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Manual for the Use of Stable Isotopes in Wetland Science

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84

MANUAL FOR THE USE OF STABLE
ISOTOPES IN WETLAND SCIENCE

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INTERNATIONAL ATOMIC ENERGY AGENCY
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FOREWORD

This publication was developed to support the technical cooperation project entitled Enhancing Wetland Management and Sustainable Conservation Planning under the Regional Co-operative Agreement for Research, Development and Training Related to Nuclear Science and Technology. The project brings together representatives of 14 countries in the Asia and the Pacific region — Australia, Bangladesh, China, Indonesia, Laos, Malaysia, Mongolia, Myanmar, New Zealand, Pakistan, the Philippines, Sri Lanka, Thailand and Viet Nam — with a common interest in the sustainable management of wetlands supported by nuclear science.

Wetlands provide fundamental ecosystem services across the Asia and the Pacific region, supporting freshwater and marine fisheries, sustaining biological diversity and providing an efficient sink for atmospheric carbon. The project aims at enhancing Member State capabilities in the sustainable management of wetlands by training partners and regional organizations in the use of stable isotopic techniques to clarify the movement of carbon between components of wetland systems. These techniques have been applied to answer basic questions posed by natural resource managers such as, What sources of primary production drive the productivity of fisheries? What are the trophic interactions on which healthy fisheries depend? How do the management of hydrology and the input of pollutants influence the integrity of wetland ecosystems? How much carbon is permanently sequestered and stored in wetland soils, and what is the source of this carbon?

As outlined in the introductory sections of this manual, stable isotopes lend themselves to answering several of these significant questions. They serve as natural markers allowing energy sources to be traced through ecosystems, making clear the dependencies between ecosystem components and habitats, and the influence of human disturbances. The widespread uptake of isotope ratio mass spectrometry (IRMS) technology has opened the door for greater application of isotopic techniques in coordinated and targeted research and monitoring programmes.

The manual has been structured to facilitate online training of scientists and technicians with limited prior knowledge of wetland environments or isotopic techniques. A problem based pedagogy is adopted, with training focused on case studies illustrating common problems and widely applied techniques. Step-by-step instruction is provided in appropriate experimental design, common sampling methods in wetlands, sample preparation and the operation of IRMS. An introduction is provided to the interpretation of results, including the application of source mixing models and their limitations.

The IAEA officer responsible for this publication was I. Tolosa of the Marine Environment Laboratories.

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1. INTRODUCTION

1.1. BACKGROUND

A major impediment to the ‘wise use of wetlands’, the central tenet of the Ramsar Convention, is the determination of the quantitative value of wetland ecosystem services and their vulnerability to anthropogenic and environmental changes. Management and conservation of wetlands is hindered by inadequate quantifiable information on the ecosystem services provided by wetlands and their dependences on primary habitat. As a result, adaptation planning and management for wetlands is hindered, leading to significant social, environmental, and economic impacts. This is particularly alarming for the Asia Pacific countries, as many of the largest wetlands in the world, such as Kerala Backwater in India, the Sundarbans in Bangladesh, and Wasur National Park in Indonesia, are located in the region. In recognition of the importance of healthy wetlands to healthy human populations, and the important role played by stable isotope techniques defining key supporting processes, “the Agency” established a program of training under the Regional Cooperation Agreement to equip Member States in the Asia–Pacific region in tools applicable to wetland science and management. This manual was developed to support training programs offered under the project RAS7037 “Enhancing wetland management and sustainable conservation planning in the Asia–Pacific”.

1.2. OBJECTIVE

The objective of this publication is to assist in the development and implementation of research programs applying isotopic techniques to commonly encountered issues in wetland management. The manual is designed to provide sufficient detail to convey the key principles in the application of isotopes in wetland science, the design of wetland research projects, issues relating to sampling within wetland environments, the preparation and processing of samples, and the interpretation of results.

1.3. SCOPE

The scope of this publication is to serve as a guidebook for the design and implementation of research programs applying stable isotopes in wetland environments. The publication reviews the important applications of stable isotopes to the monitoring and management of wetlands and key processes supporting ecosystem services of fisheries production and carbon sequestration. The design and implementation of sampling programs and field campaigns are described, as is the preparation of samples for stable isotope analysis in the IRMS. Additional training is suggested to develop technical competency in the running of the EA-IRMS instrument. The manual introduces the most commonly applied tools in the statistical interpretation of stable isotope data.

1.4. STRUCTURE

The first section reviews the science of stable isotopes and their application in environmental research and monitoring, with a particular focus on wetland environments. This provides a foundation for the subsequent presentation of sampling design principles. The second section considers sampling techniques associated with commonly encountered wetland management issues. This is achieved through three case studies, each targeting a question of central importance: determination of trophic interactions within wetlands; monitoring of wetland restoration; and the measurement of carbon stocks within wetland. The third section covers the preparation and processing of samples within the EA-IRMS, the instrument used to determine the stable isotope ratios in samples, including the full range of quality assurance protocols. The final section provides guidance in the interpretation of stable isotope data, providing links to the commonly used isotope mixing models and explanation of their application.

2. STABLE ISOTOPES IN THE ENVIRONMENT

2.1. STABLE ISOTOPES

Stable isotopes are different forms of the same element, being different in the number of neutrons, but identical in the number of protons. They are stable in that they are nonradioactive and do not decay. This property makes them important environmental tracers, and there are a range of exciting applications of stable isotopes in environmental research. They have been extensively used in food chain studies, but applications in restoration monitoring and the emerging field of 'Blue Carbon' have been important recent developments.

Natural abundance of isotopes changes in response to physical and biological processes. Because isotopes diverge between light and heavy forms (heavy isotopes having the more neutrons), rates of reaction and incorporation into biological structures may vary. These differences are small, in the order of parts per thousand, but are remarkably consistent and measurable using an IRMS.

The ratio of stable isotopes is expressed with reference to a standard; the more positive (or enriched) the ratio the higher the proportion of the heavier isotope. Similarly, if the ratio is depleted in the heavier isotope, the ratio becomes more negative, as illustrated in the example below for carbon. The ratio is expressed as parts per thousand, or parts per mil.

$$\delta^{13}C_{\text{sample}} = \left(\frac{^{13}C/^{12}C_{\text{sample}}}{^{13}C/^{12}C_{\text{standard}}} - 1 \right) 1000\% \quad (1)$$

Fractionation is the term given to the changes in isotope ratio that result from these reactions. As explained below, different types of plants (trees, grasses) may incorporate carbon from the atmosphere into their organic structures using different photosynthetic pathways, resulting in different ratios in the isotopes of carbon. This ratio will change again as the plant organic carbon is incorporated into a herbivore. The predictable nature of fractionation makes stable isotopes particularly useful in understanding food chain (trophic) linkages in the environment, as well as patterns in the movement and migration of animals, the complexity of food chain structures, and the importance of habitat types in contributing to energy budgets within ecosystems. These studies are therefore foundational to the proper understanding of ecosystems, and their informed management.

2.2. HOW ISOTOPE RATIOS VARY IN THE ENVIRONMENT

The most commonly used stable isotopes in environmental research are:

- Carbon: ^{12}C and ^{13}C (^{13}C being the heavier isotope, having the same number of protons as ^{12}C , but 7 neutrons rather than 6);
- Nitrogen: ^{14}N , ^{15}N ;
- Oxygen: ^{16}O , ^{17}O , ^{18}O ;
- Sulphur: ^{32}S , ^{33}S , ^{34}S , ^{36}S ;
- Hydrogen: 1H , 2H .

The isotopes of hydrogen and oxygen are commonly used in paleoenvironmental investigations of climate. This is because the ratio of the isotopes of oxygen and hydrogen in water vary in a way consistent with ocean and atmospheric temperature and the rate of evaporation. Indeed, stages of warmer and colder climate over the past few million years are called 'marine isotope stages' because of these predictable associations between globally cold conditions and oxygen isotope ratios.

In this manual we will focus primarily on the applications of carbon and nitrogen in environmental studies. This is because in most situations, the stable isotopes of carbon and nitrogen are sufficient to answer important questions about the structure of wetland ecosystems including:

- What plants are contributing most significantly as primary food sources in the ecosystem?;
- What are the plants and organisms contributing most to the productivity of fisheries?;
- How complex is the ecosystem structure? How many levels are there in the food chain from plants to the top predators?;
- How successful has our restoration program been in restoring ecosystem function?;
- What sources of organic carbon are being stored in wetland soils and does this amount to an emissions offset?

While the stable isotopes of sulphur in some situations can provide further discrimination in relation to these questions, most studies have utilized carbon and nitrogen in a way sufficient to provide the answers required.

Plants can be classified into three groups based on their method of photosynthesis:

- C₃ plants. Most plants (including nearly all trees) utilize this ‘standard’ method of photosynthesis. CO₂ is incorporated directly from the atmosphere into the Calvin cycle, in which energy and organic carbon are stored as sucrose;
- C₄ plants (most tropical grasses) modified this pathway. These plants first concentrate CO₂ as malic acid, allowing the ‘priming’ of the Calvin cycle with an elevated concentration of CO₂. This was thought to have evolved during periods of very low atmospheric CO₂ concentrations (as periodically occurs during ice ages). As a result, and in contrast to C₃ plants, these species gain little additional benefit from elevated levels of atmospheric CO₂ found in today’s atmosphere;
- Crassulacean Acid Metabolism (CAM: mostly succulents). These plants modify the pathway in a different way again, being adapted to arid conditions. They photosynthesize during the day but take up CO₂ at night to minimize water loss. This means that the CO₂ needs to be temporarily stored, which occurs (like the C₄ plants) in the form of malic acid (see Fig. 1).

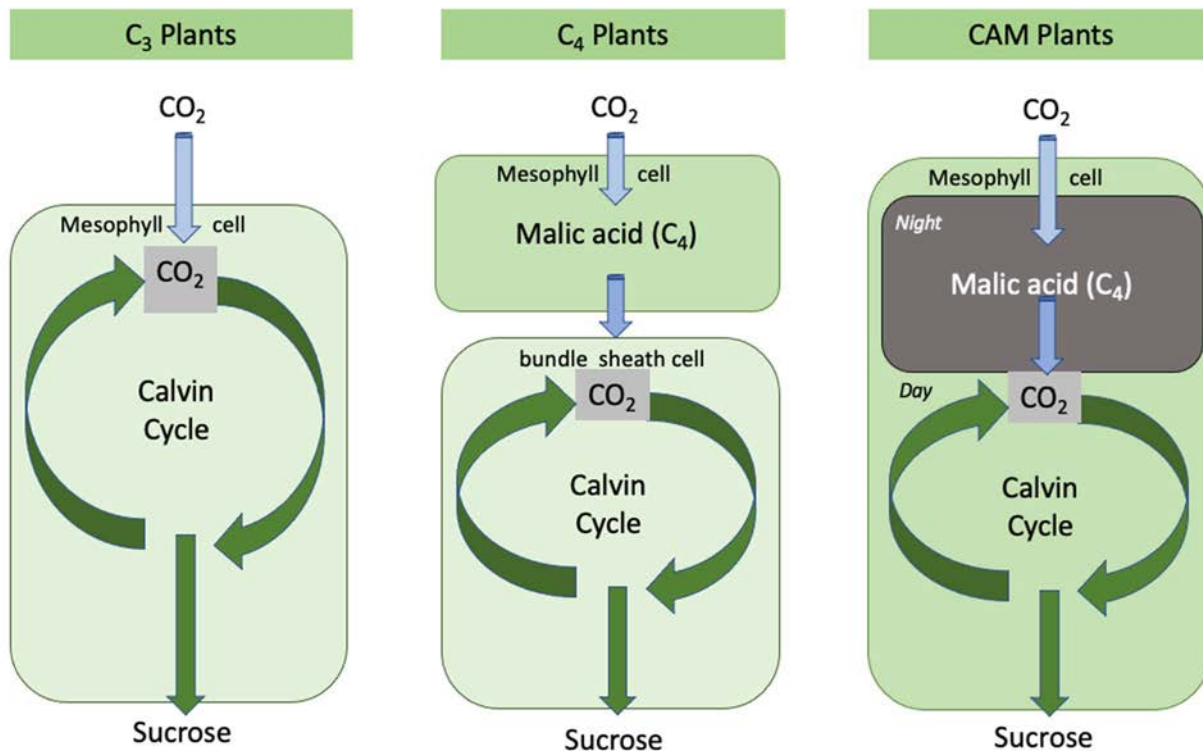


FIG. 1. Different photosynthetic pathways in plants. (Courtesy of N. Saintilan, Macquarie University, Australia.)

The different pathways of CO₂ uptake and incorporation into sucrose lead to differences in the isotopic ratio of carbon. In particular, plants utilising the C₄ photosynthetic pathway are highly enriched in the ratio of ¹²C and ¹³C. That is, the ratio (expressed as δ¹³C) is more positive (meaning there is more of the heavier isotope) for C₄ plants than for C₃ plants.

As a rule of thumb, plants of these types show the following ranges in isotope signature:

- C₃ plants: -37‰ to -20‰
- C₄ plants: -12‰ to -16‰
- CAM plants: -10‰ to -20‰

CAM plants are less commonly found in wetlands than C₃ and C₄ plants. Wetlands show a good mix of C₃ and C₄ species, meaning that the difference in isotopic composition often allows good discrimination between their ecological effects.

One further discrimination occurs between terrestrial and marine environments (see Fig. 2). This occurs because marine plants (including marine phytoplankton, seaweeds and seagrasses) take their carbon dioxide directly from the ocean waters in a dissolved form. This leads to a different carbon isotope ratio in the resulting plant organic structures. Usually, though not always, this makes marine plants (and subsequently marine ecosystems), highly enriched in δ¹³C compared to terrestrial systems. Because coastal wetlands (mangroves and tidal marshes) occur at the interface of the terrestrial and marine environments, the difference in isotope ratios of terrestrial and marine plants is an important tool in analysis of ecosystem structure.

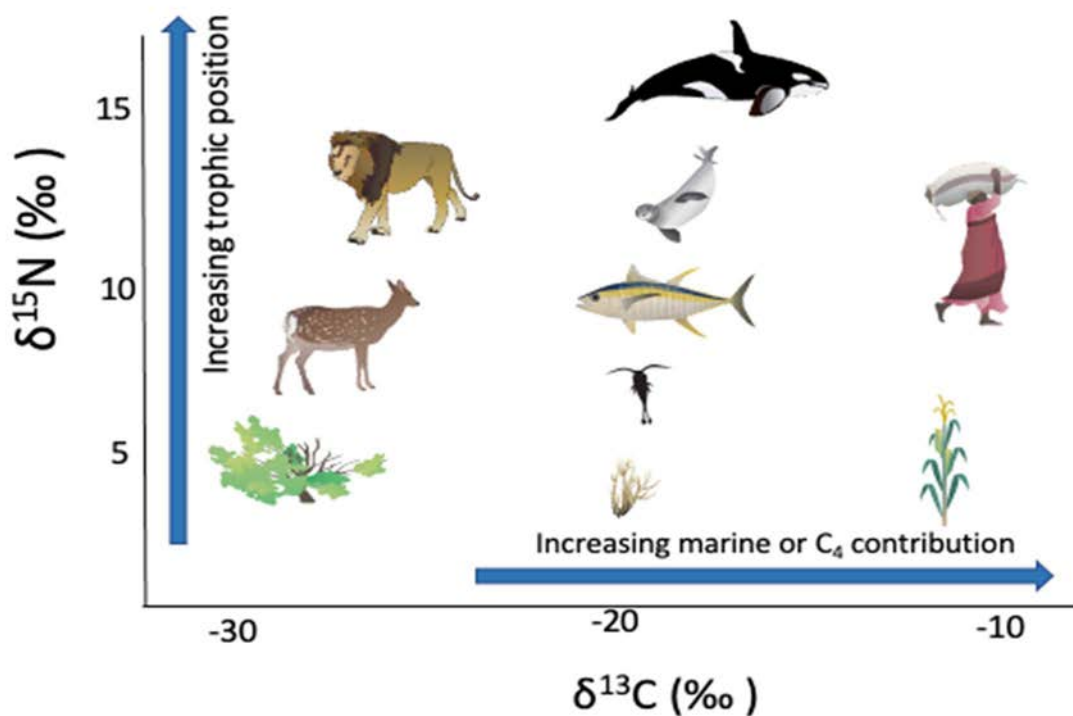


FIG. 2. Differences in isotopic ratios of terrestrial and marine organisms. (Courtesy of D.Mazumder, ANSTO, Australia.)

As explained above, the term ‘fractionation’ is used to describe changes in isotopic ratios which occur as a result of a physical or biological reaction. When an organism feeds on another, the stable isotopes of carbon and nitrogen are incorporated into the predator, but with a small and often predictable amount of fractionation. There is little fractionation in the carbon isotopes between prey and predator, usually in the range of 1 to 1.5‰. The predator inherits the carbon isotope signature of the prey, with a slight enrichment due to this fractionation. This allows the ‘basal’ or ‘autotrophic’ plant material to be identified all the way through successive trophic levels.

There is greater fractionation in the isotopes of nitrogen between trophic levels, usually in the range of 3.0 to 4.0‰. This makes the nitrogen isotopes particularly useful in interpreting where an organism sits in the trophic structure of an ecosystem. Given the ~3.5‰ enrichment between trophic levels, an organism highly enriched in relation to basal food sources is likely to be higher in the food chain than herbivores (which feed directly on the plants) or omnivores (which feed on both plant and animal matter). For this reason, while carbon isotopes are used to identify autotrophic sources powering the ecosystem, nitrogen isotopes are used to identify the trophic (food web) structure of the ecosystem.

