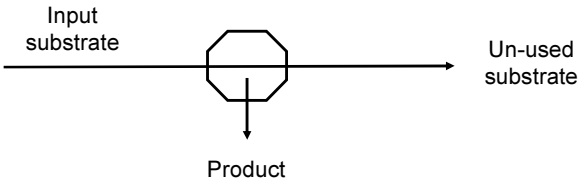
In closed systems the substrate remains in the system, if there is fractionation during the transformation to product the substrate becomes increasingly heavier as the reaction proceeds. The instantaneous product will also become increasingly heavier, however the accumulated product will reflect the mass balance of the reaction and therefore show a more gradual increase in the isotope values.

If all the substrate is converted to product there is no resultant fractionation in the product, the product will have the same δ value as the substrate irrespective of whether the system is open or closed. As all the substrate is converted it will cease to exist and it will therefore not have an isotopic value.

A good analogy here is plants grown in a stream system (open) vs. plants grown in a tank (closed). In a stream system nutrients will be entering the system continuously; in a tank system nutrients will be added once then depleted over time. Many ecological systems will be a combination of open and closed systems.

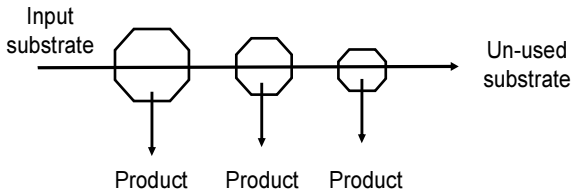
There are equations to describe these processes. In open systems the equations are relatively simple and linear, however in closed systems there are effectively two products, the long term accumulated product and the instantaneous product. This makes the mathematics a little more complex. When the fraction of substrate reacted (f) is near zero then the systems are very similar and changes in δ values of residual substrate are essentially identical.

Figure 13. Open system.

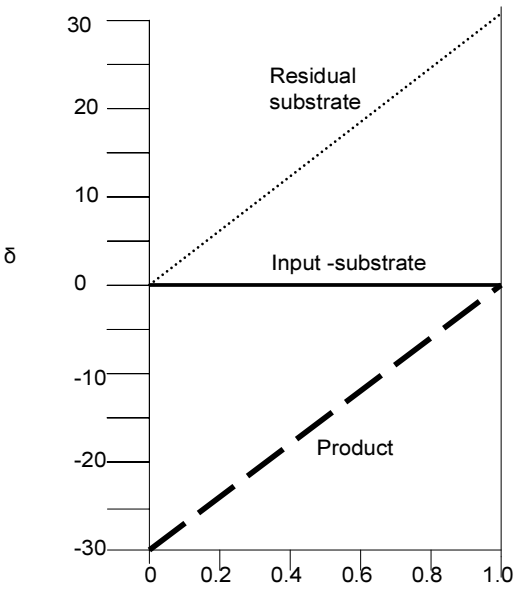


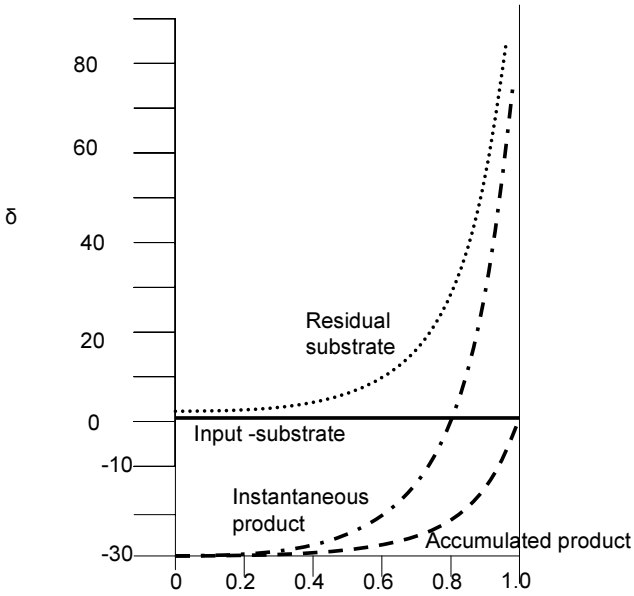
One time use of a substrate which is continually replenished.

Figure 14. Closed system.



Successive use of substrate which diminishes in amount as converted to product.





Equations for an open system

$$\delta_{rs} = \delta_{input} + \Delta * f$$

$$\delta_{p} = \delta_{input} - \Delta * f$$

Where Δ is fractionation, f is fraction reacted, rs is residual substrate, p is product.

Equations for a closed system

$$\delta_{AP} = \delta_{input} + \Delta * ((1-f)/f) * \ln(1 - f)$$

$$\delta_{IP} = \delta_{input} + \Delta * [1 + \ln(1 - f)]$$

$$\delta_{RS} = \delta_{input} + \Delta * \ln(1 - f)$$

AP is accumulated product, IP is instantaneous product.

6. ISOTOPE UNITS AND TERMINOLOGY

Isotope ratio mass spectrometry can accurately measure isotopic ratios, rather than absolute values thus equations and isotope values are usually derived from the ratios or R values obtained from the mass spectrometer. R is the ratio of the heavy to light isotopes of a particular element.

The measurement of isotopic composition for a particular element is commonly based on the ratio of the less abundant isotope of interest to the more abundant isotope. In most cases this is the heavy to light isotope ratio.

$$\text{Atom \%} = \left[\frac{\text{Moles of heavy isotope}}{\text{Moles of heavy isotope} + \text{Moles of light isotope}} \right] \times 100$$

For nitrogen this is:

$$\text{Atom \% } {}^{15}\text{N} = \left[\frac{\text{Moles of } {}^{15}\text{N}}{\text{Moles of } {}^{15}\text{N} + \text{Moles of } {}^{14}\text{N}} \right] \times 100$$

$$\text{Atom \% } {}^{15}\text{N} = \left\{ \frac{{}^{15}\text{N}}{{}^{15}\text{N} + {}^{14}\text{N}} \right\} \times 100$$

In enrichment experiments, values are generally reported in atom % or atom % excess. In nitrogen enrichment experiments in particular values are reported as atom % excess (APE), i.e. enrichment minus universal value for natural abundance. Natural abundance is the concentration of isotopes found in nature.

$$\text{APE} = \text{Sample Atom \%} - \text{Reference Atom \%}$$

For N this is:

$$\text{Atom \% } {}^{15}\text{N}_{\text{excess}} = \text{Atom \% } {}^{15}\text{N}_{\text{sample}} - \text{natural abundance of air}$$

The atom % ^{15}N abundance of air is 0.3663

A compound with an enrichment of 5.000 atom % ^{15}N can be also described as 4.6337 atom % ^{15}N excess.

Natural abundance terminology

In natural abundance studies due to the small isotopic differences caused by kinetic and equilibrium isotope effects the units used are delta values, these are values which are standardized against internationally agreed standards and are defined in molecules per thousand or per mil values.

A lower-case delta (δ) value is defined as the isotopic ratio of a sample standardized to the isotopic ratio of a defined reference:

Where R_S is the isotopic ratio of the sample and R_R is the isotopic ratio of the reference standard.

$$\left[\frac{(R_S - R_R)}{R_R} \right] \times 1000 = \delta_{S/R} \text{ or } \left[\left(\frac{R_S}{R_R} \right) - 1 \right] \times 1000 = \delta_{S/R}$$

. In natural abundance studies, values in the range of –100 to 100 δ are more common.

Samples that are depleted in the heavier isotope due to discrimination against the heavier isotope have negative deltas: the more negative the greater the discrimination against the heavier isotope.

Example

$\delta^{15}N_{Air}$ =12 per mil (or ‰) means the that the sample was analysed against a reference material and was found to contain twelve ^{15}N molecules per thousand more than Air the accepted zero point for expression of nitrogen-15 in per mil notation.

6.1 Two pool mixing models.

Two-pool mixing models are one of the most widely used models in isotope science and once understood can be used to perform a variety of tasks from determining pool size to determining the proportion of diet derived from a specific organism etc.

The basis of the two-pool mixing model is mass balance and that matter cannot be created or destroyed. The analogy of colours mixing may help understand the principles behind two pool mixing models, e.g., red wine for labelled compounds and water for unlabelled compounds.

For example if red wine is mixed 1:1 with water the colour intensity is half. The same with isotopes; if 50 atom % excess KNO_3 is mixed 1:1 with regular KNO_3 0 atom % excess (or 0.3663 atom %) then the resultant mixture will be 25 atom % excess.

If red wine is mixed 1:9 with water then the colour intensity will be one tenth, again the same with isotopes; if 50 atom % excess KNO_3 is mixed 1:9 with natural abundance KNO_3 then the resultant mixture will be 5 atom % excess or one tenth of the original enrichment. If lysine with a value 10‰ is mixed 1:1 with lysine with a value of 0‰ the resultant mixture is 5‰.

The isotopic composition is of a mixed sample is determined by the isotopic signature and contribution of the original sources.

For example if half of the sample comes from a source with 5 atom % excess and half from a source with 0 atom % excess the resultant sample will have an enrichment of 2.5 atom % excess. If one knows the isotopic composition of the sources and the sample one can determine the proportional contribution of sources to the sample. This is known as a two pool mixing model and the majority of isotope applications use these models.

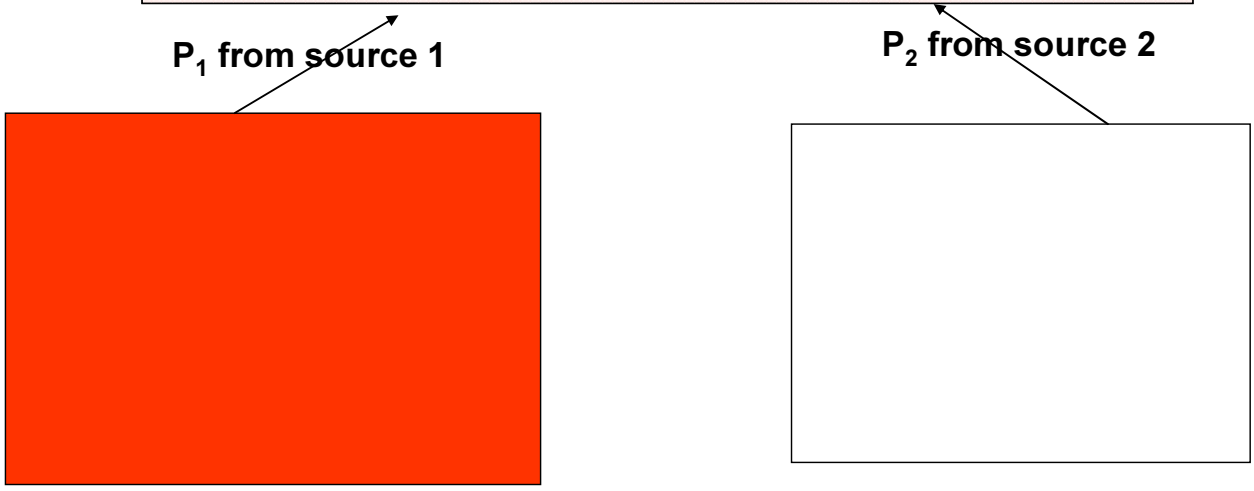
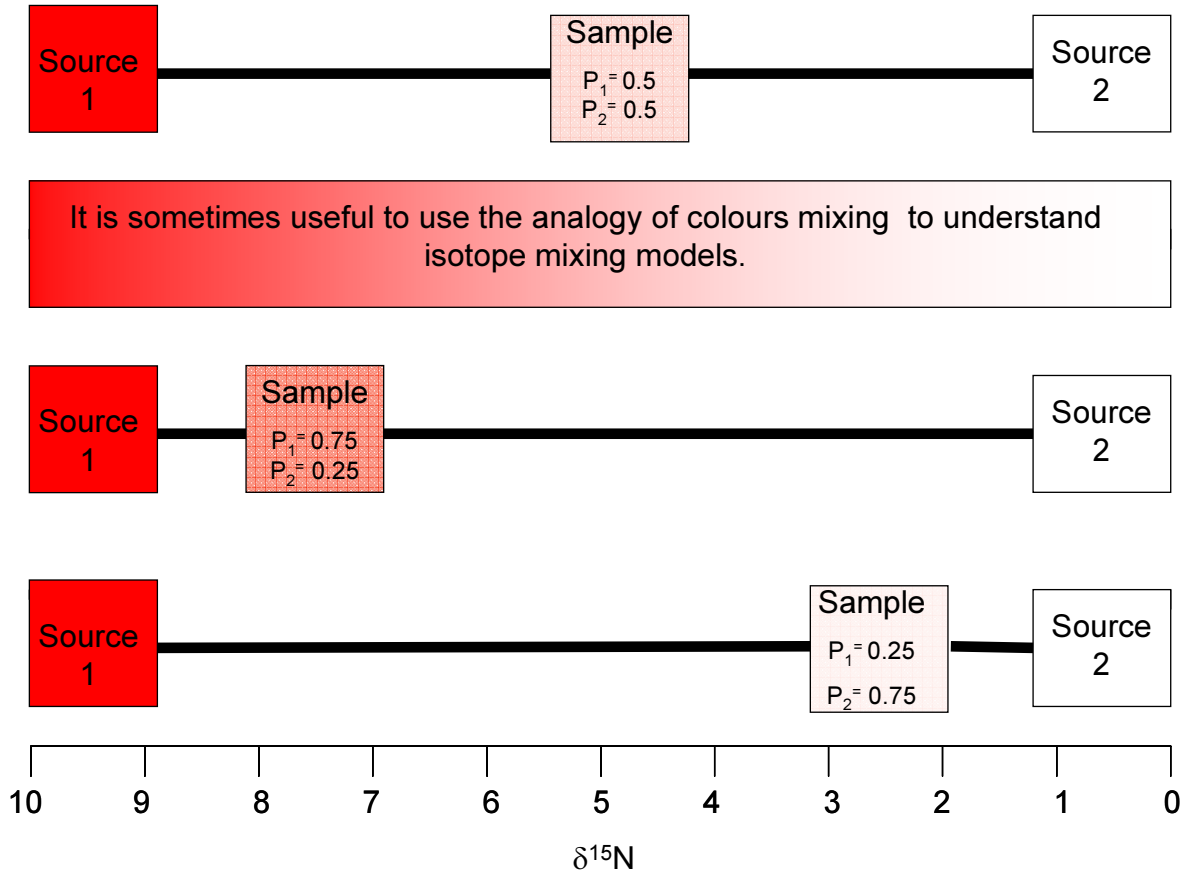
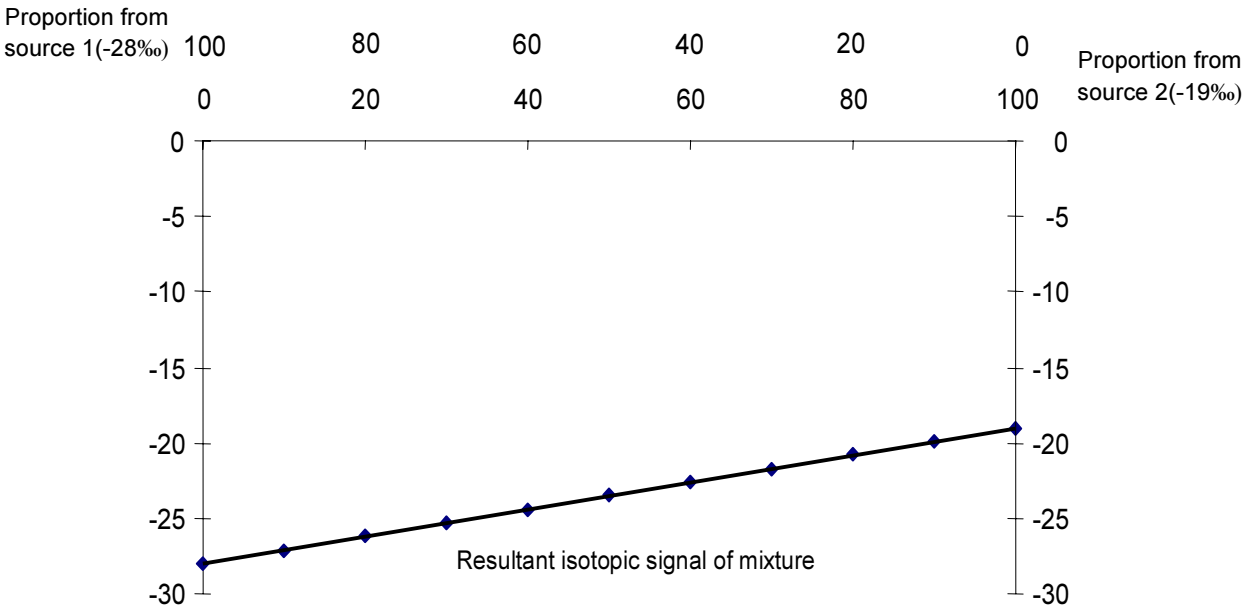


Figure 15. Using the colour analogy to understand isotope mixing models.



This can also be shown mathematically when two different sources are mixed, the resultant mixture will change linearly with the proportion of either source contributing to the final mixture.

Figure 16. Mathematical representation of two source model.



6.2 Two pool mixing model equations.

For enrichment studies it is simple to visualise mixing of two sources of very different isotopic value, which leads to the resultant dilution of the enriched isotope added, if the second source is of natural abundance.

In this example atom % excess values were used however it would have also been possible to use atom % values but then all values must be calculated using atom % and all results reported as atom %.

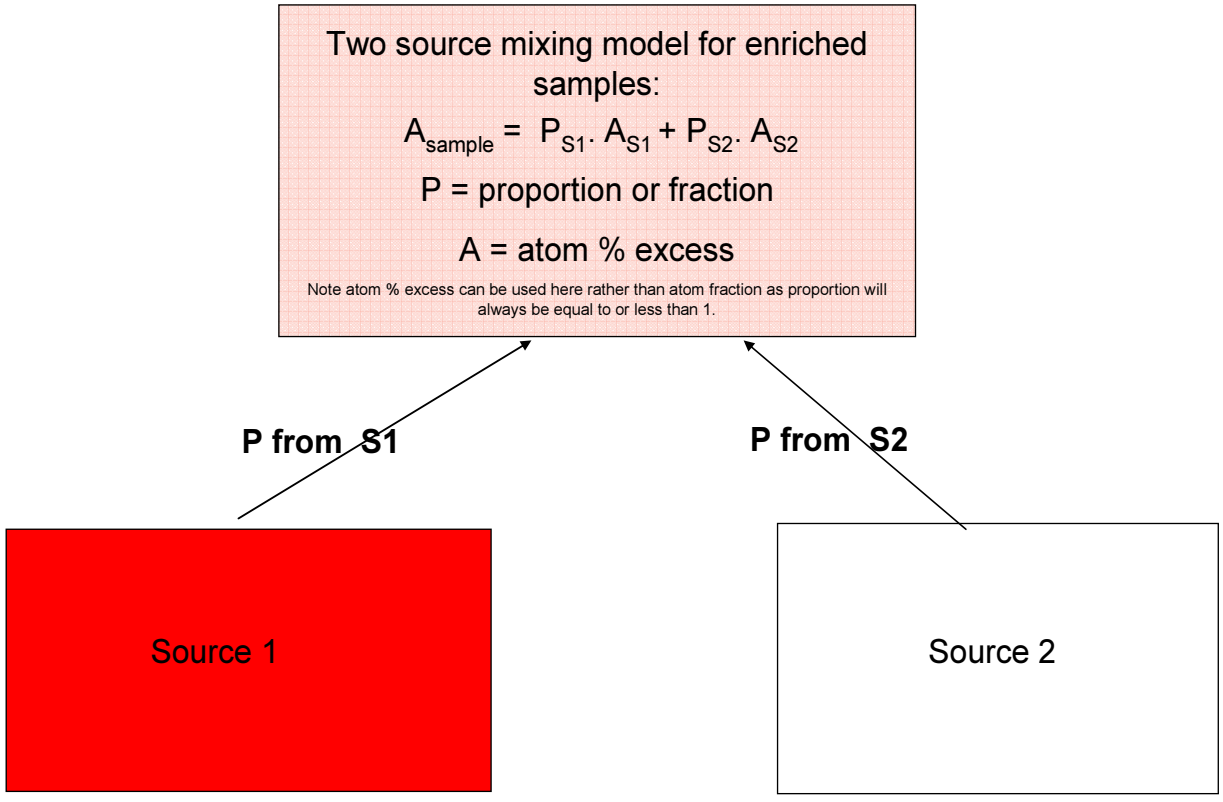
If the product is derived from only the two sources it is possible to calculate the proportion of product derived from each source by knowing only the isotopic value of the sources and the final product. As the product (1) will be made up of a proportion from source 1 (P1) and the remaining proportion of the product will be derived from source 2 (P2).

$$P1 + P2 = 1 \quad \text{or} \quad P2 = 1 - P1$$

These equation are useful as they do not require that we know the pool sizes of the sources and/or the absolute pool size of the product. For example in a feeding experiment it may be useful to know an insect has fed 20% on source 1 and 80% from source 2, but it is not necessary to know the absolute food intake and turnover. If we use highly enriched substances it is not necessary to take into consideration fractionation effects.

