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13

Stable isotopes

James R. Ehleringer and C. Barry Osmond

13.1 INTRODUCTION

The use of stable isotopes at natural abundance levels is rapidly emerging as a powerful approach for understanding a number of physiological processes and food web and environmental interactions in ecology, especially in physiological ecology. The analysis of stable isotopes developed as an outgrowth from geochemical investigations and represents a relatively new approach within ecological studies. In the 1970s, the principal application of stable isotopes in physiological ecology was for the measurement of $^{13}C/^{12}C$ ratios to identify the photosynthetic pathway of a species. Today stable isotopes are being applied to a broader range of questions, including nitrogen fixation, water-use efficiency and water-source studies. Our ecological understanding of the applications of stable isotopes is still in its infancy, but recent advances suggest that the rapid expansion of stable isotope studies into new areas is likely to continue in the coming decade. The purpose of this chapter is to introduce stable isotopes and the techniques for their measurement, as well as sampling and preparation procedures. Although several ecological applications of stable isotopes are presented, the discussion is by no means complete and may represent only a fraction of the applications developed in the next several years.

13.2 NATURAL ABUNDANCES OF STABLE ISOTOPES OF ECOLOGICAL INTEREST

Most elements of biological interest have two or more stable isotopes, although one isotope is usually present in far greater abundance than other forms (Hoefs, 1980). Table 13.1 lists the average abundance of the elements used in ecological studies. Isotopic abundances of these elements are by no means uniform in nature, but most of the variation between biotic and abiotic components is within 1% of the values in Table 13.1. In addition to the five light elements, strontium isotopes are assuming greater importance in understanding ecological transport processes (Rundel *et al.*, 1988) and have therefore been included, although they will not be discussed further in this chapter.

Table 13.1 Average terrestrial abundance of the isotopes of major elements used in environmental studies (from Fritz and Fontes, 1980)

Element	*Isotope*	*Average terrestrial abundance (%)*
Hydrogen	^{1}H	99.985
	^{2}H	0.015
Carbon	^{12}C	98.89
	^{13}C	1.11
Oxygen	^{16}O	99.759
	^{17}O	0.037
	^{18}O	0.204
Nitrogen	^{14}N	99.63
	^{15}N	0.37
Sulfur	^{32}S	95.0
	^{33}S	0.76
	^{34}S	4.22
	^{36}S	0.014
Strontium	^{84}Sr	0.56
	^{86}Sr	9.86
	^{87}Sr	7.02
	^{88}Sr	82.56

13.3 STABLE ISOTOPE MASS SPECTROMETRY

13.3.1 Mass spectrometers

Mass spectrometers are instruments which measure the mass-to-charge ratio of a substance. In a mass spectrometer, the compound is first ionized under high-vacuum conditions and then deflection of its ions is measured while subject to a magnetic field. Most isotope ratio mass spectrometers are capable of measuring only low-molecular-weight compounds (usually <64). The compounds are introduced into the instrument as gases, most often as H_2, CO_2, N_2 and SO_2, permitting measurement of the isotope ratios of H, C, N, O and S in organic and inorganic materials. Heavier elements, such as strontium, are measured with a thermal emission isotope ratio mass spectrometer.

In an isotope ratio mass spectrometer, the pure gas (H_2, CO_2, N_2, SO_2) is introduced at an inlet at one end of a flight tube. At this point, the gas is then ionized in an ion source which knocks an electron from the outer shell of the compound. The beam of ionized gas is accelerated and deflected along the flight tube by a powerful magnet (Fig. 13.1). Because the ions have different mass-to-charge ratios, light and heavy ions containing different isotopes will be deflected differently and sorted by the magnetic field. At the opposite end of the flight tube are a series of collectors (Faraday cups) which are positioned to capture the charged ions of different mass (Fig. 13.2). DC amplifiers attached to the Faraday cups convert the ionic impacts into a voltage, which is then converted to a frequency. The absolute intensity of the signal on the Faraday cup is not critical because this depends on the amount of gas introduced into the mass spectrometer and other factors. Rather the critical parameter is the ratio of the signals measured by the different Faraday cups.

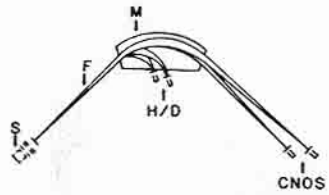

Fig. 13.1 Principal features of an isotope ratio mass spectrometer, including flight tube (F), ionizing source (S), magnet (M), Faraday cups for detecting hydrogen isotopes (H/D), and Faraday cups for detecting C, N, O and S isotopes (CNOS).

Only two Faraday cups are needed for hydrogen isotope measurements, since the only ionized gases are $^{1}H^{1}H$, $^{2}H^{1}H$. However, for CO_2 and N_2, three cups are used in the

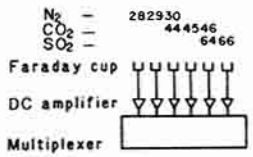

Fig. 13.2 Schematic diagram of Faraday cups and amplifier systems.

measurements. Three isotope forms of nitrogen gas ($^{14}N^{14}N$, $^{14}N^{15}N$ and $^{15}N^{15}N$) require one Faraday cup to detect each form. Carbon dioxide is more complicated since two elements and five isotopes can lead to light isotopic forms of the gas. Potential complications arise because several forms have the same mass and as many as six different masses (44 through 49) should conceivably be measured to detect all the forms. Fortunately ^{17}O is present in only trace amounts, assumed to be a constant percentage of the ^{18}O abundance and forms with more than two isotopes are present at low frequency. It is usually assumed that the different combinations are present in equal proportion to their isotopic abundances, so only three masses (44, 45 and 46) need be measured to calculate the $^{13}C/^{12}C$ and $^{18}O/^{16}O$ ratios.

One of the first requirements of an isotope ratio mass spectrometer is a good vacuum system which maintains extremely low levels of contaminating molecules. The flight tube operates at a vacuum of approximately 10^{-8} Torr ($\approx 10^{-5}$ Pa). Since the mean free path of a gas molecule is inversely proportional to the pressure, a vacuum that low will insure a mean free path length of over 500 m. This means that ions traveling down the flight tube (usually 0.5–1 m in length) will not collide with other gas molecules and be scattered.