The application of stable isotopes in environmental research is complicated by the behaviour of organisms, which rarely feed exclusively on one food source. A fish feeding on both C₄ and C₃ autotrophic sources will incorporate the stable isotopic signature of both, and the resulting isotopic signature of the fish will be a mixture of the two (see Fig. 3). Statistical techniques, termed

'mixing models' have been developed to help tease apart the varying contributions of different potential food sources to the isotopic signature of an organism.



FIG. 3. Mixing of isotopic sources in a sample organism. (Courtesy of D.Mazumder, ANSTO, Australia.)

2.3. RESEARCH APPLICATIONS OF STABLE ISOTOPES IN THE ENVIRONMENT

One important application of nitrogen isotopes is in the field of pollution studies. Nitrogen is an important pollutant, both as a by-product of agricultural systems and urban sewage. Nitrogen from anthropogenic sources is usually enriched in relation to background or natural nitrogen isotope ratios. This makes the nitrogen isotopes useful in determining the extent to which an ecosystem is being influenced by human derived sources of nitrogen, and subsequently the extent to which this is influencing ecosystem trophic structure. Below we describe a case study where these isotopes were used for this purpose in a coastal wetland setting [1].

The Sydney region (Australia) consists of a number of estuaries with varying levels of urban, industrial and agricultural development (see Fig. 4). The Parramatta River, on which Sydney Harbour is situated, is one of the most densely populated estuaries in Australia. Botany Bay to the south, is fed by the Georges River, also densely populated. To the north, the Brisbane Water estuary represents a more natural system.

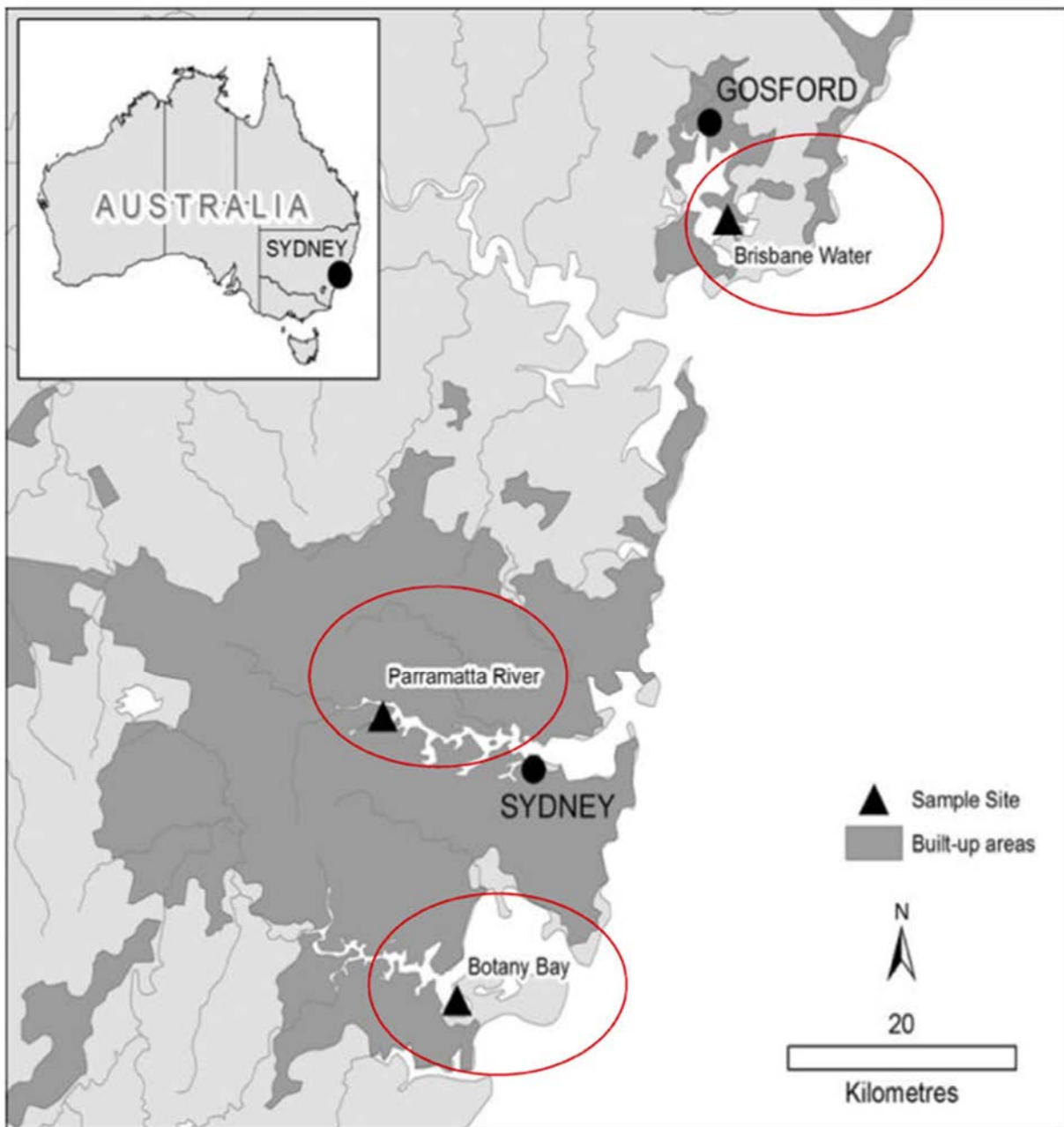


FIG. 4. Location of Sampling Sites (reproduced from Ref. [1] with permission courtesy of Elsevier).

Previous research had suggested that estuarine wetlands in the region had demonstrated sediment organic matter to be a basal food source (Fig.5). This ‘scum’ on the surface of the wetland incorporated microphytobenthos (microscopic algae growing on the surface of the wetland), along with decomposing plant material. This was the primary food source of the most important herbivore in the ecosystem, the grapsid crabs which feed at low tide. These crabs produced copious amounts of larvae during the spring tides, which was an important component of the diet of the glassfish, the most common small fish in the estuary.

In sampling between these three estuaries, the study tested a number of clear hypotheses: first, that the ecosystem structure varied between estuaries consistently with variation in the signature of sediment organic matter (SOM), the proposed basal food source; second, that the more polluted estuaries would be enriched in $\delta^{15}\text{N}$, reflecting the high contribution of anthropogenic N in these situations; third, that this pollution may be impacting the ecosystem structure, as measured by the number of trophic linkages to higher level predators.

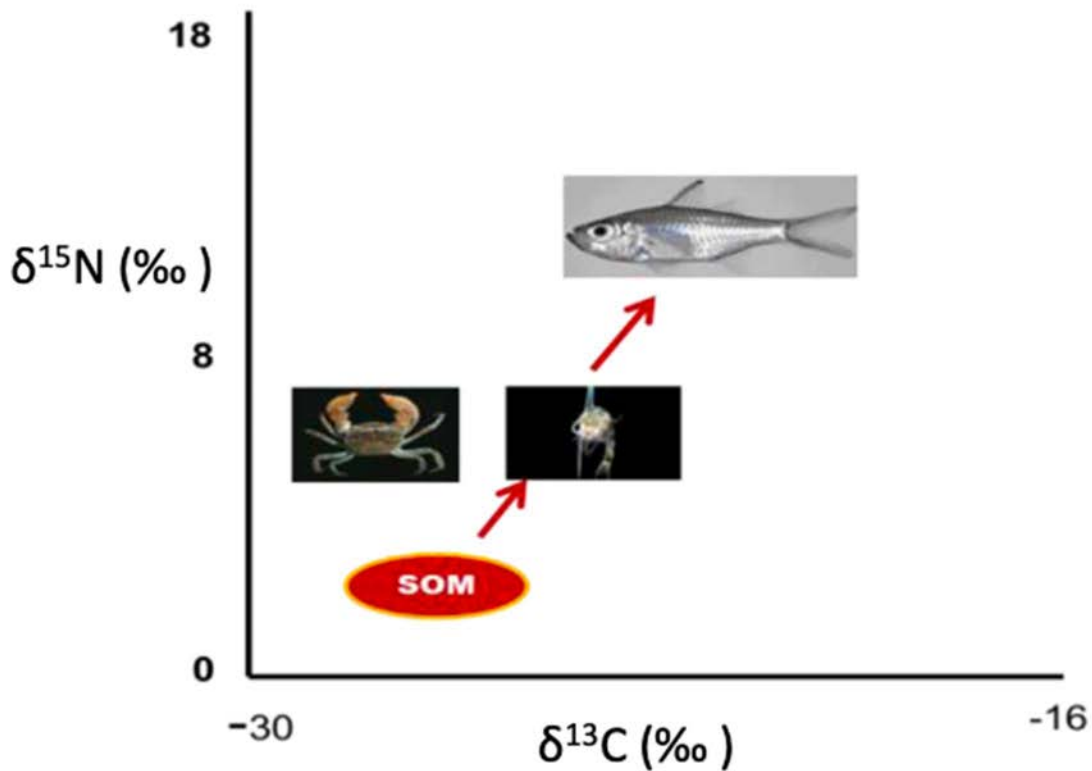


FIG. 5. Schematic of basal ecosystem structure . (Courtesy of D.Mazumder, ANSTO, Australia.)

The result confirmed the importance of SOM as a basal food source in these estuaries (Fig. 6), even as the signature of SOM varied between settings (reflecting differences in the isotopic signature of contributing algae and plant material in the carbon isotope, and the high levels of anthropogenic nitrogen input in the polluted estuary in the nitrogen isotope). In each case, the isotopic signature of the grazing crabs strongly suggested their dependence on this food source.

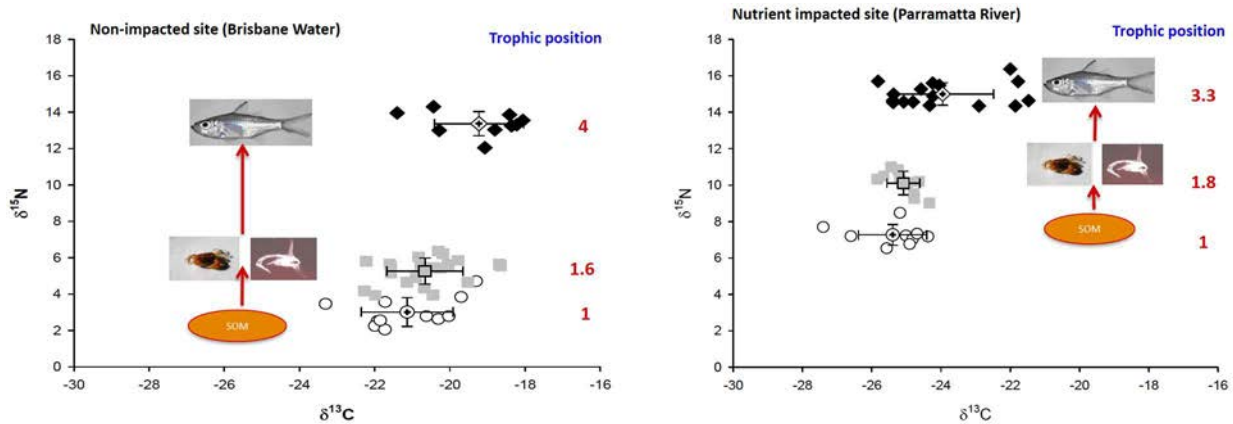


FIG. 6. Differences in isotopic signatures between a polluted and pristine estuary (adapted from Ref. [1] with permission courtesy of Elsevier).

In both cases, the glassfish was also aligned to the carbon isotope signatures of the basal source (SOM) and the crabs, allowing for a 1.2‰ fractionation between levels. Interestingly, the glassfish were feeding higher in the food chain than in the polluted estuary, suggesting that other food sources being accessed by the glass fish in the pristine estuary were absent in the polluted estuary: the food chain had ‘flattened’.

2.4. STABLE ISOTOPE APPLICATIONS IN WETLANDS

The case study described in Section 2.3 illustrates several important applications of stable isotopes in wetland ecosystems. In summary these include:

- Carbon isotopes are useful in identifying basal, autotrophic food sources contributing to the ecosystem. There is a slight enrichment in $\delta^{13}\text{C}$ between ecosystem levels, due to fractionation between predator and prey;
- Nitrogen isotopes are useful in determining the position of an organism within a food chain. There is an enrichment of 3.4‰ between predator and prey in $\delta^{15}\text{N}$;
- Anthropogenic sources of nitrogen are highly enriched in $\delta^{15}\text{N}$. This can be used to determine the influence of anthropogenic N pollution within ecosystems.

Below we consider some further applications of isotopes in wetland systems, building upon the above principles. These three themes will be developed throughout the manual, as we return to each in more detail in subsequent sections.

2.4.1. Understanding wetland food chains

We have introduced in Section 2.3 a program of research based in eastern Australia which has documented the trophic relationships between component of the wetland and estuary ecosystem. The studies utilized the contrasting signatures of the dominant saltmarsh grass, *Sporobolus virginicus*, a C_4 grass with a highly enriched $\delta^{13}\text{C}$ signature, and the mangrove *Avicennia marina*, a C_3 tree with a highly depleted $\delta^{13}\text{C}$ signature. Stable isotope analysis of available food sources suggested that the herbivorous crabs were feeding on sediment organic matter, the $\delta^{13}\text{C}$ signature of which being influenced more strongly by saltmarsh than mangrove.

Within these wetlands the grazing crabs were a keystone species, meaning that the higher trophic orders were highly dependent on the contributions of crabs. Predatory fish and birds fed on adult crabs, but the monthly release of crab larvae into the spring tide created important feeding opportunities for small glassfish which, in turn, were important to the diet of higher predators, including commercially important fish. Figure 7 below provides a schematic of these interactions. Video 1 features the sampling and analytical components of this research program.¹

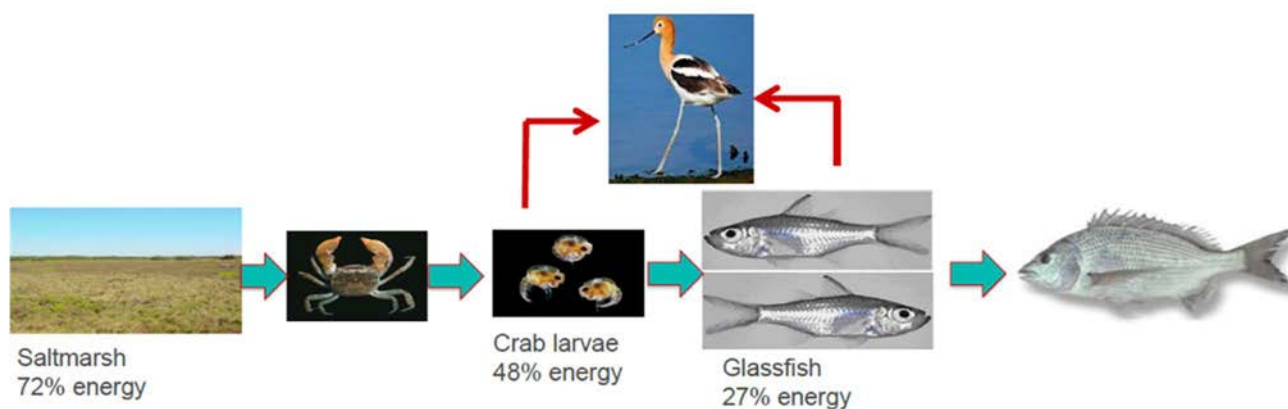


FIG. 7. Transfer of energy between components of the coastal wetland ecosystem, as illustrated by stable isotope analysis. (Courtesy of D.Mazumder, ANSTO, Australia.)

2.4.2. Monitoring restoration success

There are several approaches to monitoring the success of a restoration program. These may simply document the restoration of the target habitat, using vegetation survey or remote sensing. However, the restoration of ecosystem function may take far longer than the restoration of hydrological conditions and the plants characteristics of a wetland type. If the interest is determining the restoration of ecosystem function, it is important to sample organisms occupying the restored habitat, and ideally their interactions. Stable isotopes are an excellent way of determining whether organisms within an ecosystem are interacting in a way which resembles their trophic associations in a reference system.

In southern China, the saltmarsh plant *Spartina* is an invasive species, smothering native mangrove wetlands. The contrasting isotopic signatures of *Spartina*, a C₄ plant, and the C₃ mangroves facilitate an understanding of both ecological and trophic impacts of this invasive species, but also the time required for restoration of the natural trophic exchanges in restored wetlands. This case study is described in detail in Section 3.

2.4.3. Documenting blue carbon sequestration

The effects of elevated concentration of greenhouse gasses in the atmosphere have become increasingly apparent in recent decades, and projections provided by the Intergovernmental Panel on Climate Change are strongly suggesting the importance of controlling global temperature increases to within 1.5 degrees Celsius. Achieving this target will require a range of mitigation actions to be

¹ Detecting the transfer of energy in wetland systems (Sydney Catchment Authority)
<https://vimeo.com/littlegeckcomedia/gsls-saltmarsh-and-mangrove-ecosystems/video/325125452>

implemented, including energy efficiency measures, transition to renewable energy sources, and changes to the management of forests, agriculture and natural landscapes. The forestry and agricultural sectors account for approximately one quarter of all greenhouse gas emissions.