Example equations

A mosquito has both a larval diet and an adult diet. In the laboratory we can control these diets and use these controlled experiments to determine the proportion of the larval diet that contributes to adult carbon status. For example if we feed a mosquito larva with a diet of carbon with an isotopic value of 1.0843 atom % (-24.5‰) and an adult with a sugar diet of 2 atom % and the adult mosquito has a isotopic value of 1.450 atom % after two-weeks then it is simple to calculate the proportion of the mosquito carbon derived from larval diet. By combining the two equations:



$$A_{sample} = P_{S1}.A_{S1} + P_{S2}.A_{S2}$$

$$P1 + P2 = 1 \text{ or } P2 = 1-P1$$

We can derive a simple expression:

In this example:

$$\text{proportion derived from source 1} = \frac{(A_{sample} - A_{source2})}{(A_{source1} - A_{source2})}$$

$$\text{proportion derived from source 1} = \frac{(0.01450 - 0.010843)}{(0.020 - 0.010843)}$$

$$\text{proportion derived from source 1} = 0.3999$$

Because in this example there are only two possible sources of carbon

P2 = 1-P1

Then the proportion derived from P2 = 1-0.3999 or 0.6001. If we round these up and convert them to percentages we see that the mosquito derived 40% of its current carbon from the sugar diet and 60% from the larval diet.

6.3 Two pool mixing models using natural abundance.

Two pool mixing models also work for natural abundance or delta values, however care must be taken to account for fractionation effects such as trophic isotopic enrichment especially when trying to determine proportion of diet derived from two distinct food sources. It is recommended in these cases to subtract the ”standard” trophic enrichment value Δ from the consumer value.

$$\delta_{sample} - \Delta = P_{S1}{}^{*}\,\delta_{S1} + P_{S2}{}^{*}\,\delta_{S2}$$

$$\delta_{sample} - \Delta = P_{S1}{}^{*}\,\delta_{S1} + (1-P_{S1})^{*}\,\delta_{S2}$$

$$\text{proportion derived from source 1} = \frac{((\delta_{sample} - \Delta) - \delta_{source2})}{(\delta_{source1} - \delta_{source2})}$$

In this example the average delta values in ‰ were:

δ web building spiders = -21.5 (consumer)

δ terrestrial insects = -26.5

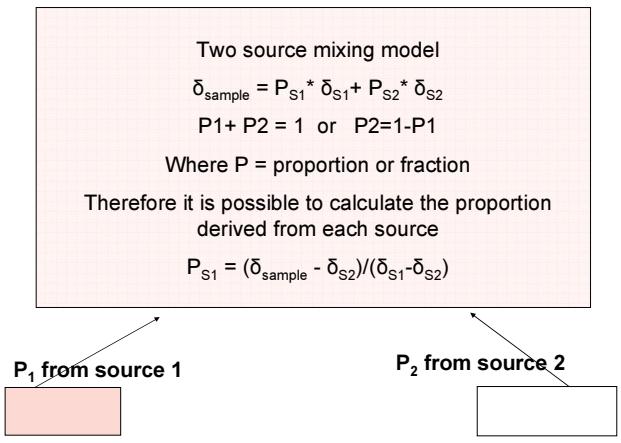
δ aquatic insects = -19.2

Δ of trophic shift = 1.0

$$\text{proportion derived from source 1} = \frac{((-21.5 - 1) - (-19.2))}{((-26.5) - (-19.2))}$$

$$\text{proportion derived from source 1 terrestrial insects} = \frac{3.3}{7.3} = 0.4520 \text{ or } 45.20\,\%$$

Assuming there are only two possible food sources, the remaining contribution to the diet is from aquatic insects. In this example 100 - 45.20 or 54.80% is derived from the aquatic insects.



6.4 Multiple tracers to determine more than two sources.

If there are more than two food sources it is possible to determine contribution from each food source by using additional isotopes and simultaneous equations. Basically two sources can be determined using one isotope, three using two isotopes and four using three isotopes. Generally the number of isotopes available plus one gives the possible number of sources which can be determined. The isotopes used are usually C, N, S and in some marine environments Cl.

However, there must be sufficient isotopic distinction as shown in the Figure 16. If the two sources are for example –10‰ and –20‰ and the sample is -15‰ then it is simple 50:50 split. If however the third source is also –15‰ then a second isotope is required to calculate the contribution from the third source, usually this resolves the problem. However the sources may again fall on the straight line in which case a third isotope may be required or the use of additional label may be useful.

Equations for three source model

$$\delta J_D = f_A \delta J_A + f_B \delta J_B + f_C \delta J_C$$

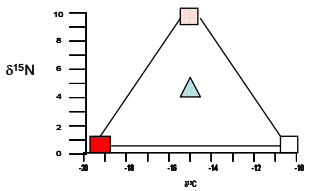
$$\delta K_D = f_A \delta K_A + f_B \delta K_B + f_C \delta K_C$$

$$1 = f_A + f_B + f_C$$

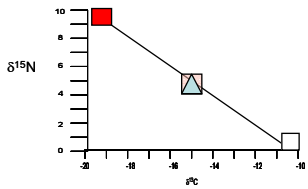
Where δJ, δK are the isotope ratios of the two elements, e.g. ^{15}N and ^{13}C and A, B, C, and D subscripts represent the three food sources and the consumer respectively (Schwarcz 1991).

Using simple algebra it is possible to solve these equations and to determine the contribution from each fraction. An additional isotope would give the possibility of solving for another source. In reality there may be a greater number of sources, so modelling is required to predict feasible solutions. See:http://www.epa.gov/wed/pages/models/stableIsotopes/isosource/isosource.htm.

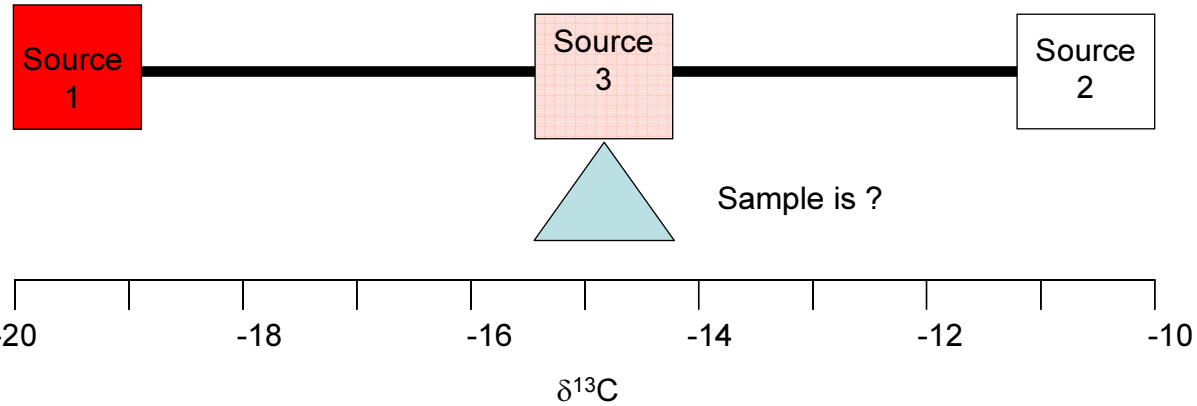
Figure 17. Example of three source modelling using stable isotopes.



Possible to use second isotope to resolve mixing equation.



Not possible to use second isotope to resolve mixing equation, adding an enriched isotope may solve problem.



6.5 Two pool mixing models and mass balance equations.

Mass balance equations can be extremely useful as they allow the isotopic value of unknowns or the pool size of unknown entities to be calculated.

If we add a quantity of substance of a known isotopic ratio to an unknown quantity of substance with a known isotopic ratio and if we assume complete and unbiased mixing then it is possible to calculate from the isotopic dilution, the size of the original pool. This is a useful method in soil science, geology etc.

However in entomology mass balance equations can serve another purpose. Many insect parts are small and therefore to get them above the detection limit of the mass spectrometer it may be useful to spike them with a element of a known mass and isotopic ratio. As the spike is added to the tin cup complete mixing is assumed.

In this example a spike of sugar is used to allow measurement of a minute spermathecae sample from a mosquito, by bringing it above the detection limit of the mass spectrometer.

Spike example calculations:

Atom % sample	= 1.0917
Mass C in sample	= 28.52
Atom % sugar spike	= 1.0843
Mass C in sugar spike	= 22.316
Mass C in spermathecae	= 6.204
Atom fraction	= atom %/100

$AF_{Sample}.\ Mass_{sample} = AF_{spike}.Mass_{spike} + AF_{Spermathacae}.\ Mass_{spermathacae}$

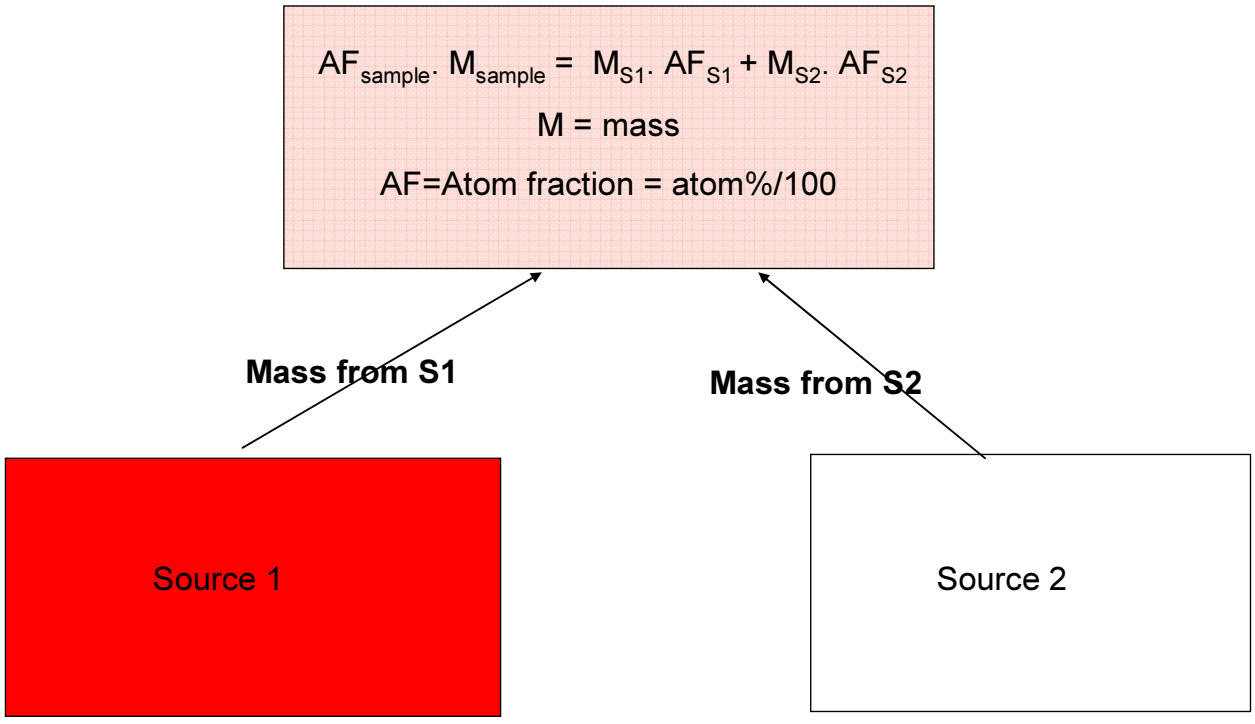
$28.52 * 0.010917 = 22.316 * 0.010843 + 6.204 * X$

$0.31137 - 0.241972 = 6.204 * X$

$0.069398 / 6.204 = 0.011186$

Atom % spermathecae = 0.01186*100 = 1.1186

or when converted to delta value 6.71‰ vs. PDB which was significantly above natural abundance of around -25‰ ^{13}C vs. PDB



6.6 Mass balance equations using delta values.

It is also possible to use delta values for mass balance equations. However some care must be taken as they are not absolute values. Delta values 100‰ below or above zero should be converted back to atom % or atom fraction values and re-calculated to avoid making minor errors. Also utmost care should be taken to account for all minus signs in the values and equations when using delta values. In the case of micro-enrichment techniques it is always prudent to convert back to atom % or atom fraction values.

The example shows how delta values can be used to calculate the isotopic signature of the spermathecae when a spike is used.

The advantage of using the delta values is that they are in ranges that are more familiar and therefore it is easier to spot obvious errors.

Sample calculations

δ_{Sample}	= -17.52
Mass C in sample	= 28.52
δ_{spike}	= -24.502
Mass C in sugar spike	= 22.316
Mass C in spermathecae	= 6.204

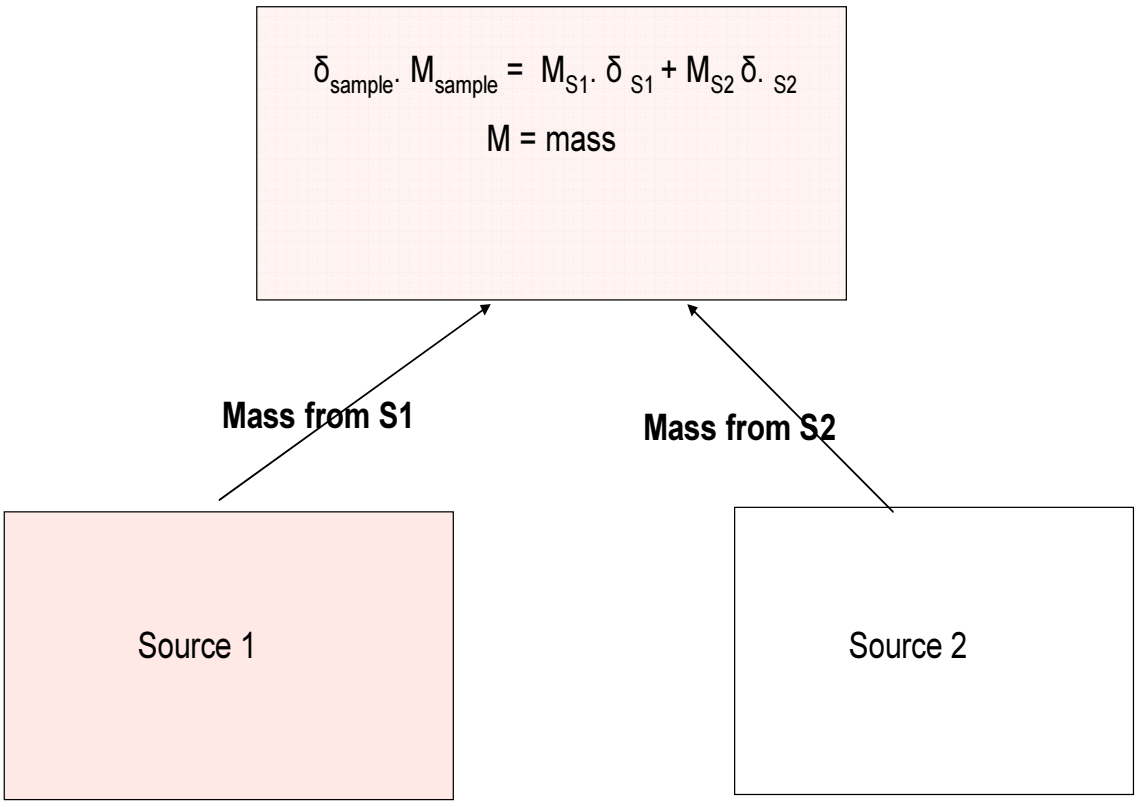
$\delta_{Sample}.\ Mass_{sample} = \delta_{spike}.Mass_{spike} + \delta_{Spermathacae}.\ Mass_{spermathacae}$

28.52*-17.52 = 22.316*-24.502+6.204*X

-505.089-546.78 = 6.204*X

41.46/6.204 = 6.719‰

The spermathecae had a δ value of 6.719‰ which was significantly above natural abundance of around -25‰.



6.7 Propagation of error in mixing models.

One of the main problems with the mixing models is estimating the error or uncertainty associated with the final contribution, where two sources may have their own associated error term or uncertainty. A general rule of thumb is that the more isotopically distinct the two sources are, the smaller the error or uncertainty associated with the final contribution value.

Calculating the error can be achieved in a number of steps.

Measure each value in the equation and its errors or uncertainty associated. 95% confidence limit is usually two times the standard deviation if the data are normally distributed.

e.g.

$P_{S1} = (\delta_{sample} - \delta_{S2})/(\delta_{S1} - \delta_{S2})$

Value ± Error (95% confidence limit) for δ sample = 5 ± 0.9‰

Value ± Error (95% confidence limit) for δ source 1 = 0 ± 1.1‰

Value ± Error (95% confidence limit) for δ source 2 = 10 ± 1.0‰

P = (5-10)/(0-10) = 0.5

The errors must be propagated in the numerator and denominator terms using the formulae for subtraction. If A = B - C there are errors in both B and C (e_B and e_C) the propagated error for A is

$e_A = (e_B^2 + e_C^2)^{0.5}$

Error in numerator = $(0.9^2 + 1^2)^{0.5} = 1.345$

Error in denominator = $(1.1^2 + 1^2)^{0.5} = 1.487$

A propagation of errors equation must then be used for the divisions:

If $P_{S1} = N/D$ then the propagated error for Pe_p can be calculated from the equation

$(e_p/P)^2 = (e_A/N)^2 + (e_B/D)^2$

Solving these values $(e_p/0.5)^2 = (1.345/-5)^2 + (1.487/-10)^2 = 0.0945$

The final propagated error $e_p = 0.154$ for this example.

Thus 0.5 ± 0.154 or 50% ± 15.4%

It becomes apparent why the error term of the final proportion is reduced when the source values are more isotopically distinct if the exercise is redone assuming the error is identical. This is a reasonable assumption as the error associated with the source is often derived from the uncertainty associated with the accurate measurement of the isotopic value or a machine derived uncertainty or error of measurement.