The gas inlet into the mass spectrometer is symmetrically arranged for the introduction of either sample or standard gases. Gases are temporarily stored in a metal bellows and then are passed through a set of matched capillaries (one for each side) to ensure viscous flow of the gases. Precise matching of the capillaries ensures that fractionation of the gases prior to introduction into the mass spectrometer is small. A changeover valve is used to switch between the standard and sample gases. The difference in the signals between sample and standard gases is used to calculate the isotope ratio for the sample. Measurements of the absolute ratios (i.e., 45/44 and 46/44 for CO_2) on a given gas cannot be made with the same precision as a comparison between two samples, so the difference between the sample and standard ratios is of interest in the isotope ratio calculation.

13.3.2 Delta units and standards

The differences in the equilibrium and kinetic characteristics of isotopic species are usually small (on the order of a few percent), and thus absolute variations in isotopic abundances based on physical factors may be small. Enzymatic discrimination for or against an isotopic species will affect the absolute abundances, but again these variations are on the order of one or two percent. Therefore, in any isotopic analysis very precise analytical techniques are required. Most often, it has been found that measuring the absolute isotopic composition is not as reliable and/or convenient as measuring isotopic differences between a sample and a given standard. This is because, while obtaining high precision in absolute isotopic composition of a sample is not difficult over the short term, machine drift has a time base of minutes needed for replicate measurements (Hayes, 1983). In contrast, analyses based on the measurement of the differences between a defined standard and sample provide high precision and repeatability over both short-term and long-term periods. The differential analysis approach allows very small differences in the isotopic composition of two samples to be accurately and reliably determined.

Isotopic composition of a sample is therefore usually expressed with the differential notation (Friedman and O'Neil, 1978). That is:

$$\delta X_{std} = (R_{sam}/R_{std} - 1)1000 \quad [0/00]$$

where δX_{std} is the isotope ratio in delta units relative to a standard, and R_{sam} and R_{std} are the isotope abundance ratios of the sample and standard respectively. Multiplying by 1000 allows the values to be expressed in parts per thousand, or as more commonly expressed on a 'per mil' (‰) basis. Since the isotopic composition of two samples will not differ extensively in their absolute values, the differential notation allows one to focus on the differences between samples.

There are presently four accepted isotopic standards for the five principal light elements of biological interest. These are Standard Mean Ocean Water (SMOW) for hydrogen and oxygen, PeeDee Belemnite (limestone) (PDB) for carbon, atmospheric air for nitrogen and the Canyon Diablo meteorite (CD) for sulfur. Estimated absolute ratios of these standards are listed in Table 13.2. While there is some variance in the estimates of the absolute ratios in these standards, the use of the differential or deviation from standard measurement approach overcomes these and provides far greater precision and long-term reliability. The original supplies of both SMOW and PDB have been exhausted and replaced by other materials which had been carefully compared to the original standards. These standards are available to investigators for calibration of working standards in each mass spectrometer laboratory. The International Atomic Energy Agency in Vienna has mixed various waters together to produce V-SMOW (Vienna SMOW), which has an isotopic composition nearly identical to that of the original SMOW. The National Bureau of Standards provides a graphite, NBS–21, with a carbon isotope of −28.10‰ on the PDB scale. The latter standard is not easily combusted so working standards of a chemical composition similar to those of the unknowns should be selected. Greatest accuracy will be obtained with standards having an R value similar to that of the unknowns. Thus in carbon isotope analyses, inclusions of sucrose standards (such as the Australian National University Radiocarbon Dating sucrose standard −10.5‰; Chinese Radiocarbon Dating Charred Sucrose standard −24.4‰ or other beet sucrose source) among every ten or so unknowns insures contained appropriate calibration.

Table 13.2 Isotopic compositions of primary standards (from Hayes, 1983)

Primary standard	*Isotope ratio*	*Accepted value (×10⁶) (with 95% confidence interval)*
Standard Mean Ocean Water (SMOW)		
	$^{2}H/^{1}H$	155.76±0.10
	$^{18}O/^{16}O$	2005.20±0.43
	$^{17}O/^{16}O$	373±15
PeeDee Belemnite (PDB)		
	$^{13}C/^{12}C$	11237.2±9.0
	$^{18}O/^{16}O$	2067.1±2.1
	$^{17}O/^{16}O$	379±15
Air		
	$^{15}N/^{14}N$	3676±8.1

13.3.3 Resolution and precision

Resolution on modern mass spectrometers is approximately 0.005–0.01‰. However, this value often exceeds the precision that mass spectrometers can provide. The precision in isotopic measurements is dependent on the particular element of interest and depends on three factors. First is the precision of the isotope ratio mass spectrometer itself, which is calculated from repeated measurements of the same sample gas, second is the amount of gas injected, and third is the precision of the sample preparation (conversion of element from sample form to a gas which can be injected into the mass spectrometer), which will be discussed in a later section. The internal precision is usually defined as two times the standard error of 10 analyses of a single gas sample. Standard inlets for modern gas isotope mass spectrometers have an internal precision of approximately 0.01‰ for 100 μl of CO_2, 0.02‰ for 200 μl of N_2 and 0.2‰ for 200 μl of H_2.

13.3.4 Automation and other recent advances

A number of recent advances allow for analysis of small sample sizes and for large sample throughput. However, precise isotope measurements require adequate sample gas pressure in the mass spectrometer. This is achieved by reducing the volume within the inlet system, usually by means of a variable metal bellow in conjunction with a 'cold-finger' which freezes the gas into a small volume. With a cold finger attachment, the above precision can be achieved with only one-tenth the amount of gas.

In terms of sample throughput, there have been two recent advances. The first is the use of automatic gas-handling equipment at the front end of the mass spectrometer. Such systems are computer controlled and allow for 36 or more samples to be analyzed in succession with high precision and without operator intervention. The sample cycle time in such a situation will be approximately 20–30 min, meaning that as many as 50 or so samples could be analyzed per day. A very recent development is the use of CHN elemental analyzers coupled to the mass spectrometer. In this approach, an organic sample is first combusted and its elemental composition determined, then the gases are sent directly to the mass spectrometer. Such systems are still in their early stages of testing; it is thought that 100–300 samples per day could be analyzed using this approach without significant loss of precision.