Many opportunities for reductions in emissions can be realized through the better management of agricultural and natural landscapes. Incentives for the preservation of natural carbon sequestration values of forests have been developed through policy initiatives such as the “Reduction in Emissions from Deforestation and Degradation plus the enhancement of carbon stocks through afforestation” or REDD+. Under these initiatives, local community custodians are paid to preserve their forests rather than seeking economic opportunities through their exploitation. In doing so, carbon is preserved in living biomass rather than being lost to the atmosphere (an ‘avoided emission’). Avoided emissions can be quantified and are of commercial value. Companies and governments seeking to offset their emissions are willing to pay to purchase carbon credits associated with conservation and afforestation schemes, a win for nature and for ‘triple bottom line’ reporting (and marketing).

While these mechanisms have been in existence for some time for forests and agricultural practices, it has only been recently that emissions reduction policies have been developed for coastal wetlands. However, the carbon locked in natural mangroves, tidal wetlands and seagrasses, termed ‘Blue Carbon’, is emerging as one of the most promising fields of natural carbon capture and storage, as coastal wetlands support much high carbon stores on a per capita basis, compared to terrestrial forests (see Fig. 8 below).

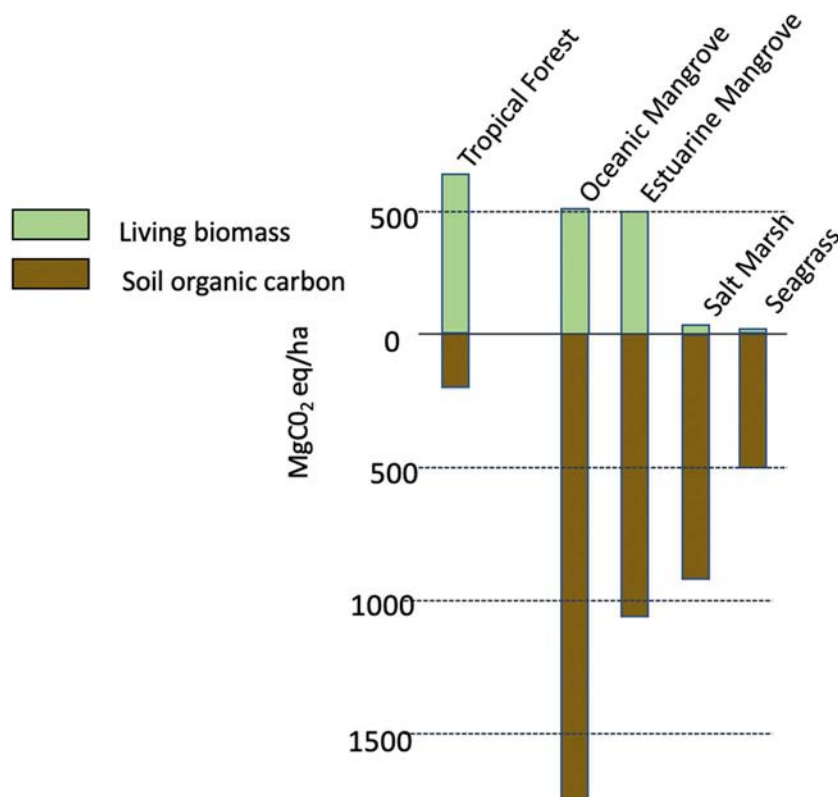


FIG. 8. Comparison of carbon stores in soil and living biomass across Blue Carbon ecosystems, compared to terrestrial forests (data from Ref. [2]). The unit is tonnes (Mg) of carbon dioxide equivalent per hectare. (Courtesy of N. Saintilan, Macquarie University, Australia.)

As shown in Fig. 8, most of the carbon occurring in these systems is locked up in the soil — less than ten percent on average is contained within aboveground plant biomass. This is because the soils of saturated, saline coastal wetlands are a uniquely efficient preserving environment, particularly under the influence of slowly rising sea level. The absence of oxygen inhibits the decomposition of organic carbon and its conversion into carbon dioxide (oxygen diffuses 10 000 times more slowly in water than in air). Also, the saline conditions of the ocean waters inhibit the bacteria which promote the release of methane to the atmosphere, and instead encourage sulphur reducing bacteria which inhibit methane generation. This makes saline coastal wetlands uniquely important in overall emissions reduction.

Unfortunately, losses of coastal wetlands across the Asia–Pacific region have been amongst the highest on the planet. Up to one fifth of mangroves globally have been cleared for aquacultural developments, and the methods of clearance and pond construction are particularly prone to carbon loss. Up to half of the carbon stored in the upper metre of soil is lost to the atmosphere when the soils are formed into pond bund walls. Fortunately, as awareness of the importance of mangroves to coastal fisheries, coastal protection and carbon sequestration has become better known, increasing emphasis has been placed on their protection across the region, and the rate of mangrove forest loss has slowed in recent decades.

Blue Carbon represents an additional opportunity to promote mangrove conservation and restoration. Video 2 provides an introduction to mangrove Blue Carbon and the broader context of mangrove conservation and restoration.²

Given the importance of soil carbon to the Blue Carbon value of coastal wetlands, it is relevant to understand where the carbon has come from. This knowledge directly informs our understanding of the rate of carbon sequestration for which the wetland is responsible. For example, two contrasting sources of carbon might be envisioned. First, organic carbon may enter the wetland as suspended particles in the tidal water. This is called ‘allochthonous carbon’; carbon sourced from elsewhere. It is becoming increasingly apparent that allochthonous carbon may be an important contributor to carbon accumulating in the wetland. For example, the use of carbon-14 dating in wetland soils often shows a comparatively high contribution of ‘old’ carbon, suggesting that carbon is being reworked and deposited in coastal wetland sinks. A second important source of carbon is that captured on site, through plant photosynthesis. This is termed ‘autochthonous’ carbon (see Fig. 9). This carbon source is clearly a net reduction in atmospheric CO₂, being taken from the atmosphere and stored primarily belowground in plant root material, where it may be contained for hundreds or even thousands of years. Distinguishing between allochthonous and autochthonous carbon sources in coastal wetlands is a key knowledge gap important to the further development of Blue Carbon policy.

² Introduction to mangrove blue carbon (Environmental Justice Foundation)
<https://vimeo.com/505750631/504434bce0>

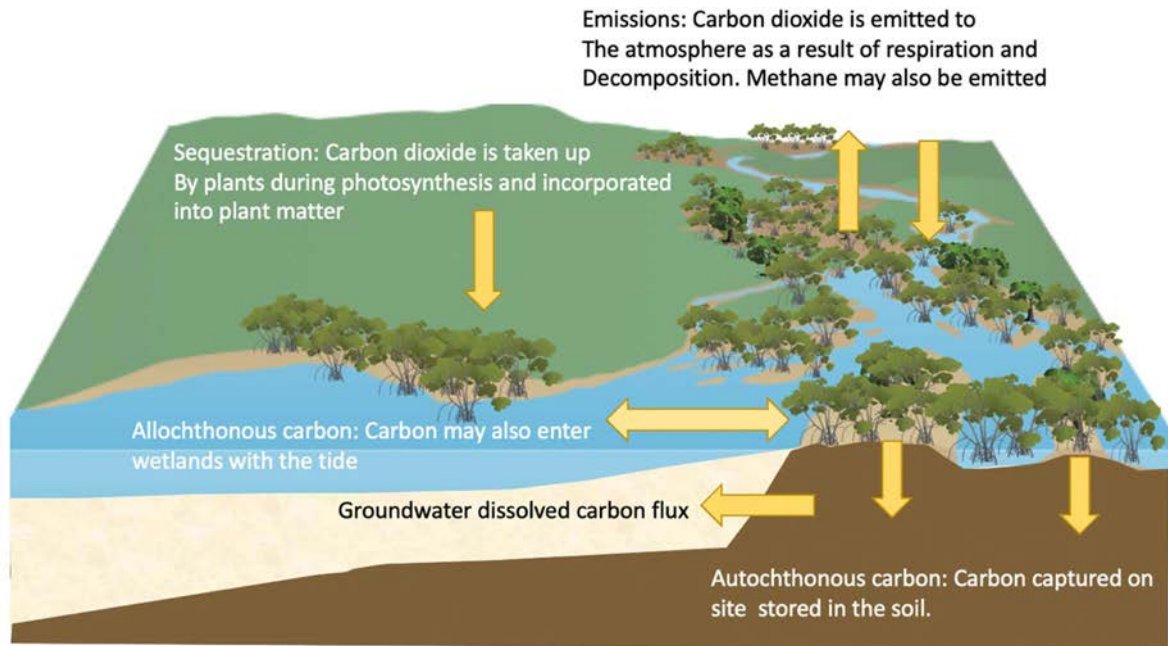


FIG. 9. Carbon fluxes in a coastal wetland. (Courtesy of N. Saintilan, Macquarie University, Australia.)

2.5. DESIGN PRINCIPLES OF A SAMPLING PROGRAM

The availability of scientific tools should never drive the research question, and in many situations stable isotopes may not be the most effective tool to address management concerns. Further, there may be situations where the application of stable isotopes will be unable to answer the question being posed [3]. This might occur under three circumstances:

- The sources of autotrophic carbon have little discrimination in their isotopic composition. For example, it will not be possible to differentiate the contribution of different species of mangroves in most situations because their isotopic signatures overlap;
- There is large spatial variability in the diet composition of a highly mobile species. Stable isotopes provide a ‘time integrated’ signature of diet, usually reflecting food sources taken over a period of weeks to months (though some tissues with a higher turnover may reflect diet over a period of days). Careful thought needs to be given to the research question in these circumstances, and sampling account for this variability;
- There is large temporal variability in the diet of a species or the isotopic values of diet items. If, for example, a species is moving from consuming crab larvae during the spring tide and copepod zooplankton during the neap tides, how will these be reflected in the isotopic composition of the consumer? These considerations do not preclude the application of stable isotopes, but an understanding of this variability will be helpful in framing the research question and the approach to sampling.

2.5.1. Sampling questions and sampling design

The assessment of ecosystem structure using stable isotopes should be informed by a prior knowledge of the behaviour of target species, including their feeding behaviour if possible. The more you know about the movements of the animal in the habitat and whether it is, for example, a herbivore, omnivore (feeding on both plant and animal matter) or a predator is helpful in restricting and targeting the range

of materials to be sampled and analysed. The scientific literature is a useful starting point, for instance keeping in mind the interesting similarities between the diet and trophic position of fishes in the Malaysian and Australian mangrove forests outlined above. Gut content analysis is also a useful tool to independently verify the findings of stable isotope analysis, and the two methods are commonly deployed in tandem. However, there are two important distinctions between gut content and stable isotope analysis: first, not all elements found in the gut are consumed by the animal and incorporated into their tissues; second, the gut content reflects the immediate feeding history of the organism (past few hours) while stable isotope analysis integrates feeding activity over a period of weeks to months.

Some microscopic food sources can be difficult to independently characterize using stable isotope analysis, given difficulties in sampling at quantities required of the technique (typically several grams are required). Examples include elements of organic carbon found on the marsh surface, including microphytobenthos, and microbial food sources utilising detrital and faecal carbon on the marsh surface. For this reason, a bulk sample is usually collected, and termed ‘Sediment Organic Carbon’, though care should be taken to determine how homogenous this signature is through the wetland.

When a range of dietary sources are utilized by a species, stable isotope mixing models can be used to quantify the proportional contribution of each dietary source to the overall diet of the organism. These mixing models are discussed in detail in Section 5 of the manual.

Consideration needs to be given to the tissue of the organism to be sampled and analysed, as different tissues have different turnover rates, reflecting diet over different times periods. Bone may change little over the life of the organism and reflect conditions in the early development of the organism [4]. This provides an interesting contrast to using metabolically active tissues with shorter turnover times. Muscle tissue, the most commonly used tissue in stable isotope analysis, integrates the signature of dietary sources over a period of months. Liver tissue and blood plasma may turn over in a matter of days and provide a more immediate picture of short-term feeding habitats [5]. Fish otoliths (ear bones) are laid down sequentially over time. Bird feathers provide a fixed isotopic signature for the time they are formed. Using a combination of these materials can provide information on changes in diet over the life cycle of the organism [6,7].

2.5.2. Identifying possible carbon sources

Careful consideration should be given to potential food sources, as this is the foundational element of any assessment of ecosystem trophic structure. This should include consideration of autotrophic sources of carbon (i.e. plant and algal species fixing energy at the base of the ecosystem) along with herbivores and detritivores that transfer this energy to higher levels of the ecosystem. Video 3 introduces potential autotrophic sources of carbon within the mangrove forest of Towra Point in eastern Australia.³

The choice of autotrophic sources will depend on the likely diet of species (some prior research is recommended). Ideally, a range of sources with good isotopic discrimination will be available. However, the number of potential sources should be kept as low as possible to allow mixing models the best chance of correctly representing the relative importance of food sources: a balance therefore needs to be established between inclusion of possible sources and exclusion of extraneous material [3].

³Potential autotrophic sources of carbon within the mangrove forest of Towra Point in eastern Australia. <https://www.youtube.com/watch?v=i6vYaxBHjBI>

The isotopic composition of some sources will vary over space and time in relation to environmental conditions. Autotrophic isotopic composition often varies along salinity gradients, and this is true for mangroves also [8]. It should not be assumed that a mangrove species has a ‘characteristic’ isotope signature. Root and plant material should be collected from the location within which consumer organisms are sampled.

In some studies, the interest may be in the diversity of food items being sampled by organisms across space and time, in which case individual food items may not need to be sampled and attribution of diet to specific sources may not be required. In these cases, comparison is made in the dimensions of the ‘trophic niche’, with the implication that a low variation in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ between organisms represents relatively homogenous diet, while a high variability suggests the same species is feeding across a range of food sourced. The following case study illustrates this approach.

The impact of hydrological regulation on the habitat of overwintering geese is an important management consideration in the wetlands of the Yangtze River in China. Here two species of geese, the bean goose *Anser fabalis* and the lesser white-fronted goose *Anser erythropus* feed on wetland plants exposed during water draw down. Management authorities were concerned to know how the availability and quality of wetland habitat influenced geese movements and feeding across the landscape. This study (see Ref. [9]) combined radiotracking of birds with isotope analysis of blood to test the hypotheses that poor habitat conditions led to (a) an increase in foraging distance and (b) a greater range of food sources accessed (increased trophic niche width). The radiotracking showed much greater distances being traversed by the geese under poor habitat conditions, though the niche trophic dimensions were similar between years (see Fig. 10), suggesting that the geese were seeking similar food sources over longer distances rather than increasing dietary range within the same area. These results helped focus management attention on the required resources.

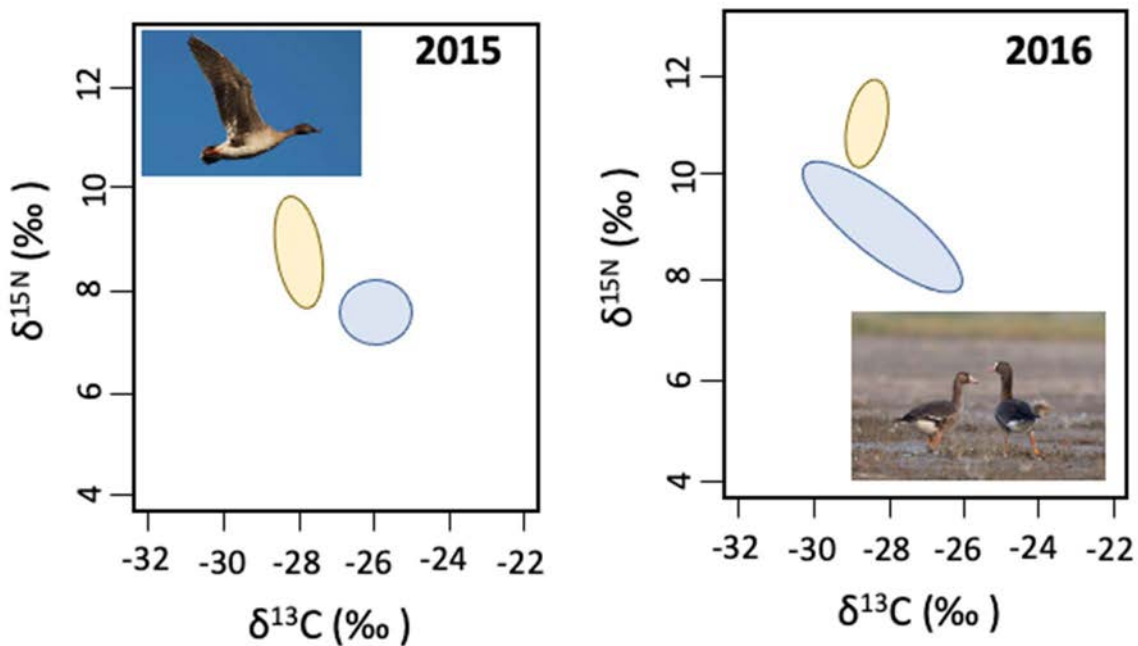


FIG. 10. Trophic niche dimension of the lesser white fronted goose (yellow shading) and bean goose (blue shading) in Dongting Lake, China (reproduced from Ref. [9] with permission).

2.5.3. Representative sampling and replication

Individuals from the same species in the same location may vary in their feeding habits and histories. How much variability exists at a population level can only be determined by representative sampling with sufficient replication. This is particularly important if Bayesian mixing models are to be used, because the Bayesian models will incorporate variability between replicate samples as a source of uncertainty. We will look further into these models in Section 5.