PS1 = (δsample - δS2)/(δS1 - δS2)

Value ± Error (95% confidence limit) for δ sample = 50 ± 0.9‰

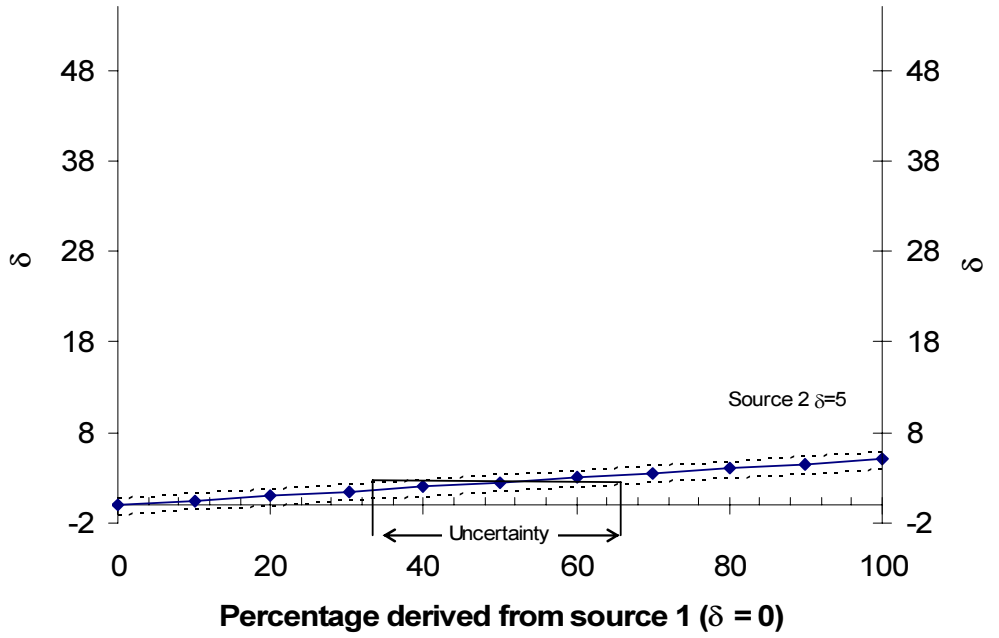
Value ± Error (95% confidence limit) for δ source 1 = 0 ± 1.1‰

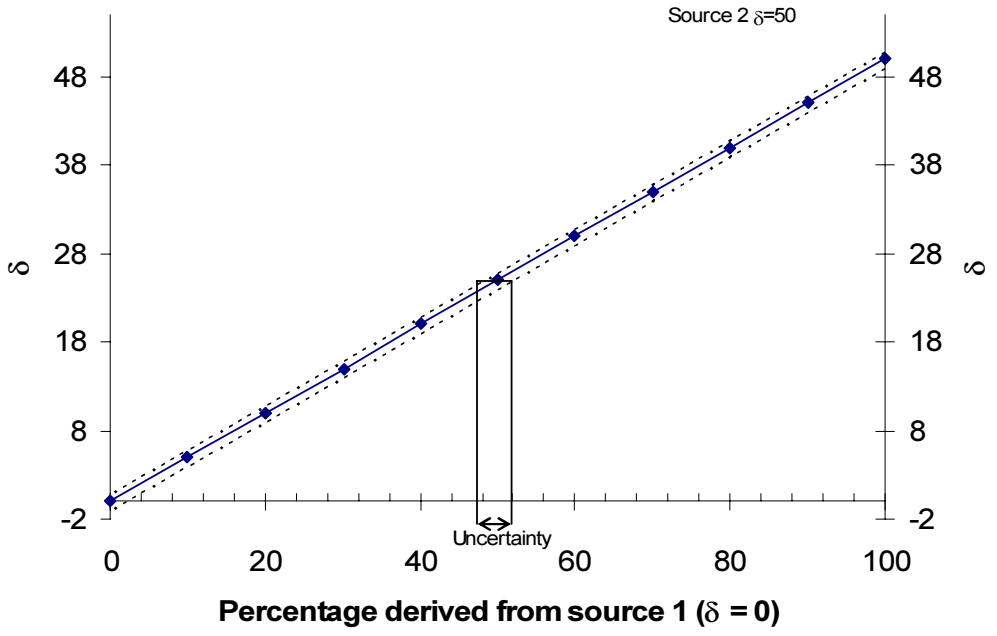
Value ± Error (95% confidence limit) for δ source 2 = 100 ± 1.0 ‰

P = (50 - 100)/(0 - 100) = 0.5

If the number of food sources exceeds the number of elemental isotope ratios by more than one there is no unique solution. There are Euclidean distance equations which can be used to determine the contribution of a number of food sources when the number of isotopes is less than the source number plus one, however these solutions are not unique and must be used with caution and supplementary evidence, such as gut contents and observation (Phillips 2001).

Figure 18. Propagation of error in mixing models.





It is simple to see how if we assume the same level of error or uncertainty of the source values in both examples (in this instance 0.923), how the use of more isotopically distinct samples yields a more accurate estimation of the proportion derived from source.

Another approach to calculate uncertainty is to use minmax solutions which give a range of possible solutions. Minmax solutions are obtained by sequentially setting fraction derived from source one and two to zero and calculating resultant maximum and minimum values.

7. MOVEMENT AND POPULATION STUDIES

7.1 Estimating population size.

Estimating the size of an insect population is vital for conservation or control programmes, such as those employing the sterile insect technique. Mark, release and recapture is a widely used method in ecology in which insects are marked, released and recaptured in the environment studied. Population size is estimated by comparing the number of released marked individuals recaptured to the number of unmarked individuals recaptured. Marking insects can be difficult, however it is possible to mark insects intrinsically using stable isotopes. Some insects that are reared on C_4 sugar based diets will be isotopically different in ^{13}C signatures from the wild populations that feed on C_3 plants. Alternatively insects can be marked by adding an enriched compound to the diet, this can be done by incorporating minute quantities of labelled isotope compound such as ^{15}N labelled glycine to the rearing diet. For conservation purposes labelling may be done *in situ* or after capture of individuals and feeding them labelled isotope. All methods require some preliminary studies to optimise and validate the labelling methodology. More details on marking are given later in this chapter.

$$N = \frac{n1n2}{m}$$

N = estimate of population size
n1 = number of marked individuals released
n2 = number of captured individuals on second visit
m = number of marked individuals recaptured

A known number of marked insects is released and after an adequate amount of time has elapsed that allows the insects to mix randomly in the unmarked population; a sample of insects is then recaptured. These individuals are then analysed for their isotopic value and the population size estimated using the Lincoln-Peterson model. This model assumes that the population is "closed", i.e. that within the study period no animal dies, emigrates or immigrates. The model also assumes that the marker will not be lost and that it will be possible to detect the marker.

Figure 19. Population monitoring trap for tsetse.

Example calculations

A manager of an SIT programme releases 20,000 fruit flies marked using the isotopic difference in the factory C_4 sugar based diet compared to the diet of the wild flies. The factory flies will have a ^{13}C value of around -19‰ and the wild flies will have a signature of around -27‰. The manager returns to the site and captures 100 individuals and sends them for isotope analysis. 20 of the individuals have an isotopic value of around -19‰ and 80 have values of around -27‰.

$$N = \frac{n1n2}{m} \qquad N = \frac{20{,}000 * 100}{20}$$

The manager concludes that there is a wild population of 100,000 individuals.

Preliminary experiments to assess the confidence limits of the isotopic signature of factory flies are necessary (see later Section).

7.2 Natural abundance marking.

For release recapture experiments it is possible to label insects using nature's isotopic variations. Innate differences in isotopic signature are the result of different rates of reaction at an enzymatic level which can result in slight variations in isotopic composition. One of the most commonly applied uses is the difference between C_3 and C_4 plants (see Table 4), which photosynthesise using different biochemical pathways, consequently the ratio of $^{12}C/^{13}C$ in their tissues differ by about 14‰. C_3 plants are generally found in temperate climates with lower light intensity and have typical isotopic values of around -27 +/- 2‰ but range between –25‰ and -35‰ vs PDB depending on the species and environmental conditions (O'Leary 1988). Plants such as maize, millet, sugar cane etc. have C_4 Hatch-Slack photosynthetic pathways (Hatch and Slack 1966) and thrive in sub-tropical environments.

C_4 plants have isotopic values of around -13.1 +/- 1.2‰ and range between -7 to -18‰ vs PDB with maize about -11‰ vs PDB (O'Leary 1988). In a number of insect species the larval isotopic signature is sufficiently retained in the adult tissue but this depends on the biology and life cycle of the insect. This isotopic signature can then be used as a marker. Insects raised on C_3 plants will have a different signature to wild insects feeding on C_4 plants. Dalecky *et al.* (2006) used this to track European corn borer (ECB) populations and movement. They raised ECB populations on C_3 soybean and wheat plants and then released these C_3 moths into C_4 corn fields, the isotopic difference allowed them to track both population size and dispersal of the ECB. The advantages of this method is that it does not require application of an isotope, however tests must be carried out to determine that changing the feeding regime does not affect the longevity and fecundity of the insects.

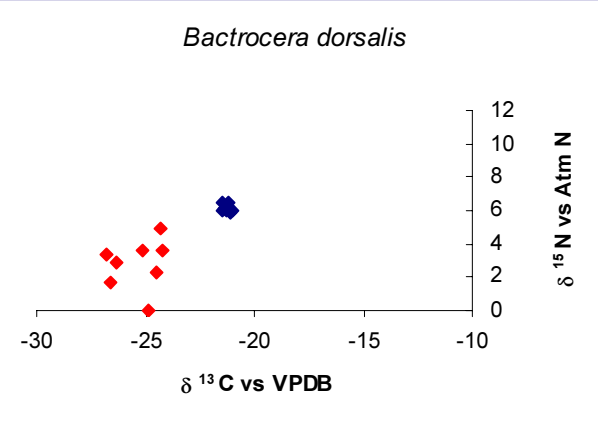
This method works well if 1) the larval isotopic signature is retained in adult life, 2) if marked populations are easily statistically distinguishable from the wild population and 3) if the insect is not very host specific and can be fed on different diets without effecting its biology and fecundity.

Table 4. Common C_3 and C_4 plants.

Photosynthesis	Common name	Scientific name
C_3	Wheat, common	*Triticum aestivum*
C_3	Rice	*Oryza sativa*
C_3	Cotton	*Gossypium L*
C_3	Potato	*Solanum tuberosum L.*
C_3	Alfalfa*	*Medicago sativa L.,*
C_3	Sunflower	*Helianthus annuus L.,*
C_3	Soybean*	*Glycine max (L.) Merr.,*
C_3	Barley	*Hordeum L.,*
C_3	Apple	*Malus x domestica*
C_3	Pears	*Pyrus communis*
C_3	Oranges	*Citrus sinensis*
C_3	Lemons	*Citrus limon*
C_3	Grapes	*Vitis vinifera, (wine)Vitis labrusca, (table)*
C_4	Maize (corn)	*Zea mays L. ssp.*
C_4	Sorghum	*Sorghum sps*
C_4	Millet finger	*Eleusine coracana*
C_4	Sugar cane	*Saccharum*

Mass reared *Bactrocera dorsalis* flies fed on a C_4 based sugar diet showed significant differences in isotopic signatures to flies caught in the wild. This can be a useful technique for mark release recapture type experiments as C_4 cane sugar is cheaply available and easily integrated into mass reared diet.

Figure 20. Intrinsic marking.



Blue squares factory reared flies, red diamonds wild flies.

Figure 21. Trapping Bactrocera dorsalis *in the field.*

7.3 Enrichment marking.

Labelling studies are often referred to as enrichment studies because an isotopically enriched compound is added to the system (Unsicker *et al*. 2005). The slight difference in mass of isotopes of the same element can result in slight variations in isotopic composition of substrate compared to product and although these differences are important in natural abundance studies (see above), in enrichment studies this discrimination is generally irrelevant. Thus it is important to consider prior to undertaking a labelling study whether you are sure you want to use a label in the area, as subsequently it will not be suitable for natural abundance studies. It may be worth pinpointing labelled areas using a GPS system, noting exactly where the label has been applied and ensuring this information is published.

Labelling insects with stable isotopes is an effective marking method for population studies and tracking. Commercially available compounds enriched with stable isotopes such as glucose or amino acids can be easily integrated into the feeding regimes of laboratory reared specimens prior to release at reasonable cost (Table 2). It is also possible to grow plants in ^{15}N labelled fertilisers or in ^{13}C enriched atmospheres and then feed the labelled food to the target organism.

For labelling purposes preliminary laboratory-based insect-specific and isotope-specific studies are required to ensure that the isotope label is fixed into structural body tissue of the target organism and that the isotope label is not “lost” through metabolic turnover within the time period of the recapture event. It is also important to ensure there are no toxic effects of labelled compound addition (n.b. the addition of the compound rather than the label *per se* may cause detrimental effects).

Stable isotope marking of insects fulfils the marking criteria set out by (Hagler and Jackson 2001; Hobson and Clark 1992) of: retention, not affecting the insect’s fecundity or behaviour, durable, non-toxic, easily applied, clearly identifiable and inexpensive. In insect studies in particular the costs of the enriched feeding compounds are not prohibitively expensive and the small size of most insects means that whole insects can be analysed or specific insect body parts, which reduces sample preparation time and thus cost.

For example we have estimated that it would cost between 150-250 USD to label one million mosquitoes with ^{13}C labelled glucose in the larval stages and because the mosquitoes fit neatly into the tins cups used for isotope analysis there is minimal sample preparation time. In the case of mosquitoes, the larval diet is spiked with a low concentration of highly labelled glucose solution which is retained throughout the adult mosquito life (Hood-Nowotny 2006). It has also been shown that ^{15}N is a suitable marker for mosquitoes, if detection limits of the mass spectrometer system are above 10 µg N which is approximately the amount of N in a mosquito. N could be used as a marker in other larger insects successfully as it is cheaper to buy and easier to analyse it is therefore an obvious choice as a marker. The advantage of using N rather than C is that N does not turn over as rapidly as C, as a result of carbon metabolism, specifically during respiration.

8. CONSIDERATIONS WHEN MARKING INSECTS

8.1 Target values.

Target enrichments of approximately 2-3 standard deviations (SD) above the isotopic abundance of the natural population should ensure that insects are sufficiently labelled and distinguishable from the native population with a 95-99.9% certainty. Therefore initial survey data of the isotopic variation of the native population is necessary. Large variations (of more than 10‰) in isotopic signatures of wild populations especially if clustered could suggest distinct populations with different habitats or feeding strategies. In terrestrial habitats, biological $\delta^{13}C$ values are generally negative, therefore marking up to 0‰ would usually be sufficient to distinguish marked from wild populations and would negate the need for extensive surveying.

8.2 Background values.

Consideration of the sources of environmental isotopic variation that may have an impact on the insect's isotopic signature is essential. This will be dependent on the isotope and the insect's interaction with the environment. Figures 27 and 28 show some general variations in the natural isotopic signatures. Therefore it is useful to collect insect samples prior to release of marked insects.

8.3 Marking strategy.

Minute additions of highly labelled compounds will have less impact on the insects and the system to be studied. Enrichments of 80-95% of added compounds are recommended as the cost of labelled substrates steeply above this threshold. In marking experiments such high purity enrichments are usually unnecessary as the label will be diluted out by other unlabelled sources. It is generally not advisable to label with deuterium above 30 atom % as it can interfere with some

8.4 Time of labelling.

Labelling when structural tissues are formed either in juvenile or active growth stages is recommended, as structural tissues such as chitin usually retain the isotopic signature throughout the insect's lifespan and turn over at a slow rate. This should be validated with controlled experiments. In some insects such as locusts it may be more difficult to label as the exoskeleton is discarded as the insect grows (Webb *et al.* 1998). Therefore a labelling strategy must be tailored to the insect and the goals of the study.

8.5 Turnover.

The rate and nature of biochemical turnover of the insect will have a significant impact on the retention of the isotopic label. If the insect is labelled in juvenile stages and then released as an adult, the label will be diluted as the insect feeds on unlabelled resources which could bring it back below the 2SD threshold. To assess turnover, controlled time course experiments must be undertaken to guarantee that the label will be detected for the duration of study period.

8.6 Toxicity.

Some compounds that we want to add to the diet may prove toxic or growth retardant therefore it is always best to test whether the addition of the unlabelled compound to the diet has any effect on growth and fecundity prior to buying the labelled compound.

8.7 Loss of label.

Labelling with carbon compounds obviously results in large losses of carbon due to respiration. Labelling with nitrogen compounds will result in less systemic losses, but may not be the isotope of choice, as the amount of N in the insect will be about ten

times less than carbon and for small insects may be below the detection limits of the mass spectrometer system (typically 10-15 µg N).

8.8 Labelling uniformity.

To get an evenly labelled population is desirable; this is achieved through isotopic homogeneity in the feeding substrate. In an aquatic system in the laboratory, addition of the label in a soluble form ensures the most uniform method of application. Labelling can be done in the laboratory or *in situ* (Hershey *et al.* 1993).

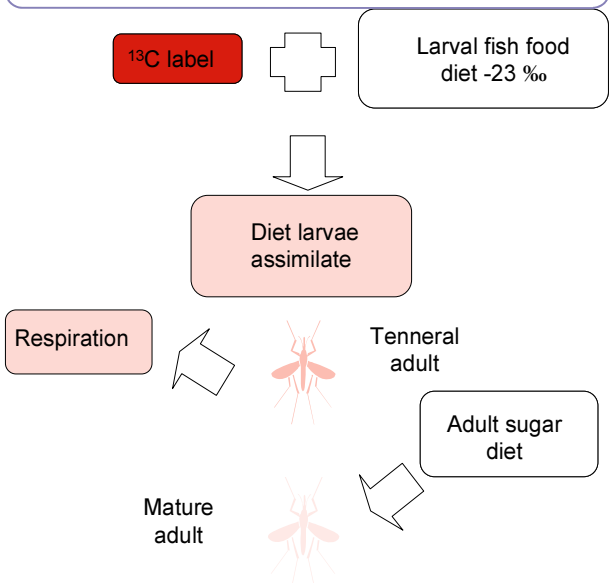
In some cases it may be necessary to feed insects their normal food rather than attempting to develop artificial diets. This can be achieved by labelling the host plant, or secondary consumer species (see Section 16).

9. PRACTICAL EXAMPLES OF ENRICHMENT STUDIES

All labelling methods will require some trial and error, and call for informed guess work, as information on the turnover of tissues etc is not always readily available, although it maybe elucidated using isotopes (see Section 13.4). The entire life cycle of the insect must be considered when attempting to establish the correct labelling strategy and some basic strategic questions answered. At which point in the life cycle is the insect to be labelled? Is this feasible and does it fulfil the goals of the study etc? Making a rough sketch of the life cycle and possible inputs may be useful.

Having established a marking strategy it is necessary to calculate approximately how much label is required.

Figure 22. Schematic of C inputs to male mosquito diet.



9.1 Labelling mosquitoes with ^{13}C.

Experiments were designed to label adult mosquitoes; the final goal was to use the marking system for sterile insect technique (SIT) purposes. A simple easily integrated and low cost solution was required. It was assumed that an SIT programme would release male pupae and not adults therefore any marking had to be carried out in the larval stages with detection in the adults.

