Handbook of Stable Isotope Analytical Techniques, Volume-I
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CHAPTER 9

Preparation of Ecological and Biochemical Samples for Isotope Analysis

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9.1 Introduction

Stable isotope analyses have proven to be a critical source of information for delineating processes in ecological and ecosystem studies (Rundel et al., 1989; Lajtha & Michener, 1994), which can encompass the study of habitats and the interactions of organisms with their environment. The main power of isotopic analyses resides in the ability to study both specific processes and also to trace sources of materials and flows of energy through complex ecological webs. Biological processes, such as photosynthesis and metabolism, fractionate materials and change isotope compositions. For example, plants fractionate carbon during CO_2 uptake and therefore carbon isotope studies are a useful tool to study the photosynthetic process. Conversely, at the whole organism level, the fractionation associated with animal feeding is much smaller. As biological or inorganic matter that transfers between components in a particular ecosystem may have unique isotopic compositions (e.g., Fry & Scherr, 1984; Michener & Schell, 1994; Dittel et al., 1997; Chamberlain et al., 1997; Kelly, 1999), isotopes may be useful as indicators of food web structure and trophic level status. For example, the living components of all terrestrial communities, including humans, as well as many marine communities rely on the primary production of organic matter by photosynthesis to provide energy for consumers. Organic matter and energy are then passed along food chains, with energy being dissipated at each trophic level. Stable isotopes can be one of the most effective tools for tying together these ecological processes that affect, in turn, affect the biosphere, the atmosphere, and the geosphere at both local and global scales (e.g. Gearing, 1991; Quay et al., 1989; Paerl & Fogel, 1993).

This chapter is intended as an overview of the application of stable isotope techniques to addressing ecological questions. We offer suggestions for ecologists who might be considering including isotope analyses to their work, as well as for more experienced isotope geochemists who are interested in expanding their research into a new field. The chapter expands on previous work including the several books that

have been published on isotope application to ecological studies (Rundel et al., 1989; Lajtha & Michener, 1994; Griffiths, 1998), and specific texts on methodology for stable isotope use in environmental studies (Coleman & Fry, 1991; Knowles & Blackburn, 1993; Boutton & Yamasaki, 1996). Although techniques in measuring stable isotopes have changed to more automated devices, the information included in specific chapters of these books (i.e., Ehleringer, 1991; Boutton, 1991) is still appropriate. The focus of this chapter is on the proper sampling protocols for accurately determining the natural abundance and distribution of stable isotopes, particularly carbon and nitrogen, in ecological systems and does not address methods that include the addition of isotopically labeled compounds at enriched levels. Furthermore, methods for the analysis of H isotopes in different sample matrices are presented elsewhere in this book and

BOX 9.1 - Case studies of an aquatic ecosystem

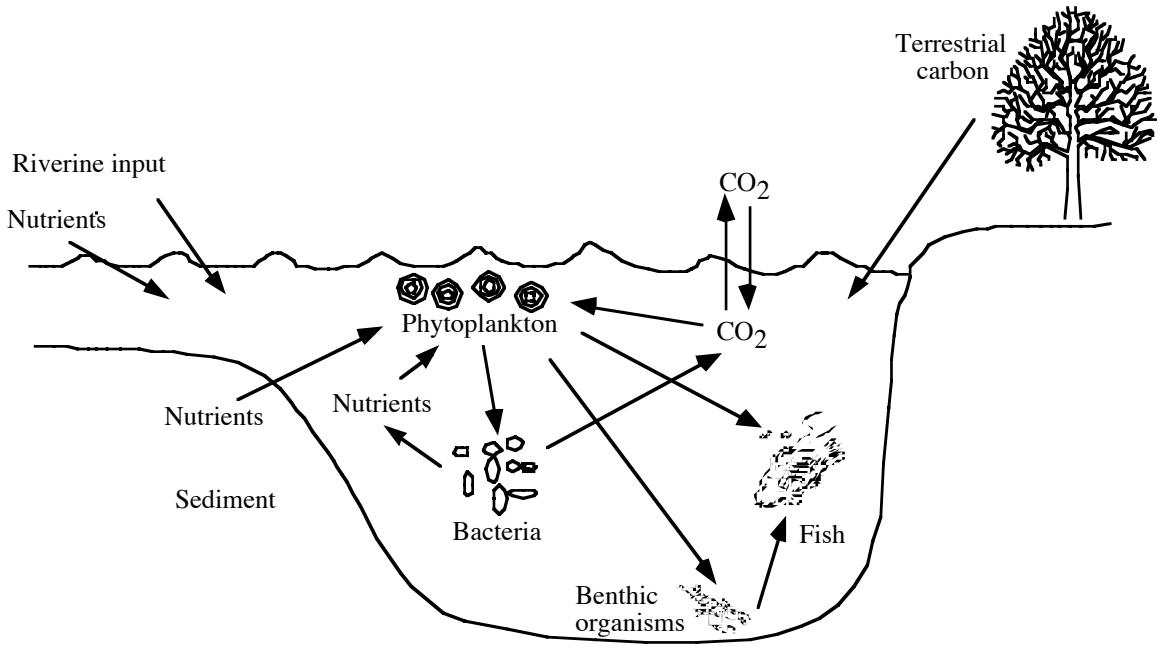

Figure 9.1 - A simplified representation of the complex interactions and pathways of energy transfer in an aquatic ecosystem. Examples of external energy and substrate sources are also represented.

Case study 9.1 - ***Role of terrestrial carbon in aquatic food webs***

Terrestrial carbon enters aquatic ecosystems via land runoff and may be a significant source of carbon to the consumers of the food web. The size and importance of this external source of carbon to the aquatic food web can be assessed by measuring the stable carbon isotope ratios of different elements of the food web. The isotopic composition of phytoplankton will reflect an aquatic signal (e.g. lacustrine or oceanic signal), whereas isotope analysis of dissolved and particulate organic matter will indicate whether such materials are terrestrial in origin. Measuring the isotopic composition of larval fish, whose diets include phytoplankton and particulate organic matter, will indicate the relative importance of terrestrial carbon to the aquatic food web. Furthermore, analysis of planktivorous and canivorous fish and generalist top feeders (e.g. trout, bass, and birds such as gulls) will confirm the role of terrestrial carbon in the aquatic ecosystem. Such approaches were employed in studies of the aquatic food web in Loch Ness in Scotland (Jones et al., 1998), and in an Australian tropical stream (Bunn et al., 1997).

Case study 9. 2 - ***Nutrient loading in aquatic ecosystems***
Management decisions on issues of water pollution frequently revolve around high concentrations of nutrients, their sources and how they affect water quality. A research study can consider a study of phytoplankton blooms in a lake, and ask the question, "What is the source of nutrients supporting a phytoplankton bloom in a lake?" A study of this sort requires measuring the concentration and distribution of nutrients such as phosphate, nitrate and ammonium, in conjunction with measuring levels of bacterial activity and phytoplankton productivity. Nitrogen isotope measurement of phytoplankton and different sources of nutrients will aid in determining the major source of nutrients which supports the phytoplankton bloom.
Dissolved inorganic nitrogen has at least four major sources: terrestrial runoff, regeneration from benthic processes, atmospheric deposition, and regeneration in the water column by bacteria. To best characterize each source, their concentrations and $\delta^{15}N$ values should be monitored periodically (weekly to monthly) over the course of the year as temporal variations occur. In conjunction with these measurements, bacterial activity should be measured to determine the relative importance of these processes over the year. In addition, it is important to determine the dominant species of phytoplankton which comprises the bloom, and also the change in algal population over the year. Isotopic compositions of the different algal species collected will provide insights into the temporal utilization of different nutrient sources with respect to time. The overall result of this type of combined study will indicate the relative importance of particular nutrient sources to phytoplankton productivity in the lake. Examples of these types of studies include those by McClelland et al. (1997) and Cifuentes et al. (1988, 1989).

the reader is referred to the chapters by *Horita & Kendall* (*Chapter 1, this volume*) and *Volume II, Part 3, Chapter 1-2/3* on hydrogen isotopes. Our chapter is presented in three sections: Section I deals with the development of experimental approaches and the potential uses of stable isotopes, while Section II describes different methods of storage and preparation of samples for isotopic analysis. In Section III we outline chemical methods for bulk and compound specific isotope analyses of biological materials pertinent to ecological studies.

9.2 Section I: Stable isotope biogeochemistry in ecological research

Before undertaking a study in this field, a clear understanding of the ecological questions that are of relevance is of the utmost importance. In any particular ecosystem, the number of plant, animal, water, soil, and air samples available for analysis is almost infinite, and although methods for analyzing bulk isotopic ratios are becoming more and more automated (Barrie & Prosser, 1996; Fry et al., 1992; Wong et al., 1992b), resources can easily be wasted when trying to investigate a large-scale ecosystem. Conversely, nothing is more vexing to the scientist, and to the community, as isotopic results that are incomplete in the sense that sources and sinks, and interconnections between matter pools have not been adequately characterized either chemically, biologically, or isotopically.

Once hypotheses have been established, the site needs to be assessed biologically, chemically, and physically. Literature surveys are a good source of biological inventories, but the most useful information usually arises from the studies and observations of ecologists who have often worked for many years at a particular site. For example, the U. S. National Science Foundation funds a series of Long Term Ecological Research Sites (LTER) and in recent years, some of these sites have been the focus of

isotopic investigations (e.g. Fry, 1991).