13.4 SAMPLE PREPARATION

Few special precautions are necessary for preparing and storing plant samples for later determination of their isotopic composition. Leaves have been commonly used for most measurements in the past, although there is now increasing interest in the isotopic composition of other tissues and plant parts. Changes in chemical composition which accompany long storage and slow drying of living material should be avoided. It is best to freeze tissue at time of collection and then to freeze-dry it, or dry as quickly as possible at moderate temperature, to avoid loss of organic materials. The isotope ratios of organic material are determined on dried tissues that have been ground to pass a 40 mesh screen. Only a small amount of tissue is required for the analysis, and so the grinding ensures that the sample is homogeneous, and minimizes variation in isotope composition that might exist within the tissue or in any bulked sample. Finely ground material also burns more uniformly. Isotopic composition of water samples requires that the water be immediately sealed in filled glass vials to ensure that isotopic fractionation due to distillation does not occur between the time of collection and later analysis.

The necessity for sample homogeneity cannot be emphasized enough. In most cases

there will be a greater variance in the repeated analysis of the same 'bulk sample' than in repeated analysis of an individual sample through the mass spectrometer. This is partly because the amount of tissue required for an analysis is usually quite small. In most cases, less than 3 mg of dried organic material or water is used for D/H, $^{13}C/^{12}C$ and $^{18}O/^{16}O$ measurements (slightly more is required for $^{15}N/^{14}N$ analyses). With a coldfinger option on the mass spectrometer, less than 0.1 mg will be used in the analysis, further compounding this source of variation.

13.4.1 $^{13}C/^{12}C$ in organic matter

The $^{13}C/^{12}C$ of organic materials is analyzed as CO_2 in the mass spectrometer. Originally, the quantitative production of CO_2 from organic matter required repeated cycling of an O_2 atmosphere and the combustion products through a furnace and traps to absorb contaminant products such as nitrogen and sulfur oxides (Craig, 1953). This was a slow process in which only one sample could be prepared at a time. The O_2 pressure bomb method described by Osmond *et al.* (1975) was similarly time-consuming and susceptible to incomplete combustion. These methods have been replaced by semiautomatic combustion trains such as the ISOPREP-13 (VG Instruments, Oxford, UK) and those based on elemental analyses in which high-efficiency combustion and small volume accelerate the process. Batch preparations of CO_2 for $^{13}C/^{12}C$ analysis can be done by an in-vial combustion technique similar to that described originally by Buchanan and Corcoran (1959). Dried organic material, cupric oxide and silver foil are sealed under vacuum in a Vycor glass tube. The sealed tubes are then heated in a furnace at 850°C for 4 h and then allowed to cool slowly for another 12 h. After combustion the sealed tube contains CO_2, H_2O and N_2. The tube is then cracked under vacuum and the gases are separated by passing them first through an ethanol–dry ice trap, to freeze out the water, and then through a liquid-nitrogen trap to freeze out the CO_2. The remaining gas (primarily diatomic nitrogen) is pumped away. The clean CO_2 is then cryogenically moved into a vial to be then transferred to the mass spectrometer. Organic samples should be analyzed soon after they have been combusted, because H_2O and CO_2 will slowly interact with the copper present in the tube to form copper carbonate and this may affect the isotopic composition of the remaining CO_2. The variance in sample preparation with this technique is less than 0.05‰.

13.4.2 D/H in water samples

Hydrogen isotope ratios are measured after the hydrogen in water is reduced to diatomic hydrogen through one of two possible procedures. The first involves the reduction of water to hydrogen by passing the water vapor through a uranium furnace at 750°C with a Toepler pump and then trapping the hydrogen on activated charcoal at liquid-nitrogen temperatures (Bigeleisen *et al.*, 1952). This is a relatively time-consuming process, in which all samples must be individually processed. A second speedier method uses zinc as the catalyst at temperatures of 420°C (Coleman *et al.*, 1982). A clear advantage of using zinc as the catalyst is that the process can be batched so that large numbers of samples can be prepared simultaneously. In the batch procedure developed by Hayes and Studley (personal communication), water samples (usually in a capillary) are inserted into Vycor tubes (previously backfilled with nitrogen), zinc catalyst is added, and the tubes are then frozen, evacuated and sealed. The tubes are then heated at 500°C for 30 min in a heating block. The only gas then remaining in the tubes is diatomic hydrogen, which can be directly taken to the mass spectrometer for analysis. The precision of this technique is approximately 0.8‰.

Water in plant tissues can be recovered by lyophilization or azeotrophic distillation from dry toluene. In the final method, leaves or other tissues collected in the field are enclosed in small sealed containers (e.g. plastic bags) and frozen in liquid N_2 or on solid CO_2 (Sternberg *et al.*, 1986). Care should be taken to minimize transpiratory losses between removal from the plant and freezing. Tissue water is subsequently recovered by freeze-drying with a double liquid-N_2 trap in the vacuum line between the sample and the pump (Farris and Strain, 1978). A less convenient sampling procedure involves rapid transfer of the tissue to a flask of sodium-dried toluene, followed by azeotrophic distillation of the water in the laboratory (Leaney *et al.*, 1985). This method is useful, however, for the recovery of soil water for isotopic analysis.

13.4.3 D/H in organic matter

If the purpose of the investigation is to relate long-term average δD values of organic matter to average soil/meteoric water δD values, the most acceptable method is to purify cellulose and to nitrate it. This method ensures that the exchangeable hydroxyl groups of cellulose do not experience further isotopic fractionation during processing (Mann, 1971). The cellulose is extracted with sodium chlorite/acetic acid, then washed with sodium hydroxide and acetic acid (Wise, 1944). Finally, the cellulose is nitrated with nitric acid and acetic anhydride (Bennett and Timell, 1955; Epstein *et al.*, 1976; DeNiro, 1981; Yapp and Epstein, 1982). Nitration can be applied to other fractions such as sucrose (Dunbar and Schmidt, 1984). The nitrated material is then combusted using the same combustion technique described above for carbon. Following this, the combustion tube is cracked under vacuum and the water separated by passing the gases through an ethanol–dry ice trap to freeze out the water. The remaining gases are pumped away. The water from combustion is then reduced to diatomic hydrogen through one of two possible procedures as described previously for D/H measurements of water.