There is no fixed number, no right or wrong answer to the question of how many replicate samples to take. The number of individuals which should be sampled will depend on the degree of variability between individuals, which may not be known prior to sampling. It can be assumed, for example, that leaves of mangroves in the same area are likely to exhibit low variability, and therefore a low number of samples (~6 leaves) may be sufficient to characterize this variability. Take care, however, to sample separate trees. Sampling six leaves from the same tree is a classic example of pseudoreplication, as variability within an individual is likely to be lower than variability between individual trees.

Crabs and other infauna tend to feed in a very restricted home range, often a few metres or less [10]. For this reason, variability in isotopic composition for a species may be small in an homogenous habitat, but may vary if there is a mosaic of habitat with different autotrophic signatures [11, 12].

Schooling fish may show more consistent isotopic signatures within the school than solitary predatory fish. Mazumder et al. [13], used statistical techniques (power analysis) to determine the sample size necessary to characterize the isotopic signature of glassfish (*Ambassis jacksoniensis*) in an estuary in eastern Australia. They found that in this case five individuals were sufficient to represent the variability within the population, and there was little additional benefit in sampling a high number. However, this should not be used as a rule of thumb for other finfish, and erring on the side of higher numbers (10–20 individuals) is preferable to sampling too few. However, logistical and cost constraints may necessitate tradeoffs between sampling of range of species and within species replication.

3. SAMPLING TECHNIQUES

3.1. INITIAL CONSIDERATIONS

3.1.1. Safety considerations in planning a sampling campaign

Safety is the primary consideration in the planning of any field or laboratory activity. There are many potential hazards associated with fieldwork, and while these should not preclude field sampling campaigns, potential risks should be identified and mitigated prior to the commencement of fieldwork. In this section we will consider hazards which might be encountered in wetland research, and some of the protocols which might be deployed to keep ourselves and our colleagues safe (see Table 1 below).

TABLE 1. HAZARDS ASSOCIATED WITH WETLANDS AND THEIR MITIGATION

Hazard	Mitigation Strategy
Drowning	Wearing a life jacket or floatation device in deep water
Extreme temperature	Conducting fieldwork at times of reduced temperature. SPF50 sunscreen regularly reapplied. Rehydrating in the field: min 2L per person per day. Wearing appropriate clothing. Ensure adequate shade. Emergency contact
Exposure to cold	Avoiding fieldwork at times of extreme cold. Shelter available. Appropriate clothing (warm and waterproof). Emergency contact
Injury due to falling	Emergency contact. First aid kit. Sufficient food and water. Registering field location prior to departure
Bushfire	Field sites monitored for bushfire, Appropriate communications at hand. Avoiding periods of high bushfire danger
Hazardous wildlife	Consider likely wildlife and poisonous fauna in the area. Protective clothing as appropriate. Other protective equipment as necessary. Remaining on marked paths were practicable. Appropriate first aid kit with at least one participant trained in their use
Contaminated environments	Appropriate PPE worn, including rubber boots or waders, nitrile gloves

Your participation in fieldwork should be registered with your home institution, with report back procedures at the end of each day in place. If travelling long distances to a field site, car should be taken to avoid fatigue. Frequent rests or change of drivers every two hours is recommended.

3.1.2. Ethical issues and permits

The treatment of animals is an important consideration in field and laboratory work, as is the protection of sensitive ecosystems and populations from unsustainable sampling. Most jurisdictions prescribe the conditions under which live samples can be taken. This will include humane methods of euthanasia, and field sampling should consider the volume of containers, ethanol or similar, required to appropriately euthanize and preserve biological samples.

Fisheries authorities may need to approve sampling of fish, which may limit the number, type and size of samples being taken, along with the proposed sampling methods. Liaise with your local fisheries authority to determine what approvals might be required and their reporting requirements.

Many of the wetland systems within which isotope studies may be conducted are protected reserves. These locations may have their own requirements for authorized entry and sampling. Local reserve managers should be contacted and provided with the objectives of the research program, the proposed sampling dates, and the proposed method of sampling.

3.1.3. Reiteration of principles of sample design

In the previous section we considered some principles to be applied to the design of a sampling strategy. To briefly reiterate, consideration should be given to the following:

- Carefully define the research question, including the geographic and temporal scope;
- Research the probable sources and flows of carbon within the system being considered, drawing upon available literature or expert opinion. Develop a conceptual model prior to sampling;
- Consider what the important autotrophic carbon sources might be, and how they might vary in space and time;
- Decide on what tissues will be sampled (that is, how metabolically active and the length of time over which the tissue integrates dietary isotopic signatures);
- Decide on the level of replication which may be required to adequately characterize sample variability.

In the following section we will give detailed consideration to several case studies which exemplify the application of these principles.

3.2. CASE STUDY. TROPHIC STRUCTURE OF MANGROVES

We will now consider three detailed case studies which exemplify the principles of stable isotope application in wetland environments. These further develop the themes introduced in Section 2: understanding the trophic structure of wetlands; monitoring wetland restoration success; and documenting sources of organic carbon accumulation in ‘Blue Carbon’ habitats.

In the first of these case studies, we illustrate a collaborative research program involving two partner countries in the Regional Co-operative Agreement (RCA): Australia and Malaysia.

3.2.1. Framing the question

In this case study [14], principles of stable isotope analysis are used to determine similarities and differences in the trophic structure of mangrove forests in very different settings as shown in Fig. 11. The Matang mangrove forest in Malaysia is a high biomass forest near the centre of mangrove biodiversity. By contrast, the mangrove at Towra Point in New South Wales, Australia, is near the southern latitudinal limits of mangrove ecosystems and supports lower biomass and diversity of species. Given these differences, to what extent was the trophic structure different between the two systems? How did the length of food chain differ between basal sources of production and the top commercially important predators? What species of herbivore and omnivore were important in transferring energy between basal production and these predators?

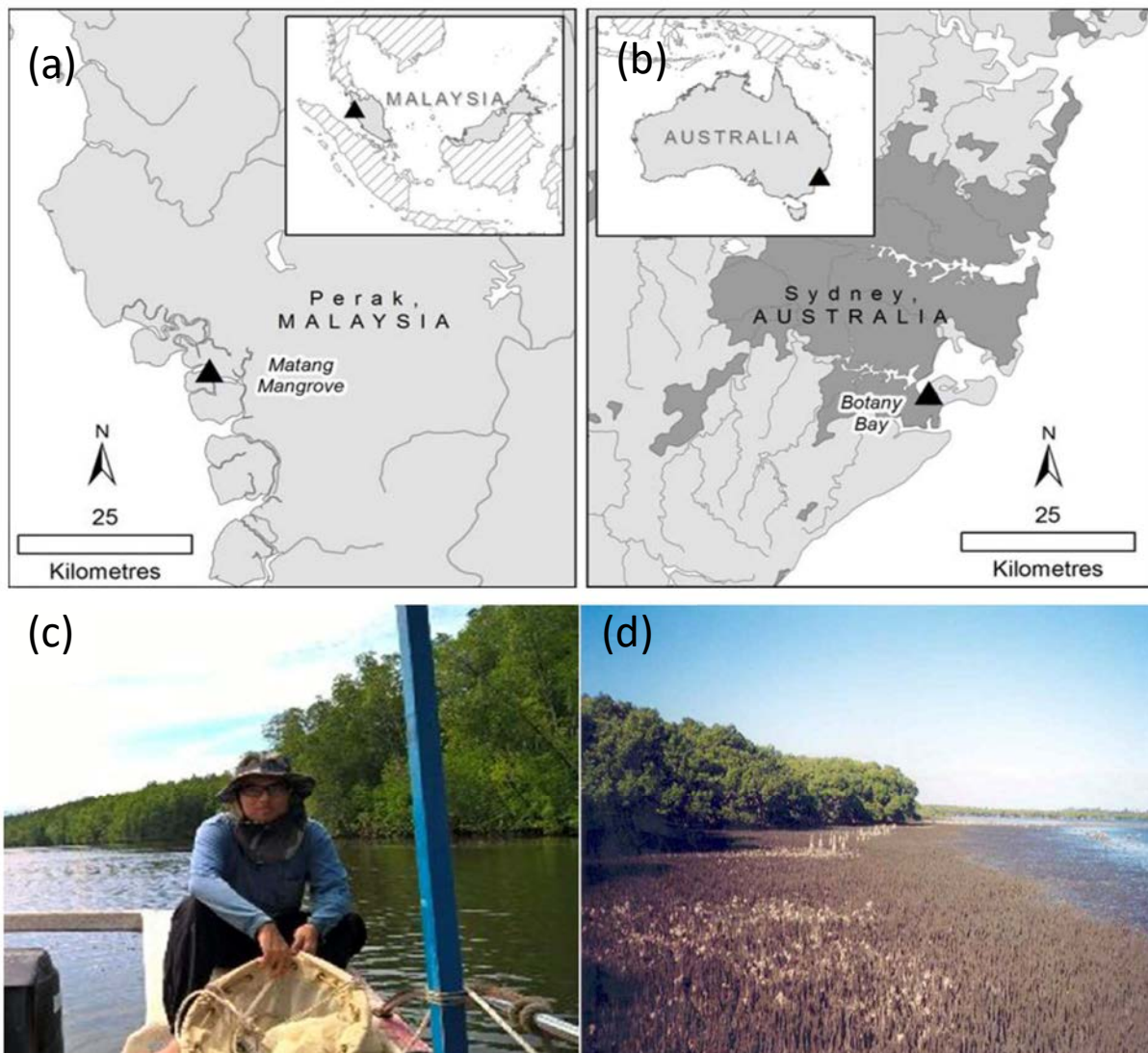


FIG. 11. Two sites where the trophic structure of mangrove ecosystems have been studied and compared (a) the location of the Matang mangrove forest, Malaysia (b) the location of Towra Point, Australia, (c) the seaward edge of the Matang mangrove forest, and (d) the seaward edge of the Towra Point mangrove forest. (Courtesy of N. Saintilan, Macquarie University, Australia.)

While this question was explored in just two systems, it would be interesting to compare mangrove trophic structure more broadly across the Asia–Pacific region. Regional cooperation in sampling and analysis would help determine whether the organisms inhabiting mangroves organize in predictable trophic associations. This would greatly facilitate the setting of management objectives for mangroves within the region and allow the development of monitoring protocols targeted at ecosystem function. The results of the Australia–Malaysia comparison provide encouraging results suggesting that broadscale comparisons might be made.

3.2.2. Experimental design

The study employed common sampling protocols between Malaysia and Australia to sample the most important elements of the mangrove ecosystem. No attempt was made in this study to determine the population density of sampled organisms. Though see Ref. [15, 16] for these methods.

In each mangrove, an inventory of common species was made, with five replicates of each species retained for isotope analysis. The sampling methods are described below. Potential autotrophic basal sources included the dominant mangrove species (*Avicennia marina* and *Aegiceras corniculatum* at Towra; *Rhizophora apiculate* and *Bruguiera parviflora* at Matang), along with sediment organic matter sampled as a surface scrape from the mangrove.

Stable isotopes of carbon and nitrogen were used to identify the position of each species in the food chain. As shown in Fig. 12, not all organisms caught in the Matang forest were deriving their carbon from the basal mangrove sources, but the key ecosystem links between important species were common between Matang in Malaysia and Towra in Australia.

While species differ between the mangroves, there is a degree of ‘trophic equivalence’ between the systems, with similar species occupying similar trophic positions. In both systems, crabs (*Parasesarma*) and shrimp (*Penaeus*, *Macrobrachium*) are important in transferring energy from basal sources to predatory fish. Predatory fish and crabs of the same family (the mullet *Liza* and the mud crab *Scylla*) occupy the same trophic position in both systems. Even when different families, the ‘glassfish’ (*Parachela* and *Ambassis*) occupy the same trophic position.

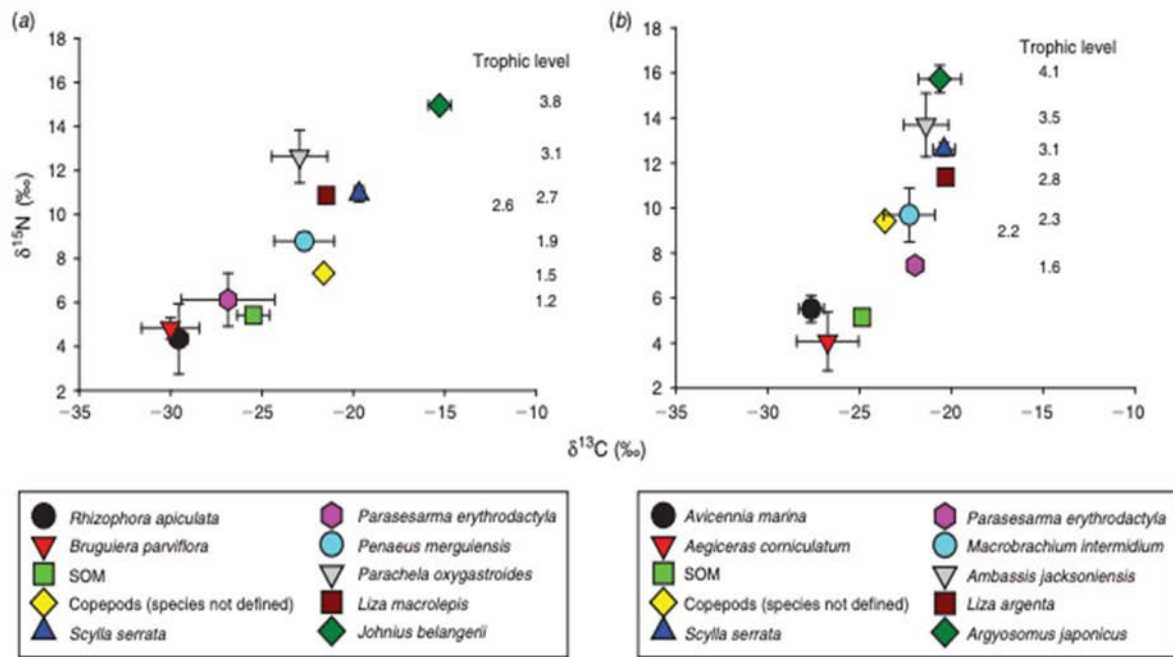


FIG. 12. Position of important species in the ecosystem structure of (a) Matang forest Malaysia and (b) Towra Point mangrove, Australia (reproduced from Ref. [14] with permission).

The trophic position of each organism was calculated as the difference between the nitrogen isotopic values of consumer and basal carbon source, divided by the usual nitrogen isotope fractionation between ecosystem levels ($\sim 3.4\text{‰}$). The equation used in this case was:

$$\text{Trophic Position} = 1 + (\delta^{15}N_{\text{consumer}} - \delta^{15}N_{\text{basal consumer}})/3.4 \quad (2)$$

The use of trophic position, standardized against basal isotopic signatures, allows for comparison of ecosystem structure between systems and locations.

3.2.3. Techniques for sampling biota

3.2.3.1. Finfish

There is a diverse range of sampling gears designed to catch fish in shallow water environments, and here we consider three of the most commonly deployed: the seine net, the fkye net and the pop net. All three have strengths and weakness, and their appropriate use will depend on the environment being sampled and the nature of the research question.

The seine net, sometimes called a dragnet is commonly used in commercial fisheries and is an efficient sampling approach in shallow wetlands with a bare surface (see Fig. 13). The net consists of a weighted base and a buoyant top extending along two long wings. The net is pulled across the marsh using ropes extending from the wings, often to a marsh edge or beach, at which point the net is removed from the water and fish collected. The net is unsuitable in sensitive habitats likely to be damaged by the dragging of the net, or habitats with complex hard structures, such as the root system of a mangrove forest.



FIG. 13. Deployment of the seine net in an estuarine setting. (Courtesy of N. Saintilan, Macquarie University, Australia.)

For this study the fyke net was utilized as an efficient sampling method in wetlands (Fig. 14). The fyke net has several advantages for use in shallow wetland environments. First, unlike large seine nets, the fyke net can be deployed in complex structural habitats such as mangrove forests with interlocking root systems without damaging the habitat or the net. The fyke net is particularly useful in areas with gently flowing water such as tidal systems, allowing fish to be funnelled into the net on the flood or ebb tide. The net is easy to deploy and retrieve. Video 4 shows the deployment and retrieval of the Fyke net.⁴

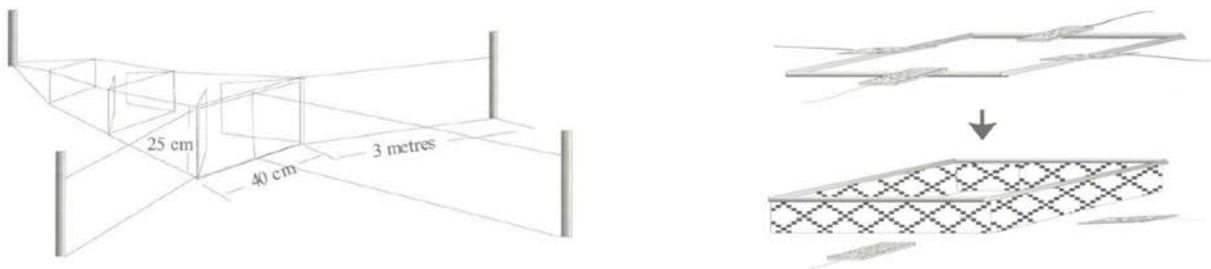


FIG. 14. Configuration of a typical Fyke net (left) and Pop net (right). (Courtesy of N. Saintilan, Macquarie University, Australia.)