As in any new scientific endeavor, the first iteration of a large-scale isotopic project will very likely involve some unforeseen problems associated with the analytical design. Many factors need to be considered in an attempt to reduce these potential problems, thus we suggest an approach that can be tailored to the specific questions being addressed. Illustrative examples of how this general approach might be applied to investigations of aquatic (Figure 9.1) or terrestrial (Figure 9.2) ecosystems are also described in Box 9.1 and Box 9.2 respectively.

In general, the first step to approaching the isotopic study of an ecosystem requires the consideration of issues that are specific to natural abundance stable isotope analysis of ecosystems and their components, and takes place before actual sampling of

BOX 9.2 - Case studies of a terrestrial ecosystem

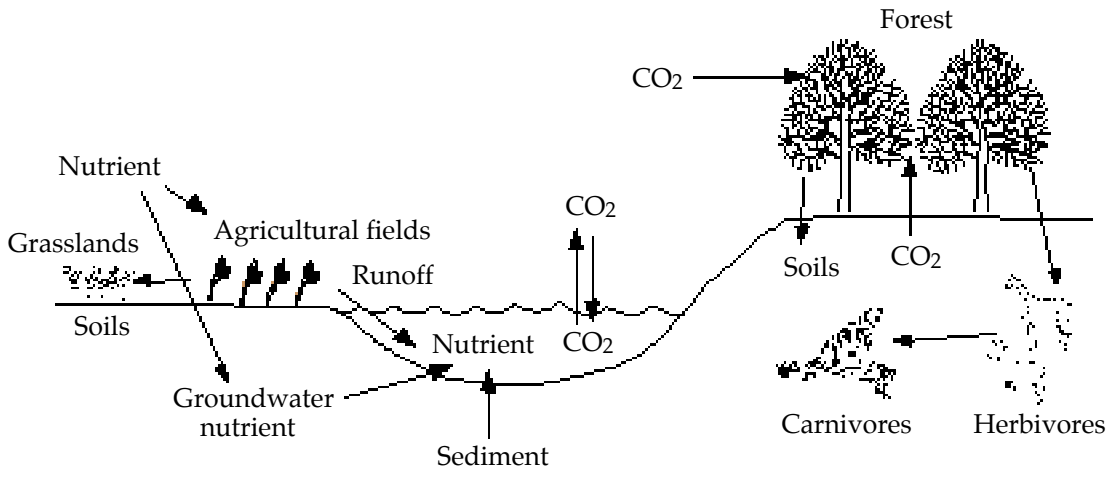

Figure 9.2 - Schematic of the major components of a typical terrestrial ecosystem. Representative examples of different trophic levels and particular pathways of energy and matter transfer are shown. A well defined stable isotope study can aid in understanding the complex interactions between these components.

Case study 9.3 - ***The impact of atmospheric carbon dioxide on a terrestrial ecosystem***
A question relevant to the present day surficial environment is whether a signal of increasing CO_2 concentrations in the atmosphere can be found in terrestrial plants, animals, or soils? This question provides an opportunity to study changes in CO_2 concentrations over several different timescales by designing a protocol which involves the analysis of carbon from a variety of "pools". For example, the $\delta^{13}C$ value of plant tissue is related to both the isotopic composition and concentration of atmospheric CO_2. This isotopic ratio of plants is set during a single growing season, whereas the $\delta^{13}C$ value of tree rings provide excellent yearly signals. At longer timescales, the isotopic composition of leaves is passed up the food chain to herbivores, such as deer. Collagen in their bones, or apatite in their teeth take several years to form and will therefore contain an integrative isotopic signal. As a result, a longer-term record of $\delta^{13}C$ values more in line with the tree ring study can be determined. Lastly, the carbon isotope composition of soil carbonates which are formed over thousands of years, provide a record of long-term changes in climate and CO_2 concentration.

material begins. It is important to consider the different factors that affect the stable isotope composition of material. Biological and biochemical processes fractionate isotopes and therefore have a substantial effect on the isotope composition of organisms. On the other hand, the fractionation associated with the consumption of organic matter by higher trophic level organisms is small, facilitating the use of isotopes in source identification. Therefore, the isotopic composition of a particular material in an ecosystem will depend on both its source isotopic composition and process-related frationations associated with its formation.

Case study 9.4 - ***Assessment of the effect of fertilization nitrogen on agricultural crops***
The primary question is the following: Are nutrients completely available for crop growth? There is also a secondary concern whether fertilizer pollutes ground water and affects other plant and aquatic ecosystems. For this study, characterizing the isotopic and chemical concentrations of separated nitrogen-containing nutrients (i.e. nitrate and ammonium) is critical. A sampling regime should be implemented which includes the collection of plants, crops, soil, and groundwater samples. In conjunction with concentration data, nitrogen isotopes can be used to trace the transport of nutrients and fertilizer nitrogen species into plants and groundwater. Initially, baseline values of concentration and isotopic composition of the nitrogenous nutrients in potential sources, such as groundwater, should be sampled. After application of the fertilizer, soil and groundwater nutrient concentrations should be monitored over time, preferably over the entire course of the growing season, and also subsequent seasons. If there is evidence of elevated nutrient concentrations, then more complicated analysis of the $\delta^{15}N$ of dissolved nitrogen species should be added to the study (see Box 8.3). Assessing plant uptake of nutrients involves sampling and analyzing the bulk nitrogen isotope composition of plants, both crop and indigenous species, in the agricultural fields on a monthly or weekly basis. In addition, different parts of the plants should be sampled in order to determine the degree of isotopic heterogeneity in a large plant sample. Lastly, at least 5-10 samples of the crop plants from various locations in the field should be collected in the event that the fertilizer application was not uniform.

Case study 9.5 - ***Determination of dietary sources***
Stable isotopes are increasingly being used to investigate the feeding ecology and behavior of animals and birds. The question may be "What is the relative contribution of freshwater fish to the diet of a particular bird," or "Does the diet of an animal change over the seasons". In addressing these questions, it is imperative that the potential diet sources under investigation exhibit distinct isotopic compositions (i.e. d values of sources differ by more than 2‰). For example, marine species of fish ($\delta^{13}C$ = -17‰ ± 4) have isotope compositions which are substantially different to freshwater species ($\delta^{13}C$ = -24‰ ± 4). In such diet studies, potential dietary sources are collected and analyzed and compared with particular tissue samples from the consumer. Similar sample types should be measured (e.g. feathers, muscle tissue, teeth), as the isotopic composition of individual tissue types can vary significantly within a single organism (Gearing, 1991; Tieszen et al., 1983). The carbon isotope composition of an organism should reflect its diet and the nitrogen isotope composition may indicate the trophic level of the organism in the ecosystem. It is important to understand the limitations of these methods, as there can be considerable sources of isotopic variability in the diet and the particular tissue analyzed. Gearing (1991) provides a general review of the study of trophic relationships with stable isotopes and addresses many of the issues of isotopic heterogeneity in sample types. There is a wealth of studies employing stable isotopes in elucidating feeding strategies of a large variety of organisms including birds (Bearhop et al., 1999), fish (Doucett et al., 1999), whales (Hobson and Schell, 1998), crabs (Fantle et al., 1999) and primates (Schoeninger et al., 1998).

1. *Differentiation of sources using isotopic ratios*: Because stable isotopes are most often used as tracers of different sources in an ecosystem (Case study 9.1), the first consideration should be to establish whether sources truly have unique or even distinct isotopic ratios (e.g. Farquhar et al., 1989a; Quay et al., 1992). As a rule of thumb, if the δ values of sources differ by less than 1‰, a major isotopic study designed to differentiate their movement in an ecosystem should probably be reconsidered (e.g. Benner et al., 1987). While current instrumentation and methods are capable of distinguishing much smaller differences in isotopic composition (0.2‰ for $\delta^{13}C$ or 0.3‰ for $\delta^{15}N$), in complex systems involving multiple interactions, the reality of distinguishing sources of materials with such small differences is a concern. Overall, spatial and temporal heterogeneity in δ for a single material, and isotopic variation between specific compound classes in organisms, makes distinguishing and tracing particular sources of organic matter virtually impossible at the level of less than 1‰. Therefore, it is important to study ecosystems where the ratio of signal (distinct isotope ratios) to noise (e.g. isotopic heterogeneity) is favorable.

2. *Isotopic heterogeneity in individual samples*: The most important variable that can blur isotopic signals is heterogeneity within a single sample. Individual samples may be isotopically heterogeneous on spatial scales from meters to nanometers, and therefore sampling of material requires careful consideration of these factors. For example, within a single animal, individual tissues (e.g. muscle, blood, hair) have different isotopic compositions (Tieszen et al., 1983), and in a study on isotopic heterogeneity of leaf tissue, we observed that the $\delta^{13}C$ value of plant mesophyll is consistent over a leaf, however the veins and petioles show significant deviation. In addition, individual materials are isotopically heterogeneous on a molecular scale with different biochemical compound classes (e.g. proteins, lipids and carbohydrates) having a range of isotopic compositions. This molecular heterogeneity results from different process-related fractionations that occur during the biosynthesis of specific compound classes. Since the development of continuous flow gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) for the isotopic analysis of individual compounds (Hayes et al., 1990), the literature is filled with publications defining the breadth and scope of isotopic ratios on the compound specific level. Not only are different biochemical fractions quite distinct in their isotopic compositions (range from 3-5‰), but the individual compounds that comprise the pools also have almost an order of magnitude greater variation (20-30‰) (e.g., Blair et al., 1985; Rieley et al., 1991; Abrajano et al., 1994; Fantle et al, 1999). These isotopic variations between individual compounds generally result from different isotopic fractionations during their biosynthesis. Furthermore, the heterogeneity in such biochemical isotopic fractionations can be overprinted by modification of physiological processes, such as the variations in the $\delta^{13}C$ value of phytoplankton as a function of dissolved CO_2 concentrations (Bidigare et al., 1997; 1999). These physiological variations can result in shifts of C or N isotope ratios of the bulk material by at least 5‰ (e.g., Ambrose, 1991; Bird et al., 1995; Buchman et al., 1996; Cifuentes et al., 1988). Therefore, unless a very specific biochemical pool if sampled from a physiologically invariant source, then the likelihood of source vari-

ations is very real.