If the purpose of the investigation is to understand the dynamics of hydrogen isotope fractionation processes during photosynthesis in different plants and environments, then there is little point in concentrating on cellulose and its nitration. The δD value of lipids, for example, vary markedly from that of cellulose (Smith and Epstein, 1971a; Estep and Hoering, 1980; Sternberg *et al.*, 1984a). Estep and Hoering (1980) showed that saponification and separation procedures for lipids did not significantly alter the isotopic composition of standards. The δD value of sucrose from C_3 plants is different from that of C_4 plants (Smith, 1975) and it is unlikely that this is an artifact of commercial purification procedure. The δD value of organic hydrogen obtained from water of combustion of dried plant material varies markedly with metabolic pathway and environment (Ziegler *et al.*, 1976; Estep and Hoering, 1981). The δD value of cellulose nitrate from CAM plants (but not that of lipid), differs from that in C_3 and C_4 plants growing in the same location (Sternberg *et al.*, 1984a). These experiments show that water collected by combustion of dried organic fractions from plants, if checked with controls passed through the same purification procedures, is likely to yield valuable information on the relationship between the physiology and biochemistry of water in photosynthetic reduction.

13.4.4 $^{15}N/^{14}N$ in organic tissues

The $^{15}N/^{14}N$ of organic materials is determined from the isotope composition of N_2 produced from ammonium sulfate prepared by the Kjeldahl method (Kohl *et al.*, 1971; Hauck, 1982). In this reaction, the ammonia is mixed with sodium hypobromide to produce diatomic nitrogen via the Rittenberg reaction. This is a slow process, involving many steps

and in which only one sample can be prepared at a time.

Organic material is now routinely prepared for $^{15}N/^{14}N$ analysis using a batch mode combustion technique similar to that described above for carbon (Minagawa *et al.*, 1984). Dried organic material, cupric oxide and silver foil are sealed under vacuum in a Vycor glass tube. The sealed tubes are then heated in a furnace at 850°C for 4 h and then allowed to cool slowly for another 12 h. After the combustion process has been completed, the sealed tube contains CO_2, H_2O and N_2 which are separated by passing them first through an ethanol–dry ice trap to freeze out the water and then through a liquid-nitrogen trap to freeze out the CO_2. After the CO_2 has been frozen out, the nitrogen gas is then trapped on activated charcoal in a vial at liquid-nitrogen temperatures. The clean N_2 is then transferred to the mass spectrometer for analysis.

13.4.5 $^{18}O/^{16}O$ in water samples

Largely because of adsorption and condensation problems, the isotopic composition of water is not directly measured in mass spectrometers. Instead, the $^{18}O/^{16}O$ composition of waters is usually determined by equilibration with CO_2 (Compston and Epstein, 1958); ^{18}O and ^{16}O composition at equilibrium is known. A known volume of water, typically 3–5 ml, is placed in a small vessel of approximately twice that volume, air is removed from the vessel and replaced by CO_2. After allowing the vessel to equilibrate for 8–36 h in a constant-temperature water bath, a portion of the CO_2 is withdrawn and analyzed in the mass spectrometer. Since the molar fraction of oxygen in the water is so much greater than that in the CO_2, the $^{18}O/^{16}O$ ratio of the CO_2 takes on the value of the water (after correcting for a known liquid–gas phase equilibrium fractionation). Although this procedure can be batched in that numerous vessels can be equilibrating simultaneously, it is nonetheless a relatively slow process.

A promising approach to measuring the $^{18}O/^{16}O$ ratios in small volumes of water is to react guanidine hydrochloride with water to produce CO_2. This technique has been used successfully by Dugan *et al.* (1985) and Wong *et al.* (1987) to measure $^{18}O/^{16}O$ ratios on 10 µl water samples. The guanidine hydrochloride and water are heated in an evacuated sealed tube at 260°C for 16 h. The two gases formed in this reaction are NH_3 and CO_2. Upon cooling, the CO_2 combines with NH_3 to form an ammonium carbamate. The CO_2 is released from the ammonium carbamate by reacting it with phosphoric acid, trapped by freezing the CO_2 at liquid-N_2 temperatures and injected into the mass spectrometer for analysis. The clear advantage of the guanidine hydrochloride method is that very small sample sizes can be used. The precision of this technique is similar to that of the H_2O–CO_2 equilibration method (Wong *et al.*, 1987). Both methods have a standard deviation for sample preparation of approximately 0.2‰.

13.4.6 $^{18}O/^{16}O$ in organic tissues

The $^{18}O/^{16}O$ ratios in plant organic matter are usually determined on purified cellulose (Burk, 1979). The best method, like those for $^{13}C/^{12}C$ and H/D, is an in-vial combustion technique, in which cellulose and mercuric chloride are combusted in sealed tubes at 850°C in a muffle furnace. The gases produced are CO_2, CO and HCl. The CO is converted to CO_2 by electric discharge and then the gaseous HCl is removed by trapping it in isoquinoline. The CO_2 is frozen out into a vial at liquid-nitrogen temperatures and then transferred to the mass spectrometer for analysis. The combustion process can be batched; however, the later steps involving conversion of CO to CO_2 and trapping out of the HCl are slow and cannot be batched. On-line methods, based on elemental analyses are being developed.

13.5 SAMPLE VARIABILITY

13.5.1 Variation among tissue types and within cellular components

Individual plant tissue components may vary considerably in their carbon and nitrogen isotope ratios. Pectins, hemicellulose, starches and sugars are typically heavier (have more ^{13}C) than cellulose and lignins; lipids tend to be lighter than other cell components (Deines, 1980; O'Leary, 1981). Amino acids and other nitrogenous compounds may differ in their nitrogen isotope ratios (Shearer and Kohl, 1988). Consequently, when carbon or nitrogen isotope ratios of different organs are compared, there can be systematic variations dependent on tissue composition (O'Leary, 1981; Farquhar *et al.*, 1988; Shearer and Kohl, 1988). However, since the correlations among different organ types (leaves, roots, seeds, wood, etc.) remain high, interplant comparisons will remain valid so long as the ecological comparison is made using similar tissues. For the most part, ecological comparisons have been made using whole leaf tissues.