One disadvantage of the fyke net is that it is difficult to determine the density of fish in a habitat if this is a component of the research question. If one intention of the study is to compare fish density between wetland habitats, the ‘pop’ net is recommended. These nets consist of a floating frame (PCV is usually used), to which is attached the netted enclosure wall, pegged to the surface of the marsh.

⁴ Deployment and use of the Fyke net

<https://www.youtube.com/watch?v=k1ARntlWRiM>

On deployment, the floating frame is held to the marsh surface using several weights, which can be pulled from the frame using ropes to release the net. See [17, 18] for a description.

3.2.3.2. *Infauna*

Crabs are an important component of the coastal wetland and the approach to their sampling will depend on the size and habitat of the species. For large species, such as the mud crab *Scylla serrata*, commercial crab traps, baited (e.g. with cat food), are the most effective approach. Care should be given to the safe handling of this species. For smaller grapsid crabs, the pitfall trap (Fig. 15) is inserted into the marsh, flushed with the surface. This can be as simple as a disposable cup. Baiting may increase the effectiveness of the method. Usually the trap will be effective within a few hours of deployment. Some species (including Sesarmids) will habituate under fallen branches and other debris, and can be easily retrieved from the surface. Some infauna, such as benthic shrimp, may require destructive sampling of the sediment and subsequent sorting. Snails are important herbivores in most wetland systems and can be easily retrieved by hand from the surface of the marsh and marsh plants.



FIG. 15. A pitfall trap. (Courtesy of D.Mazumder, ANSTO, Australia.)

3.2.3.3. *Plankton*

Plankton consists of both algal material (phytoplankton) and animal material (zooplankton), both being important in the transfer of energy within an ecosystem. Plankton are collected using a plankton net, being a fine meshed net, hand towed, with a collecting vial at the base. This vial can usually be detached and the contents transferred to a container (see Fig. 16). We can see the sampling of zooplankton in a saltmarsh in the beginning of video 5.⁵

⁵Sampling of zooplankton in a saltmarsh
<https://vimeo.com/littlegeckomedia/gsls-saltmarsh-and-mangrove-ecosystems/video/325125452>



FIG. 16. Deployment of the zooplankton net within a mangrove. (Courtesy of D.Mazumder, ANSTO, Australia.)

Within wetlands, the timing of zooplankton availability may be related to tidal or seasonal cycles. For example, crabs release their larvae during monthly spring tides, and these are the best opportunities to sample crab larvae for isotope analysis. Under these circumstances, a bulk sample of nearly uniform crab larvae can be obtained from the wetland.

3.2.3.4. *Autotrophic carbon sources*

Most autotrophic carbon sources are sedentary and easily sampled. However, there are two common mistakes made in collecting candidate autotrophs. First, there is common misconception that root material will contain the same carbon and nitrogen isotope signature as the stem and leaves of the plant. In fact, mangrove roots are often enriched in $\delta^{13}\text{C}$ in relation to the stems, even for the same individual plant [8]. Leaves and root material should be collected, as both may be contributing to the diet of wetland animals. Second, there are several important autotrophic carbon sources which may be neglected because they are found on the surface of the marsh and may be cryptic. These include algae attached to plant stems and above ground roots, unicellular algae associated with the marsh surface (microphytobenthos), or seagrass wrack washed into the wetland. Video 3 introduced in Section 2.5.2, covers a range of possible sources of autotrophic carbon in a coastal wetland. Plant material, when sampled, can be retained in sealed, labelled bags prior to laboratory analysis.

3.3. CASE STUDY. EXPLORING RESTORATION SUCCESS

The use of carbon and nitrogen isotopes to explore restoration success of coastal wetlands is illustrated in a study conducted in the south-east coast of China [19].

3.3.1. Framing the question

Invasions of biological species pose a significant threat to local community structure and ecosystem functions in the coastal wetlands of China. For instance, the invasion of US cordgrass (*Spartina alterniflora*, a C₄ grass) has seriously damaged ecosystem structure and functions of coastal wetlands in eastern coastlines of China (Fig. 17) [20]. Thus, great efforts have been made to control the spread of these exotic species. In China, ecological replacement using native mangrove species combined with physical treatments has become an effective method in controlling the spread of invasive *S. alterniflora* in southern China, but how can we evaluate such restoration successes?

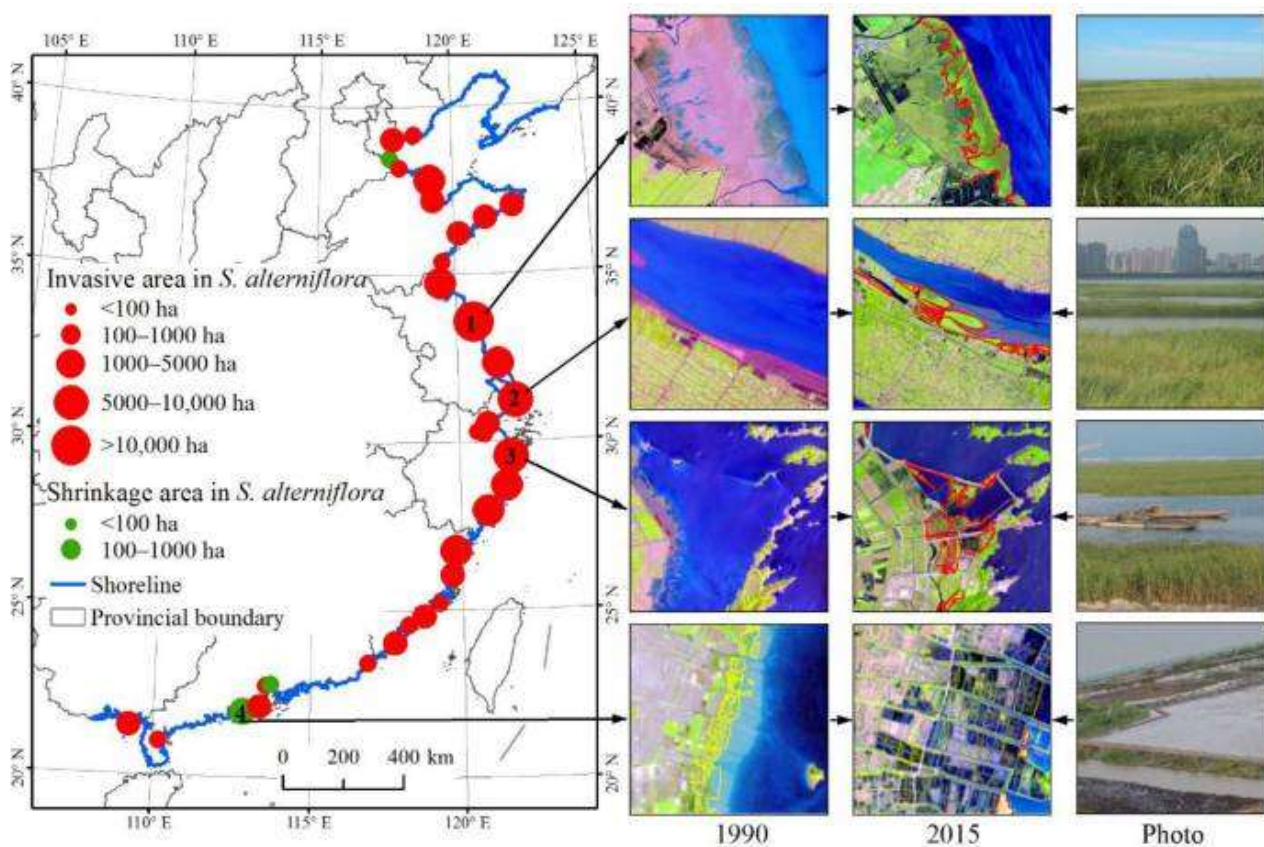


FIG. 17. Changes in the distribution location and area of invasive *S. alterniflora* between 1990 and 2015 in the eastern coastlines of China. Satellite images (composited from bands: red, green, and blue) provide examples for notable expansion and shrinkage of *S. alterniflora* with comparison with the photos taken in 2015 (reproduced from Ref. [20] with permission).

As explained above, stable isotopes provide quantitative information on the food sources of consumers and have been used to assess the effects of environmental degradation and restoration strategies on food web function [19]. So, this study aimed to address the following questions:

- Do the effects of *S. alterniflora* invasion on the diet selections of benthic macrofauna differ among different wetland ecosystems?;
- Following the removal of *S. alterniflora* and planting native mangrove species, how many years it will need to restore trophic relationships of selected benthic macrofauna?;
- How the recovery rates of trophic relationships of selected benthic macrofauna differ among their taxa and feeding types?

3.3.2. Experimental design

3.3.2.1. Principles

All mangroves (e.g. *Kandelia obovata*) are C₃ plants, which have much lower $\delta^{13}\text{C}$ (-27‰) than C₄ plants, including US cordgrass (-13‰). The nitrogen isotopic ratio is a good indicator for trophic level, with an increase of 3‰ every trophic. Thus, carbon and nitrogen stable isotope ratios for dominant macrofauna, as well as their potential food sources can be analysed to quantify relative contributions of the different carbon sources to the diets of benthic macrofauna and isotopic niche width, using the Mix SIR model developed within a Bayesian modelling framework. The results could be used to indicate changes in the diets of macrofauna in three restored mangrove ecosystems with different invasion histories following the removal of *S. alterniflora* in southern China, as an indicator of success.

3.3.2.2. Site selection

This study was conducted at three wetland ecosystems in the Fujian province of China (Fig. 18) with detail coordination information as bellows:

- Minjiang Estuary in Changle (N 26°01.93', E 119°37.71')
- Meizhou Bay in Quangang (N 24°14.56', E 118°53.31')
- Dazhou Island in Yunxiao (N 23°55.72', E 117°26.43')

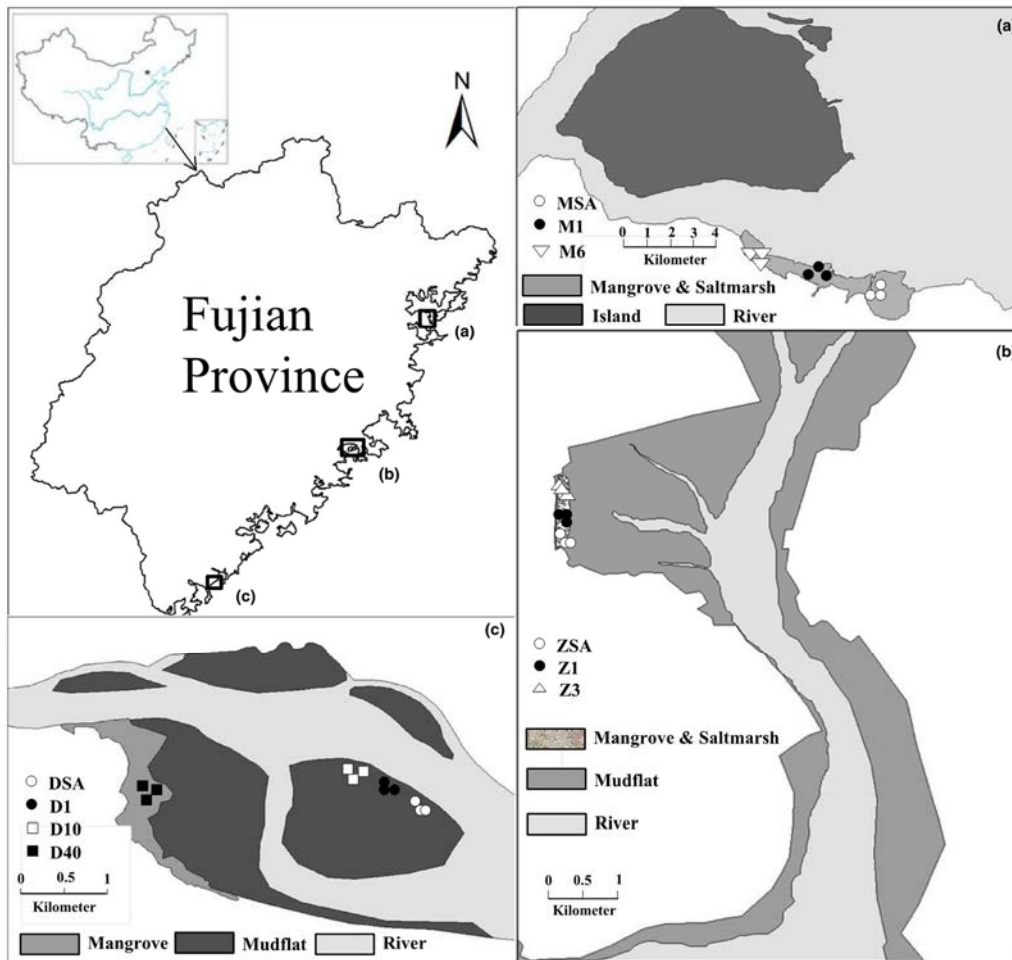


FIG. 18. Map of sampling sites in three different regions of Fujian province, China: (a) Minjiang Estuary, (b) Meizhou Bay, (c) Dazhou Island (reproduced from Ref. [19] with permission).

3.3.3. Deployment (methods, techniques)

3.3.3.1. Field sampling

The samples of primary producers and consumers were collected from three study sites shown in Fig. 18. The relevant information on the samples collected are given in Table 2. Litter from *K. obovata* or *S. alterniflora*, surface sediment organic matter (SOM) and particulate organic matter (POM) in tidal water were selected as four potential carbon sources for the benthic macrofauna studied. POM samples were extracted from seawater collected offshore (at least 2 km from shoreside) of each study region (three replicates from each region). The seawater was filtered onto precombusted (450°C, 6 hr) Whatman GF/F glass fibre filters to extract POM. SOM samples were collected by scraping surface sediments (0.2 cm) where benthic microalgae could be clearly observed. There were four replicates for each vegetation community at each sampling site. The POM and SOM samples were treated with hydrochloric acid to remove carbonates before carbon stable isotope analysis. The crab and gastropod species were handpicked during low tide periods by pooling 5–10 individuals together as one sample, with at least three samples collected from each site. The muscle tissue samples of selected benthic macrofauna were washed with distilled water and then dissected manually prior to stable isotope analysis. To ensure homogeneity, the animal samples were first freeze-dried using a vacuum freezer, then pulverized into a fine powder using a mortar and pestle.

TABLE 2. INFORMATION OF THE REGIONS, SAMPLING SITE, MAIN GASTROPOD AND CRAB SPECIES AND THEIR FEEDING TYPES FOR THE SAMPLES COLLECTED (from Ref. [19])

Region	Site/vegetation	Year of invasion	Species collected	Feeding type	
Meizhou Bay	ZSA	1980s	<i>Cerithidea cingulata</i>	Arboreal herbivore	
			<i>Cerithidea ornata</i>	Deposit feeder	
			<i>Littorina melanostoma</i>	Arboreal herbivore	
			<i>Sesarma plicata</i>	Herbivore	
			<i>Uca arcuata</i>	Deposit feeder	
	Z1	2010	<i>Cerithidea cingulata</i>	Arboreal herbivore	
			<i>Sesarma plicata</i>	Herbivore	
			<i>Uca arcuata</i>	Deposit feeder	
	Z3	2007	<i>Cerithidea cingulata</i>	Arboreal herbivore	
			<i>Cerithidea ornata</i>	Deposit feeder	
			<i>Littorina melanostoma</i>	Arboreal herbivore	
			<i>Sesarma plicata</i>	Herbivore	
Minjiang Estuary	MSA	2002	<i>Helice latimera</i>	Herbivore	
			<i>Chiromantes dehaani</i>	Herbivore	
			<i>Sesarma plicata</i>	Herbivore	
	M1	2010	<i>Helice latimera</i>	Herbivore	
			<i>Chiromantes dehaani</i>	Herbivore	
	M6	2006	<i>Helice latimera</i>	Herbivore	
			<i>Chiromantes dehaani</i>	Herbivore	
			<i>Sesarma plicata</i>	Herbivore	
	Dazhou Island	DSA	1990s	<i>Cerithidea cingulata</i>	Arboreal herbivore
				<i>Littorina melanostoma</i>	Arboreal herbivore
				<i>Metaplex longipes</i>	Deposit feeder
				<i>Sesarma plicata</i>	Herbivore
<i>Uca arcuata</i>				Deposit feeder	
D1		2011	<i>Cerithidea cingulata</i>	Arboreal herbivore	
			<i>Metaplex longipes</i>	Deposit feeder	
D10		2000	<i>Cerithidea cingulata</i>	Arboreal herbivore	
			<i>Cerithidea ornata</i>	Deposit feeder	
			<i>Littorina melanostoma</i>	Arboreal herbivore	
			<i>Metaplex longipes</i>	Deposit feeder	
			<i>Sesarma plicata</i>	Herbivore	
			<i>Uca arcuata</i>	Deposit feeder	
			<i>Uca arcuata</i>	Deposit feeder	
D40		1970s	<i>Cerithidea cingulata</i>	Arboreal herbivore	
	<i>Cerithidea ornata</i>		Deposit feeder		
	<i>Sesarma plicata</i>		Herbivore		
	<i>Uca arcuata</i>		Deposit feeder		

3.3.3.2. Laboratory analysis

The carbon and nitrogen stable isotope ratios for all plant litter, animal tissue, POM and SOM samples were analysed using an isotope ratio mass spectrometer (IRMS, Finnigan Delta V Advantage, Thermo Fisher Scientific, Inc., USA) coupled with an elemental analyser (Flash EA 1112HT, Thermo Fisher Scientific). Two laboratory standards (i.e., glycine and urea) were run at the beginning and end of each run with three replicates to ensure IRMS precision and accuracy and once after 12 samples to correct any instance of instrument drift. The analytical precision for $\delta^{13}\text{C}$ and analysis were better than 0.1‰ and 0.2‰, respectively.