Mosquitoes have three distinct stages of development: a larval stage in which they filter feed in an aquatic system, a non feeding pupal stage and a sugar feeding adult stage. Any addition of labelled carbon in the larval stages will be diluted out by non-labelled sugar carbon sources in the adult stages. These considerations must be built into the estimation of the amount of label required and the assumptions made tested experimentally.

Experiments were set up which assumed that the mosquito would be 50% structural tissue and 50% tissue that turned over rapidly. It was also assumed that only about 1% of the label added to the water would be assimilated and retained by the mosquitoes as a high percentage is lost in microbial and mosquito respiration. From this a base rate value, an isotope addition of glucose was calculated, treatments were set up with the base rate isotope addition, ten times the base value and one tenth of the base value.

Zero addition controls and unlabelled glucose controls with the same concentration of glucose as the labelled treatments were also set up to ensure that the addition of the compound *per se* had no detrimental effects on mosquito development or survival.

Isotope dilution from the conventional larval food source in this case fish food was also taken into consideration in the calculation and dilution was accounted for during the entire larval feeding period.

For C or N dilution only the carbon or nitrogen in the food source should be calculated. If no data are available most plant material has a range of 1-3% N and 42-50% C. Accurate data can be obtained from the elemental analyser and it is usually best to get this at the outset of the experiment.

Calculations

In experiments where there is little prior experience, it is often good to get a rough target value. The calculations were then designed around these assumptions. We also need to account for all sources of carbon in the larval diet.

Experimental set up:

Larval fish food 40% carbon

Larvae fed 0.25 mg /larval fish food per larva

Larvae fed for 10 days

400 larvae per tray.

µg C in a mosquito

Larval fish food isotopic value of -23‰ ^{13}C or 1.0859521 atom % (0.010859521 atom fraction)

^{13}C glucose label 40% carbon, 99 atom % (0.99 atom fraction)

Final target enrichment of final mosquito 100‰ ^{13}C or 1.22099 atom % (or 0.0122099 atom fraction)

Initially the amount of carbon in the larval food needs to be calculated.

Amount of fish food per day * no of days fed * no of larvae in tray * % C in fish food = Total C fed to larvae

$$A_T = \frac{A_1M_1 + A_2M_2}{M_1 + M_2}$$

Rearranging the equation gives:

$$M_2 = \frac{A_TM_1 - A_1M_1}{A_2 - A_T}$$

Where A = atom fraction and M = mass

A1 = 0.0108592

A2 = 0.99

AT = 0.0110011

M1 = 0.4g

M2 = ?g

$$M_2 = \frac{0.0110011 * 0.4 - 0.0108592 * 0.4}{0.99 + 0.0110011} = 0.000553$$

= Molar Mass glucose/Molar mass of C in glucose*amount of C required

= 180/72*0.000553 = 0.001381g of 99 atom % glucose per tray

or 1.38 mg per tray. Because these amounts are difficult to weigh and because it is easier to add and store the label as solution, a solution was made up in water and an appropriate aliquot of solution used.

Methods and results

In initial experiments dried glucose was mixed with the larval fish food, but it proved difficult to obtain a homogeneous labelling. The experiment was modified to include a treatment where solutions of glucose were added to the larval trays. This strategy fitted in well with normal mosquito rearing and could be simply and easily implemented. Feeding was standardised and identical in each tray to ensure uniform labelling across replicates.

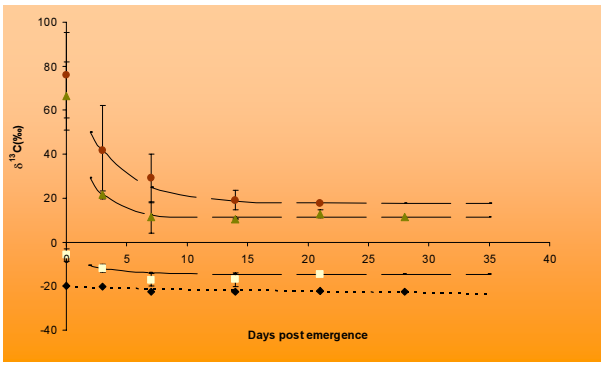
In systems were there is little or no experience of using isotopes it is often good to determine a target value and then to set up treatments with ten times greater the amounts and a tenth of the amount. This should give a good chance of hitting the desired target value.

Highly enriched glucose solutions were accurately made up using a five figure balance and volumetric flasks. These flasks were then permanently marked and designated isotope flasks and not used for other purposes in the laboratory to avoid any cross contamination.

Three replicate trays for each treatment were set up and L1 larvae counted into each tray manually, to reduce the experimental uncertainty. The glucose treatments were added as a single dose, unlabelled treatments were set up first to avoid cross contamination. Pupation rates and survival rates were monitored in each tray daily. Mosquitoes that emerged on a single day from all trays were sampled and caged for the isotope longevity experiments, again to reduce uncertainty.

Approximately similar numbers of adult mosquitoes were put into separate holding cages and fed with un-labelled sucrose solution *ad libidum.* Adult male mosquitoes were sampled 0, 3, 7, 14, 21 and 28 days after emergence. Mosquitoes were knocked out with CO_2 and loaded into tin cups and lightly sealed to avoid escape. Samples were dried at 60°C.

Figure 23. Mosquito $\delta^{13}C$ values over time following larval labelling with ^{13}C glucose. T1-T3. 0.175, 0.875 or 1.75 mg of 99 % ^{13}C glucose added per tray as solution. T4 regular fish food no label added.

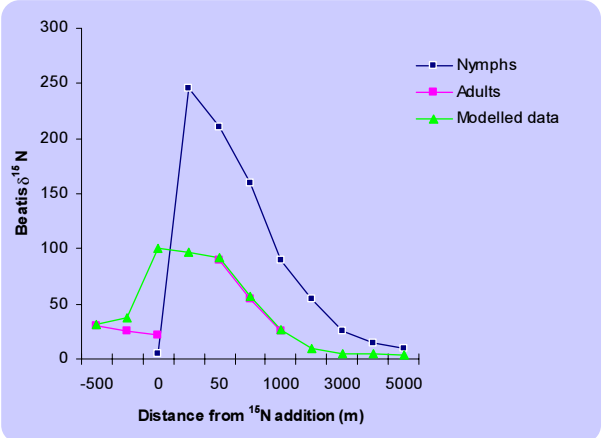


n.b. It is good practice to wash ones hands between handling treatments and when washing all equipment, the unlabelled first rule applies, again to avoid cross contamination. The results show that using the two highest concentrations of labelled glucose in the larval medium, adult mosquitoes could be easily differentiated from unlabelled individu-

9.2 Labelling mayflies with ^{15}N.

It is possible to label populations of insects in the field using stable isotopes safely and easily. Mayfly dispersal was studied by point labelling a section of a river with ^{15}N in the form of ammonium chloride solution constantly dripping into the river using a peristaltic pump (^{15}N addition rate of 0.89g day^{-1}). The addition of the label did not significantly increase the concentration of ammonium in the river, an important consideration when studying ecological systems. The label was rapidly taken up by the native algae, a major food source of the mayfly larvae. Abundance of the mayfly larvae was monitored at 16 points along a 4.2 km transect and abundance of adults monitored at three upstream sites and three downstream sites. Dispersal of the labelled adult flies was modelled after recapturing the adult flies and determining their isotopic composition.

Figure 24. ^{15}N abundance of mayfly nymphs and adults following point source labelling of the river section.



It was shown that adults have essentially the same $\delta^{15}N$ as the nymphs from which they emerge. Adults captured upstream of the labelled site had $\delta^{15}N$ above natural abundance and although $\delta^{15}N$ signals decreased exponentially downstream adult values were essentially lower than nymph values at given sites suggesting dilution by upstream movement of less-enriched adult flies. The study concluded that downstream larval drift does occur and does not deplete upstream populations because it is compensated for by upstream adult movement. In fact they estimated upstream flight movement was up to 1.9 km. The study demonstrates how a complex system can be examined using isotopes.

For further details of this project follow see:http://ecosystems.mbl.edu/ARC/data_doc/streams/streamsdefault.htm

9.3 ^{15}N in situ labelling of stoneflies in the field.

Using a point source approach it is also possible to study insect dispersal using stable isotopes. Briers *et al.* (2004) quantified the extent and rate of dispersal of stoneflies (*Leuctra inermis*) from a point source labelled with ^{15}N in a Welsh stream system.

They showed that the population dispersed up to 1.1 km away from the point source, significantly greater distances than had been previously reported using direct studies.

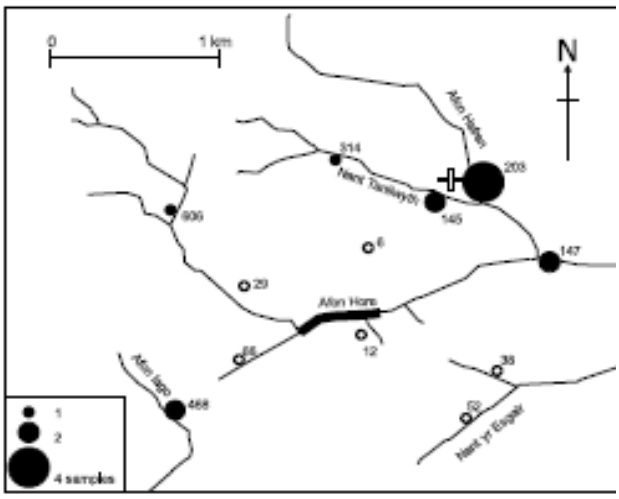
In this study the researchers used a pooling of individuals approach, as the isotope analysis was more time consuming and costlier than the isotope added. That is by adding a greater amount of “cheap” label they could detect the label in batches. Individuals were marked to up to 200‰ which meant that in a sample of 10 pooled flies the isotopic signature of one marked fly in the sample would be easily detectable. This pooling approach is particularly useful in range studies where a large number of samples need to be analysed.

A pooling or bulking approach may also be useful for studying smaller insects which may be under the detection limits of the IRMS systems.

The use of pooling or bulking can significantly reduce the number of measurements required, however the detection limits of the system must first be established. This is fairly simple using mass balance equations to calculate the minimum enrichment of an individual so that the labelling and sampling protocol can be designed around this figure.

Map of the study site in which circular symbols (open and closed) with adjacent numbers indicate the positions of trapping sites and the total catch of *Leuctra inermis*. Filled circles are trapping sites where marked samples were taken; their size is proportional to the number of marked samples. The cross indicates the location of a weather station from which data were obtained. The enlarged section of Afon Hore (source population) indicates the length of stream in which detectably marked *L. inermis* larvae were found.

Figure 25. Study site map.



10. NATURAL ISOTOPE MARKERS FOR INSECT MOVEMENT STUDIES

Using naturally-occurring stable isotope markers is useful as it does not require the pre-marking of individuals. Specific geographical regions have distinctive isotope profiles as a consequence of biogeochemical processes. These can be used to trace the origin and movement of insects. The most commonly used isotopes are the isotopes of (H, O, C, N and Sr). δ^2H generally decreases with increasing latitude and altitude due to rainfall patterns and temperature differences over large geographical ranges. $\delta^{18}O$ is higher in summer than winter above latitudes of 30° and generally decreases with distance from the sea (for detailed maps of isotopic signature of rainfall see Bowen *et al.* (2005) and http://isohis.iaea.org). $\delta^{18}O$ and δ^2H from precipitation and snow varies systematically across the globe. $\delta^{13}C$ decreases with increasing latitude and altitude, and drier habitats are more enriched (less negative) than wetter habitats with predominantly C_3 plant species.

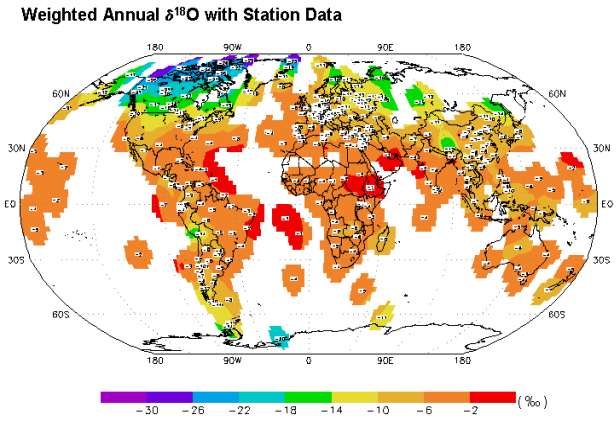
The oxygen and hydrogen isotope signatures of an insect will accurately reflect the signature of its water source, which is usually dependent on the signature of the weighted average of local precipitation. However, processes of evaporation and infiltration can lead to isotopic discrimination, and ground water and water bodies may thus have significantly different signals from the weighted average of precipitation. The carbon isotope signatures reflect the insect's diet, although the $\delta^{15}N$ signature can also be affected by water availability and nutritional stress.

Both will give some indication of the organism's position in the food web (see section on food web structure).

Using these latitudinal differences in $\delta^{13}C$ and δ^2H of food and water sources the natal origin of monarch butterflies (*Danaus plexippus* L.) over-wintering in Mexico) was determined (Hobson *et al.* 1999). Initially, isotopic values in the butterflies were compared with their host plants and water sources, simply by rearing butterflies on test milkweed plants irrigated with water of known isotopic composition. The researchers then assessed whether geographical patterns in the δ^2H and $\delta^{13}C$ values were evident. A coordinated effort of rearing and sampling of adult butterflies, cultured from eggs reared on rain-fed milkweed host plants, was undertaken at different locations across the butterfly's ecological range throughout the USA. They then tested whether the resolution of the isotopic patterns was sufficient to infer natal origins, and isotopic maps were drawn. The signature of the host plant and adults were analysed for δ^2H and $\delta^{13}C$ and the natal origins of the butterflies over-wintering at 13 locations across Mexico were identified using isotopic signals. (Wassenaar and Hobson 1998)

This technique is somewhat limited to long-range migrations or to studies of movement across isotopic boundaries.

Figure 26. Global ^{18}O distribution pattern.



11. RESOURCE CAPTURE AND UTILISATION

11.1 Isoscapes.

Natural abundance studies are based on the small differences in isotopic composition brought about by fractionation or discrimination, for or against the heavier isotopes due to kinetic and equilibrium isotope effects. Although stable isotope forms of the same element are chemically identical, as determined by the number of protons, the rates of reaction can be very slightly different for the heavier atoms. These differences are in the order of parts per thousand or more commonly referred to as per mil (‰). In nature values are typically in the range of -100 to +100‰.

These small differences lead to distinctive isotopic landscapes in the biosphere as the main cycles of N, C, S, H and O have characteristic fractionation factors associated with the dominant transformation processes (Figures 27 and 28).

The main processes of fractionation that dominate in the landscape differences are photosynthesis, differences in C_3 and C_4 photosynthetic pathways, leading to differences in ^{13}C signatures, distillation effects on the isotope ratios of rainwater leading to latitudinal and cross continental isotopic gradients of deuterium and ^{18}O and nitrogen fixation, de-nitrification and ammonia losses.

An understanding of the isotopic landscape or isoscape could be useful in studying a number of key processes in the biology and ecology of insects, such as migration, natal origin, movement, dietary uptake etc. The beauty of natural abundance studies is that the ecosystem can be studied *in situ* with no inputs which affect the interactions. The disadvantage of natural abundance studies is that there are not always significant differences in isotopic signatures of the systems we would like to study, which can make interpretation difficult.

Figure 27. $\delta^{15}N$ values in natural systems. Redrawn from Peterson and Fry 1987.

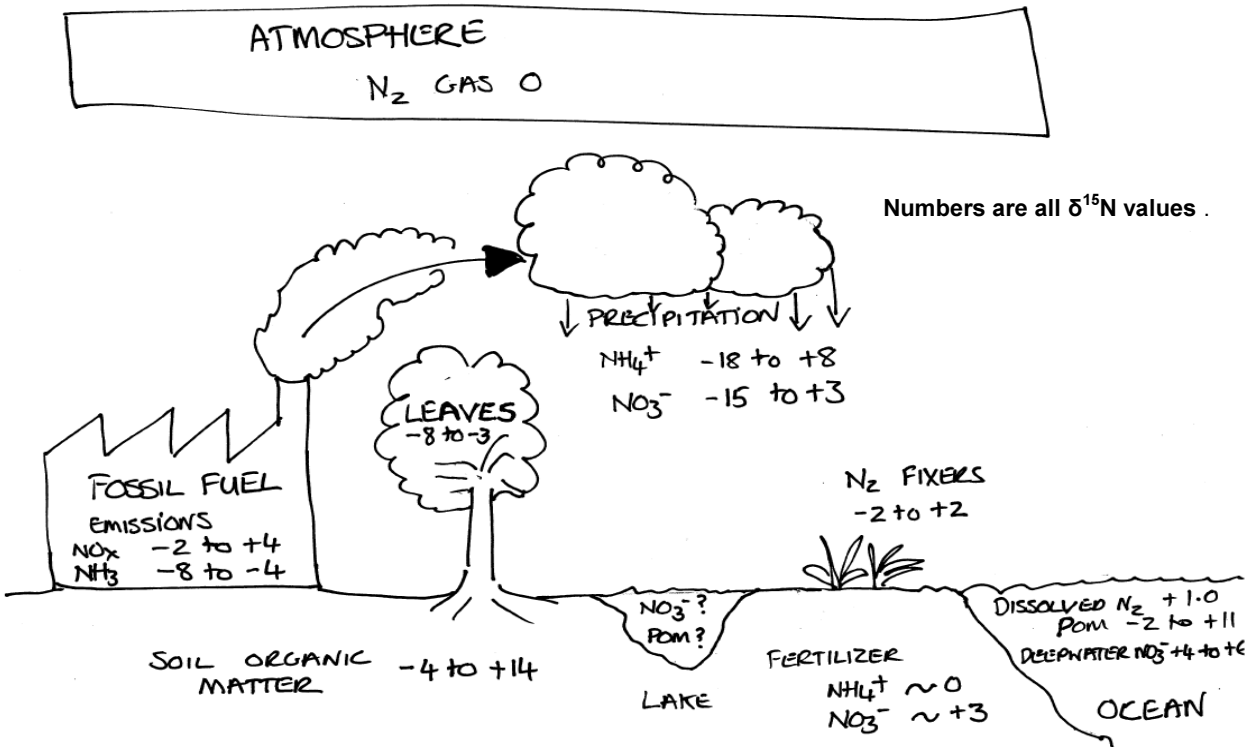
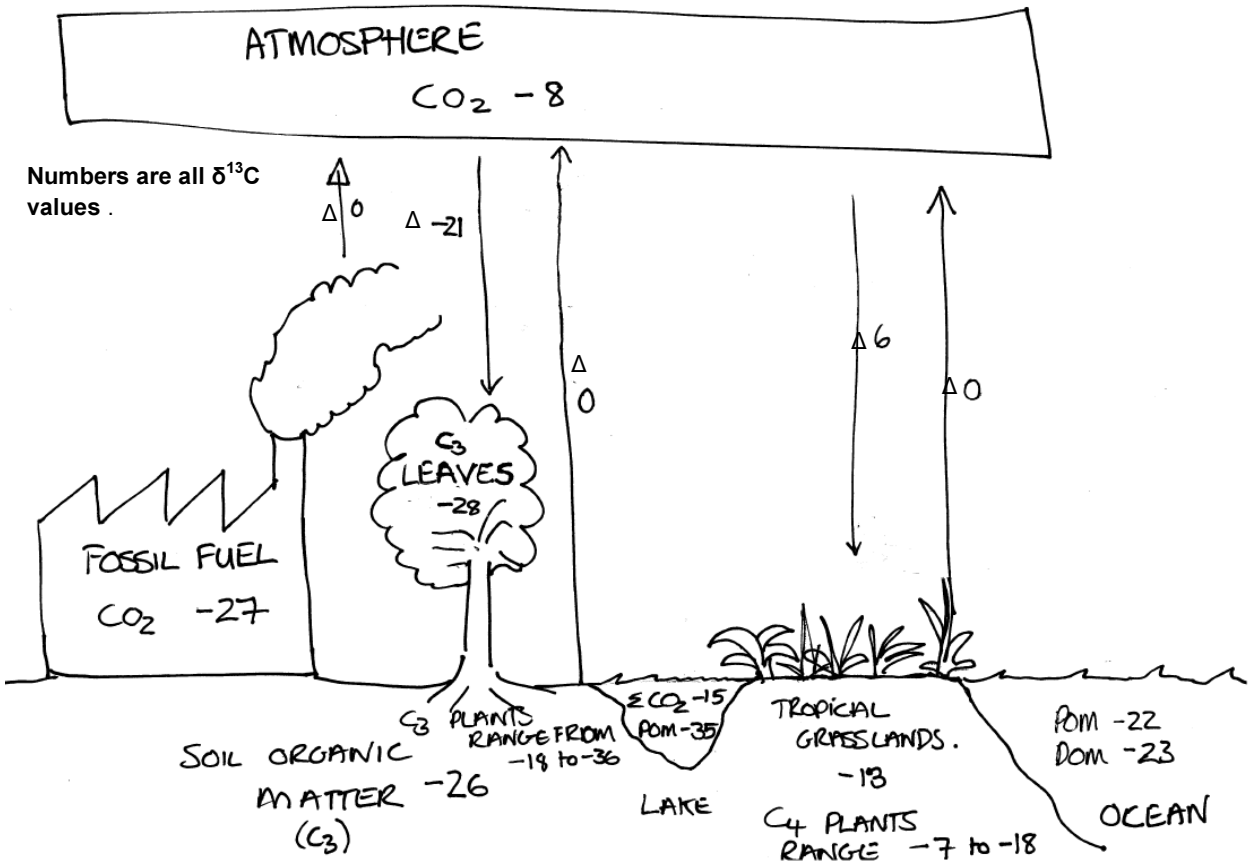


Figure 28. $\delta^{13}C$ values in natural systems.
Redrawn from Peterson and Fry 1987.