3. *Spatial variation*: Sample heterogeneity governed by physiological or biochemical changes, can be exploited for delineating ecosystem dynamics. For example, the stable carbon isotope compositions of vegetation and air in forest ecosystems are influenced by height. With increasing height, the canopy air becomes progressively enriched in ^{13}C, as the influence of soil-respired CO_2 and tropospheric CO_2 changes. Therefore, as the carbon source for photosynthetic fixation (i.e. canopy CO_2) changes, the isotope values of leaves of individual trees vary as a function of height (Buchmann et al., 1997). Similarly, the isotopic composition of leaves may change with overall elevation (Sparks & Ehleringer, 1997).

4. *Temporal variation*: The stable isotope composition of components of an ecosystem can vary on a multitude of different timescales from seconds to thousands of years. The temporal variations of an ecosystem and how it responds on a diel, seasonal, or longer time spans rely specifically on isotopic fluctuations (Case study 9.2). In such studies, researchers hypothesize or predict that isotopic variations will be linked to specific biological or chemical processes (Cifuentes et al., 1989; Fogel et al., 1999). Such studies have also led to discoveries of the importance of particular pools of matter or organisms that were previously considered unimportant in the functioning of an ecosystem (e.g. Stapp et al., 1999). In addition, the isotopic composition of individual materials may reflect a specific time period and this parameter is an important one to consider (e.g. Ben-David et al., 1997; Johnson et al., 1998). For example, carbon isotope ratios in leaf tissue reflect a single growing season (e.g. Brooks et al., 1997), whereas the carbonate in apatite of teeth of animal feeding on leaves will have an isotopic signal reflecting several years of an animal's life (0-5 years) (Lee-Thorp & van der Merwe, 1991). Trees with significantly sized trunks can live for several centuries; thus long-term trends are recorded in the $\delta^{13}C$ values in growth rings (Stuiver & Braziunas, 1987; Bert et al., 1997). The $\delta^{13}C$ values of soil carbonates, which form over thousands of years, reflect long-term climate change that occurs on continental scales (e.g., Cerling, 1992). If one study included all of these carbon pools, the leaves, teeth, wood, and soil carbonates, multiple ecological processes occurring over very different time signals could be discovered (Case study 9.3).

Considering all the issues discussed above and incorporating the experiences of several researchers, we summarize below an approach to a general ecological investigation that involves stable isotope techniques:

Step 1: After defining the research questions or hypotheses, decide whether there is a good possibility that stable isotopes will be able to provide answers to the questions. Is there a good "isotope signal to noise ratio"? Which isotopes will be the most useful? Are there methods already established to analyze the stable isotopes in the ecological materials that are part of the study design?

Step 2: Design a survey of the ecosystem to measure baseline isotopic values in the pools that are of interest. Is the study site accessible year round or seasonally? Does the ecosystem vary seasonally, and most importantly, does the scientific question involve studying the ecosystem over a period of time? Is special sampling equipment required? Are permits needed for sampling or importation(e.g. CITES) across international borders?

Step 3: Begin the planning stage for collecting, processing, and analyzing samples. Assemble the equipment needed for the collection keeping in mind the storage needed until samples are analyzed (Section II). A list of all reagents, bottles, and chemicals that are needed in the field can be extremely helpful. Ancillary analyses, for example nutrient concentrations, are important considerations that are often overlooked.

Step 4: Preliminary sampling trip and isotopic analyses: Take the first set of samples, if the opportunity exists to travel easily to the ecosystem to be studied. Collection of 2-3 times more samples than you intend to analyze will allow you to choose from this array of samples at the time of isotope analysis. If the sampling site is remote, check the availability of equipment at the site, and confirm that all necessary items of equipment and chemicals are on hand. Attempt the first set of isotopic analyses. The first set of analyses should determine whether the analytical procedures are robust, if sources have distinct isotopic compositions and whether the stable isotope techniques used will address the research objectives.

Step 5: Rethinking the experiment and the sampling protocols: Often, the first field collection is an adventure that is problematic and incomplete. Analyze the initial data and reformulate the questions and experimental design to reflect the reality of the ecosystem. Consider adding additional parameters, sample types, or time points, at this stage, that now are more important, and eliminate those that will not contribute to the final results. In many instances, the material that is collected consists of a complex mixture (e.g. soil, sediment, filtered material) and therefore a more refined approach to isotope analysis may be required. In such cases, measurement of the isotopic composition of individual organic compounds can provide unique information for studying complex interactions where physical separation and even biological separation are difficult (for example the transfer of dissolved organic carbon from phytoplankton to bacterioplankton). The methods and techniques for such analyses are discussed in section 9.3.3.

9.3 Section II: Methods of sample collection and storage

Different types of samples require different methods of collection and storage, and the choice of methods will be influenced by the location of the ecosystem, the availability of cold storage, and the kinds of analyses to be performed. Whatever methods are chosen, it is imperative that they do not affect the isotopic integrity of the material being sampled. Isotopic ratios of organic material are susceptible to alteration if degradative processes take place during storage. Samples that are poorly preserved are at

risk of possible bacterial decay, which may result in mineralization or solubilization of organic carbon. Alternatively, contamination can alter isotopic ratios, especially of samples destined for compound specific isotope analysis. Contamination while sampling is minimized through the use of gloves and minimal handling of materials. Geochemical materials with ancient organic matter are sampled and stored at room temperature (e.g. light hydrocarbons in petroleum samples), whereas other materials require more refined techniques and, in some cases, specialized collection equipment is required.

9.3.1 Biological materials

Biological sampling is based on capturing and preserving the isotopic and biochemical composition of an organism at a particular point in time. In order to accomplish this, living processes must be effectively inhibited, because at death, organisms release enzymes (e.g. proteases, nucleases, and lipases) that specifically target all of the biochemical classes of compounds. Cold storage is the best method for slowing down living reactions, while also inhibiting degradation pathways. If liquid nitrogen is available on the site, quick-freezing and storage in an enclosed vessel will preserve all of the high molecular weight biochemical information, as well as any isotopic information. Dry ice or freezing in a conventional –20°C freezer are also acceptable methods of cold storage, and are often available on oceanographic research vessels or nearby laboratories. Prior to freezing biological material it is important to rinse any adhering sediment or salt off of the organism with distilled or deionized water. The use of fixatives (e.g formalin or ethanol) to preserve samples is not recommended, as we have found that tissue preserved by the addition of these reagents has an altered carbon isotope ratio, most likely because of binding between the solvent and the tissue.

In remote areas, there are several ways to overcome the inconvenient access to cold storage. First, long-life liquid nitrogen dewars can be purchased that maintain liquid nitrogen temperatures of –196°C for at least 30 days. The dewars are initially expensive, but are reusable and should provide several years worth of service. For this method to be useful, there must be a source of liquid nitrogen available. Most universities have reasonably priced liquid nitrogen reservoirs, and dewars such as these require filling only one time. Transporting liquid nitrogen to the field requires careful planning and prior to using such methods the reader is encouraged to understand the potential hazards associated with working with cryogenic liquids. Safety information is available from gas supply companies and also the National Research Council (1981). An alternative to cold storage is drying in the field. Plants can be effectively dried in plant presses on herbarium paper. We have tested this method with samples taken in the Australian desert, which is characterized by low humidity and high temperatures. Drying occurred in 2-3 days, with no change in plant appearance. Care should be taken to use clean blotter paper rather than newspaper, as the ink from the newsprint could contaminate the plant sample. Last, some plant presses are connected to a source of heat to promote drying; a simple light bulb should be chosen over heated charcoal.

An isotopic comparison of samples preserved by drying, with those preserved using liquid nitrogen revealed no difference in $\delta^{15}N$ or $\delta^{13}C$. Keep in mind that one aspect of drying that may cause complications is the use of heat to accelerate drying, which could act to increase the activity of degradative enzymes. Another complication often encountered in tropical regions is high humidity, which will increase drying times. A third and simple technique to dry and preserve samples in the field is to use desiccants (e.g. silica gel). The sample can be wrapped in foil and placed in a sealed jar or sample bag that contains a desiccant. With frequent replenishment of the desiccant, this method can remove a significant amount of water.

Storage of animal tissue is a separate problem, because drying tissue can attract insects that might consume it. As a result ample consideration should be given to the type of tissue to be studied. For example, collagenous or keratinous material from bone, horn, nails, or feathers can be easily rinsed with distilled water and dried without any chemical or isotopic alteration. Teeth are also very resistant tissues that are especially desired because they contain well preserved organic and inorganic isotopic reservoirs. However, if tissue samples are needed, they should be frozen immediately, preferably in liquid nitrogen or put on ice as soon as possible.