3.3.3.3. Data interpretation

The $\delta^{13}\text{C}$ values of *S. alterniflora* litter were significantly higher those of the native mangrove species *K. obovata* ($p < 0.01$) at all three study regions, whereas the $\delta^{13}\text{C}$ values of POM or SOM were between those of *S. alterniflora* and *K. obovata* litter at each site (Fig. 19). The $\delta^{15}\text{N}$ values of POM were significantly lower than those of SOM at both Meizhou Bay and Minjiang Estuary (both $p < 0.01$), whereas no such differences were at Dazhou Island. The litter of *S. alterniflora* had higher $\delta^{15}\text{N}$ values than any of the other food sources within each region, ($p < 0.01$).

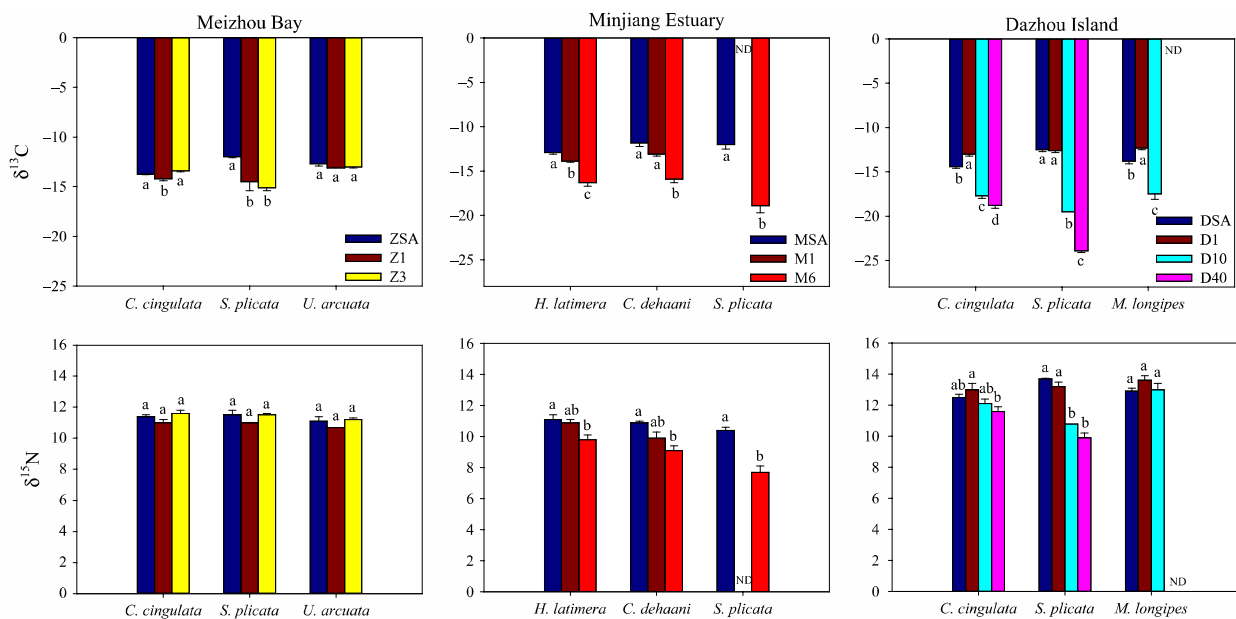


FIG.19. Comparison in the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of selected mangrove macrofauna species among different sampling sites in three study regions. Different letters denote significant differences at $p < 0.05$ (reproduced from Ref. [19] with permission).

At the unrestored *Spartina* sites (MSA, ZSA and DSA), the $\delta^{13}\text{C}$ values of all the species and sites that had been restored for a year were similar to those of *S. alterniflora*, indicating that the organic matter from *S. alterniflora* was the dominant carbon source for these microbenthic macrofauna species (Fig. 20). At the Meizhou Bay site, the tissues from *C. cingulata* and *U. arcuata* (two dominant mangrove crabs) from different habitats all had similar $\delta^{13}\text{C}$ values to those of *S. alterniflora* litter, and the $\delta^{13}\text{C}$ values of the herbivore *Sesarma plicata* decreased significantly

after restoration ($p < 0.05$). There was little or no change in the $\delta^{13}\text{C}$ values of benthic macrofauna following one year of restoration at the Minjiang Estuary and Dazhou Island sites but decreased subsequently with restoration time. However, there were significant differences in the $\delta^{15}\text{N}$ values for macrofauna between the invasive *S. alterniflora* and restored mangrove forests that were over 6 years old at the Minjiang Estuary and Dazhou Island sites ($p < 0.01$).

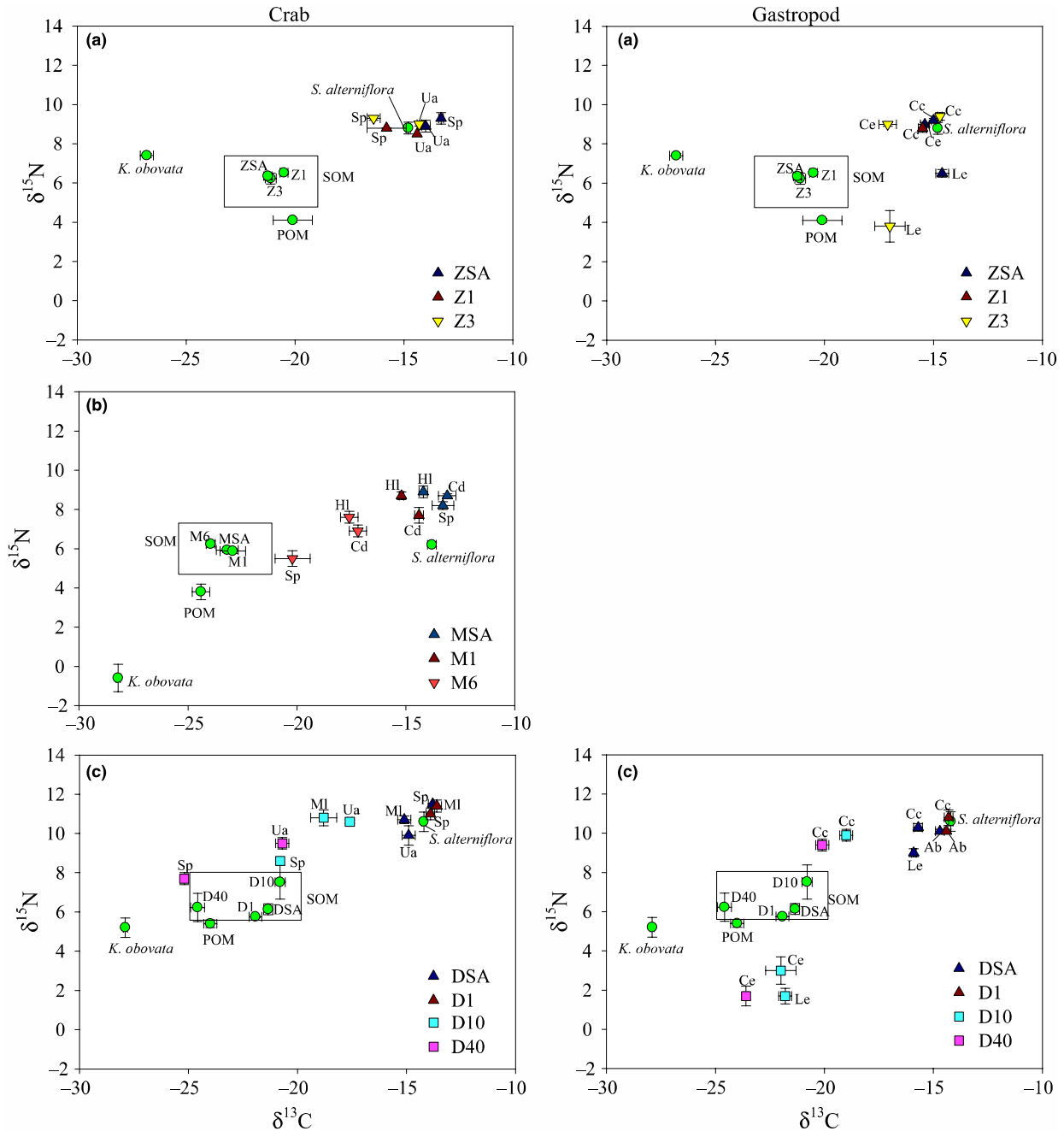


FIG. 20. The dual plots of $\delta^{13}\text{C}$ vs. $\delta^{15}\text{N}$ for the crabs, gastropods, and their potential food sources: (a) Meizhou Bay, (b) Minjiang Estuary, (c) Dazhou Island. Ab: *Assiminea brevicula*, Cc: *Cerithidea cingulata*, Cd: *Chiromantes dehaani*, Ce: *Cerithidea ornata*, Hl: *Helice latimera*, Le: *Littorina melanostoma*, Ml: *Metaplex longipes*, Sp: *Sesarma plicata*, Ua: *Uca arcuata* (reproduced from Ref. [19] with permission).

Based on the results from isotopic sourcing calculation, the *Spartina*-derived detritus contributed to >80% of the organic carbon sources of selected dominant macrofauna in the *S. alterniflora* communities in all three regions (Fig. 21). *S. alterniflora* communities had lower convex hull areas and standard ellipse areas than natural mangrove forests, indicating significant resource competition among different benthic? species. Replacing *S. alterniflora* with the native mangrove species *K. obovata* could reverse the diets of these macrofaunal species, causing a shift from homogeneous *Spartina*-dominated diets to more heterogeneous algae-based diets. It could take several decades to restore food web interactions to a pre-impacted state. Even the diets of macrofauna in mature mangrove (>40 years) remained affected by the *Spartina*-derived organic matter.

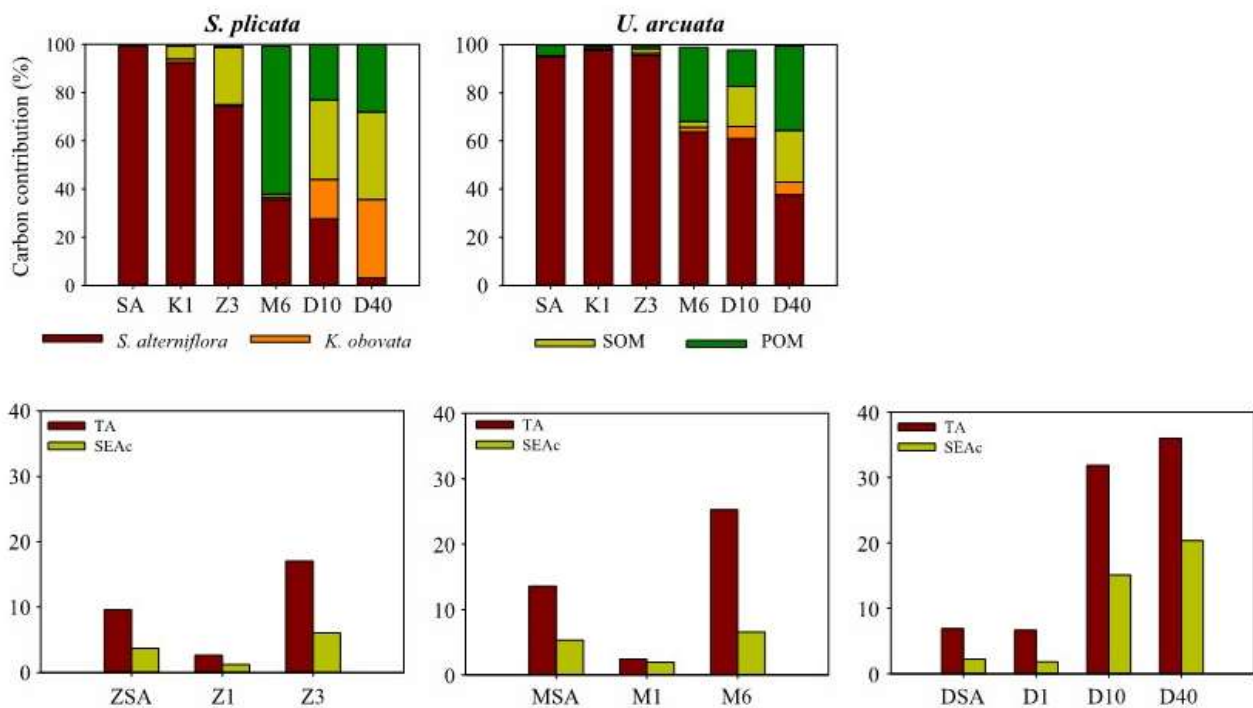


FIG. 21. The Convex Hull Area (TA) and Standard Ellipse Area (SEAc) for the benthic macrofaunal communities in the *Spartina alterniflora* community and mangrove forests with different restoration ages (from Ref. [19]).

3.3.3.4. Conclusions

Ecological replacement of invasive *S. alterniflora* using native mangrove species could restore food web function gradually. However, this removal and replacement approach is a long process and requires significant manpower and resources. Furthermore, the native ecosystem will continue to be influenced by the cordgrass as long as large areas of wetland nearby are occupied by *S. alterniflora*. Consequently, preventing the colonization of *S. alterniflora* should be a priority for coastal ecosystem management in southern China. Stable isotope analyses provide quantitative assessments about mangrove wetland restoration in southern China, where mangrove wetlands have been seriously invaded by US cordgrass [21].

3.4. CASE STUDY. APPLICATIONS IN BLUE CARBON ASSESSMENT

We introduced the concept of Blue Carbon in Section 2, outlining the climate change mitigation policy context and the opportunity this represents for mangrove conservation and restoration. Here we provide a more detailed consideration of how stable isotopes can be used to identify the sources of carbon accumulating in these important Blue Carbon habitats. We provide information on protocols commonly deployed in the sampling of blue carbon and provide two case studies which show how these analyses have been implemented and how the results have informed wetland management.

3.4.1. Stable Isotopes and Blue Carbon: allochthonous and autochthonous sources

As discussed in Section 2, the source of carbon accumulating within a coastal wetland is an important consideration in accounting for the carbon sequestration value. Figure 22 illustrates some key potential sources of carbon in a tidal wetland: tidal accretion (allochthonous carbon); marsh ‘cannibalization’, the reworking of the eroding marsh fringe and redeposition on the marsh interior platform; and in situ sequestration, the autochthonous accumulation of carbon through the fixation and burial of atmospheric CO₂. Each of these sources may have distinct isotopic signatures, and the application of stable and radioisotopes has been used to fingerprint carbon contributions.

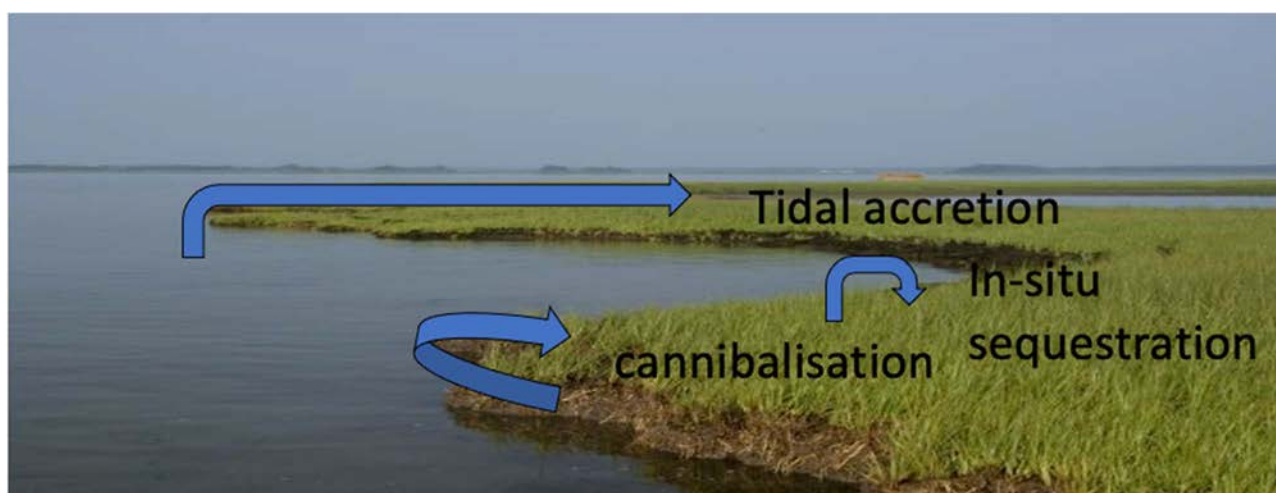


FIG. 22. Provenance of soil carbon in a coastal wetland. (Courtesy of N. Saintilan, Macquarie University, Australia.)