13.5.2 Environmentally induced variation

It is now commonly accepted that the carbon isotope ratio of C_3 and CAM-inducible photosynthetic plants is very much influenced by environmental factors (discussed in greater detail below). This necessitates a certain caution in that if the interest is beyond simply determining the photosynthetic pathway of a plant, care must be taken in sampling to insure that the tissue samples are from similar environmental regimes. It is incorrect also to assume that carbon isotope ratios of leaves, for example, are constant within a single plant (such as a tree) if leaves are exposed to different microclimates. For example, Ehleringer *et al.* (1986) have shown that there is a strong correlation between leaf carbon isotope ratio and light environment, so that leaves developing within the canopy or under the shade of other canopies can differ widely in the isotopic values (Fig. 13.3).

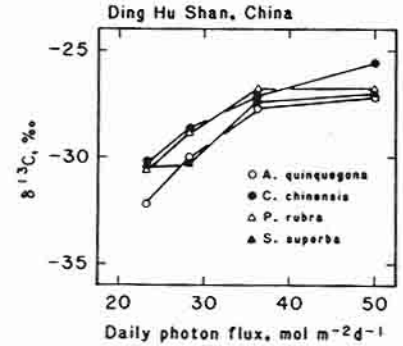

Fig. 13.3 The correlation between leaf carbon isotope ratio and the light environment in which that leaf developed for several tree and shrub species in a monsoonal tropical forest in China (redrawn from Ehleringer *et al.*, 1986).

13.5.3 Sample requirements

Sample sizes will of course depend on the specific research question posed, and will be very much affected by the magnitude of the differences to be resolved, and on the extent of both environmental and genetic heterogeneity. The precision of the mass spectrometer and preparation methods have been presented earlier and these values set the minimum error associated with a sample. Few studies have examined the isotopic variability in ecological situations, but of those available the indication is that three to five individual sample replicates may be needed to characterize an ecological situation. Since there are trade-offs between sample size and the cost of the research, one alternative is to bulk samples, thereby reducing interplant variability. However, it is likely that genetic variation in isotopic composition exists within natural populations, and that by lumping samples together to form a single

sample observation, valuable information on the structure of the population will be lost.

13.6 APPLICATION OF STABLE ISOTOPES IN ECOLOGICAL STUDIES

13.6.1 Photosynthetic pathway determination

Perhaps the first ecophysiologically related uses of stable isotopes were those of Bender (1968, 1971) and Smith and Epstein (1971b), who showed that carbon isotopic composition could be used to distinguish between C_3 and C_4 photosynthetic pathway plants. This area developed rapidly and over the next several years the phylogenetic and ecological distributions of the C_3 and C_4 photosynthetic pathways were established (Smith and Brown, 1973; Card *et al.*, 1974; Troughton *et al.*, 1974; Osmond *et al.*, 1975, 1982; Smith and Turner, 1975; Webster *et al.*, 1975; Eickmeier and Bender, 1976; Winter *et al.*, 1976; Mooney *et al.*, 1977; Rundel *et al.*, 1979; Winter, 1979; Hattersley, 1982, 1983). During this time, it also became apparent that CAM plants were often intermediate between C_3 and C_4 $\delta^{13}C$ values (Bender *et al.*, 1973; Osmond *et al.*, 1973; Lerman *et al.*, 1974) and that a number of CAM succulents exhibited large environmentally related variations in their $\delta^{13}C$ values (Troughton *et al.*, 1977).

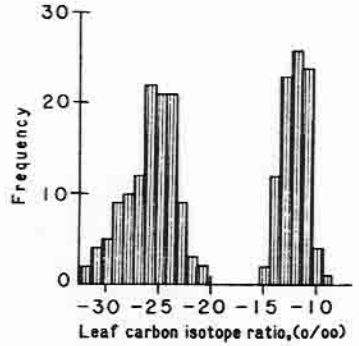

Fig. 13.4 Frequency histogram of carbon isotope ratios for different species of C_3 and C_4 plants (redrawn from Deines, 1980).

We now know that the $\delta^{13}C$ of plants can vary from −7 to −35‰ with C_4 plants having values of −7 to −15‰, CAM plants −10 to − 22‰, and C_3 plants −20 to −35‰ (Fig. 13.4). The sources of these variations in carbon isotope composition in land plants are principally associated with the photosynthetic carboxylation enzymes, with second-order differences being due to diffusional fractionations, and differences in the $\delta^{13}C$ value of the atmospheric CO_2 fixed in photosynthesis (O'Leary, 1981). In aquatic plants often the latter factor may be the major source of variation (Osmond *et al.*, 1981; O'Leary, 1984). The primary carboxylase of C_3 photosynthesis, ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco), discriminates strongly against ^{13}C (approx. −29‰ with respect to the source CO_2; Whelan *et al.*, 1973; Roeske and O'Leary, 1984). The primary carboxylase of C_4 photosynthesis, phosphoenolpyruvate carboxylase (PEPCase), discriminates much less strongly against ^{13}C (approx. 2‰ with respect to source CO_2; Reibach and Benedict, 1977; O'Leary *et al.*, 1981). Diffusional contributions to carbon isotope fractionation during CO_2 exchange are observed during CAM (O'Leary and Osmond, 1980) and during CO_2/HCO_3^- uptake in aquatic plants (Raven *et al.*, 1982). Small variations in source isotope composition due to changing atmospheric CO_2 concentration (progressively enriched with CO_2 from fossil sources at about −30‰) and larger variations due to respiratory CO_2 sources in dense rainforest canopies (Medina and Minchin, 1980) or in aquatic plants (Osmond *et al.*, 1981) have been detected. These sources of variation have been integrated into functional models for CO_2 fixation in C_3 photosynthesis (Farquhar *et al.*, 1982b) and in C_4 photosynthesis (Farquhar, 1983).