The isoscape is a unique isotopic pattern embedded in the environment. The more we understand the processes which govern the isoscape the greater our ability will be to model and predict the isoscape and use it for ecological and agricultural studies.

Advances in stable isotope mass spectrometry techniques have meant that it is now possible to analyse a number of isotopes simultaneously giving a greater range of tools to work with. However the majority of studies still focus on the use of carbon and nitrogen isotopes. Advances in ICP-MS for isotope ratio measurement of the heavier isotopes such as calcium, strontium and lead, provide us with tools which allow us to link the ecology to the geology of a landscape. These techniques could be extremely useful in insect movement studies.

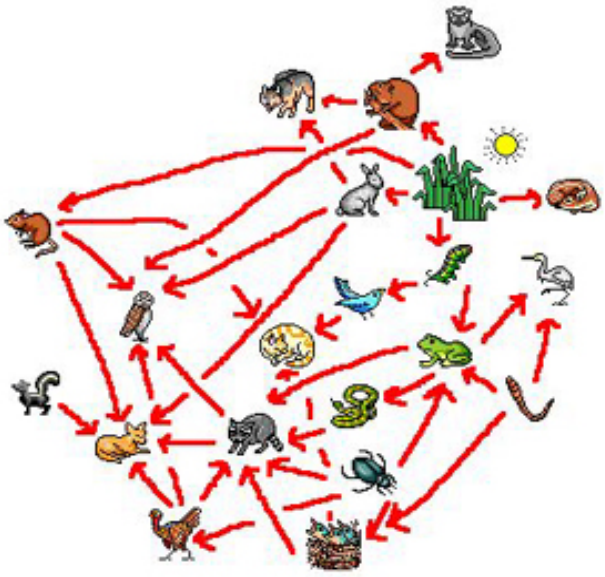
One of the most successful ways the intrinsic isotopic signature has been used is to look at the ecosystem as a whole to determine trophic structures in specific habitats. As insects are often key species at the lower levels of many terrestrial and freshwater foodwebs, they have often been included in these studies, but are not necessarily the key focus of the study.

The advantages of using stable isotopes in insect food web analysis are clear as small insects in particular are simple to prepare for analysis. In many cases it is a question of just popping them into a tin cup.

11.2 Food web re-construction.

Stable isotope analysis of carbon and nitrogen are powerful tools to study food web structure and the energy flows therein. Isotopic techniques have distinct advantages over traditional techniques such as gut content analysis and observation as they allow for long term studies and are generally less time consuming; however they are also most revealing when used in conjunction with the traditional techniques and knowledge.

Figure 29. Complex food web Source:www.tburg.k12.ny.us/mcdonald/web.jpg



Food webs are generally very complex and as with the modelling of all complex systems conceptual simplification is usually necessary to describe and predict them. One way is to rank organisms into trophic levels. However this is complicated by the fact that some organisms feed across trophic boundaries; to account for this, those species are assigned an effective trophic level, which is a fractional number. Another way to assign a species status is to estimate the mean number of trophic links of the species to a basal species i.e. a primary producer. A further simplification is to study the energy flows of all the chains. Traditional methods of studying food webs and energy flows therein are difficult and extremely time consuming. From a practical perspective each predator-prey interaction requires detailed data of the dietary contribution and energy transfers, which are often impossible to obtain. Having painstakingly obtained such data, the information is aggregated into a single number, which is the fractional trophic level of the species.

You are what you eat.... plus a few per mil

Isotopic analysis of organisms in a food web provides information about trophic relationships "who eats whom" and will reflect what a particular organism has eaten. Animal tissues generally reflect the isotopic composition of the animal's diet plus a few per mil, as animal tissues are slightly enriched in heavier isotopes compared to diet. This is a not fully understood phenomenon, but is fairly ubiquitous in all organisms, terrestrial, aquatic, vertebrate and invertebrates. The isotopic shift from diet to consumer is generally constant among organisms and for a particular element. Although $^{13}C/^{12}C$ ratios have been used to study food web structure $^{15}N/^{14}N$ ratios are more suitable, as enrichment with trophic level is greater than with carbon.

11.3 Trophic enrichment.

Consumers are typically enriched by about 2.3 ± 0.18‰ $\Delta\delta^{15}N$ (mean ± SE) and 0.5 ± 0.13‰ $\Delta\delta^{13}C$ (mean ± SE) (where Δ denotes the change in isotope ratio between diet and consumer (McCutchan *et al*. 2003)). These are slightly different from the values reported earlier in the literature, $\Delta\delta^{15}N$ +3‰ and $\Delta\delta^{13}C$ +1‰, which were based on smaller data sets (DeNiro and Epstein 1978, 1980; O'Leary 1988).

$$TL = 1 + (\delta^{15}N_{Consumer} - \delta^{15}N_{Primary\ producr}) / 2.3$$

Where TL is the trophic level. Trophic enrichment factor 2.3 could be substituted for 3.0 depending on which reference one prefers to quote.

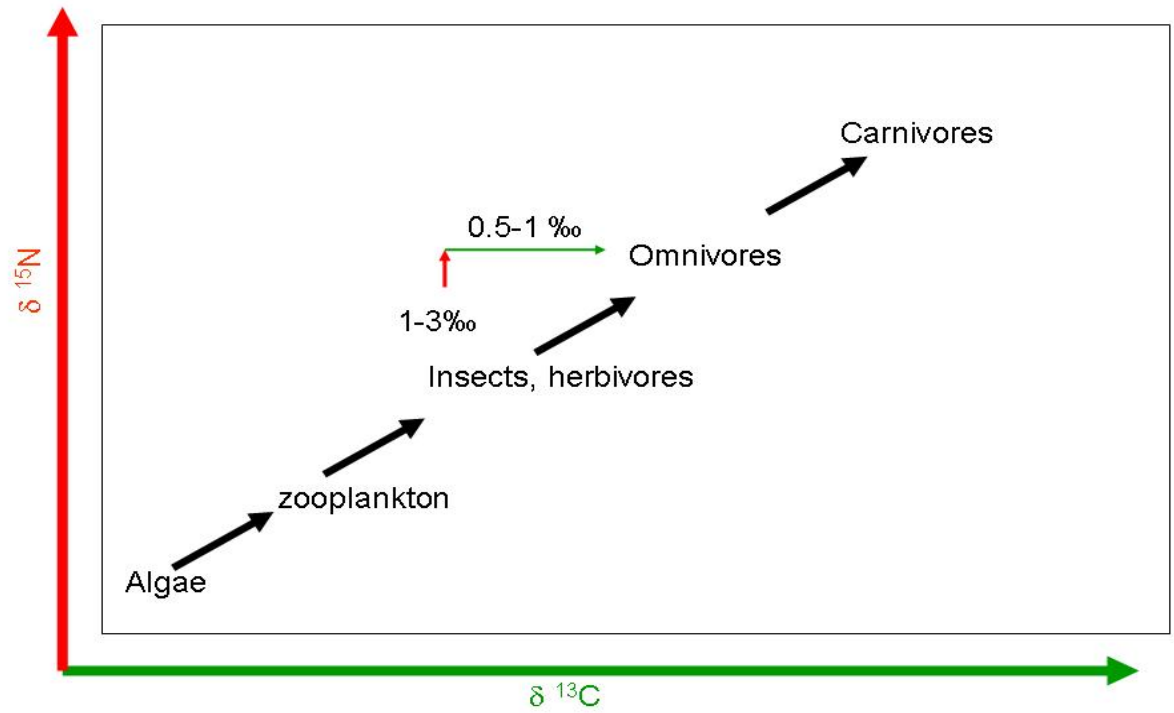
Trophic estimates and trophic position of consumers derived from isotopic data alone should be interpreted with some caution, as isotopic signatures of the consumers are a function of source mixing as well as trophic enrichment (Kwak and Zedler 1997). Observed isotopic shifts may also be the result of sample preparation.

For example, bulk samples may exhibit less isotopic shift than specific tissues such as muscle tissue (McCutchan *et al.* 2003).

Carnivores and fluid feeders may exhibit different isotopic shift patterns to consumers with plant or algal diets (McCutchan *et al.* 2003). Some studies have observed isotopic differences between starved and non-starved individuals and which may be identified using traditional techniques (Schmidt *et al.* 1997; Scrimgeour *et al.* 1995). Possible contributions from biological atmospheric nitrogen fixation in a system should be ruled out or accounted for, as these organisms will have an isotopic signature that tends towards that of atmospheric nitrogen (0‰ ^{15}N). Isotopic signatures can also be used to identify atmospheric nitrogen input into the food web as demonstrated by Tayasu *et al*. (1998) in their study of biological fixation of atmospheric nitrogen as a nitrogen source in termites. All the above factors should be taken into account when constructing isotopic food webs.

Isotopes of sulphur are also increasingly being used in a multi-isotope approach and trophic shifts in high protein diets have been shown to be around +2.0 ± 0.65‰ and in low protein diet -0.5 ± 0.56‰ (McCutchan *et al.* 2003; Bowen *et al.* 2005; Kwak and Zedler 1997; O'Leary 1988). ^{15}N enrichment increases predictably with trophic level and is the most commonly used food web construction.

Figure 30. Simplification of isotopic trophic shift.



Identifying primary producers in the food web is dependent on the ability to differentiate potential sources isotopically and may require the use of a multi-isotope or a multi-disciplinary approach.

Most studies conclude that $^{15}N/^{14}N$ ratios reflect trophic structure despite isotopic differences among species (Neilson *et al.* 1998; Ponsard and Arditi 2000). Using the technique slightly differently, isotopic food web analysis may be a useful way of studying the qualitative and quantitative shifts in food webs due to environmental or anthropogenic disturbances. Isotopic pyramids constructed before and after a disturbance event may prove useful in identifying keystone species and developing management strategies for protection (Caquet 2006).

Isotopically labelling primary consumers with enriched compounds can also be useful in food web studies as this approach may provide clearer pathway distinctions in complex systems (Section 9).

12. FOOD SOURCES

Specific insect-food source relationships are difficult to study but essential, especially in pest management or species conservation. Tracing energy flows from plants to insects, or insect to insect usually requires tedious methods such as direct observation, gut content analysis, pigment tracing or radioactive methods. Stable isotopes offer a safe, rapid, and direct technique to study feeding behaviour both in the laboratory and the field.

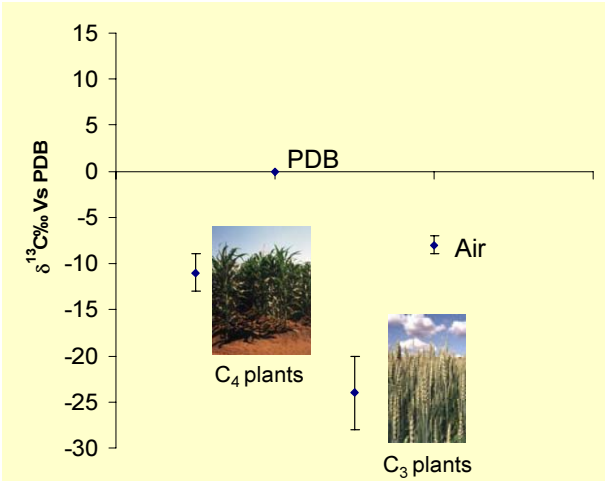
12.1 Natural abundance methods to determine food preference.

Natural abundance methods in which primary food sources have inherently different isotopic signatures such as C_3 and C_4 plants may be used to study food preferences in some insect species, however when there is no natural difference in isotopic signal, enrichment studies can be extremely useful.

Using a natural abundance approach, the food preferences of a variety of insects have been determined using the differences in isotopic signatures of C_4 and C_3 crop plants. Leaf hoppers (Cicadellidae) were shown to prefer the C_3 species with isotopic values in the range of the C_3 plant values, whilst lace bugs (Tingidae) and tortoise beetles (Chrysomelidae) had values which were indicative of feeding on C_4 plants (Petelle *et al.* 1979). Other innate isotopic differences occur between terrestrial and aquatic plants and between nitrogen fixing plants and non-fixing plants. These signals could also be useful to study food preference.

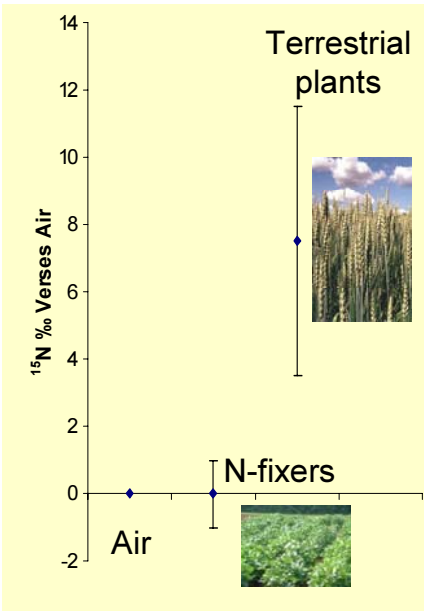
The beauty of using natural abundance techniques is that it is not necessary to alter the system studied in any way however it also requires a degree of luck in that there must be an isotopic difference in the food sources studied. Natural abundance techniques are limited as they are dependent on the presence of isotopic differences of food sources to determine feeding strategies.

Figure 31. Range of $\delta^{13}C$ values in terrestrial ecosystems.



Also there is a problem as often C_4 plants have a higher C:N ratios than C_3 plants which may lead to differential development rates. Labelling of primary producers or prey with enriched isotopes is an alternative method to study food preference. Almost identical labelled and unlabelled primary producers or compounds differing in key components can be grown or formulated

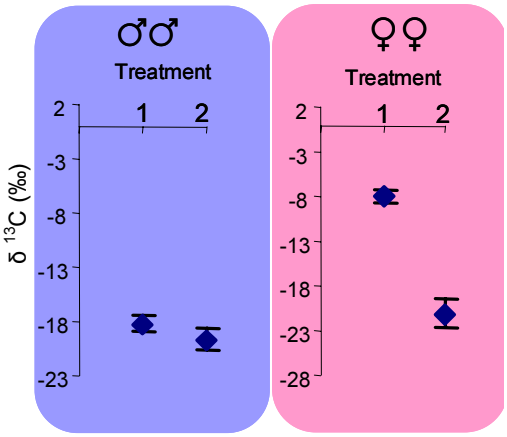
Figure 32. Range of $\delta^{15}N$ values in terrestrial ecosystems.



12.2 Food preference of mosquitoes.

Enrichment techniques have been used to study feeding preference of laboratory mosquitoes. Adult *Anopheles arabiensis*, were fed sugar diets supplemented with methyl-paraben (MP), an anti-microbial agent widely used in food and pharmaceutical products. In a simple two treatment experiment, mosquitoes were offered a choice of sugarwater and sugar water with MP. ^{13}C glucose was added as a tracer in Treatment 1, to the un-adulterated sugar and in Treatment 2 to the methyl-paraben amended sugar.

Figure 33. Isotopic composition of mosquitoes, 7 days after emergence, following sugar feeding.



Similar isotopic values of the mosquitoes in the two treatments would have inferred no preference, however higher enrichments were seen in mosquitoes in Treatment 1, suggesting there was significant aversion to the methyl-paraben amended sugar water, particularly from the females which are known to be more sensitive to odours.

It was possible to determine the amount of C derived from the labelled sugar water using the equation below. Using this data is was possible to calculate the amount of sugar water taken up when offered a choice of sugar waters or when forced to drink sugar water plus methyl paraben.

$$\%CLSW = \left(\frac{\delta^{13}C_c - \delta^{13}C_i}{\delta^{13}C_c - \delta^{13}C_A} \right) \times 100$$

Where:

$\delta^{13}C_i$ is the isotopic value of the sampled mosquito.

$\delta^{13}C_a$ the isotopic value expected if the mosquito only consumed labelled SW (29.6‰).

$\delta^{13}C_C$ the isotopic value of expected if the mosquito only consumed an unlabelled diet (-25‰).

Red is % total carbon in mosquito derived from MP amended sugar water when offered a choice.

Red is % total carbon in mosquito derived from MP amended sugar water when offered no choice.

Here the data show that when the mosquitoes are offered a choice of sugar water they prefer the control however when given no choice they derive up to 50% of their carbon after seven days.

Similar techniques could be used to study insect food preferences, after manipulation with pheromones or companion planting etc, in laboratory or semi field settings.

12.3 Plant - insect symbiosis.

Using the minute quantities and highly enriched forms of N, Fischer *et al.* (2003) studied the symbiotic relationship of ants (*Piper fibriulatum* and *P. obliquum*) and their host plant species in natural settings. In a pulse labelling experiment ants were fed with highly labelled ^{15}N glycine *in situ* and the label was traced into the ant colony. In further pulse chase experiments they followed the fate of nitrogen excreted by the ants and transferred back to the host plant species. These studies show how isotopes can answer specific questions in ecology.

Figure 34. Ants used in ^{15}N feeding studies.