9.3.2 Aquatic particulate material

One of the most frequently used methods for collecting aquatic material for isotopic analysis is filtration. There are, however, many things that should be considered prior to venturing into the field. First, how much material needs to be collected for the desired analysis? Second, will the filter interfere with the analysis, or contaminate it, in any way? Third, what is the particle size of interest? For on-line isotopic methods for determining bulk C, N, H, and O isotope ratios of organic material, less than a milligram of material is needed, even including replicate analyses. In contrast, for compound specific work, especially if a target molecule (e.g. cholesterol) is sought, 5-10 mg of organic matter is a reasonable amount to collect to allow for replicate analyses. For example, if the environment that is to be studied is rich in biological material, typically a liter of water is filtered for bulk analysis.

For sampling larger amounts of material or in regions where there is a very low amount of particulate biological material, large filters are required and often each scientist constructs filtration devices that fit individual needs. Large volume filtration apparatus have been designed to operate in situ, to be connected with complex pumping system, or to be contained in pressurized vessels (Wakeham & Volkman, 1991). The size of the filter can be determined by the user, and for most materials sheets of glass fiber or polycarbonate can be cut to fit the design of the filter holder.

At this stage the type of filter material is an important consideration. For a filter to be compatible with an analytical technique it should not contaminate the sample. Most researchers use glass fiber filters, which can be cleaned by heating (450°C for 4 hours), and conventional filter holders (47 mm in diameter), which can be readily obtained from scientific supply companies. Filters containing particles (e.g. GF/F filters) are wrapped in clean aluminum foil, stored frozen, until they are dried or pro-

cessed in the laboratory prior to isotopic analysis. If the method requires a specific amount of weighed material, the filter should be chosen that will allow the sample to be recovered quantitatively and with ease (e.g., a Nuclepore filter is the perfect choice, because material can be readily rinsed off the surface of the filter).

The particle size is the last consideration for filtration. By the simplicity of their design, filters are only crude ways of separating material by size. The most common filter is the GF/F filter (glass fiber) with a pore size of 1 mm, however, as filtration proceeds, material is trapped in the fibers of glass, so that the effective or nominal pore size is reduced to 0.7 mm. A GF/F filter can effectively trap most phytoplankton, many bacteria, and all zooplankton. Typically, a much larger size filter or mesh is used to separate zooplankton from phytoplankton and bacteria. Mesh sizes > 100 mm will separate the majority of zooplankton, and filter sizes > 20 mm will remove a majority of the microzooplankton. The sample trapped between 1 mm and 20 mm is the material typically captured on a GF/F filter. It will usually include phytoplankton of all types, detrital material, some attached bacteria, and microheterotrophs including flagellates and rotifers. Unfortunately, many studies in aquatic ecology are aimed at defining trophic relationships among these different groups of organisms, and thus simple filtration fails to provide adequate separation. In such cases, a compound specific approach may be useful, especially if the organisms under investigation produce source-specific biological marker compounds, as outlined in section 9.4.3. However, if the concentration of these source-specific compounds is low, then substantially larger volumes of material would be required, collection of which may not be logistically feasible in the field.

9.3.3 Collection of water samples

Water samples collected for the isotopic analysis of ammonium or nitrate should be frozen immediately. Typically one liter of water is collected, and if the sampling location is very turbid or biologically-active (e.g. a river or lake), it is best to filter the sample through a pre-combusted GF/F filter. Seawater samples from the open ocean that contain very little particulate matter can be collected and frozen directly. In addition, a subsample (approximately 25 mls) should be frozen or analyzed for nutrient concentrations immediately (Solarzano, 1969; Strickland & Parsons, 1972). For isotopic analysis, the frozen sample will be returned to a laboratory for subsequent analysis, however long-term storage should be avoided, because concentrations of ammonium, for example, are often lower after storage. If a freezer is not available in the field, water samples should be kept cold on ice. If no method of refrigeration is available, adding 1 ml of 2 N HCl to 1 liter of water will generally preserve the sample by halting bacterial growth and ensuring that the pH of the solution favors the ionic state of ammonium.

9.3.4 Storage of samples

In the laboratory, samples are typically dried and stored in a freezer prior to isotopic analyses. The preferred method of drying is freeze-drying, but alternatives such as oven drying may be employed. Freeze-drying employs a vacuum to remove water from tissues that are kept frozen throughout the procedure, thereby minimizing the

possible degradative processes caused by heat drying. However, freeze-dryers typically are not resistant to acid, so that samples can not be pre-treated with acid (e.g. samples containing carbonate carbon) and subsequently dried. For such acid-treated samples, a similar type of drying, which was developed for biological tissues and is termed centrifugal vacuum drying, can be employed. In these instruments, samples are placed into a centrifuge, and as vacuum drying proceeds, the sample is concentrated in the bottoms of the centrifuge tubes. These drying units are generally built with chemically resistant housing and traps so that samples treated with acid or solvents can be dried readily.

The most common alternative to freeze-drying is oven drying. Most ecological laboratories house large drying ovens maintained at 80°C with or without flowing dry gas. Drying biological tissue, that is clearly dead and no longer metabolically active, can be accomplished at temperatures between 50° and 80 °C. At lower temperatures, flowing an inert gas such as nitrogen, argon, or helium over the sample both minimizes oxidation, and also decreases drying time, as water is swept efficiently from the oven. Vacuum ovens offer another alternative means of drying, and often desiccants (e.g. P_2O_5 or Silica Gel) are added to increase the efficiency of water removal.

9.3.5 Sample preparation for isotope analysis

The initial preparation of samples for isotope analysis is dependent on the type of analysis to be performed. If the isotope composition of the whole sample is sought, then after drying the material, little further preparation is required. Samples that may contain carbonate carbon, however, should be treated with dilute HCl (0.1N) and thoroughly rinsed with distilled water prior to drying. Carbonate carbon is typically more enriched in ^{13}C than organic carbon and may confuse analyses of organic material if not removed prior to isotope analysis. The carbonate present in dissolved materials or on filters can be removed by incubating the sample for 24 hours in a glass dessicator over fuming HCl. Carbonate can be removed from sediments, which can contain up to 90 wt% $CaCO_3$, by acidifying samples with either concentrated HCl in silver sample cups (Nieuwenhuize et al., 1994) or with a modified HCl vapor phase method (Yamamuro & Kayanne, 1995).

Complex sample matrices (e.g. soils, leaf litter, stomach contents) should be ground to a fine powder using a mortar and pestle, as the necessity for sample homogeneity cannot be overemphasized. Material frozen with liquid nitrogen in a mortar, or crucible, can be easily ground to a fine powder using a glass pestle. Alternatively, commercially available grinders can be used which pound samples to fine powders under cryogenic conditions (e.g., Spex Mill, Wig-L-Bug). Such equipment is very useful when working with materials such as wood, bone, soils and sediments.

If a compound specific approach is to be undertaken then substantial preparation, including specific chemical and biochemical techniques may be performed prior to isotope analysis. These techniques are discussed with relation to specific analyses later in this chapter.

9.4 SECTION III: Stable isotope analysis of ecological samples

The present chapter concentrates on techniques to measure the isotopic composition of important nutrients, bulk organic matter and the three major classes of organic compounds (lipids, amino acids and carbohydrates). The reader is also directed to *Chapters 8 and 23* for additional methods on such subjects. In addition, it may be important to measure the isotope composition of inorganic material in the ecological setting under investigation. The specific techniques required to measure these parameters are discussed in other chapters on isotopes in hydrology (*Chapter 1*), dissolved inorganic carbon (*Chapter 10*) and atmospheric gases (*Chapter 14*).

9.4.1 Isotopic analysis of ammonium

Measurement of the two major N-containing nutrients, ammonium and nitrate, is important for nitrogen isotope studies. The analysis of dissolved nitrate is described in a *Chapter 15* (applications in Liu et al., 1996; Brandes & Devol, 1997; Chang et al., 1999). There are several methods for ammonium analysis, and in all of them, NH_4^+ is separated from solutions or samples by either gas phase transfer (i.e., distillation or diffusion) or adsorption and separation by a substrate (e.g., molecular sieve Zeolite

BOX 9.3 - Nitrogen isotope analysis of ammonia

Two distinct methods of measuring ^{15}N-NH_4^+ are available. One method is a modification of the ammonia diffusion method and is described in detail in Holmes et al. (1998). The second method utilizes steam distillation and zeolite adsorption to measure the nitrogen isotopic composition of ammonia (Velinsky et al., 1989). The steam distillation unit requires a hardy refrigeration unit, a source of ammonia-free water for steam generation, a condenser, and a receiving flask. All of the glassware used in this method should be cleaned and rinsed with 1% HCl and then rinsed thoroughly with distilled water. After the glassware for distillation and receiving is assembled, and prior to preparing the first sample, 200 mls of pure ethanol is distilled into the receiving vessel. This rinse is followed by a subsequent wash with 200 mls of distilled water. Both solutions are discarded after distillation. This washing and rinsing procedure is repeated between each sample. The recovery flask is then prepared as follows: a pre-combusted Pyrex Pasteur pipette is attached to the output tube from the condenser unit. The flask contains 25 mls of dilute HCl (0.00I N), and care is made to have the tip of the pipette underneath the surface of the acid solution.

A filtered water sample (250 mls) is added to the distillation flask with 1 ml of 10N NaOH. The amount of NaOH added will depend on the salinity and the buffering capacity of the environmental water. Once the NaOH is added to the solution, the NH_4^+ is converted to NH_3, so distillation should begin immediately. It is essential that >99% of the ammonia is distilled, and during all phases of this procedure, individual steps should be checked for yields by conventional ammonium analysis (Strickland and Parsons, 1972). It is important to ensure that the pH of the acid trap remains below 6.