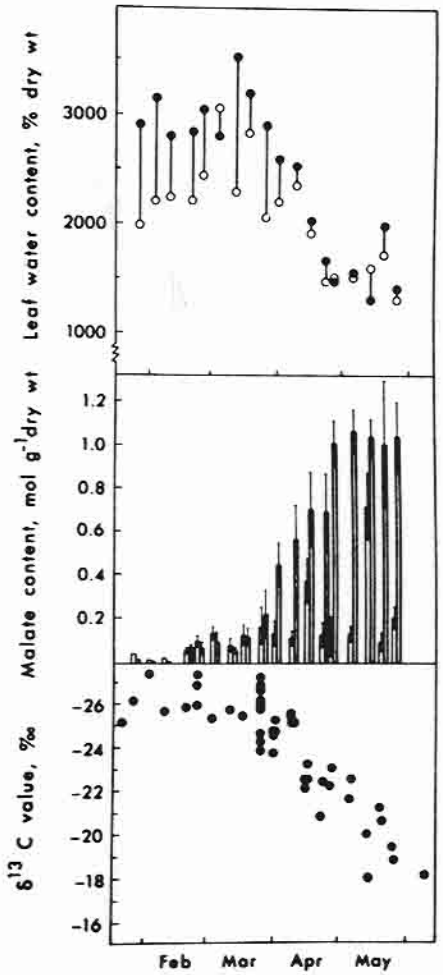

Fig. 13.5 Seasonal courses of leaf water content, malate content and leaf carbon isotope ratio of *Mesembryanthemum cristallinum* (redrawn from Winter *et al.*, 1978).

Within a species these sources of variation can indicate ecophysiologically significant changes in function. In CAM plants for example, the proportion of carbon fixed in the dark (by PEPCase) or in the light (by Rubisco) is directly indicated by changes in $\delta^{13}C$ values (Bender *et al.*, 1973; Osmond *et al.*, 1973). In field studies, the water-stress-dependent induction of dark CO_2 fixation by CAM (Fig. 13.5) has been elegantly shown with correlations between $\delta^{13}C$ value and nocturnal acidification (Winter *et al.*, 1978). In C_3 plants, changes in $\delta^{13}C$ value along environmental gradients such as salinity can be correlated with increased diffusional limitations associated with stomatal closure (Guy *et al.*, 1980; Farquhar *et al.*, 1982a). These relationships are discussed below. In some C_4 plants growth under low-nitrogen nutrition leads to more negative $\delta^{13}C$ values, indicating impaired function of the CO_2-concentrating mechanism (Wong and Osmond, 1988). Other correlations with nutrients (Bender and Berge, 1979), temperature and light (Smith *et al.*, 1976) have yet to be evaluated in terms of function. There are even examples of differences in photosynthetic pathways between organs of a species (e.g. C_3 leaves on CAM stems, Lange and Zuber, 1977), and of the relative contribution of different carboxylation pathways to the composition of different tissues such as guard cells and mesophyll (Nishida *et al.*, 1981) and root cells and nodules (Yoneyama and Ohtani, 1983).

Measurements of $\delta^{13}C$ value on individual different species of succulent plants, for example, have been used to indicate changes in ecophysiological functions along environmental gradients (Osmond *et al.*, 1975; Eickmeier and Bender, 1976). The $\delta^{13}C$ value of biomass has been used to monitor the contributions of C_3 and C_4 plants in communities along elevational gradients (Tieszen *et al.*, 1979b) and correlates closely with percent of species with the C_4 pathway (Fig. 13.6). One especially important application, where most other techniques fail, is estimation of belowground biomass due to C_3 and C_4 plants, such as in competition studies with C_3 and C_4 plants (Wong and Osmond, 1988).

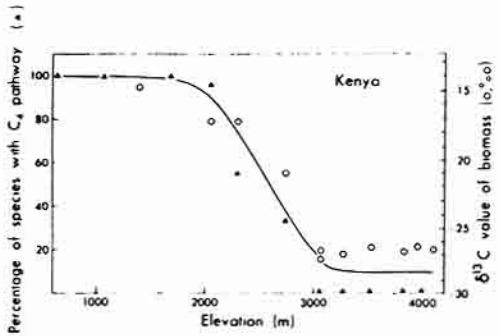

Fig. 13.6 Changes in the percentage of C_4 photosynthetic pathway plants and in carbon isotope ratio of plant biomass along an elevational transect in Kenya (redrawn from Tieszen *et al.*, 1979b).

13.6.2 Water-use efficiency in C_3 plants

The previously mentioned studies were largely survey-type investigations seeking to delineate biochemically based phenomena. In 1980 three independent approaches to the integration of physical (stomatal diffusion) and biochemical (discrimination of carboxylations) processes of carbon isotope discrimination were published (Farquhar, 1980; O'Leary and Osmond, 1980; Vogel 1980). These led to better models of carbon isotope discrimination (O'Leary, 1981), and to the recognition of a relationship between $\delta^{13}C$ value and intercellular CO_2 concentration (Farquhar *et al.*, 1982b). These theoretical interpretations can be tested by direct 'on-line' analysis of CO_2 fractionation in leaves during conventional gas exchange (Evans *et al.*, 1986). Supporting evidence has been obtained from several sources (Farquhar *et al.*, 1982b; Fig. 13.7; Bradford *et al.* (1983) for tomatoes; Farquhar and Richards (1984) for different wheat cultivars; Ehleringer *et al.* (1985) for desert shrubs and their parasitic mistletoes). Variation in the intercellular CO_2 concentration may account for much of the intraspecific isotopic variation observed, as well as the known variation that seems to be associated with water stress and growth humidity levels (Shomer-Ilan *et al.*, 1979; Winter *et al.*, 1982).

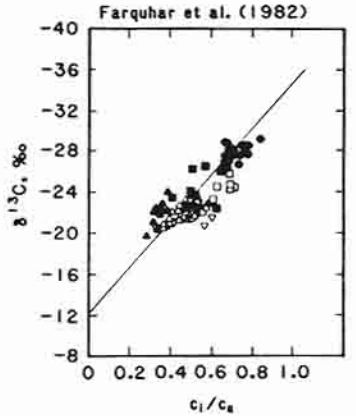

Fig. 13.7 Relationship between intercellular CO_2 concentration (c_i) and leaf carbon isotope ratio (redrawn from Farquhar *et al.*, 1982b). c_a is ambient CO_2 level. (Symbols represent different species.)

What is extremely useful about the relationship between intercellular CO_2 concentration (c_i) and carbon isotope ratio is that c_i is also related to water-use efficiency (molar ratio of photosynthesis to transpiration) as can be seen from the equations below:

$$A = [(c_a - c_i)g]/1.6$$
$$E = \Delta w g$$
$$A/E = (c_a - c_i)/(1.6\Delta w)$$

where A is photosynthetic rates, E is transpiration rate, g is leaf conductance to water vapor, c_a is ambient CO_2 level, c_i is intercellular CO_2 level, and Δw is leaf to air water vapor concentration gradient.