12.4 Feeding strategy comparisons.

Natural abundance isotopic techniques may also be useful to determine differences in feeding strategies within species which exploit different ecological niches. For example Trimble and Sagers (2004) showed that *Azteca constructor* Emery ants were more opportunistic foragers of phylosphere fauna at lower elevations in Costa Rican forests whilst at higher elevations they relied more heavily on the resources supplied by their host plant species. In addition, specific contribution of orchid extrafloral nectars to ant diets have been calculated using isotopic techniques (Fisher *et al*. 1990). Stable isotope analysis can be a useful tool in assessing ecological and temporal dietary patterns of insects. However there are complications: 1) dietary components can be assimilated with different efficiencies, b) isotopic fractionation can alter values in tissues in relation to source, c) metabolic routing can disproportionately distribute source elements among different tissues.

13. DIET CONTRIBUTION AND ISOTOPE MIXING MODELS

An organism's isotopic composition will directly reflect what it has eaten, e.g. obligatory herbivore with a sole diet that has a signature of -26‰ will also have an isotopic signature of around -25‰, taking into consideration trophic shift. If an organism has a mixed diet with differential isotopic composition then it is possible to use mixing models to predict the percentage contribution from each dietary source. Mixing models are based on mass balance equations and therefore using the simplest forms it is usually only possible to determine n+1 sources for n isotopes. For example if data are available for two isotopes, say carbon and nitrogen, one could determine the contribution from three food sources, if the isotopic value of all three food sources are known and the isotopic value of the target organism is known. Mixing models are covered extensively in Section 6.1 onward.

13.1 Mixing models.

Mixing models are based on mass balance equations and are one of the most powerful tools in isotope science and used in areas as diverse as soil science and animal nutrition. These models are subject to a number of assumptions; primarily that the system is in isotopic equilibrium, secondly that the diet composition and isotopic values of the food resources are constant and finally that the isotopic turnover of consumer tissue is rapid and reflects temporal changes. If all these assumptions are fulfilled then using simple calculations it is possible to derive % contributions from specific diets be they enriched or natural abundance levels.

The greater the isotopic difference between diets the less uncertainty associated with the calculation of contribution. Care should be taken when the isotopic differences between two different food sources is small. McCutchan *et al.* (2003) demonstrated that calculated contribution from a specific source may differ by as much as 30% depending on whether a trophic shift of +0.3 or +1.1‰ is assumed in experiments where food sources differ by only 4‰.

13.2 Use of two source mixing model.

Akamatsu *et al*. (2004) used the inherent isotopic differences between aquatic and terrestrial diets of riparian spiders to study their diet composition. Due to the lower diffusion velocity of CO_2, carbon fractionation is relatively small in aquatic environments (Yoshioka 1997), thus aquatic plants usually have higher $\delta^{13}C$ values compared with terrestrial plant species. Using two-source mixing models the feeding preferences of the spiders were determined. The two-source mixing models determine the contribution of two different sources of substrate in this example the aquatic or terrestrial nature of the diet:

$$\delta^{13}C_{spider}-\Delta = \delta^{13}C_{aquatic\ insect} \times f + \delta^{13}C_{terrestrial\ insect} \times (1-f)$$

Where Δ is isotopic fractionation in C during the feeding process and f is the relative contribution of the respective insects to the diet. Using these techniques it was shown that more than 50% of the diet of web-building spiders consisted of aquatic insects.

13.3 Host identification.

Using multiple isotope ratios can provide useful information on specific insect host diets. Isotopic differences in plant species, are dependent on photosynthetic pathways e.g. C_3, C_4 differences or terrestrial versus aquatic, the way in which they obtain N, e.g. nitrogen fixer versus non-fixer and also marine versus terrestrial or their water uptake strategy, e.g. surface water versus ground water acquisition. This technique is most useful when there are a limited number of possible host species and there is an isotopic difference between possible host species.

Using the natural differences in C and N isotopic signals Markow *et al*. (2000) distinguished four different host plants of Drosophila, they were then able to isotopically segregate seven wild-caught Drosophila species, suggesting it would be a useful technique to study Drosophila resource ecology.

This technique may be particularly useful in understanding the usefulness of refuge plants for insect conservation in agricultural settings or feeding strategies of economically important insects.

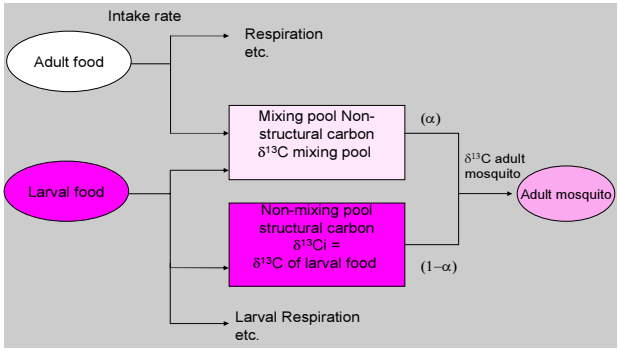
13.4 Resource turnover.

Diet switching experiments have been extensively used to study the temporal dynamics of different tissues especially in vertebrates but these techniques have also proved useful in insects. For example in a study of two predacious ladybeetles using aphids fed on C_3 soybean or C_4 maize the isotopic signature in the body fat and reproductive organs changed rapidly suggesting high metabolic rates as expected. However in the more metabolically inert tissues such as wings and cuticle, changes in the isotopic signature were slower (Gratton and Forbes 2006).

In insects with distinct larval stages structural tissue may reveal information about larval food sources and habitats, as evidence suggests that signatures from larval diets are “fixed” in the insect (Hood-Nowotny *et al.* 2006). For example European corn borers essentially retain their larval food $\delta^{13}C$ signatures in the wing tissues with only minimal variations (< 5%) owing to adult diets (Ponsard *et al*. 2004). This information could be used to trace back juvenile habitat of the insect and control or protect the site. It has been demonstrated for insects that grow from a nymph form and moult, e.g. locusts, that structural chitin turned over fastest of all components studied (Webb *et al*., 1998). Thus to use these techniques to study natal origin, metabolically stable tissues or body parts must be identified and turnover rates established through specifically designed laboratory experiments. These physiological differences stress the importance of species-specific complimentary isotope studies in establishing underlying mechanisms prior to field application of isotope techniques.

Bulk isotopic values of organisms raised on monotonous diets reflect the isotopic value of the diet whilst the bulk isotopic value of organisms utilising more than one dietary source are a weighted average of the isotopic values of the different sources. Isotopic ratios in the tissues of organisms are a consequence of food source signature and tissue-specific fractionation and turnover processes, and therefore can reveal substantial information about the diet of the individual (Chamberlain *et al.* 2004; Ostrom *et al.* 1997). Uptake and turnover rates of specific resources can be calculated if a number of organisms are initially uniformly labelled and the change in enrichment, due to dilution effects of the label from unlabelled sources and loss of label through excretion or respiration is monitored over time (Figure 35) (Hood-Nowotny *et al.* 2006; O'Brien *et al.* 2000).

Figure 35. Model of resource turnover in mosquito, where larval food source was labelled.

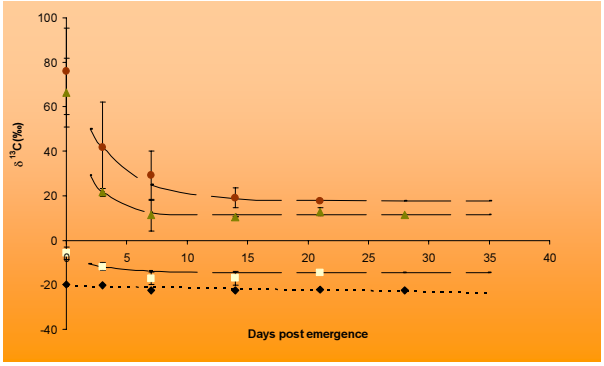


It has been established that turnover rates vary between body tissues (Tieszen *et al.* 1983) and that different tissues have distinctly different isotopic signatures relative to bulk values (Hobson and Clark 1992).

Isotopic ratios in the tissues of organisms are a consequence of food source signature and tissue-specific fractionation and turnover processes, and therefore can reveal substantial information about the diet of the individual. By switching the isotopic signature of the diet, it is possible to study the nutrient uptake of carbon, nitrogen, or sulphur, turnover, and assimilation in even the smallest of insects. The isotopic values are retained in an isotopic memory related to the Source values, growth, and the rate of turnover (Tieszen *et al.* 1983).

Examination of isotopic values of different tissues reveals the temporal history of diet intake, whereas metabolically active pools such as blood or haemolymph provide information on recently acquired food resources. Tissues that are more inert and do not turnover so rapidly through out the insect's life such as cuticle provide longer time integration of the feeding history, depending on the physiology of the insect.

Figure 36. Isotope signatures in mosquito, where larval food source was labelled.



14. MATING STUDIES

In some insect species, it is difficult to study mating behaviour and for SIT understanding the male's mating success in the field is critical (Ferguson *et al.* 2005). Techniques that label sperm (spermatozoa and accessory gland fluid) could vastly improve knowledge of mating behaviour. Although radioactive isotopes have been used in the past to study the fate of semen, by exposing young larvae to radioactive solutions and following the semen into spermathecae (Dame and Schmidt 1964; Tantaway *et al.* 1967; Smittle *et al.* 1969; Young and Downe 1978), current restrictions would preclude using these methods in the field. Other possibilities to study mating behaviour include the use of mutant strains, but these must be developed for each stock and there are social and ethical issues which need to be addressed prior to release of transgenic insects. Therefore stable isotopes could prove to be a simple and ubiquitous tool to study sperm transfer in insects.

14.1 Natural abundance methods.

Studies of mating behaviour using natural abundance techniques have been undertaken in insect species where the spermatophore is of sufficient mass as to be within the detection limits of the mass spectrometer system and there is a natural difference in isotopic signal. Ponsard *et al.* (2004) used the isotopic difference in the C_4 maize crop to the native C_3 host plant species to study sperm transfer and assortative mating in sympatric host races of the European corn borer. The authors tested a number of hypotheses in the laboratory, does adult wing isotopic signal reflect larval plant host signal, does adult feeding change the signal of the wing, does the egg mass signal reflect larval host signal and is the isotopic signature of the larval host retained in the spermatophore. They tested these hypothesis using diet switching experiment with C_3 and C_4 host plants and adult sugar diets. They showed that the isotopic signature of the wing tissue essentially reflects the larval diet and there is little influence of adult diet on this signature, they also showed that egg mass and spermatophores reflected larval diet signatures. These experiments were then used as the basis of a field campaign to determine whether there is mating between moths reared on different host species.

Moths retained the signature of their natal host, e.g. a moth grown on a C_3 weed retained a C_3 wing signal, but it was possible to trace (by analyzing the female spermatophore) whether she had been inseminated by a moth which had been reared on a maize C_4 host. This allowed them to study whether there was reproductive isolation based on host specificity (Malausa 2005).

14.2 Using natural abundance.

One of the difficulties in an SIT programme is the determination of the number of females a sterile male has inseminated, as egg counts and hatch rates are difficult to obtain in the field. One method which has potential is the use of the natural isotopic differences in the C_3 and C_4 plants to determine insemination rates in the field.

Most important insect pests targeted for SIT feed on C_3 plants which have an isotopic signature of around –28‰, therefore indigenous insects raised on these species will have an isotopic siganture of around –28‰. Artificial diets are often based around sugar as the primary carbon souce. It is possible to use C_4 cane sugar in these diets which gives the insects reared in the factory an isotopic value of around –10‰, although if the diet is not solely cane sugar this value could be a high as –19‰.

If the spermathecae or spermatophore of the female is large enough for direct of measurement using an elemental analyser

isotope ratio mass spectrometer system (approx 10 µg C) then it is possible to trace the proportion of the female population that has been inseminated by the sterile males, by determining the isotopic signature of the dissected spermathecae or spermatophore. If the isotopic value is around –28‰ it suggests the insect has been inseminated by native insects or not inseminated, and if the value tends towards the value of the factory fly then it suggests that it has been insemminated by a factory reared fly. Obviously laboratory based experiments would be required prior to using this technique on a large scale.

Using observed data from factory reared fruit flies, field collected flies and simple isotopic mixing models it is possible to make a calculation of the isotopic signature of the spermathecae and to see whether it is worth proceeding with testing the method.

Table 5. Modelled isotopic values of spermathecae.

Percentage of "spermathecae" C derived from male	Percentage of "spermathecae" C derived from female	Observed isotopic signature of factory reared males reared on C_4 based diet.	Observed isotopic signature of female in the field	Calculated isotopic value of spermathecae
20	80	-19	-28	-26.2
50	50	-19	-28	-23.5
80	20	-19	-28	-20.8

The calculations show that insemination should be detectable if the male contributes as little as 20% to the total carbon mass of the spermathecae or spermatophore .

Dissecting spermathecae or spermatophores can be done under a standard dissection microscope using a small volume of carbon free buffer. The spermathecae can then be brushed onto a glass slide to check for insemination and then on to a small glass fibre or quartz filter disc, which is then put in a tin cup and dried at 50°C for 24 hours prior to isotopic analysis. This can also be done with frozen insects.

14.3 Enrichment studies.

Given that, in most species there is not a neat C3, C4 dietary difference, the addition of labelled isotopes maybe an alternative. This approach maybe particularly useful for SIT, as target insects are usually reared under controlled conditions which would allow isotopic manipulation of the diet.

The principle of the technique is that the males are labelled with an isotope so that it is possible to trace the sperm in the female spermathecae. The spermathecae are dissected and examined to determine whether insemination has occurred and subsequently the spermathecae is analysed for isotopic ratio. If the sensitivity of the IRMS set up is such that the small sample size of the spermathecae precludes direct measurement, a spiking approach in which a known standard of known enrichment can be added to the sample. Using this approach it is possible to detect insemination in extremely small insects such as mosquitoes.

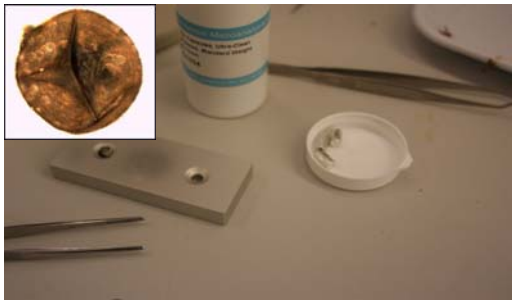
Most IRMS systems can easily determine differences of about 1.0‰. However a target difference of 5‰ (or two standard deviations of the control) between inseminated and un-inseminated individuals would lead to more robust conclusions. Based on these values and whether a spike would be used or not it is possible to calculate the amount of label required to be able to trace sperm in the female spermathecae.

14. 4 Semen transfer in mosquitoes.

In experiments conducted with mosquitoes, sufficient labelling was achieved by adding 0.25 g of 99 atom % ^{13}C glucose as a solution per tray (30 x 40 cm) to one litre of deionised water. The amount of ^{13}C-glucose added was based on the total amount of carbon in the diet (a commercial fish food AquariCare Koi Floating blend USA) which was 40% C.

Up until pupation 1.0 g of food was added per tray (0.25 mg x 500 larvae x 8 days). As the carbon in the glucose was also 40%, 0.25 g of 99 atom % sucrose was added to give a target enrichment of the male mosquitoes of approximately 20 atom %. This resulted in sufficient labelling in the spermathecae. The spermathecae were captured using a very thin paint brush on to fibre glass discs (produced using a standard office hole-punch from fibre glass filter papers) and placed in standard tin cups. Due to the small sample size of the spermathecae the samples were then spiked with unlabelled sucrose solution which contained 25 µg carbon pipetted onto the disc. The samples were then dried and the tin cups closed and analysed for isotopic ratios using IRMS. Using this technique it would be possible to study the mating effectiveness of sterile versus native males in a semi field setting. Using a multiple isotope approach it would also be possible to study multiple mating scenarios etc.

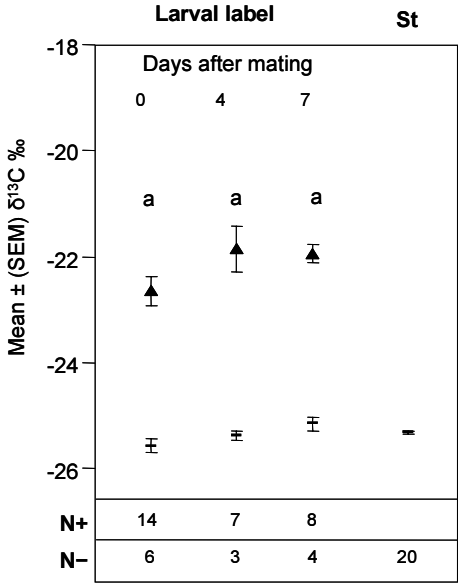
Figure 37 Tins cups for sample collection and close up of spermathecae.



There were no detrimental effects to the mosquitoes of adding glucose to the larval trays at these concentrations; this was determined by longevity and physiological experiments.

The experiments showed that in mosquitoes label added at larval stage was the most effective means of sperm labelling. Experiments also showed that in larval labelled systems the amount of label transferred was effectively the same in young or 7 day old mosquitoes, which are important considerations when considering the practical use of the technique.

Figure 38. $\delta^{13}C$ ‰values of inseminated (triangles) and un-inseminated (bold line) spermathecae of females mated with labelled males (20 APE). Females were isolated for 0, 4, or 7 days after mating to test the persistence of the label in the spermathecae.



This technique could also be undertaken using the isotopes of N (Helinski *et al.* 2008) or O and H, or using a multiple isotope approach. However initial experiments to determine the limits of the experimental set up similar to the one above must be conducted. N would be the obvious choice for multiple mating experiments as it is cheap and well conserved in the life span of most insects.

It should also be stressed that the spermathecae of mosquitoes are extremely small, this example shows what could be achieved Most agriculturally important insects will have larger spermathecae which would allow for easier dissection and direct measurement using mass spectrometry, i.e. without the use of a spike. This would probably allow for the use of low level labelling or natural abundance differences in diet described earlier in Section 14.2 to be used as the basis for detection of sperm transfer. For further details see Helinski *et al.* (2007).

15. STABLE ISOTOPES IN INSECT BIOCHEMISTRY AND PHYSIOLOGY

Studying the biochemistry of insects can be extremely difficult given their small size and the detection limits of the analysis equipment. Often samples must be bulked together to get enough material to analyse and this results in greater measurement uncertainty. Using conventional techniques to measure concentrations of compounds gives us an instantaneous net measurement of a biochemical, but tells us little about the flux and dynamic nature of the biochemistry within the insect. Stable isotopes however can reveal the gross fluctuations of compounds in insect biochemistry and can be used to look at biochemical turnover, production and assimilation pathways.

To study the isotope-biochemistry of insects compound-specific studies are required, these differ from bulk analysis which basically analyses the entire insect. The isotopic ratio of a compound reflects the chemical and physical processes of synthesis and degradation, and the source of the elements. In compound-specific studies the compound of interest is isolated and the isotope ratio of the compound measured. This can be done off-line using modified conventional purification procedures followed by isotope ratio analysis of purified samples with an elemental analyser. It can also be done on-line following some degree of isolation and derivatisation using either gas chromatography combustion isotope ratio mass spectrometry systems (GC/C/IRMS) or High Performance Liquid Chromatography systems (HPLC) linked to an isotope ratio mass spectrometer (irm-LC/MS). GC/C/IRMS systems are useful for complex mixtures of compounds that can be vaporized easily; the peaks coming off the GC can be either combusted or pyrolysed to form gaseous forms that can then be bled into the mass spectrometer for isotopic analysis of C, N, H or O.

The irm-LC/MS systems are more useful for compounds of high molecular weight and that have greater polarity. One big disadvantage of the irm-LC/MS systems described here is that it can only analyse carbon isotopes, however future developments in post HPLC preparation may eventually allow for N isotopes to be measured. Carbohydrates and amino acids exhibit good separation and appear to yield consistent isotopic ratios in the nano-gram range using irm-LC/MS systems.