After distillation is completed, the receiving flask is removed from the unit and the ammonium is adsorbed onto a zeolite molecular sieve. A bed of zeolite is made on a GF/F filter (cleaned by preheated to 550°C for 2 hours) and the ammonium solution passed through the filter. Gravity filtration takes up to 60 minutes but results in approximately 100% recovery. The filtered zeolite is placed in a drying oven at 50°C for no less than 48 hours. If a dry gas can be passed through the oven, water vapor is more efficiently removed. After drying, the zeolite can be scraped off the filters and analyzed by combustion in either a sealed tube or by EA-IRMS. The precision and accuracy of this method is about ±0.5‰, with the error probably originating from slight isotopic fractionation of the NH_4^+ onto the zeolite. For EA analysis, the zeolite should be dried in the presence of either He or Ar, because N_2 from air can be trapped within the pores of the zeolite, which interferes with the analysis.

W-85) (Box 9.3). All of the procedures that have been developed are relatively simple, however, attention must be paid to almost all of the details, because large isotopic fractionations occur in the transfer of ammonium [NH_4^+] or ammonia [NH_3] between different solutions. For example, there is a 30‰ isotope effect between dissolved ammonium and gaseous ammonia. Therefore, to minimize this potential fractionation during distillation or diffusion it is essential that all (>99%) of the ammonium/ammonia in solution is transferred to the receiving solution.

Smaller isotope effects are associated with adsorption. In many of the employed methods distillation and adsorption are often coupled together (e.g. Velinsky et al., 1989), and sloppy techniques with either process can result in decreased analytical precision and accuracy. Moreover, with adsorption methods substantial losses in recovery are possible. Usually, the amount of the sample that is necessary for isotopic analysis requires the methods to be as efficient as possible, with 20 mg of N being the lower limit for routine analysis. Surface water NH_4^+ concentrations in most aquatic ecosystems are below 5-10 mM, such that 200 to 350 mls of water need to be processed for acceptable precision. Therefore, it is essential to prevent contamination of NH_4^+, which can arise from many sources, as ammonia is a gas. In particular, investigators should be keenly aware of any enriched tracer work that is being done in the same laboratory or ship, and if so, separate areas and glassware are warranted. The other sources of possible contamination are from cleaning solutions, smoke, and distilled water.

The isotope composition of low levels of ammonium in marine waters can be measured using an adaptation of the ammonia diffusion method. Large volumes of water (up to 4L) are used and during the process, ammonium is converted to ammonia, which is trapped on glass fiber filters (Holmes et al., 1998). To determine the appropriate volume of water required for analysis, it is necessary to know how much ammonium is in the sample (Solarzano, 1969; Strickland & Parsons, 1972; Holmes et al., 1999). This method may be initiated in the field or on-board ship, although diffusions generally last two weeks. Two distinct advantages of this method are that multiple samples can be run concurrently and that the labor involved per sample is minimal.

9.4.2 Isotope analysis of bulk organic matter

The isotope composition of bulk tissue samples can be measured using one of two methods. In the first, the organic material is placed in a quartz tube with reduced copper and copper oxide (CuO), sealed under vacuum and combusted in a muffle furnace at 900°C. The resulting gases are purified on a vacuum line and are then analyzed by conventional IRMS (for a thorough review see Boutton, 1991).

The second more common approach uses a continuous flow system, which comprises an elemental analyzer (EA) directly attached to an IRMS (Fry et al., 1992). Samples are weighed into tin or aluminum boats and loaded into a multiple sample carousel on the EA. The whole sample including the metal boat is combusted and the resulting gases (CO_2, N_2 and H_2O) are separated on a gas chromatography column. These gases are then introduced into the IRMS, and both $\delta^{13}C$ and $\delta^{15}N$ values can be

obtained from the same sample. Typical sample sizes range from 100 - 200 mg for animal tissue to 1- 2 mg for sediments. It is good laboratory practice to analyze a standard after every 5 samples to check precision, and ideally the standard should be similar in elemental composition to the samples of interest. Examples of standards include mesquite leaves for the analysis of plant material and collagen for muscle tissue analysis. The EA-IRMS system has many advantages over the traditional sealed tube method, including a rapid throughput of samples (up to 80 samples per day) and sample sizes of less than 100 mg (e.g. organic rich material such as muscle tissue). It should be noted that results from EA-IRMS analysis differ from those of off-line techniques, as a result of an isotopic fractionation which occurs during the introduction of the gases into the mass spectrometer. Therefore, a correction should be applied to the EA-IRMS results which should be calculated on a daily basis through the analysis of known, preferably NBS, standards.

In some instances, such as the analysis of muscle, the isotope composition of fat-free tissue may be required to simplify trophic web analysis (e.g. Hesslein et al., 1993). Removal of fats or lipids from whole tissue will affect the $\delta^{13}C$ values as lipids are typically depleted in ^{13}C relative to bulk material. The techniques employed to remove lipids range from vigorous Soxhlet extraction (e.g. Focken & Becker, 1998) to mild solvent extraction (Hesslein et al., 1993). If the goal of the study is to measure fat-free tissue, then the extraction method employed should aim to remove fats and little else. Generally, this can be easily achieved by a mild extraction technique, such as immersion of the sample in hexane, with gentle agitation and decanting of the solvent. More vigorous techniques may remove other organic compounds, such as the more polar compounds (e.g. cholesterol), which will in turn affect $\delta^{13}C$ values of the "fat-free" tissue. In addition, vigorous extraction of blood samples may affect $\delta^{15}N$ values, as compounds such as uric acid and urea may be preferentially extracted (Bearhop et al., 2000).

Stable isotope analysis of bulk organic matter has been widely used in ecological research (reviews include Griffiths, 1998; Lajtha & Michener, 1994; Ehleringer et al., 1993; Rundel et al., 1989; Peterson & Fry, 1987). For example, the isotopic composition of plant organic matter can be related to changes in physiology, associated with differences in water use efficiency (Bert et al., 1997; Johnson & Tieszen 1994; Read et al., 1992; Farquhar et al., 1989a). Environmental parameters such as light, water, salinity and air pollution (Buchmann et al., 1996; Du et al., 1998; Farquhar et al., 1989a) mitigate $\delta^{13}C$ values as well. Stable isotopes of animal tissue are also used to investigate trophic structure, migration, and metabolism (Hobson & Clark, 1992; Koch et al., 1995; Ben-David et al., 1997; Anderson & Polis, 1998; Witt et al., 1998; Schmutz & Hobson, 1998; Wainwright et al., 1998; Jones et al., 1998; Schoeninger et al., 1998; Alisauskas et al., 1998; Marra et al., 1998; Hansson et al., 1997; Nelson et al., 1998; Ostrom et al., 1997).

9.4.3 Compound specific isotope analysis of major biochemicals

The isotopic composition of individual organic compounds in ecological and biological samples can be extremely useful in deconvoluting processes and interactions

between organisms in complex systems. In studies where bulk isotope analysis may provide an overview of the whole ecosystem, targeting specific molecules will provide information on the role of individual organisms. This approach relies on different organisms synthesizing different molecules. Through numerous chemical and biochemical procedures, these molecules can be separated and their isotopic compositions analyzed individually. The approach is particularly powerful in studies where physical separation of material is difficult such as in the determination of sources to water column particulates in lakes. Filtering particulate matter from the water column provides an overview of the multiple sources to the lake and the isotopic composition of the complete filter reflects an average signal of input. The isotope composition of specific molecules will provide more detailed information pertaining to the importance of different sources. The $\delta^{13}C$ of individual n-alkanes indicate different types of higher plant sources (Spooner et al., 1994; Rieley et al., 1991), whereas the isotopic composition of a particular suite of sterols may provide information on phytoplankton inputs (Canuel et al., 1997).

A more recent application of compound specific isotope analysis is aimed at elucidating particular metabolic pathways in organisms. The isotope composition of individual compounds is dependent on the enzymatic reactions which occur during their biosynthesis, and therefore retains a signal of their pathway of synthesis. Several studies have exploited these techniques to elucidate the pathways of lipid synthesis in organisms such as bacteria (Teece et al., 1999; Summons et al., 1998; van der Meer et al., 1998) and pigs (Stott et al., 1997a).

The compound specific approach requires a significantly greater effort to prepare a sample for analysis, and in some cases this additional work may not be worthwhile. For example, the preparation of a single sample for the analysis of the $\delta^{13}C$ values of individual amino acids by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) may take up to three days in the laboratory. Typically, the isotope composition of samples are measured in triplicate with each analytical run being up to 60 minutes in length. As a result of these protracted periods of preparation and analysis, it is difficult to acquire large data sets. Secondly, GC-C-IRMS systems are not abundant and are not simple off-line attachments which are free of problems. As with any analytical instrument, these systems require continual monitoring and maintenance, however the results obtained from such analyses are often worth the hard work involved. Therefore, in determining whether such a compound specific isotope approach is warranted, several questions should be addressed:

- Can bulk isotope analysis provide the answer?
- Is the determination of sources or biochemical pathways important?
- Are there multiple sources (pathways) that cannot be resolved by bulk techniques?
- Do the source organisms (pathways) synthesize different molecules?
- Are these compounds specific to the source organism (pathway)?

Once the specific compounds of interest have been determined, the question of analysis techniques need to be addressed. The primary technique to analyze the isotope composition of individual compounds is gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS). The sample is injected into a gas chromatograph and the compounds of interest are separated on a well-chosen chromatography column. The column eluent passes through a micro-volume furnace where the material is combusted and the resulting CO_2 (or N_2) is introduced into the mass spectrometer (Hayes et al., 1990; Eakin et al., 1992).