Also, changes in the isotopic composition of carbon with depth within the wetland soil provide with an indication of the residence time, or permanence of soil carbon. If, for example, there is a strong shift in the isotopic signature in the upper few centimetres of the soil, this might be a clue that components of the soil organic carbon depositing on the surface do not last long. We call this less permanent carbon ‘labile’, and the more long-lasting carbon ‘refractory’.

To summarize, stable isotopes are useful in helping to identify the provenance of carbon, that is, the contributing source, and the permanence of carbon, how long it survives in the soil. Both are important considerations in carbon accounting. In the example above, if there was a strong match between the carbon stable isotope signature of the plants and the carbon within the soil, we could deduce that autochthonous inputs are likely to be the dominant source. If there is a strong difference

in the stable isotopic composition of the wetland plants and the soil carbon, allochthonous inputs are likely dominant. Radiometric analysis adds a further insight: if the ^{14}C signature is old, organic carbon is likely to be sourced from marsh cannibalization if stable isotopes match the wetland plants, or labile allochthonous material if stable isotopes do not match the plants. In either case, inclusion of this carbon in emissions calculations might consist of ‘double accounting’.

Let’s consider some of the processes that influence variation in carbon between autotrophic sources. We previously introduced the important differences between C_3 and C_4 plants in their stable isotope composition (see Fig. 23). Essentially, many (but not all) tropical grasses utilize the C_4 photosynthetic pathway. This includes many families of saltmarsh (e.g. *Spartina*, and *Sporobolus*; two of the most common saltmarsh families). Without going into the details of this photosynthetic pathway, one outcome is that plants which photosynthesize in this way are strongly enriched in $\delta^{13}\text{C}$ compared to other plants.

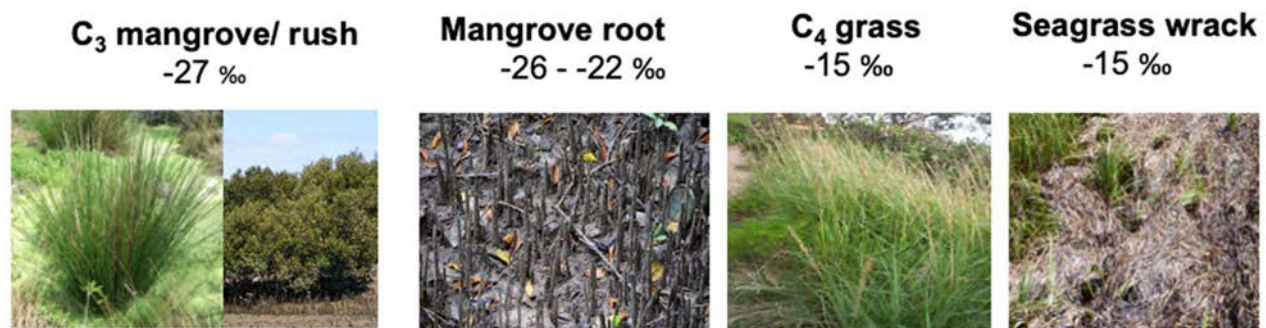


FIG. 23. Variation in $\delta^{13}\text{C}$ amongst common coastal plants. (Courtesy of N. Saintilan, Macquarie University, Australia.)

All mangroves utilize the ‘normal’ C_3 photosynthetic pathway, and one problem this presents to provenance studies is that mangroves are similar to each other in their isotopic signatures and may be similar to terrestrial trees and C_4 marsh plants. However, the distinction between mangrove and saltmarsh is useful. This has been used where the two plant types grow together to unravel some interesting aspects of the food web (see Section 3.2).

For Blue Carbon studies, it is critically important to measure the carbon isotopic signature of both the leaves and the roots of the mangrove, because these can differ substantially [8]. Figure 24 below is derived from an Australian study and shows how widely the $\delta^{13}\text{C}$ values of roots differ to leaves, with stems providing intermediate values. Clearly, to characterize the possible sources of carbon in a wetland, both the leaves and roots should be sampled. Many studies have neglected to do this and drawn incorrect conclusions about the importance of mangrove root material in carbon accumulation.

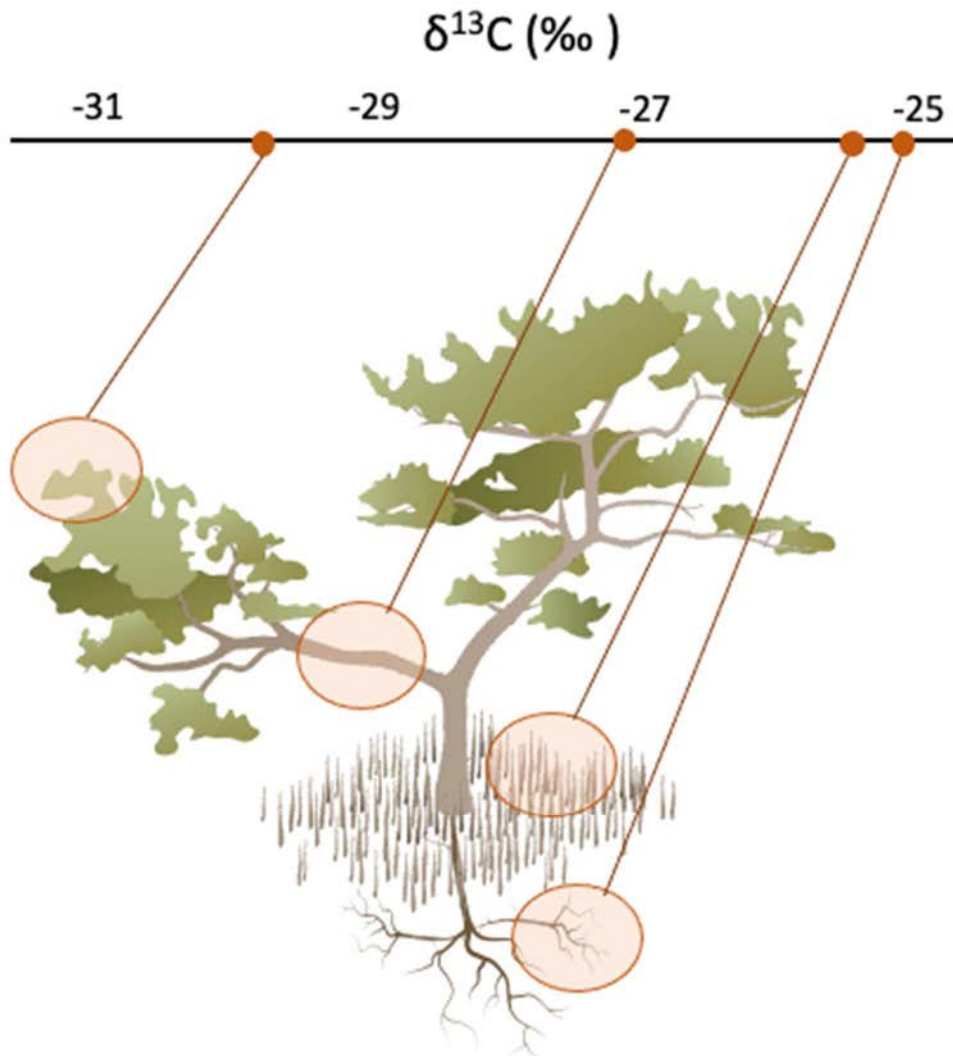


FIG. 24. Fractionation of carbon isotope signatures between mangrove plant parts (data from Ref. [8]). (Courtesy of N. Saintilan, Macquarie University, Australia.)

Small variation in the stable isotope signatures of mangroves can also occur in relation to salinity. This is because mangroves tightly regulate their water loss under conditions of environmental stress, this influences the rate of CO_2 diffusion into the plant which leads to preferential isotope uptake. It is important therefore to sample plants in the vicinity of the study, rather than presuming that sampled or published values from elsewhere will be identical to the same species within the study location.

One further important difference in isotopic composition occurs between marine and terrestrial/intertidal plants. Seagrasses are highly enriched in $\delta^{13}\text{C}$ compared to mangroves and terrestrial plants and may be even more enriched than C_4 plants. This is because their carbon is incorporated from the surrounding marine waters, and the process of taking up this carbon leads to strong isotopic enrichment. This is important not only in trophic studies, but also in Blue Carbon research for several reasons. First, seagrasses are important Blue Carbon ecosystems in themselves, often showing high soil organic carbon and burial of carbon for centuries. Second, the seagrass leaves are subject to erosion during storms, and seagrass wrack may be a locally important source of carbon

accumulating within intertidal wetlands. Where seagrass grows in the vicinity of a blue carbon study site, it should be sampled as a potentially important contributing autotrophic source.

3.4.2. Sampling Considerations

A useful resource on each of these steps is the Conservation International's Blue Carbon Manual⁶, a practical guide to field sampling for Blue Carbon analysis [22].

Planning your project will consist of four elements:

- Conceptualization: getting clear on what the aims and objectives of the project are;
- Sampling in the field: collecting the materials that will allow you to answer your research question;
- Sample preparation and laboratory analysis;
- Analysis and application of the results.

It is important from the outset to be clear on the goals of your research project. If, for example, you want to understand the sources of carbon contributing to carbon accumulation in a mangrove forest, there are several steps to consider (see Fig. 25 below).

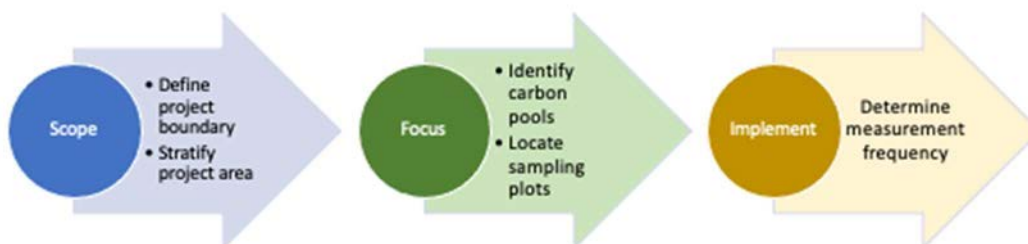


FIG. 25. Steps in the development of a Blue Carbon sampling program (reproduced from Ref. [22] with permission).

The boundary of the project will be set by the scope of your research question. If it is to characterize sources of carbon contributing to carbon accumulation in a mangrove forest, you will need to sample sufficiently within the forest to characterize the spatial variability (temporal variability is soil carbon stores is usually small). Characterising spatial variability may involve stratifying sampling, particularly if different vegetation communities or soil properties are encountered. If the forest is homogenous in characteristics likely to be controlling blue carbon, then random sampling of locations is preferred. Some sampling approaches are shown below in Fig. 26. Linear designs are usually deployed to explore variability along a gradient. For example, a hypothesis might be that the contribution of a carbon source decreases away from that source. Random deployment is the best way

⁶ <https://www.thebluecarboninitiative.org/manual>

of sampling replicates increasing the number of samples used to characterize variability in an homogenous area. Your Smartphone is the most convenient way of generating a random number grid for this type of sampling.

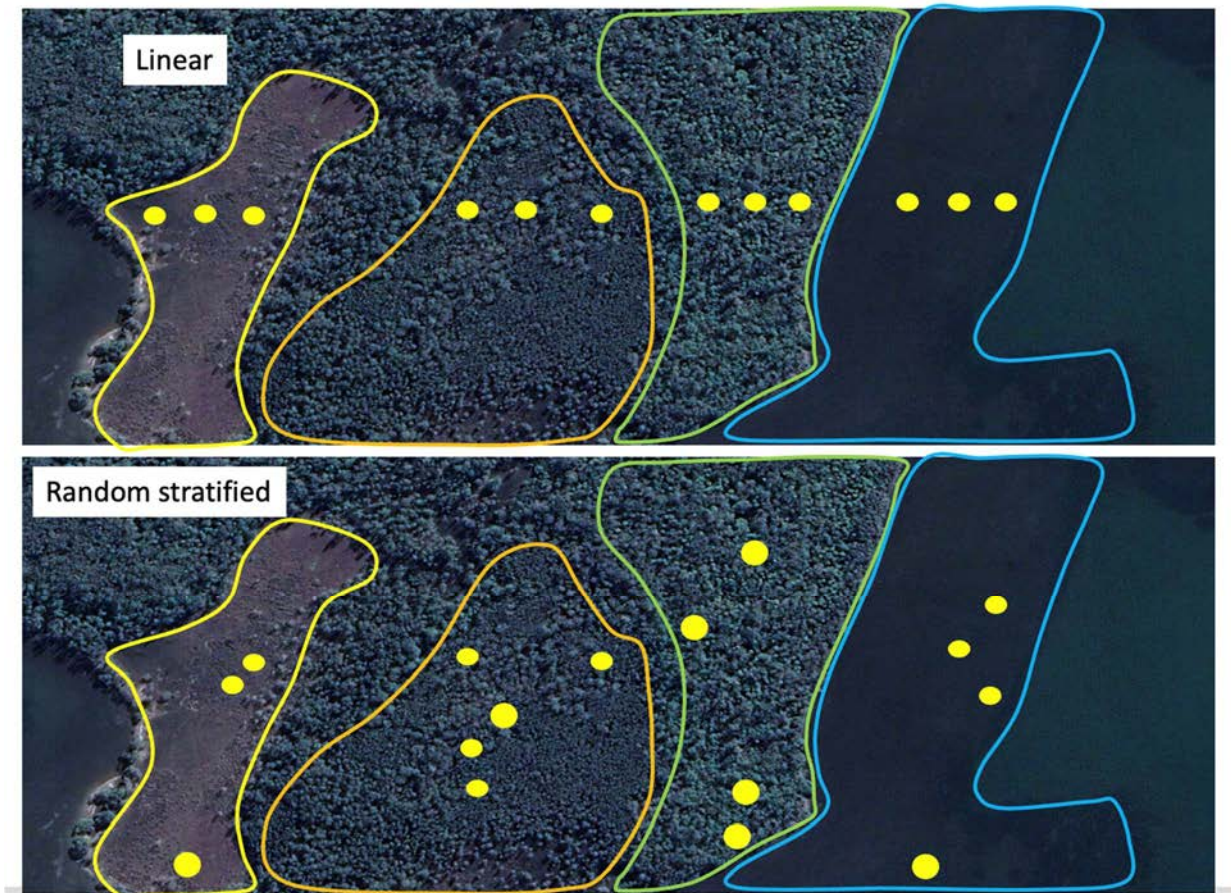


FIG. 26. Approaches to sampling carbon within mangrove environments. (Courtesy of N. Saintilan, Macquarie University, Australia.)

3.4.3. Sampling and processing soil cores

There is a standard protocol to sample soil carbon to a depth of one metre. There are a few reasons for this, but the most important is that the upper metre of soil is that depth likely to be disturbed by human interference, for example, but draining or dyking a wetland. Knowing how much carbon there is to that depth gives a conservative estimate of the carbon lost to the atmosphere as a result of land use conversion.

Sampling to one metre depth can be challenging in coastal environments, particularly if the intention is to retrieve an undisturbed soil core. The most common approach is to use a Russian D-Section peat corer. In Video 5, Dr Karen McKee demonstrates the use of the Russian peat corer.⁷ Usually only one or two extensions are required to have a continuous core extending to one metre.

⁷ <https://youtu.be/CcDUNgcZdVw>

In this video, samples are transferred to PVC casing (cut in half and then resealed) for transport back to the laboratory. However, it is also possible to subsample the sediment core in the field if the depths intended to be analysed have been defined. The schematic below (Fig. 27) provides common sampling depths for carbon and isotope analysis. This scheme can be followed, though keep an eye out for stratigraphic discontinuities which might indicate sudden shifts in soil conditions.

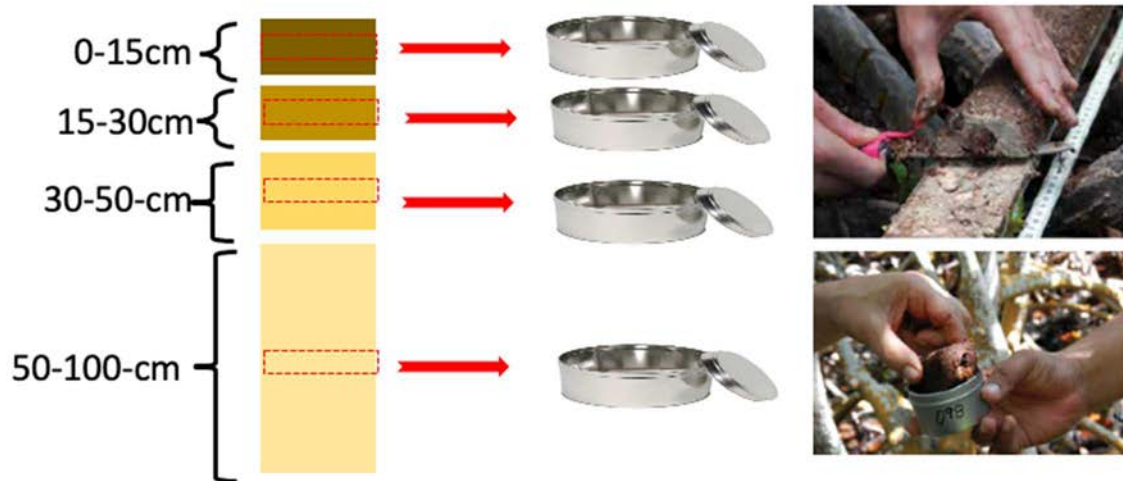


FIG. 27. Core subsampling for organic carbon. (Courtesy of N. Saintilan, Macquarie University, Australia.)