One of the main advantages of using on-line compound-specific methods is the significantly smaller sample size required for measurement. The system of analysis will depend on the target compound and physical and time restraints of sample preparation. Many compounds can not be analysed directly by GC/C/IRMS but required chemical manipulation to yield GC amenable components. Phospholipids are saponified and methylated to generate fatty acid methyl esters (FAMEs). Solvent unextractable bio-polymers such as carbohydrates, proteins, aliphatic bio-macromolecules eg cutin must be chemically or pyrolytically cleaved prior to GC/C/IRMS. All manipulation reactions must convert all substrate to product as incomplete reactions result in kinetic isotope effects (see Table 6 for common derivatisation procedures). The development of the irm-LC/MS systems has overcome a number of these analytical constraints and can be used in a complimentary manner with the GC/C/MS systems if available.

It should be noted that compound-specific isotope analyses are time intensive and instrument intensive methodologies. For example isotopic analysis times for sugars using irm-LC/MS systems can be up to one hour per sample.

Compound-specific studies can yield information about a number of aspects of insect biochemistry, biology and ecology. Some examples are given below.

15.1 Tissue turnover rates.

Turnover times of a tissue or biochemical compound can be calculated by tracking the isotopic composition following the transition from a diet of one isotopic composition to a diet of another isotopic composition. For carbon C_3 and C_4 plant sources are generally used as the two diet sources, and turnover times calculated from the change in isotopic composition after the switch from C_3 diet to C_4 diet or vice versa. However it is also possible to use micro-enriched materials, which avoid the problems of the different plant quality characteristics of C_3 versus C_4 plants. The turnover times of insect tissues or biochemical compounds could provide valuable information on how food reserves are used and converted. For example how quickly haemolymph trehalose levels are replenished following flight.

The isotopic dynamics of a biochemical pool will be dependent on initial isotope ratio and size of the pool, quantity and isotopic composition of substrate entering and product leaving the pool. There is a wealth of information to be gained using these techniques which could significantly enhance our understanding of insect resource utilisation and routing.

15.2 Temporal source distinctions.

Particular body parts or biochemical compounds can provide information on the source or origin of a particular element. Isotopic differences in the landscape due to environmental and biological processes are captured in certain tissues and may allow us to trace back the natal origin of the insect. These signals would be lost in bulk analysis but compound specific studies give a better resolution and act as a recorder of past environments the insect has drawn its nutritional resources from. For example, chitin in insects which have a distinct juvenile stage (holometabolous insects) is formed in the juvenile stage and turns over slowly in adults. The isotopic composition of this chitin can provide information on the breeding grounds of such insects, in mosquitoes it could tell us whether insects are breeding in ephemeral sites or permanent larger water bodies as the two have distinctly different isotopic compositions.

Because different tissues have different turnover times they will provide information on different temporal scales, tissues which turnover quickly will provide information on the recent diet which an insect has exploited. This again provides greater resolution and understanding of the insect biology. Adult insect diets may differ significantly from juvenile diets. Using a compound-specific approach for example sampling the haemolymph, a pool which generally turns over rapidly, may allow us to determine the source of the adult diet as distinct from the juvenile diet and given this knowledge may allow us to manage the insect appropriately either by protection or by eradication of the food source. For example for a rare butterfly species we may want to determine the adult dietary source and protect it. However for adult male mosquitoes identifying and destroying an adult sugar source may play a role in mosquito suppression management.

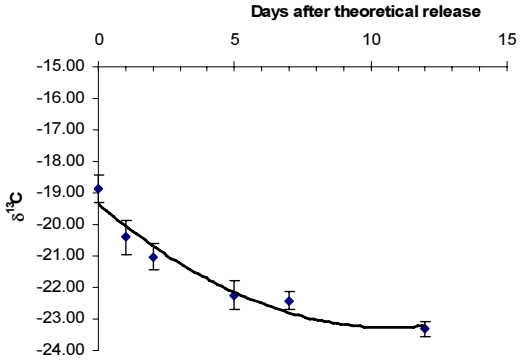
15.3 Stable isotope clock.

If turnover rates of a tissue are known and predictable, and there are isotopic differences between immature and adult feeding stages for example then the isotopic composition of a tissue or biochemical could act as an “isotopic clock”. As the adult assimilates the adult food source the isotopic value of the insect will tend towards the isotopic value of

the adult diet. Assuming rates of adult food assimilation and uptake are generally constant, i.e. if food is in plentiful supply and the insect is eating from a known single source it is possible to calculate the age of the insect based on modelled rates of tissue turnover.

This may be an extremely useful method to determine the age class of insects. Specifically it could be a very useful technique in SIT research of individuals where it is possible to feed a C_4 factory diet as opposed to the native C_3 diet. If it assumed that the flies feed in a similar manner to which they are feed in the laboratory, it is possible to track the decline in the isotopic composition of the flies and use this to determine their age based on predetermined isotope decline curves.

Figure 39. Decline in ^{13}C signal in fruit fly with switch in diet from C_4 to C_3 source.



15.4 Biochemical pathways.

Different body tissues have different turnover rates and different tissues may also exhibit distinct isotopic values relative to the bulk values. This is also true of different biochemical classes; this is because in biological reactions the fractionation is driven by the enzymatic reactions. There are specific points in metabolic pathways; these points are enzymatic steps (branch points) where isotopic discrimination against heavier isotopes occurs. Fractionation is greatest when the isotopic mass differential is more pronounced for example in enzymatic reactions involving smaller molecules such as decarboxylation or deamination.

Determination of isotopic branch points depends on both the amount of discrimination against the heavier isotope and the amount of element moving through the pathway.

These enzymatic steps can result in specific fractionation or discrimination values for a particular process. It is possible to exploit these discrimination pathways to reveal biochemical information at a molecular or compound specific level. Isotopes can indicate whether a compound has been directly assimilated or there has been *de-novo* synthesis. Generally compounds which are directly assimilated, e.g. some essential fatty acids, generally reflect their source isotopic value closely and do not show fractionation, however non-essential compounds which are synthesised *de-novo* show distinctive isotopic shifts from diet to product. Measuring isotopic composition of a specific fatty acid can track these biochemical pathways.

These studies can lead to a better understanding of the biochemistry of an organism, especially those that are almost impossible to study using conventional observation and feeding studies such as Collembola (Chamberlain *et al.* 2004).

Using switching-type experiments, it is also possible to distinguish between assimilation and synthesis pathways. For example, if all lipids are produced by *de novo* synthesis, a similar value $\delta^{13}C$ of all lipids would be expected as they are all derived from the common acetyl co-enzyme A; any deviation from this value would suggest uptake as opposed to synthesis (Chamberlain *et al.* 2004). A similar principle has also been used to study defence compounds in chrysomelild beetles (Soe *et al*. 2004) and sex pheromone biosynthesis in moths (Bjostad and Roelofs 1986).

15.5 Diets.

Another way in which these compound specific studies may be useful is in the formulation of artificial diets. By analysing and distinguishing between directly assimilated and synthesised compounds using the isotopic shift information it is possible to determine the probably essential dietary compounds such as specific fatty acids, necessary for a high productivity artificial diet.

Compound specific studies may also be used to determine dietary preferences in an ecological setting. Fatty acids derived from the diet may be identified in the consumers as some fatty acids are directly assimilated as intact molecules in significant concentrations. If there is sufficient isotopic difference between consumer and prey it is simple to reconstruct the diet.

16. LABELLING OF PRIMARY PRODUCERS

16.1 Production of ^{15}N labelled material.

Growing plants in soil with ^{15}N fertiliser is the simplest way of labelling plants or primary consumer material. This can be done *in situ* in the field or in pots in the greenhouse. ^{15}N fertiliser is usually applied to soil in water and is taken up by the plants almost immediately. (Nienstedt and Poehling 2000; 2004).

For insect marking purposes uniformity of the label in the plant is not particularly important and it may be possible to apply a single dose of ^{15}N fertilizer about one week prior to introduction of the insect, but this will require testing. Using a paper hole punch to sample plant leaf material is an excellent way of non-destructive sampling of the plant material, about 2-3 discs should be sufficient for analysis, these can be dried in the tin cups at 60ºC and analysed in the normal way. Transport tissues and newly formed structures will always have higher enrichment than older tissues that do not turnover so quickly.

If one is to study the insect/host interactions or resource turnover the plant material should be evenly labelled and therefore ^{15}N tracer should be applied regularly throughout the growing period of the plant. It is best to calculate a total fertilizer requirement and to calculate the appropriate bi-weekly doses, then to make up solution accordingly and store it in the fridge.

The target enrichment required in the insect should be considered when designing such production systems, by working back using an approximate value of 50% fertiliser efficiency (average for a normal soil). Some rough figures for N application are given below.

Labelled fertiliser is widely available in a variety of formulations, the one selected will depend on experimental design, cost and availability; ammonium sulphate, urea, and potassium nitrate are some of the most common forms of agricultural fertilisers. The required amount of fertiliser to be added depends on the fertiliser formulation used, this can be calculated from the proportion of N; an example for KNO_3 is given.

Molar mass of compound/mass of N X required application rate = mass of compound required.

For example 50 mg N kg^{-1} soil is required for 5 kg pots of soil

101/14 *50 = 360.7 mg of KNO_3 required per kg soil.

Multiplied by the amount of soil
360.7 *5 = 1803.5 mg KNO_3 or 1.8 g.

If this were to be applied to a plant for 10 weeks to get uniform labelling then 20 doses would be required, For a 5 kg pot 50 ml solution per application, giving a total solution of 1 litre. If large quantities of plant material are required growing plants in sand culture augmented with ^{15}N nutrient solution minimises wastage of ^{15}N, but requires more work to make up appropriate nutrient solutions.

When labelling biological nitrogen fixing crops (such as peas beans etc.) the percentage nitrogen fixed should be accounted for to obtain final target enrichment of the plant. N fixers can derive up to 80% of their N from the atmosphere thus causing a dilution of about 5:1 of the applied label.

Table 6. Application rates of N required.

N application rates mg N /kg soil	Fertilizer enrichment (% ^{15}N atom excess)	Predicted delta enrichment of plant material (‰)
100	0.5	680
50	0.5	350
10	5.0	680
10	10	1400
N fixer 10	10	280

Field experiments

For field experiments a figure of 2,000,000 kg soil per hectare (10,000 m^2) is a good working figure to calculate fertilizer requirement. Each m^2 of soil is approximately 200 kg of soil to plough depth. If four micro plots each of 1 m^2 are required then following using the figure above for KNO_3 application, 360.7 * 200 * 4.

16.2 Leaf or petiole labelling using ^{15}N.

Labelling with ^{15}N via leaf or petiole feeding is a technique that has been used for a number of years in plant physiology experiments (McNeill *et al.* 1998). This method achieves isotope labelling of shoots and roots *in situ* however the amount of ^{15}N solution taken up by the plants can be 1.5-2.5 times lower than with the stem feeding technique. The technique is simple and easy to use in the field where a large number of plants need to be labelled. Leaf or petiole-labelling can also be used to label roots to look at pest infestations.The principle is that a portion of the leaf or petiole is submerged in a solution containing ^{15}N (e.g. as urea), the solution is taken into the transpiration stream and the ^{15}N is assimilated by passive and active N uptake processes.

Apparatus

- Putty, plasticene (e.g. Terostat or Blutak)
- 0.5 or 1 mL micro pipette
- Sticky tape
- Scissors
- Aluminium foil and paper towels.
- Petiole feeding
- Eppendorf tubes
- Leaf feeding
- Tissues or paper towels
- Corner cut from plastic bags or leaf size plastic bags made using heat sealer
- Short sticks

Reagents

- Normal unlabelled urea to assess plant N tolerance.
- 1-2 mL of highly enriched (95-98% atom % 15N excess) urea solution at concentrations of 0.5% (w/w) is sufficient per plant.
- A single leaf feed (2 mL) in pasture plants resulted in clean root enrichment of 0.7858 atom % 15N excess.

Note: It is best to assess the tolerance of the plants to be labelled and test the method using unlabelled urea solutions, as high N concentrations can cause necrosis and death of the plants. This is done using the same procedures as for ^{15}N labelling. It is always best to add no more than about 5% of the total plant N as urea. If there are no spare plants available to test for tolerance then 1 mL of 0.5% (w/w) solution should not cause any problems in most plant species that are older than a few weeks. The practical constraints and aim of the experiment will determine the time and frequency of feeding, generally young plants take up solution more rapidly and apportion more to the root systems than older plants.

Procedure

- Make up required amount of ^{15}N solution in a volumetric flask with distilled water.
- Ensure that the plant growing conditions are good and that transpiration is maximum i.e. make sure the plants are well watered and feeding is carried out at the beginning of a photoperiod.
- Cover the soil with layer of aluminium/tin foil then paper towel (to absorb any spilt ^{15}N).
- Have solutions, pipettes eppendorfs and

Petiole/leaf tip labelling

- Fill the plastic eppendorf with required amount of ^{15}N solution (usually 1-1.5 mL) have it at the ready.

- Find the youngest newly fully expanded leaf.
- Cut the leaf tip off under water (top 1-2 mm in wheat, or tips of all the leaflets in a compound leaf such as clover) (if you are quick enough it is not necessary to cut the tip under water especially if you have many samples to do).
- Insert the cut tip(s) into the eppendorf and seal the petiole to the edge of the eppendorf with the putty, then securely place the eppendorf in the soil or on to a supporting stick (with sticky tape).
- Wash your hands and then remove the tin foil and tissue avoiding contact with the plant or soil.
- Leave the plants to take up solution (this can be 24 hours under high temperature and light intensity up to 3-4 days. It is ideal that all the solution added is taken up from each vial.
- When you remove the feed it is advisable to remove the leaf also as it may be highly labelled due to contact with the solution.

Leaf labelling

- Find the youngest newly fully expanded leaf.
- Wrap the leaf in a small amount of paper towel or tissue.
- Put the wrapped leaf still attached to the plant in the plastic bag.
- Using the pipette add the required amount of ^{15}N solution usually about 1 mL.
- Close the bag securely with sticky tape and support it with a stick so that no ^{15}N solution can escape.
- Wash your hands and then remove the tin foil and tissue avoiding contact with the plant or soil
- Leave labelling set up on the plant for one week.

Figure 40. Leaf labelling.

- Remove leaf and labelling device.

Note: although this method is quick and maybe more suitable for the field, it is not as quantitative as the other method, since it is relatively difficult to assess how much of the ^{15}N solution is left in the bag and on the leaf. The entire leaf set up can be shaken in 50 mL of water or 1M KCl to measure the urea and ammonium concentrations but this takes time.

16.3 Stem labelling using ^{15}N.

Stem labelling with ^{15}N urea solution achieves isotope labelling of shoots and roots in situ (Russell and Fillery 1996). The technique is particularly useful in greenhouse experiments however it is not so easy to do in the field. In field situations leaf labelling or petiole feeding may be more appropriate.

The principle is that a wick passed through the stem of the plant is then immersed in a solution labelled with ^{15}N. The ^{15}N solution is drawn up with the transpiration stream and it is assumed that the urea is assimilated normally in the plant. Urea is generally used as it is a non-polar un-dissociated molecule with a low salt index and a high concentration of N that is readily metabolised by plants that have the urease enzyme.

Apparatus

- Sewing needle.
- Micro drill – dentist or hobby drill with drill bits of 0.25-0.5 mm diameter (for plants which the needle does not pass through the stem easily alone).
- Thick cotton thread (embroidery thread which is 100% cotton is ideal).
- Flexible plastic tubing (thin PVC pipes from continuous flow apparatus are ideal) cut into about 2-3 cm pieces.
- Plasticene or putty (Terosat, Blutack).
- Small plastic vials with push-on or screw tops (drilled with three small holes of 2-3 mm diameter).
- 0.5 or 1 mL micro pipette.
- Aluminium foil and paper towels.
- Scissors.

Reagents

- Normal unlabelled urea to assess plant N tolerance.
- Usually per plant 1-4 mL of highly enriched (95-98% atom % 15N excess) urea solution at concentrations of 0.5% (w/w) is used, however solutions of up to 4% have been used for lupines.

Procedure

The practical constraints and aim of the experiment will determine the time and frequency of feeding, generally young plants take up solution more rapidly and apportion more to the root systems than older plants. A single stem feed (1 mL) of young plants in the greenhouse enriched the clean root systems of lupin, faba bean and field pea between 0.2294–1.3613 atom % ^{15}N excess.

Figure 41. Stem labelling.

- Make up the stem feeding vials, pass two pieces of plastic tubing through the two adjacent holes in the top of the vial.
- Make up required amount of ^{15}N solution in a volumetric flask with distilled water.
- Ensure that the plant growing conditions are good and that transpiration is maximum i.e. make sure the plants are well watered and that injection is carried out prior to a photoperiod or early in the morning.
- Cover the soil with a layer of aluminium foil then paper towel (this will absorb any spilt ^{15}N).
- Have solutions, pipettes and putty material ready as this next stage should be carried out as quickly as possible to prevent sealing of the xylem vessels.
- Pass the needle threaded with about 20 cm of with cotton through one of the pieces of plastic tubing.
- For thick stems, drill the stem with a dentist drill 1-3 cm from the soil surface.
- Pass the needle threaded with cotton through the plant stem then through the second piece of plastic tubing.
- Tie a knot at the bottom of the two tubes so that the tubing fits tightly against the stem and cut the cotton one or two cm after the knot so that the cotton reaches the bottom

- Screw or push the vial on to the top, (NB if screwing the vial on keep the top stationary).
- Add the ^{15}N solution to the vial through the 3rd hole using the pipette (note the volume of ^{15}N solution added).
- Seal the hole with the putty.
- Seal the joints between the plant and the plastic tubing with the putty.
- Cover the vial with aluminium foil to avoid condensation.
- Wash your hands and then remove the tin foil and tissue avoiding contact with the plant or soil.
- Leave the plants to take up solution for 3-4 days. It is ideal that all the solution added is taken up from each vial.

16.4 Labelling of fruits or leaves using tree injection

The tree injection technique is a relatively recent advance in ^{15}N methodology it has evolved due to the problems of labelling trees using the conventional fertilising of soil approach; it could be useful to label specific fruit bearing branches of trees or roots of trees. Basically labelled nitrogen is injected in to the active xylem of a growing tree, there then follows a period equilibration of the label. This method allows large quantities of plant material to be labelled relatively inexpensively; it also allows studies of below ground processes and could be used for investigations of soil dwelling insects (Ruess *et al.* 2004; Scheu and Falca 2000).

^{15}N is applied directly into the xylem stream.

The distribution of ^{15}N in the plant is determined by sampling through out the canopy. This technique can be used to label fruits and leaves and roots of trees. A hole is drilled in the tree and ^{15}N solution is injected into an active transpiration stream.

Suitable trees:

Trees with ring porous xylem structure are the only trees suitable for this procedure. That is, trees with a xylem that is evenly distributed through out the stem and not only on the surface. The location of the xylem can be determined easily. Cut an active fresh stem and place it in a shallow solution of Fuchsia basic for several hours. After some time has elapsed, cut the stem and examine the cross section, from this it is easy to determine where the water moves in the stem from the stain of the dye. If the xylem is only on the surface of the tree this technique will not work so well as if the xylem is evenly spaced through out the stem.