The limiting factor in compound specific analysis using GC-C-IRMS is that only volatile compounds can be analyzed as the inlet system is a gas chromatograph. There has been limited success in designing systems where the inlet system is a liquid chromatograph, though commercial experimental systems have been developed and marketed. In many cases, chemical derivatization of a compound is necessary to synthesize a component that is amenable to gas chromatographic analysis, and therefore GC-C-IRMS analysis. Volatile compounds, such as certain classes of lipids, can be directly analyzed, while amino acids and carbohydrates require extensive chemical derivatization.

The analytical procedures to separate particular classes of compounds are numerous, accordingly we will present an overview of suggested approaches. It should be noted that in order to obtain reproducible isotope values, individual compounds must be sufficiently separated on the gas chromatography (GC) column prior to mass spectrometric analysis. Isotopic precision depends on adequate, preferably baseline, separation of components. Therefore, it is important to verify both the methods of GC separation as well as IRMS techniques for the isotopic analysis of specific compounds. It is essential to check the analytical procedures used by the repeated analysis of compounds of identical chemical composition. Checking the methodology in this manner should confirm that the specific compounds of interest are quantitatively extracted from the sample, and also indicate whether any isotope fractionation occurs during analysis. Furthermore, it is good practice to add one or more "internal" standard compounds of known chemical and isotopic composition to the sample prior to extraction/analysis. These internal standards should have similar chemical properties to the compounds of interest, however should not be present in the natural sample or coelute with other compounds. The isotopic composition of these compounds can be measured in the final analyses as a check to indicate the reproducibility of the methods used. For example, *n*C36 alkane is typically employed as an internal standard in hydrocarbon analyses, and $n\mathrm{C}_{19:0}$ fatty acid used in fatty acid analyses.

The approach to measuring the isotope composition of individual compounds requires numerous steps and these should be addressed prior to sample collection (Figure 9.3).

Step 1: Collection and storage of samples for analysis should minimize contamination. GC-C-IRMS is a very sensitive technique, with nanogram quantities of material detected (cf. milligram amounts for bulk isotope analysis). Human hands

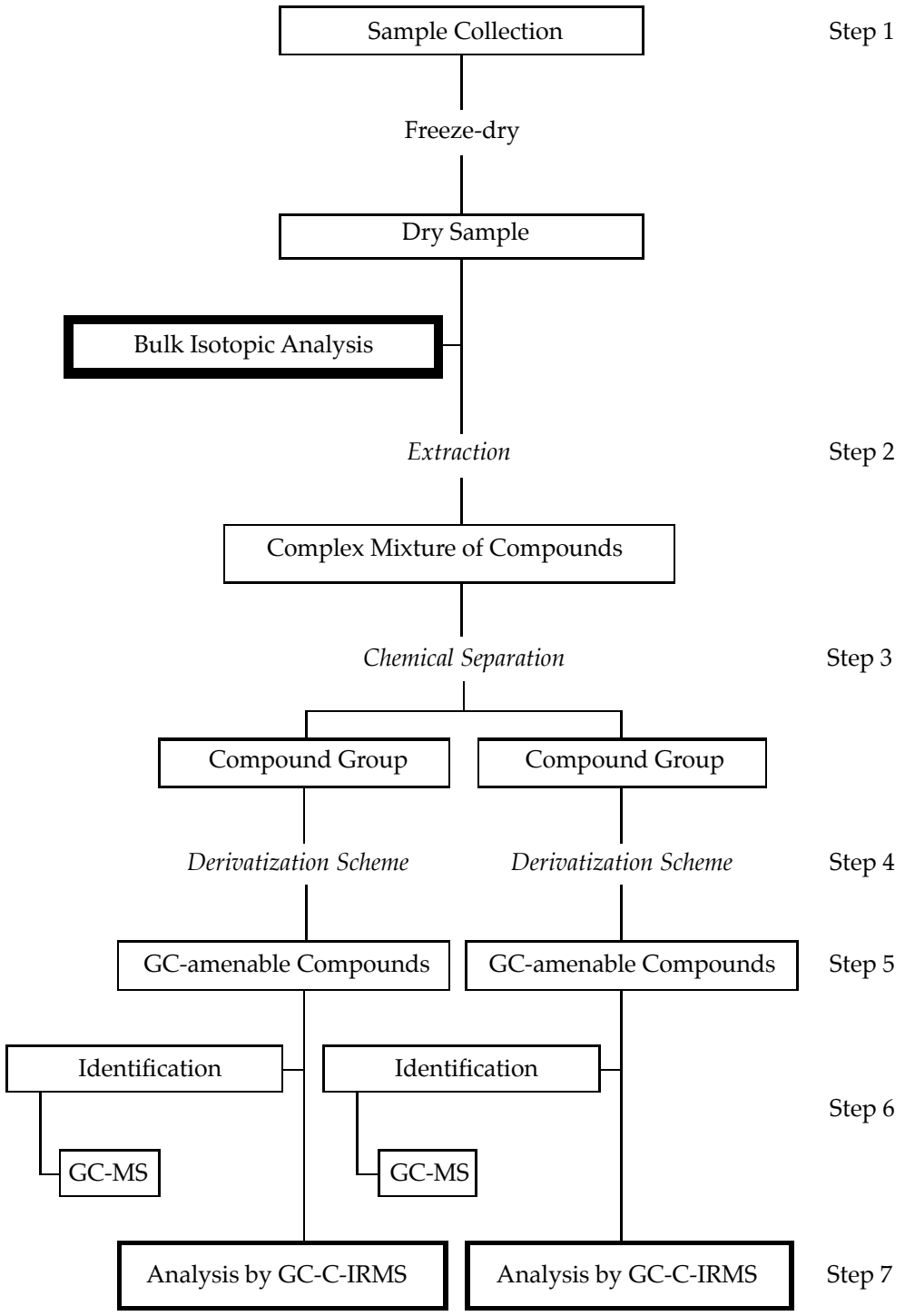

Figure 9.3 - A generalized analytical scheme for the stable isotope analysis of individual organic compounds in biological samples. The numbered steps refer to analytical procedures that are specific to the compounds of interest, and are discussed in more detail in the text.

contain many of the lipids and proteins that will be subsequently analyzed, so handling should be minimized and gloves used. The use of chemical preservatives, such as ethanol or formaldehyde, is discouraged and samples should ideally be stored in clean glassware or sterile plasticware. For most analyses, material should be freeze-dried as discussed above.

Step 2: Extraction of the compounds of interest requires the correct extraction method. Hydrophobic lipids and fats are extracted using a series of organic solvents, whereas carbohydrates and amino acids are hydrophilic and sulfuric and hydrochloric acids are employed in their extraction. The chemicals, materials and conditions differ for all compounds and selecting the correct method is paramount in the successful extraction of material. Prior to extraction, (internal) standard compounds should be added to check the methodology.

Step 3: The mixture of extracted compounds may be complex and require additional separation techniques to isolate the compounds of interest in greater purity. Several methods of separation can be used including column chromatography, thin layer chromatography, ion exchange chromatography, and electrophoresis. The particular techniques required differ with each compound class and extensive literature can be found relating to these analytical challenges.

Step 4: The mixture of compounds may be chemically derivatized to produce compounds, which can be analyzed on a gas chromatograph. Volatile lipid compounds (e.g. hydrocarbons) can be analyzed directly, such as those present in epicuticular waxes of higher plants (Rieley et al. 1991; Collister et al. 1994; Lockheart et al. 1997). However, fatty acids and alcohols require derivatization prior to analysis, in order to attain sufficient chromatographic separation required for reproducible isotopic analysis. Numerous techniques for derivatization are published and depend on the chemical functionality of the particular compounds: Fatty acids are methylated whereas alcohols are typically analyzed as the trimethylsilyl ethers.

Step 5: To acquire reproducible isotope numbers, the components of complex mixtures must be adequately separated on the gas chromatography (GC) column to reduce the influence of contaminating or co-eluting peaks during analysis. Separation of compounds using GC requires the correct choice of chromatographic column. There are a multitude of chromatographic columns available from several vendors, varying in polarity and size, and the choice of column is dependent on the chemical properties of the components being analyzed. Nonpolar columns, such as DB-1, HP-1, Ultra-1, are used for hydrocarbon analyses and a column containing a 5% phenyl substituted methylpolysiloxane phase (HP-5, CP-Sil 8C) is useful for analysis of derivatized alcohols and fatty acids.

Step 6: The identity of the specific compounds isolated should be confirmed by GC-MS prior to isotope analysis. This step will ascertain whether the separation on the chosen GC column is adequate and will also indicate whether any contam-

ination was introduced during the analytical scheme.

Step 7: Stable isotope analysis of the individual compounds by GC-C-IRMS. The analyses should be performed in triplicate to determine variations on instrumental analysis. Analysis of standards of similar chemical structure will aid in determining the reproducibility of results.

Step 8: If a compound was derivatized prior to analysis, then the isotopic composition of the original compound must be calculated. Derivatization usually involves the addition of a carbon-containing component in order to produce the volatile compound. Calculation of the isotopic composition of the original compound requires consideration of the contribution of the added carbon to be assessed if measuring $\delta^{13}C$ values. Therefore, it is essential to analyze compounds of known chemical and isotopic composition to calculate the effect of the derivatizing agent. For example, fatty acids are methylated prior to analysis and the isotopic contribution of the added methyl carbon is assessed by isotopic analysis of underivatized and subsequently derivatized fatty acid standards (see Abrajano et al., 1994). Simple mass balance calculations allow the contribution of the $\delta^{13}C$-derivatizing agent carbon to be assessed, which can then be applied to the unknown compounds to calculate the original isotope compositions.