As c_a is essentially constant, then the carbon isotope ratio should depend only on c_i and Δw. This gives us a powerful tool for estimating integrated long-term water-use efficiency by a plant. Farquhar and Richards

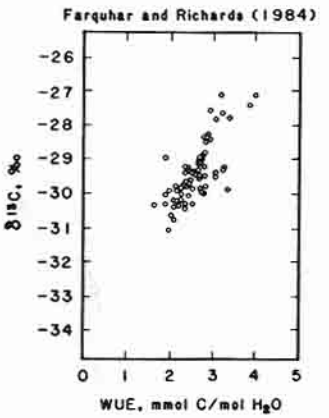

Fig. 13.8 Correlations between leaf carbon isotope ratio and the measured whole-plant water-use efficiency (WUE) (plant mass to soil water extracted) (redrawn from Farquhar and Richards, 1984).

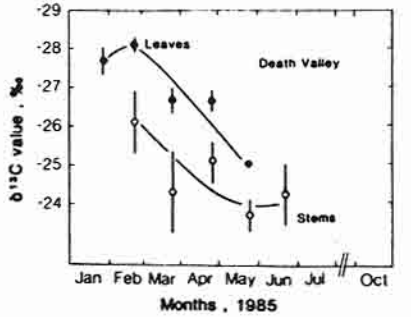

Fig. 13.9 Seasonal changes in carbon isotope ratios of leaves and photosynthetic stems of *Eriogonum inflatum* (redrawn from Smith and Osmond, 1987).

(1984) confirmed this with wheat cultivars grown under different watering regimes (Fig. 13.8). Other data suggest that carbon isotopic composition can be used to investigate growth irradiance conditions, short-term versus long-term leaf responses and variations in isotopic composition of photosynthetic structures within a single plant. For example, $\delta^{13}C$ values of leaves and stems of *Eriogonum inflatum* measured throughout the growing season (Fig. 13.9) are consistent with and confirm the lower stomatal conductance and higher Δw conditions which characterize stem photosynthesis compared with leaf photosynthesis (Smith and Osmond, 1987).

In aquatic plants much the same principles of diffusional and biochemical fractionation apply. Smith and Walker (1980) defined the problems of CO_2 and HCO_3^- diffusion in solution, and subsequent authors have demonstrated the usefulness of these approaches in relation to anatomy and water movement and biochemical pathway (Osmond *et al.*, 1981; Raven *et al.*, 1982; Keeley *et al.*, 1984). Sharkey and Berry (1985) developed an 'on-line' system to assess the significance of CO_2 concentrating mechanisms in algae using carbon isotope discrimination.

13.6.3 Water sources used by plants

There has been less research into the ecophysiological applications of hydrogen and oxygen isotope fractionation. Ehhalt *et al.* (1963) and Schiegl and Vogel (1970) identified differences in the deuterium content of organic matter that were highlighted by large variations in the isotopic composition of rain water. Perhaps for these reasons isotopic studies of plant water relations have not progressed very far. However, recent evidence suggests that isotopic analyses of xylem sap for either element may provide a signature of the source of soil moisture which a plant is using (White *et al.*, 1985). This approach has been used in studies of water balance in pines, where the objective was to separate the uptake of ground water from recent precipitation during the growing season (Fig. 13.10).

The relationships between the isotopic content of leaf water, determined by factors such as ground water isotope content, atmospheric humidity and stomatal conductance,

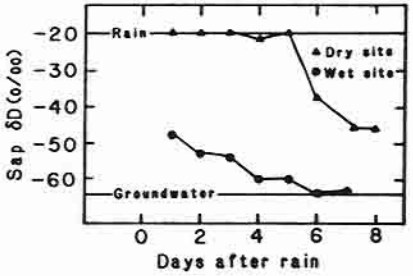

Fig. 13.10 Daily changes in the hydrogen isotope ratio of xylem water in pines following summer rains (redrawn from White *et al.* (1985).

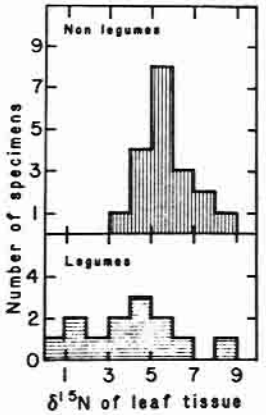

Fig. 13.11 Frequency histogram of nitrogen isotope ratios for different species of nitrogen-fixing and nonnitrogen-fixing plants (redrawn from Shearer *et al.*, 1983).

and fractionations during photosynthesis in this water are being assessed. Leaf water δD and $\delta^{18}O$ values are very dynamic (Dongmann *et al.*, 1974; Farris and Strain, 1978; Förstel, 1978; Zundel *et al.*, 1978; Leaney *et al.*, 1985), yet it seems C_3 photosynthesis fractionates to a rather constant extent in spite of the exchanges with H_2O which are potentially possible (Estep and Hoering, 1980, 1981). Comparative studies suggest (Sternberg and DeNiro, 1983) and direct measurements show (Leaney *et al.*, 1985) that different photosynthetic pathways discriminate differently against deuterium, in spite of vastly different diurnal changes in leaf water δD. However, the more negative δD values of organic hydrogen in *Sedum* spp. at higher elevation suggest that photosynthesis fractionations can reflect the changing isotope composition of ground water (Ziegler *et al.*, 1976). The δD value of organic hydrogen in CAM plants becomes less negative with water stress (Ziegler *et al.*, 1976). Recent studies show that this is a characteristic of the biochemistry of CAM not a reflection of the transpiration strategy of these plants (Sternberg, *et al.*, 1984b, 1986).