Procedure

- Prepare the pipe system.
- Cut the 2 ml syringe at approximate 2 cm and connect with a suba seal.
- Connect the pipe on one end with the body of the 50 ml syringe and on the other end with the tube connector and the needle.
- Attach the injection system to the tree with tape and flush through with sap solution (NOTE THIS IS NOT LABELLED WITH ^{15}N).
- Fill another syringe with unlabelled sap solution. (5.0 mM KCl and 4 mM malic acide adjusted to pH 5.4 with KOH or NaOH solution and autoclave at 120 °C for 20 mins).
- Measure width of the stem of the tree.
- Label wood drill at 2/3 of the diameter of the tree with a small piece of tape.
- Drill a hole (2/3 of tree diameter).
- Immediately after the hole is drilled connect the suba seal connector.
- Put a second needle in the suba seal.
- Cover the soil with paper to avoid liquid dropping onto the soil.
- Flush the system using the filled syringe with sap solution to expel all the air.

- This should lead to the solution coming out of the second needle.
- Refill the 50 ml syringe with a small amount of artificial sap solution and place the end of the pipe higher than the liquid level to avoid dripping.
- Remove the syringe body and connect the needle with the pipe system 2.
- Remove the second needle.
- Mark the level of the sap solution to determine the uptake of solution.
- Cover the open syringe with parafilm.

Figure 42. Tree injection device.

- once you have determined that there is uptake of the sap solution add the labelled ^{15}N solution to the syringe, and again keep topping up with the ^{15}N solution until you have injected the desired amount (10-100 ml)
- Keep topping up the syringe with artificial sap solution to ensure the ^{15}N gets into the tree and keep doing this until uptake stops, if there is substantial uptake it may be best to take a plastic bottle and insert the end of the tube into this. This uptake of sap solution can be as much as two litres.

Notes:

Avoid spilling the ^{15}N solution on the soil by covering with plastic or paper.

Wash the pipe system in 500 ml KCl solution when no more liquid is taken up by the plant and add 1 ml of 0.1 M HCL and back titrate with an indicator and 0.01M sodium hydroxide to determine how much ammonium wasn't taken up. In this way one can determine the total N taken up by the tree. Alternatively repeatedly add a small amount of unlabelled sap solution to the syringe and chase the label into the tree.

It is important for assessment of belowground N that contamination from falling leaves is prevented. This can be done using leaf traps or making the assessments prior to any senescence event.

16.5 Labelling using other stable isotopes.

It is also possible to use ^{13}C labelled urea in the sap solution or leaf labelling solution to achieve micro enriched plant material.

In addition it may be possible to label with enriched ^{18}O or deuteriated water by replacing the urea solution with the labelled water. Initially it would ensure that only the plant is labelled although there would be some exchange with soil water and atmospheric water after some time.

16.6 Labelling plants with ^{13}C.

A simple method for uniform labelling of plant material using a pulse labelling technique is presented.

Apparatus

A chamber can be made from Perspex or based on a rigid frame covered in CO_2 impermeable plastic held together with CO_2 impermeable sticky tape. Dimensions are usually tailored to the size of the plants in the experiment, it must be as gas impermeable as possible, unless a flow through system is intended.

Since the objective is only to label the plants and that the labelled CO_2 is usually taken up by the plants within the course of hours, it is not necessary to take time to ensure the chamber is completely leak tight. If tubing is to be used ensure it is CO_2 impermeable.

A computer fan can be used in larger chambers to facilitate circulation of the gases and avoid over heating.

Additional lighting maybe required when cloudy or in short daylight hours. Lights such as mercury vapour lamps should be used above the chamber.

The CO_2 concentration inside the chamber can be monitored by an infra red gas analyser (ADC Type 225 Mk3 CO_2 analyser or similar) but is not entirely necessary.

Figure 43. ^{13}C labelling chamber.

Labelling in the field is possible if the chamber has a suitable flange that allows for the chamber to be driven in to the soil by about 10 cm.

To avoid excessive $^{13}CO_2$ losses it is best to well water the soil prior to labelling and ensure bicarbonate mixing port or gas injection port is near the top of the chamber so that only minimal amounts of CO_2 reach the soil surface.

For labelling branches or particular fruits a CO_2 impermeable plastic bag is sealed around the branch using a combination of tape and terostat/bluetak and $^{13}CO_2$ gas injected via a “suba seal” in to the bag.

Labelled CO_2 gas can be purchased or generated from labelled sodium bicarbonate stored in evacuated vacutainers (to avoid over pressure) by the addition of lactic acid. The labelled gas is then drawn out of the vacutainer using a syringe and added to the gas tight bag as necessary.

Reagents

- 7.5 mol L^{-1} lactic acid
- 95-99 atom % ^{13}C labelled sodium bicarbonate solution 0.5 mol L^{-1}.
- Un-labelled sodium bicarbonate solution, 0.5 mol L^{-1}

Procedure

- The $^{13}CO_2$ is generated from the reaction of $NaH^{13}CO_3$ with lactic acid. A reservoir of lactic acid is taped on to the inside of the chamber. Sodium bicarbonate solution is injected through a rubber “suba seal” into the reservoir. This allows for repeated additions of labelled and unlabelled bicarbonate solutions.
- The pulses of labelled CO_2 should be administered when photosynthetic activity is expected to be highest between 9.00 and 10.00 am. Plants should be well watered prior to sealing of the chamber.
- Seal the chamber for up to one hour prior to addition of label. This should allow the CO_2 concentration inside the chamber drop from about 350 ppm to 300 ppm before the labelled CO_2 pulse is introduced.
- After 2-3 hours following the introduction of the labelled CO_2 when the CO_2 concentration has declined to approximately 180 ppm, add unlabelled sodium bicarbonate solution to flush in the label.
- Sequential $^{12}CO_2$ pulsing should be continued a number of times.
- Artificial lighting can be used, to maximize the uptake of the labelled CO_2 remaining in the chamber.

- Depending on the objectives of the experiment the chamber can be closed overnight to contain any labelled CO_2 released during respiration and additional pulses of $^{12}CO_2$ can be administered the next day before opening the chamber to expose the plants to natural conditions.
- Alternatively, if for example the objective of the experiment is to look at phloem feeding it may be appropriate to remove the chamber and conduct the experiment immediately after labelling.

If the labelled material is to be used for resource assimilation or feeding experiments in insects it is recommended that the plant material be uniformly labelled, this is achieved by repeatedly pulse labelling or continuous labelling in flow through systems with special $^{13}CO_2$ labelled air mixtures available from specialist suppliers.

16.7 ^{13}C and ^{15}N duel labelling.

^{13}C and ^{15}N duel labelling may be useful. This can be achieved by either growing plants in ^{15}N labelled medium and either pulse labelling with $^{13}CO_2$ or continuous labelling with $^{13}CO_2$

16.8 Notes on plant labelling.

As the procedures described are intended ultimately to study insect physiology rather than plant physiology, concentrations of gases etc can be estimated by calculation and although useful for repeating experiments detailed information on the gas concentrations over time etc are not of utmost importance and therefore complex gas measuring equipment is not necessary. The final isotopic enrichment of the relevant plant material used in the experiment is of greater importance in these types of studies. Production and sampling of plant material will depend on the experimental aims of the study.

It must be considered that following a labelling event the plant will continue to take up the un-labelled resource therefore the enrichment of the label will usually decline or be diluted, for example after stem labelling with ^{15}N the plant will continue to take up unlabelled soil N and this will dilute the initial ^{15}N.

For example if one is interested in studying the resource turnover of phloem sap in insects then it is important that a value for phloem sap is obtained rather than a bulk value for the whole plant, especially directly following labelling, as phloem sap will contain newly metabolised photosynthetic products derived from the labelled CO_2.

However if one is interested in using the plant material for general feeding then it is sufficient to uniformly label the plant and obtain an homogenous representative sample of the plant material. It may also be useful to use non destructive techniques to sample plant material such as the hole-punch method. These allow instantaneous measurements of isotopic enrichment.

16.9 Plant sampling.

Whole plant sampling

A sub sample should be dried in an oven at 50-60°C to constant weight and the material ground to a fine powder with the consistency of talcum powder. This can be achieved using a pestle and mortar, ball mill or rotary table top mill. It is always best to start the process with un-labelled samples and ensure the equipment is carefully washed with a combination of alcohol and acetone between samples of different isotopic signature. Once ground samples can be stored in appropriately labelled plastic vials half filled so to allow for homogenisation prior to weighing them out for analysis.

Leaf or stem sap sampling

If fresh material is to be used for the experiments a sub sample of the material should be taken which represent the whole sample.

- Take an 1.5 mL eppendorf tube, make a small hole in the base of the tube with a sharp thick needle and place the tube in a second eppendorf tube.
- Cut stem or leaf and place immediately in a 1.5 ml eppendorf pair, and microwave for 60s at 600 W.
- Place sample in freezer till frozen, about three hours (this is to break down cell structure).
- Microwave for 15s to defrost sample.
- Centrifuge eppendorf pair for five minutes at 8000 g. Sap solution will collect in the lower eppendorf tube.
- Remove 20-50 µL of sap solution and pipette on to 5 mm quartz fibre filter disc (cut using standard office hole punch), placed in a tin cup.
- For ^{13}C and ^{15}N analysis dry in oven at 50-60°C.

Non destructive hole punch sampling.

Using a standard office hole punch, punch out discs of plant material place in tin cups, dry in oven at 50-60°C, and seal. Two to three discs should be sufficient for ^{15}N and ^{13}C analysis. It is easiest to use a single hole punch, however normal hole punch will do.

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Glossary

Abundance. The relative concentration of the individual isotope in a mixture of isotopes of the same chemical element.

Atom. A particle of matter indivisible by chemical means. It is the fundamental building block of elements. An atom has a relatively heavy nucleus made up of positively charged protons and neutral neutrons surrounded by orbiting electrons with a negative charge balancing that of the protons in the nucleus. The number of electrons (from I to 92) determines the chemical characteristics of the atom. The number of neutrons and protons (from I to 238) determines the weight and isotope of the atom.

Atomic mass. Originally defined as the number of protons and neutrons in the nucleus of an atom. Now measured in atomic mass units which are exactly one twelfth the mass of a neutral atom of carbon 12 (u7l.660 x 1027 kg.)

Atomic number, Z. An integer that expresses the positive charge on the nucleus of an atom and hence defines the position of a chemical element in the periodic table. **Z** is equal to the number of protons in the nucleus of any atom that belongs to element number **Z**.

Biological half-life, **see half-life, biological**.

Charged particle. Parts of an atom possessing a small charge of static electricity. A proton has a single unit of positive charge. An electron has a single unit of negative charge. A very large number of electrons passing through a conductor is called an electrical current.

Closed system. A kinetic system in which no transfer of matter (or energy) to or from its surroundings takes place.

Decay (radioactive). The change of one radioactive nuclide into a different nuclide by the spontaneous emission of alpha, beta, or gamma rays, or by electron capture. The end product is a less energetic, more stable nucleus. Each decay process has a definite half life.

Decay chain. A series of nuclides in which each member decays to the next member of the chain through radioactive decay until a stable nuclide has been formed.

Decay product. Nuclide or radionuclide produced by decay.

Deuterium (D).A stable naturally occurring hydrogen isotope. Its natural abundance is about one part in 7000 of hydrogen. Used in the form of deuterium oxide as a moderator.

Electromagnetic radiation. A travelling wave motion resulting from changing electric or magnetic fields. Familiar electromagnetic radiation range from X rays (and gamma rays) of short wavelength, through the ultraviolet, visible, and infrared regions, to radar and radio waves of relatively long wavelength. All electromagnetic radiations travel in a vacuum with the velocity of light.

Electron. A small particle having a rest mass of 9.107×10^{-28}, an atomic mass of 1/1837 of a hydrogen atom, a diameter of 10^{-12} cm and carrying one elementary unit of positive or negative charge. The positively charged electron is called the positron, while the negatively charged electron is usually termed the electron (the term negatron is rarely used). (See also **beta particle**.)

Electronvolt (eV). A unit of energy equal to the energy acquired by a singly charged particle when it is accelerated in a vacuum through a potential difference of 1 V (see units).

Enrichment (atom per cent excess). The abundance of a given stable isotope in a labelled sample minus the abundance of that isotope in nature. cadmium (greater than approximately 0.5 eV).

Excitation. The transition of a nucleus, an atom or a molecule to an energy level above that of its ground state.

First order kinetics. A process in which the rate of change of the variable is proportional to the magnitude of the variable present at any time. If the variable is P, then: dP/dt α P, i.e. dP/dt = kP, where k is termed *the* ***first order rate*** *constant.* This behaviour is common to many processes, e.g. **the radioactive decay law,** many biological and open compartment processes

Fission. The splitting of a heavy nucleus into two (or rarely more) lighter nuclei of about equal masses, whereby one or two neutrons and a relatively large amount of energy (including some gamma radiation) are released. Fission is usually preceded by neutron capture.

Fluorescence. The luminescence that exists only as long as the energy source is exciting the phosphor. (Phosphorescence is **luminescence** that continues after the exciting source is removed.)

Gamma photon, γ photon. A gamma photon is an electromagnetic energy packet emitted at the speed of light from an atomic nucleus.

Gamma radiation. High energy, short wavelength radiation emitted from the nuclei of atoms. Less damaging than the same dose of alpha radiation, but much more penetrating. Can be stopped by thick slabs of lead or concrete.

Gamma radiation. The electromagnetic radiation emitted in the process of nuclear transition or particle annihilation.

Gamma ray. A highly penetrating type of nuclear radiation, similar to X rays, except that it comes from within the nucleus of an atom, and, in general, has a shorter wavelength. adding to the total charge collected.

Half-life, biological. The period of time during which a given biological organism physiologically eliminates half the amount of a given substance that has been introduced into it when the rate of elimination is approximately exponential (see first order kinetics).

Half-life, effective. In a biological organism, the time taken for the activity of a radioisotope to decrease to half its value as a result of both radioactive decay and physiological elimination when the rate of removal is approximately exponential (see first order kinetics).

Half life, radioactive. . The time in which one half of the atoms of a particular radioactive substance disintegrates into another nuclear form. Each radioactive isotope has a characteristic half life, e.g. U235 = 0.7038 x 10^9 years; U238 = 4.468 x 10^9 years.

Inverse tracer dilution. A method of isotopic dilution analysis in which a known amount of **trace** of natural isotopic composition is added to a sample containing a **tracee** that is labelled.

Ion exchange resin. An artificial solid material that can adsorb ions and in solution exchanges these with other ions of the same sign. Ionization. The production of ion pairs (of which one may be an electron).

Ion. An atom that has too many or too few electrons, causing it to have an electrical charge.

Ionization. The process of adding one or more electrons to, or removing one or more electrons from, atoms or molecules, thereby creating ions. High temperatures, electrical discharges, or nuclear radiations can cause ionization.

Irradiate. To expose to some form of radiation.

ISO. International Organization for Standardization, Geneva (Switzerland).

Isomer, nuclear. Nuclides having the same mass number and atomic number, but occupying different nuclear energy states.

Isomer. One of several nuclides with the same number of neutrons and protons capable of existing for a measurable time in different nuclear energy states.

Isotope dilution. A method of analysis in which a known amount of tracer is added car-

rier free or together with a known amount of **tracee** (carrier) to a sample containing a **tracee** of natural isotopic composition.

Isotope. Isotopes of a given element have the same atomic number (same number of protons in their nuclei) but different atomic weights (different number of neutrons in their nuclei. i.e. same Z, different N). Uranium 238 and uranium 235 are isotopes of uranium.

Luminescence. The property of some substances of emitting light in response to excitation (excluding incandescence). Radiofluorescence, i.e. luminescence in a phosphor because of the prompt release of the energy absorbed from ionizing radiation, is termed scintillation (q.v.). Absorption of such energy with subsequent release only after illuminating or heating the phosphor is termed radiophotoluminescence or radiothermoluminescence, respectively, and forms the principle underlying the operation of certain types of dosimeters.

Mass number. The total number of protons and neutrons (Z+ N) in the nucleus of any given nuclide. The mass number, A, is the nearest whole number to the atomic weight (mass) of the nuclide.

Mass spectrometer. An instrument that separates (ionized) atoms or molecules according to mass (actually mass divided by ionic charge), these being focused on to electrodes and registered electrically. The record is a spectrum indicating the relative intensity as a function of mass divided by the charge of each species.

Molecule. A group of atoms held together by chemical forces. A molecule is the smallest unit of a compound that can exist by itself and retain all of its chemical properties.

Natural isotope. The naturally occurring nuclide of any given chemical element. A natural isotope is either: (1) stable: (2) of very long half-life; or (3) the descendant of a very long lived precursor.

Neutron number, N. The number of neutrons incorporated in the nucleus of a given nuclide.

Neutron. A nuclear particle with no electric charge and a mass approximately the same as that of a **proton**. Free neutrons are unstable, and decay with a lifetime of about 13 min to ^{1}H (i.e. proton) and a β^- particle.

Normal distribution, Gaussian distribution. Symmetrical arrangement of replicate values that deviate randomly on either side of a mean value. This bell shaped distribution is described mathematically by the Gaussian equation.

Nucleon. One of the types of particles, comprising protons and neutrons, constituting an atomic nucleus. Hence, a commonly used name for protons or neutrons belonging to a nucleus.

Nuclide. Any given atomic species characterized by: (1) the number of protons, **Z**, in the nucleus; (2) the number of neutrons, **N**, in the nucleus; and (3) the energy state of the nucleus (in the case of an **isomer**).

Open system. A system of kinetic compartments containing at least one open compartment.

Proton. A nuclear (elementary) particle with an atomic weight (mass) of approximately one unified atomic mass unit, carrying one elementary unit of positive charge. The number of protons determines what element the atom is.

Radiation. Energy given off by atoms when they are moving or changing state. Can take the form of electromagnetic waves, such as heat, light, X rays, or gamma rays, or streams of particles such as alpha particles, beta particles, neutrons or protons.

Radioactive dating. A technique for estimating the age of an object by measuring the amounts of various radioisotopes in it.

Radioactive. Exhibiting radioactivity or pertaining to radioactivity.

Radioactivity. The spontaneous emission of radiation, generally alpha or beta particles, often accompanied by gamma rays, from the nucleus of an unstable isotope.

Radioisotope. An unstable isotope of an element that decays or disintegrates spontaneously, emitting radiation. Approximately 5,000 natural and artificial radioisotopes have been identified.

Radionuclide. A radioactive nuclide. An unstable isotope of an element that decays or disintegrates spontaneously, emitting radiation.

Rate constant. The fractional rate of reaction in first order kinetics (q.v.).

Stable isotope. A naturally occurring isotope, with no observable radioactivity.

Steady state. With respect to a specified substance, the characterization of a dynamic system in which the input and output rates of the substance are equal.

Tracee. The test object, element or compound that the investigator is endeavouring to trace.

Tracer equilibrium. The stage (real or extrapolated) at which the **tracer** has become completely and uniformly mixed with the **tracee,** so that the **specific activity** has become the same throughout the system.

Tracer. A substance (e.g. an isotope) that is mixed with or fixed to the tracee in order to follow its translocation or to identify its location.

Turnover time. Pertaining to a given substance in a steady state system, it is the time needed for the input (and thus the output) to equal the total amount of the substance in the system.

X ray. Penetrating electromagnetic radiation (**photon**) having a wavelength that is much shorter than that of visible light. These rays are usually produced by excitation of the electron field around certain nuclei. In nuclear reactions, it is customary to refer to photons originating in the nucleus as gamma rays, and to those originating in the electron field of the atom as X rays. These rays are sometimes called roentgen rays after their discoverer, W.K. Roentgen.

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