These steps are general guidelines and what follows are more specific examples of approaches to analyzing specific molecules. The approaches below are suggestions and each method can be altered or customized to the particular question being addressed.

9.4.3.1. Isotope composition of individual lipids

Lipids are a class of hydrophobic compounds that include fatty acids, phospholipids, triacylglycerides, alcohols, hydrocarbons and sterols (e.g. cholesterol). Lipids perform numerous functions including acting as a source of energy (e.g. triacylglycerides) to a protection mechanism against predators (e.g. plant wax esters). The carbon isotopic composition of individual lipids reflects both the isotopic composition of the carbon source utilized by the organism and isotopic fractionations accompanying biosynthesis. Isotopic fractionations which result from the enzyme-mediated reactions during biosynthesis of lipids are ultimately dependent on environmental conditions. Therefore, the isotope composition of individual lipids can provide valuable information on both the carbon source and potential changes in the organisms environment. Such an approach has been used in several studies to determine changes in past vegetation patterns by isotopic analysis of higher plant-derived hydrocarbons (France-Lanord & Derry, 1994; Bird et al., 1995; Lichtfouse et al., 1995; 1997) and lignin-phenols (Goni & Eglinton, 1996) in lake and marine sediments.

Extraction of lipid compounds is a relatively simple procedure, however some caution should be applied to the methods employed. Ideally, the samples should be dry, having been freeze-dried as described above. There are two main methods of lipid extraction, both of which use similar organic solvents. The choice of organic sol-

vents in the extraction of lipids is important and for most procedures mixtures of dichloromethane and methanol are applicable [dichloromethane should be used rather than chloroform - dichloromethane has similar chemical properties to chloroform but is substantially less harmful]. Glass distilled or Optima grade solvents should be used whenever possible. All glassware should be thoroughly cleaned and rinsed with solvents prior to use to minimize contamination.

One method for lipid extraction uses a Soxhlet system, in which clean boiling solvents (mixtures of dichloromethane and methanol) are refluxed through the sample for periods ranging from 30 minutes to 24 hours. This method is best employed when extracting lipids from a complex matrix such as sediments, soils and some woods, where the compounds of interest may be physically trapped or weakly bound in the matrix. For biological material, particularly living tissue (e.g. plants, muscle, and blood), a simpler and less harsh extraction scheme should be used. This second extraction method (Box 9.4) uses a mixture of dichloromethane and methanol at room temperature, with repeated mixing using an ultrasonic tank or vortex mixer (modified from Bligh & Dyer, 1959).

Once the lipids have been extracted from the sample, the task of separation and derivatization of the particular compounds of interest needs to be addressed. The total organic extract of a sediment may contain more than 80 different compounds, so that one or more clean-up steps may be required before analysis by GC-C-IRMS. The goal of these procedures is to obtain a sample fraction, which contains the molecules of interest in a mixture that can be adequately separated on the gas chromatograph. In the case of hydrocarbon analysis, a single purification step allows adequate separation of more than 20 compounds (e.g. Freeman et al., 1990; Bjoroy et al., 1991) whereas low concentrations of cholesterol may require multiple clean up steps to isolate the compound in sufficient purity (e.g. Canuel et al., 1997). Similarly, analysis of lignin-phenols, a class of compounds that are uniquely synthesized by vascular plants, requires a complex procedure of chemical oxidation and derivatization procedures prior to analysis by GC-C-IRMS (Goni & Eglinton, 1996).

BOX 9.4 - Lipid extraction of plant tissue

Freeze-dried leaves are ground to a fine powder using a pestle and mortar. A mixture of dichloromethane:methanol (1:1) (Optima grade) is added to the plant samples (15-20mg) in a precombusted glass test tube fitted with a Teflon-lined cap. The mixture is thoroughly mixed for up to 10 minutes, using a combination of ultra-sonication and vortex mixing. If required, the sample can be centrifuged (1,000g; 5 mins) to pellet the plant matter, and the solvent removed with a clean glass pipette. This procedure should be repeated 5 times to ensure complete extraction with 5ml of clean solvent mixture being used for each cycle. The extract is transferred to a round-bottomed flask and the solvent removed using a rotary evaporator. The total organic extract (TOE) is redissolved in the minimum dichloromethane required to quantitatively transfer it to a clean precombusted vial fitted with a Teflon-lined cap. The solvent is removed under a stream of nitrogen at room temperature to produce the dried TOE, which is stored in a freezer prior to further treatment.

BOX 9.5 - Isotope composition of individual fatty acids from muscle tissue

After extraction of muscle tissue using dichloromethane and methanol, the total organic extract (TOE) is subjected to mild alkaline hydrolysis to release the esterified fatty acids. A 6% KOH solution in methanol (5 ml) is added to the TOE, the tube tightly sealed and allowed to react overnight at 50°C. After cooling, 3 ml of double-distilled deionized water is added and the neutral lipids extracted three times (each 5 ml) into a mixture of hexane:diethyl ether (9:1). Neutral lipids are transferred, and stored dry, in glass vials with Teflon-lined caps. The remaining aqueous methanol phase is acidified to pH 2 with 6N HCl (Pierce Chemicals, constant boiling HCl). The fatty acids present in this fraction are extracted three times (each 5 ml) into a mixture of hexane:diethyl ether (9:1) and transferred to a clean precombusted glass test tube. Solvent is then removed under a stream of nitrogen at room temperature.

Fatty acids are subsequently converted to their corresponding fatty acid methyl esters (FAMES). 200ml of a solution of 14% BF_3 in methanol (Pierce Chemicals) is added to the fatty acid fraction, the tube tightly sealed and allowed to react for 15 minutes at 60°C. After cooling, 3ml of double-distilled deionized water and 3ml of hexane is added to the mixture. FAMES are extracted into the hexane fraction (3 x 3 ml), transferred to a glass vial and the solvent removed under a stream of nitrogen. Samples should typically be analyzed within two days of preparation using a non-polar GC column (Ultra-1).

Measurement of the $\delta^{13}C$ values of individual fatty acids (see Box 9.5) requires a single clean-up step and a subsequent derivatization step. After organic extraction of the sample, the fatty acid fraction is isolated using ion exchange chromatography and converted to the corresponding fatty acid methyl esters (FAMES). The $\delta^{13}C$ values of fatty acids have been reported in studies of plants (Collister et al., 1994; Rieley et al., 1991; Vogler & Hayes, 1980), changes in diets (Rhee et al. 1997; Trust-Hammer et al., 1998; Gilmour et al., 1995a), and in ecological studies of mussels and shrimp in hydrothermal vents (Pond et al. 1998; Rieley et al., 1999).

The techniques to analyze particular lipid compound classes are reported in the literature (e.g. Blau & Halket, 1993) and require various chemical separation and derivatization techniques. Isotopic analysis of hydrocarbons has been used in studies ranging from palaeoenvironmental reconstruction (Rieley et al., 1991; Freeman et al., 1994; Bird et al., 1995) and elucidation of input sources of vascular plants to coastal sediments (Canuel et al., 1997), to the feeding habits of insectivorous bats (Des Marais et al., 1980). The isotopic analysis of PCBs (polychlorinated biphenyls) and PAHs (polyaromatic hyrdrocarbons) may also be employed to determine sources of toxic chemicals in the environment (O'Malley et al., 1994; Ballentine et al., 1996; Jarman et al., 1998). Alcohols can be analyzed as trimethylsilyl (TMS) derivatives (Jones et al. 1991) and such techniques have been applied to elucidation of dietary sources through the measurement of $\delta^{13}C$ of cholesterol in pigs (Stott et al. 1997a) and fossil whalebones (Stott et al. 1997b).

9.4.3.2. Isotope composition of individual amino acids

The amino acids are the building blocks of proteins, and the isotopic composition of these compounds reflect their pathways of biosynthesis. Amino acids are ubiquitous compounds that give very little clue as to their origin or source by their relative abundance alone. The isotopic compositions of these molecules, however, are diverse and can be quite powerful in delineating pathways of biosynthesis or processes of

diagenesis (Macko et al. 1987; Engel et al., 1990; Engel & Macko, 1997; Fogel et al., 1997; Fogel & Tuross, 1999; Fantle et al., 1999). Predictable and fundamental isotopic patterns have been studied in a diverse group of organisms from differing ecological and geological contexts. Typically in plants, glycine and aspartic acid are the most enriched in ^{13}C with valine being the most depleted (Fogel et al., 1997; Fogel & Tuross, 1999). The distribution of isotope signatures of amino acids in higher trophic level organisms is dependant on, and indicative of, the food source. The $\delta^{13}C$ and $\delta^{15}N$ values of individual amino acids in such organisms may be predicted on the basis of their pathway of biosynthesis (non-essential amino acids) or their direct incorporation from the diet (essential amino acids) (Fogel et al., 1997).

Isotopic compositions of amino acids have been analyzed by off-line combustion after the compounds had been separated by high performance liquid chromatography (Abelson & Hoering, 1961; Macko et al., 1987; Hare et al., 1991; Balzar et al., 1997). As stated, liquid chromatography has yet to be routinely interfaced with an isotope ratio mass spectrometer, therefore a majority of the analyses are now performed on derivatized amino acids separated by gas chromatography. Tissues are hydrolyzed with HCl, dried down, and then derivatized by a number of different methods (see *Chapter 8*). The method detailed in Box 9.6 is straight-forward, robust, and a majority of the major biological amino acids can be separated for isotopic analyses. In addition, the method is suitable for chiral detection of D- and L- amino acids (Silfer et al., 1991; Metges et al., 1996). A disadvantage of this method is the addition of fluorine molecules that provide volatility can contaminate oxidation furnaces and reduce the working lifetime of these furnaces.