13.6.4 Nitrogen-fixation studies

Symbiotic nitrogen fixation in natural ecosystems is difficult to estimate by conventional means and it is even more difficult to obtain long-term estimates of the contribution of fixed nitrogen to the total nitrogen content of a plant. However, stable nitrogen isotopes can provide integrated estimates of nitrogen sources for plants (Shearer *et al.*, 1978, 1983; Sweeney *et al.*, 1978). This is because there are small differences between the natural abundance of ^{15}N between atmospheric N_2 and soil sources of nitrogen. Soil nitrogen tends to be enriched in ^{15}N (mean surface value of $\delta^{15}N$ = 9.2‰) whereas bacterial fixation of N_2 does not discriminate against ^{15}N. Thus, legumes which fix N_2 have less negative $\delta^{15}N$ values than species that do not (Fig. 13.11). The fraction of the nitrogen in a legume derived from nitrogen fixation activity can be estimated as the ratio of the difference between the $\delta^{15}N$ of the leaf minus the $\delta^{15}N$ values expected if nitrogen were derived solely from the atmosphere ($\delta^{15}N$ = 0) divided by the difference in $\delta^{15}N$ of nonnitrogen-fixing plants minus the atmospheric value. Such approaches indicate that legumes can differ widely in the proportion of nitrogen derived from soil versus nitrogen fixation sources.

13.6.5 Food web studies

DeNiro and Epstein (1976) in documenting the influence of diet on carbon isotope ratios pointed out that you are what you eat (plus a few ‰). A variety of single and multiple isotope signatures have been used to study patterns of plant–herbivore interactions and energy transfer along food chains. The majority of these have involved the use of carbon isotope ratios to investigate patterns of food selection in the diet of animals (Ludlow *et al.*, 1976; DeNiro and Epstein, 1976, 1978). Since the carbon isotope ratio in animal tissues closely parallels the ratio of the food eaten, diet selectivity between foods of different isotopic composition can be assessed. Recent studies analyzing vertebrate herbivore food preferences have further illustrated the utility of isotopic analyses for quantitatively determining the feeding preferences of different species over time. In particular, a study of large herbivores in Kenyan grasslands by Tieszen *et al.* (1979a) demonstrated that reliable estimates of both long- and short-term feeding preferences can be obtained for large numbers of animals with limited sampling efforts.

Carbon isotope ratios have also been used to explore the nature of ancient human diets. Bone collagen provides a permanent record of the diet at the time it was laid down and can be used to estimate the relative amounts of marine and terrestrial foods in prehistoric diets. Such studies have been used to trace the introduction of corn among different tribes of North American Indians (Chisholm, *et al.*, 1982; DeNiro and Hastorf, 1985).

To date there has been only limited interest in the use of nitrogen isotope ratios in food chain studies. $\delta^{15}N$ values in animals reflect the composition of their diets, but are characteristically 2–4‰ more positive at each trophic level (Schoeninger and DeNiro, 1984). This characteristic elevation in nitrogen isotope ratios along food chains is thought to be due to isotopic fractionation associated with catabolic metabolism.

One of the most exciting future developments in food chain studies using stable

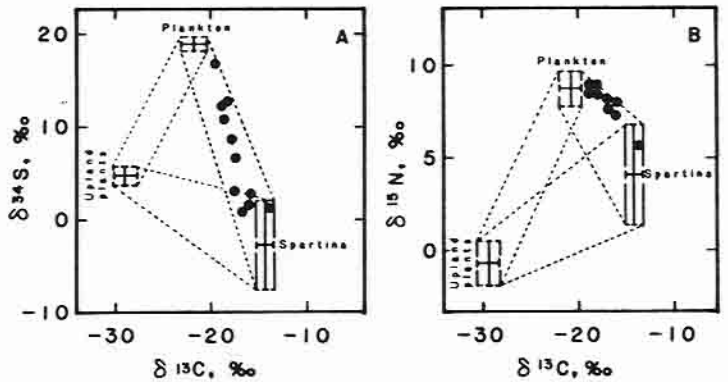

Fig. 13.12 Plots of stable isotope ratios (means and standard deviations) of C, N, and S for plankton, upland plants and marsh grass from a salt marsh and the stable isotope ratio signatures of a ribbed mussel (solid dots) feeding on detritus within this ecosystem (redrawn from Peterson *et al.*, 1985).

isotope ratios will almost certainly come from multiple element studies. Organisms that are similar in isotope ratios for one element may well differ in another. Using stable isotopes of C, N and S, Peterson *et al.* (1985) have been able to trace the flow of organic matter within a salt marsh ecosystem and to indicate clearly the detritial substances utilized by mussels downstream (Fig. 13.12). This approach may require sophisticated treatment of mathematical data in addition to the analytical needs, but it shows great promise for a wide variety of applications.

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14

Canopy structure

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14.1 INTRODUCTION

Descriptions of canopy structure are essential to achieving an understanding of plant processes because of the profound influence that structure has on plant–environment interactions. The vegetation architecture not only affects exchanges of mass and energy between the plant and its environment, but it also may reveal a strategy of the plant for dealing with long-lasting evolutionary processes, such as adaptation to physical, chemical or biotic factors, by reflecting the organism's vital activity or peculiarities in growth and development. Plant morphological studies, which are mostly qualitative, have long recognized this fact. Unfortunately quantitative descriptions of geometric features of canopies, plants or individual organs are difficult because canopies are spatially and temporally variable. The level of complexity is ever increasing as we proceed from individual organs to plants to pure stands to heterogeneous plant communities, since each higher level contains elements of the lower levels. For example, Sitka spruce needles are organized along a twig with a determined orientation distribution that varies with depth in the canopy; these shoots are organized into branches in a way that reflects developmental strategy, environmental stimulation and growth restrictions (Norman and Jarvis, 1974); these branches are organized along a stem to reveal a tree and of course trees are distributed throughout a forest. The elegance of this structure challenges the imagination to its limits. Although this elegance may teach us humility, it quickly overwhelms our quantitative aspirations so we must resort to the expediency of statistics for quantitative relations.

The influence of canopy structure on wind and radiation environments within the canopy is perhaps the most obvious. The effect of canopy structure on wind is usually described using measured normalized mean wind profiles within the canopy (Fritschen, 1985). Effects of canopy characteristics such as leaf area distribution or foliage clumping on wind usually are not quantified because of the obvious complexities associated with measurements and modeling. However, Pereira and Shaw (1980) have considered the effect of the vertical distribution of leaf area on wind profiles. Other interesting interactions between canopy structure and wind are described in Hutchison and Hicks (1985).