The isotope composition of amino acids is determined by isotope fractionations associated with the specific enzyme-mediated reactions which occur during their synthesis. Glycine is commonly the amino acid with the most ^{13}C-enriched isotopic com-

BOX 9.6 - Amino acids in bone collagen

Samples of isolated collagen (Ambrose, 1990; Koch et al., 1994) are weighed (1-3 mg), and loaded into pre-combusted hydrolysis tubes, and hydrolyzed under an atmosphere of N_2 in 1ml of 6N constant-boiling HCl (Pierce Chemical) (20 hours at 110°C). The resulting hydrolysates are dried under a stream of N_2 at 100°C, and the released amino acids esterified by 1 ml of anhydrous acidified iso-propanol (1 hour at 110°C), and subsequently acylated by 0.5 ml trifluoroacetic anhydride (TFAA) in 0.5 ml dichloromethane (10 min. at 110°C). Samples are analyzed on a GC-C-IRMS system using a non-polar GC column (e.g. Ultra-1). A suite of amino acid standards are derivatized and analyzed with each batch of samples. For carbon isotope analyses, about 2 mg of hydrolyzed protein is injected onto the column. After various splits in the injector (1:10) and the open split (1:2), about 10 ng of carbon per peak enters the ion source of the mass spectrometer. For nitrogen isotope analysis, about 3 mg of sample is injected in the splitless mode, such ca. 100 ng of amino acid is converted to N_2.

Each sample should be analyzed in triplicate with corresponding standard amino acids. Reproducibility for carbon isotopes ranges from ±0.2‰ to ±1.0‰, depending on peak size, with a typical error of ±0.4 ‰. For nitrogen isotopes, because the derivative contains no added nitrogen, values are determined directly. Standard deviations on the major peaks (e.g. proline, aspartate, glutamate, lysine, and arginine) are about ±0.5 ‰. Well-resolved but very small peaks (e.g. valine and leucine) have higher errors ±1-2 ‰.

position. It has a simple and central role in metabolism. In terrestrial plants, glycine is formed from serine during photorespiratory processes. Isotopically heavier carbon is shunted into this pathway with ^{13}C depleted carbon going into subsequent amino acids synthesized in the Krebs cycle. Glycine is a key intermediate in the formation of porphyrins and purines, thus an understanding of its isotopic composition may be critical in understanding the isotopic composition of molecules, such as chlorophyll.

Many amino acids are essential for the growth of animals and the isotopic signatures imparted to these particular essential amino acids can be retained as proteins from primary producers are digested. For example, in omnivorous animals, meat or other animal protein is the primary contributor of lysine to the diet. Therefore, the isotopic composition of lysine should indicate the source of dietary protein, whereas the bulk $\delta^{13}C$ should reflect the primary carbohydrate or energy source (Fogel et al., 1997). Furthermore, isoleucine can be 6-10‰ more positive in $\delta^{13}C$ than leucine and valine in bacterial and algal proteins. The isotopic difference between leucine and isoleucine transfers up the food chain into fish, birds, and mammals and therefore the $\delta^{13}C$ of these components can be used to trace trophic interactions (Fogel et al., 1997).

The $\delta^{13}C$ of alanine may be an indicator of the energy status of an organism. Alanine is one of the most variable amino acids in terms of isotopic composition, and derives its carbon skeleton directly from that of pyruvate. Pyruvate, and alanine, are central metabolites between the TCA cycle and carbohydrate metabolic pathways (e.g., glycolysis in case of heterotrophs or the Calvin cycle of photosynthesis). Lastly, ranges in $\delta^{13}C$ of amino acids are also useful indicators of source and processes. For example, in higher plants, a range of 25‰ in $\delta^{13}C$ is common, and in phytoplankton, the spread in $\delta^{13}C$ varies from 15 to 20‰, whereas in sharp contrast, cultured bacterial amino acids have a very narrow range of 6-8‰ (Macko et al., 1987; Fantle et al., 1999; Fogel et al., 1997; Teece & Fogel, unpublished results). The different ranges of $\delta^{13}C$ values is governed by isotope fractionations occurring during amino acid biosynthesis and such distributions may be used in future to determine sources of these compounds to natural ecosystems.

9.4.3.3. Isotope composition of carbohydrates

The largest reservoir of carbon in the biosphere resides in the carbohydrates, and can comprise up to 40% of the dry weight of bacteria and up to 70% of that of vascular plants. Carbohydrates serve as metabolic storage products, as carbon and energy sources in non-photosynthetic metabolism (e.g. starch, inulin, glycogen, mannitol), and are associated with cell walls and membranes that provide protection, stability and strength (e.g. cellulose, hemicellulose, pectins, chitin, agar, peptidoglycans, lipopolysaccharides and glycolipids).

Carbohydrates represent the major form of photosynthetically fixed carbon and are critical energy components of food webs. In the oceans, most of the organic material produced by phytoplankton is consumed by herbivorous zooplankton and protozoans in the photic zone, which are in turn consumed by higher trophic level organisms. Carbohydrates, in particular glucose, represent the major organic carbon

BOX 9.7 - Isotope composition of individual monosaccharides in zooplankton

Samples of isolated zooplankton (20mg) are added to 72% H_2SO_4 (1ml) at room temperature for 90 minutes. After dilution to 1.2M H_2SO_4, the sample is further hydrolyzed for 3 hours at 100°C. An internal standard (myo-inositol) is added to the hydrolysate, and both are subsequently neutralized to pH 6.5 with $BaCO_3$. The resulting $BaSO_4$ precipitate is washed and removed by centrifugation, and the neutralized hydrolysate reduced to 2ml by rotary evaporation. The released monosaccharides are reduced to alditols by reaction with 0.5ml of a freshly prepared solution of $NaBH_4$ (500mg in 10ml H_2O) for at least 12 hours at room temperature. The residual $NaBH_4$ is decomposed by the addition of glacial acetic acid, and after effervescence ceases (pH 5.5), a second internal standard (erythritol) is added, and the solution evaporated to dryness under reduced pressure. Boric acid is removed by repeated additions of methanol, followed by evaporation to dryness. Acetylation is performed in sealed tubes in a solution of pyridine:acetic anhydride (1:1) for 2 hours at 100°C. After the addition of water (2ml), the alditol acetates are extracted into dichloromethane (3 x 2ml) and evaporated to dryness under a stream of nitrogen. The derivatives are analyzed in triplicate by GC-C-IRMS using a polar column (e.g. BPX-70) and compared to a suite of standard compounds treated in an identical manner.

component transferred between trophic levels. The isotopic composition of glucose provides an important indicator of the processes and pathways of energy utilization in ecosystems (e.g. Moers et al., 1993).

Measurement of the isotope composition of carbohydrates is an analytical challenge, and therefore there are few published reports of analyses. Macko et al. (1990; 1991) measured the $\delta^{13}C$ of individual monosaccharides by off-line combustion of components which had been previously isolated by liquid chromatography. Carbohydrates are highly hydrophilic and the challenge to produce derivatives, which are amenable to GC analysis, requires several chemical reactions and derivatization steps (Moers et al., 1993). An approach to the analysis of individual monosaccharides is presented in Box 8.7 (modified from Cowie & Hedges, 1984), however as many steps are involved it is paramount to test these procedures with standard compounds to determine whether the reaction scheme results in isotope fractionation during derivative preparation.

In the majority of biological material, carbohydrates are enriched in ^{13}C relative to amino acids and lipids, and also bulk tissue. Individual monosaccharides can be up to 10‰ enriched in ^{13}C relative to whole tissue, such as in cyanobacterial mats (Moers et al, 1993). In herbaceous and woody pant tissues, cellulose and hemicellulose are typically enriched in ^{13}C by 1-2‰ relative to whole plant material (Benner et al, 1987). During the diagenesis of organic matter, the isotopic composition of carbohydrates provides a means to trace sources of these compounds, either resulting from new bacterial production or degradation (Macko et al., 1991). In the future, the isotopic composition of individual monosaccharides may provide additional vital information in tracing sources of organic matter and the transfer of energy in ecosystem studies. The methods for the isotopic analysis of these compounds has been established, and now provides an additional tool for investigating ecological questions on a molecular scale.

9.5 Conclusions

Because of new technological advancements in the elemental analyzers linked to isotope ratio mass spectrometers (e.g., Kornexl et al., 1999a), stable isotope analysis is quickly becoming a standard measurement for interpretation of various biological and environmental parameters. We easily imagine that in the next ten years these instruments will take their place along side of nutrient autoanalyzers, fluorescence instruments, and UV/Vis spectrophotometers. The challenge then arises for ecologists to use stable isotope tools as wisely and as carefully as those who developed the early methods, perfected the techniques, and pioneered their use. Graduate students in ecology are currently learning the "ins and outs" of stable isotope fractionation and systematics, as part of their core education.

The compound specific techniques are deeply rooted in chemistry and will probably remain in the domain of geochemists and biochemists. Chemistry departments, on the other hand, are finding that creating links to environmental and biological colleagues through interdisciplinary collaborations open up new ways of examining complex ecosystems. Students in ecology will naturally gravitate to laboratories where these more complex isotopic methods are being practiced and applied to biological problems. The ability to couple the power and speed of elemental analyzer online methods to the specificity of individual compound work will be key for the full integration of stable isotopes as essential parameters for understanding and delineating ecosystems and ecosystem processes.

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