While in the field, also sample potential autotrophic sources of carbon, placing each in sample bags for later preparation and analysis. Video 4 in Section 3.2 provides a useful guide to this.

3.4.4. Case Study: New South Wales coastal wetlands

The State of New South Wales in south-east Australia contains over 100 estuaries, each with mangrove, saltmarsh and seagrass ecosystems. In one of the first studies of its type, Saintilan et al. [23] measured the rate of carbon accumulation within several of these estuaries and used stable isotopes of Carbon to determine probable sources contributing to carbon accumulation in these wetlands.

The sampling strategy consisted in taking one metre cores in both the mangrove and saltmarsh environment within a subset of estuaries. These were selected on the basis of contrasting soil properties. In general, sandy soils hold less carbon, because they are more likely to be drained and oxygenated, encouraging decomposition and the loss of organic carbon. Two different saltmarsh communities were also analysed: the C₄ saltmarsh grass *Sporobolus virginicus*, and the C₃ saltmarsh rush *Juncus kraussii*. The mangrove environment consisted of *Avicennia marina* in nearly all locations.

In general, saltmarsh cores showed an enrichment in $\delta^{13}\text{C}$ values compared to mangrove cores in the same estuary, suggesting a contribution of the C₄ saltmarsh grasses to soil organic carbon accumulation (Fig. 28). However, even in these locations the $\delta^{13}\text{C}$ values were more consistent with mangrove root material than saltmarsh. The other consistent trend was for the surface layers to be more depleted in Carbon compared to lower down in the core. Down core depletion in $\delta^{13}\text{C}$ was nearly universal across all samples.

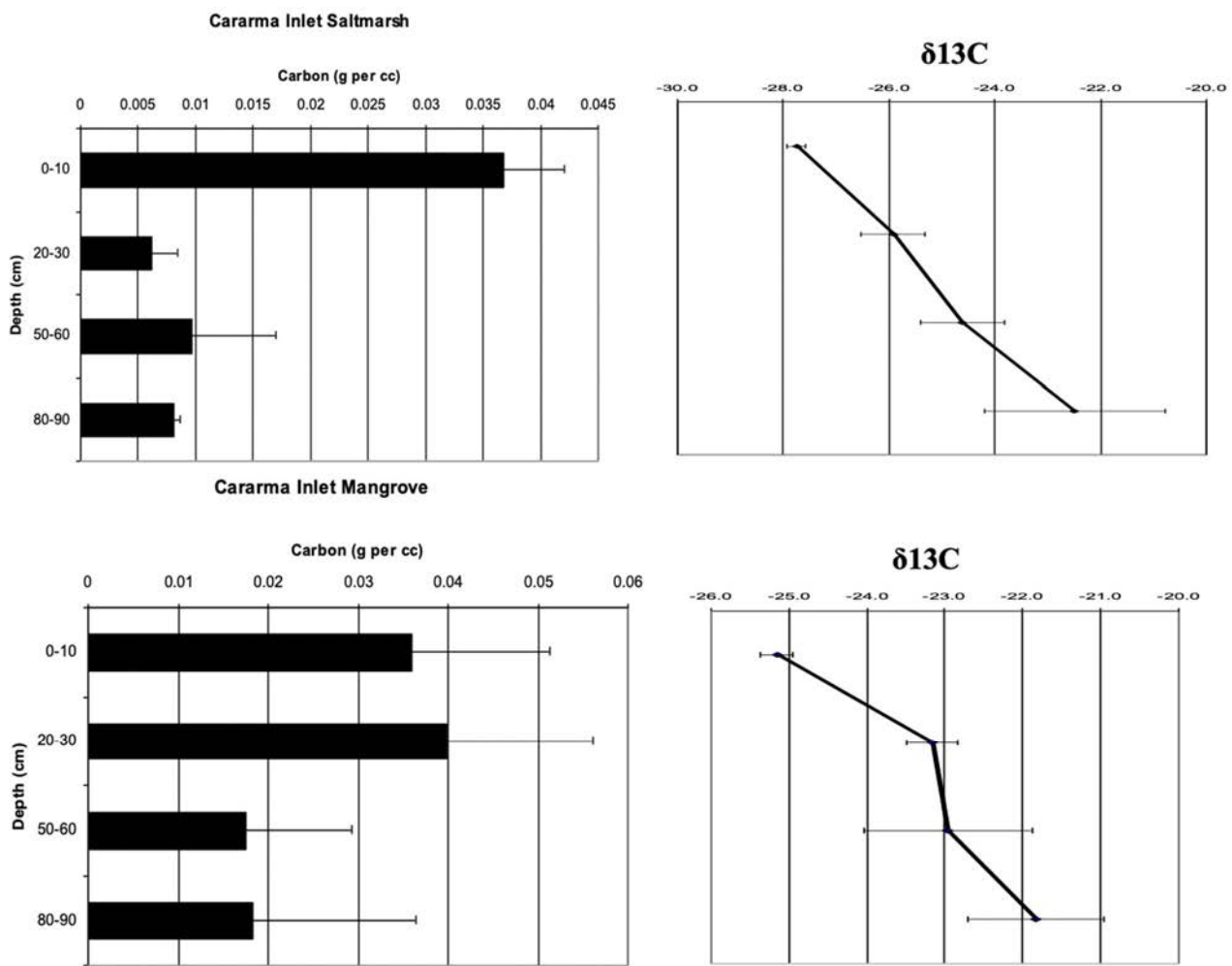


FIG. 28. Variation in Carbon concentration and $\delta^{13}\text{C}$ with depth in an Australian coastal wetland (data from Ref. [23]). (Courtesy of N. Saintilan, Macquarie University, Australia.)

3.4.4.1. Interpreting down core variability in $\delta^{13}\text{C}$

There are a few reasons why soils might become more depleted in $\delta^{13}\text{C}$. One is that mangrove roots are depleted in $\delta^{13}\text{C}$ compared to above ground parts including leaves, even in the same plant (see Section 3.4.2). Given that in many mangrove forests half or more of the biomass of the mangrove is below ground in the root system, there is good reason to think that this would be a dominant contributor to soil organic carbon. The downcore depletion observed in the New South Wales coastal wetland was interpreted as the contribution of root material giving way to an increased ($\delta^{13}\text{C}$ depleted) contribution of leaves towards the surface. One additional factor may be the change in atmospheric ^{13}C since the industrial revolution due to the burning of fossil fuels. This 'suess effect' may contribute up to 1.5 pp depletion in $\delta^{13}\text{C}$ in contemporary organic carbon [24].

3.4.5. Estuary-wide stocks and subsidies

When investigating the role of estuarine habitats in sequestering and storing carbon, it is important to understand how much of the carbon sequestered by that habitat is derived from that habitat type, versus imported from elsewhere (such as terrestrial organic matter that has been redeposited within an estuarine habitat). Without this knowledge, it is difficult to attribute what the impact of that specific habitat type is on estuarine carbon stocks or the implications if that habitat type was lost or replaced. For example, if a saltmarsh accumulated $200 \text{ g C m}^{-2} \text{ yr}^{-1}$, one could draw the conclusion that the loss of that saltmarsh would result in a loss of $200 \text{ g C m}^{-2} \text{ yr}^{-1}$. However, this loss will be offset by the sequestration occurring in any habitat type which may replace that saltmarsh and by imports of organic matter derived elsewhere (for example, from mangrove/seagrass leaf litter or terrestrially derived organic matter [25, 26]).

Here we briefly summarize a study which used stable isotopes to quantify carbon sources and cross habitat exchange of carbon in order to investigate how changes in habitat distributions may impact estuarine scale carbon stocks.

3.4.5.1. *Methods in brief*

For further methodological details please refer to [27].

This study was conducted at an estuary off the east coast of the North Island, New Zealand (Tairua estuary). The estuary is 660 ha, comprised of 451.5 ha of mud and sandflats, 41.7 ha of saltmarsh (*Juncus kraussii* being the dominant species), 36.1 ha of mangroves (*Avicennia marina*), and 130.7 ha of seagrass (*Zostera muelleri*). Sampling was undertaken at four sites throughout the estuary (Tairua Upper, Tairua Mid, Pauanui, Tairua Entrance; Fig. 29).

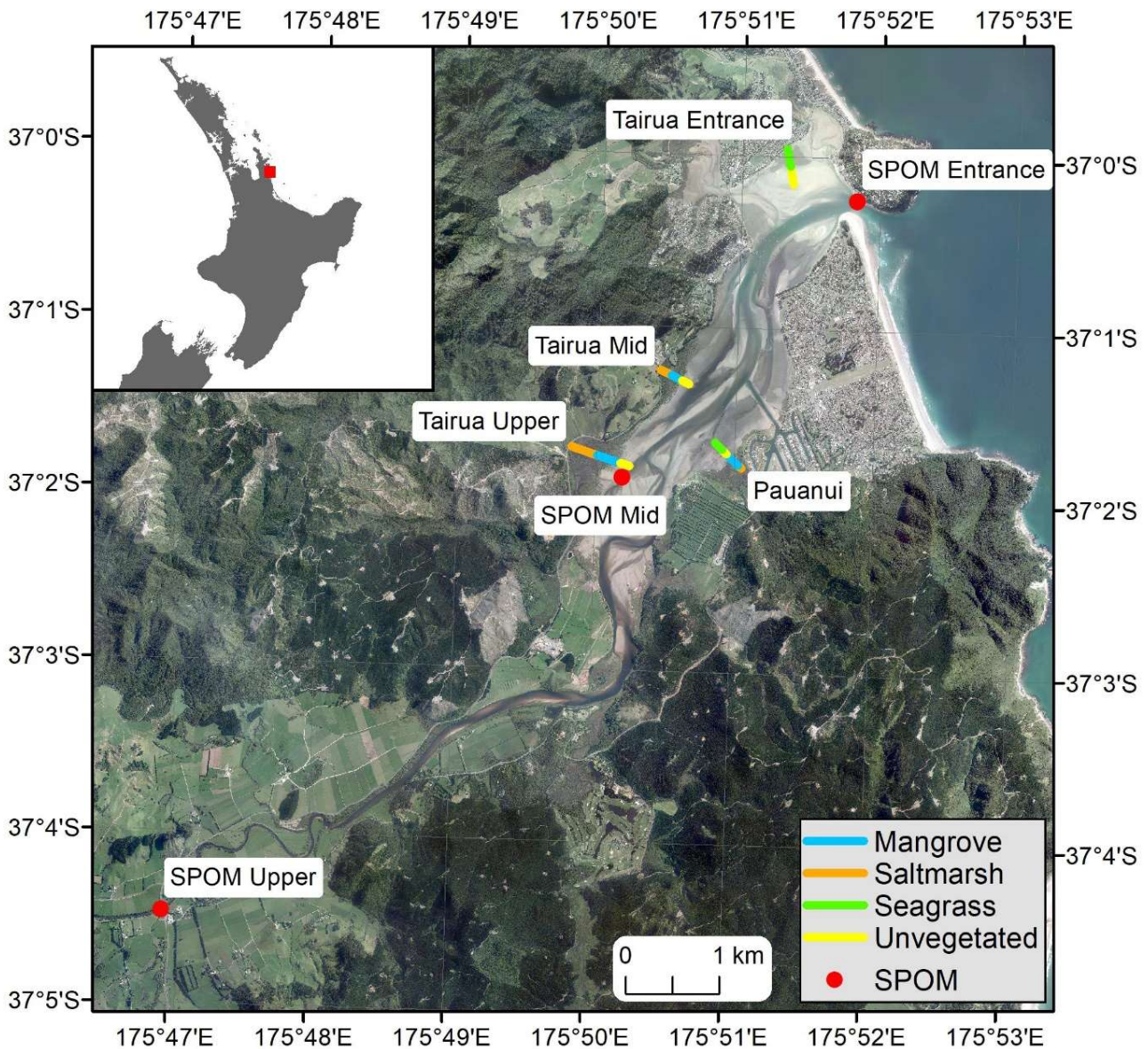


FIG. 29. Map of Tairua estuary, showing location and name of transects and SPOM collection points, color coded by habitat type. Orange, Saltmarsh; Blue, Mangrove; Green, Seagrass; Yellow, Unvegetated; Red, SPOM, Suspended Particulate Organic Matter (reproduced from Ref. [27] with permission).

A transect was established at each of the four sites, running from the upper intertidal to the lower intertidal and traversing a range of habitat types (saltmarsh, mangrove, seagrass, mud and sandflats). Three sampling points were established within each habitat along each transect and a range of measurements were collected to measure carbon stocks/isotopes (including sediment cores to 100 cm depth). Each sediment core was sectioned into several depth intervals and used to measure bulk density and C concentrations and isotopic ratios ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$) throughout the sediment column. Potential carbon sources were also collected at each sampling location, including leaves/blades of above ground biomass, root material, and microphytobenthic surface samples. Triplicate Suspended Particulate Organic Matter (SPOM) water samples were also collected at three locations throughout the harbour (Fig. 29). The contribution of each potential carbon source to sediment carbon stocks for each habitat type was estimated using a bayesian mixing model (JAGS – Just Another Gibbs Sampler) – (SIMMR package) [28].

3.4.5.2.Key results

Organic carbon stocks (above ground biomass and sediment to 100 cm) varied between habitat types, increasing from unvegetated habitats (26 t ha⁻¹) and seagrass (27 t ha⁻¹), to mangrove (46 t ha⁻¹) to saltmarsh (90 t ha⁻¹). Even though unvegetated habitats had the lowest carbon stocks per ha, when adjusted for their large extent within the estuary (68.4% of estuarine area), unvegetated habitats were found to contain the majority of estuarine carbon stocks (57%). However, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ mixing model results showed that coastal vegetation (saltmarsh, mangrove and seagrass) contributed an estimated 41% of the organic carbon within unvegetated sediments and approximately half of the carbon stocks within the estuary (51%).

3.4.5.3.Conclusions

This study illustrates the value of using stable isotopes to better understand blue carbon ecosystems. Results showed that blue carbon stocks and carbon sources varied based on habitat type, highlighting the importance of taking measurements not only in the habitat of interest, but also other habitats within an estuary. By measuring carbon stocks and sources across four major habitat types, it was possible to show that vegetated habitats (saltmarsh, mangrove, seagrass) increased total estuarine carbon stocks by 2 to 3 fold and contributed carbon to other habitats throughout the estuary (including mud and sandflats). In addition, it was found that unvegetated habitats (mud and sandflats) contained significantly more carbon than other habitat types when adjusted for their aerial extent within the study estuary.

Through the use of stable isotopes, this study highlights the interconnected nature of blue carbon ecosystems and provides information that can be used to better understand the impacts of changing habitat distributions on carbon stocks.

4. SAMPLE PREPARATION AND PROCESSING FOR IRMS

This section provides recommended instructions for sample preparation, EA-IRMS instrument operation and maintenance, sample archival, quality assurance (QA) and quality control (QC) measures and criteria, and data reporting. The content of this section is partially extracted from Refs. [29, 30, 31, 32].

4.1. PREPARATION OF MATERIALS

4.1.1. Preservation of field samples

Collected samples should be placed on ice in the field and then kept frozen until laboratory analyses.

4.1.2. Removal/preparation of tissue samples

All samples (crustaceans, fish, zooplankton, algae, vascular plants) should be washed with deionized water, labelled and put in the ziploc bags, and kept frozen until prepared for stable isotope analysis. Dissect tissue from a single specimen using clean forceps and solvent-rinsed aluminium foil.

4.1.2.1. *Invertebrate samples*

Crack the shell of snails and mussels, remove the exoskeleton of crustaceans (such as prawns, yabbies etc.) and aquatic insects and collect the white muscle tissue. For crabs, muscle tissue is collected from claws following removal of the exoskeleton.

Extremely small invertebrate (e.g. zooplankton) samples are prepared whole for stable isotope analysis. However, if possible, small invertebrates need to be kept for 24 hours in the water (filtered) from which they were sampled so as to evacuate their gut before being processed for analysis. Acid washing is also required (using 0.1N HCl for 1 h) for $\delta^{13}\text{C}$ analysis for small invertebrates with shells which are difficult to remove. Samples should be rinsed with deionized water following acidification.

4.1.2.2. *Vertebrate samples*

For large vertebrates, white muscle from the dorsal region of fish, or red muscle from terrestrial animals is usually considered for stable isotope analysis. However, selection of specific body tissue (e.g. blood, liver, kidney, stomach etc.) of an animal mostly depends on the objectives of the research. Animal samples are thawed, surfaces trimmed to eliminate scales or fur and skins, then washed with deionized water.

Usually, small animals are prepared whole for stable isotope analysis. If the animal is extremely small (<1.5 cm), remove the head and stomach, trim surfaces to eliminate scales or fur and skins, then wash with deionized water before drying.

4.1.3. Drying and Grinding

Samples are usually placed in a freezer (-25 or -80°C) for at least 1 hour or until they are frozen solid, and then placed in a freeze dryer for 24 hours to remove water [33]. In absence of a freeze dryer, samples can also be oven dried for 48–96 hours at 60°C [34].

4.1.4. Homogeneization

Due to the small sample weight used for isotopic analysis it is imperative that the samples are ground to a homogeneous powder using a pestle/mortar or mixer mill. The milling cups and balls should be solvent washed prior to use. Dry sediment, plant and biota samples can be homogenized using a mixer mill for at least 1–3 minutes using steel or agate ball bearings in a stainless steel mixer at a frequency of 15 Hz.

For the biota samples that are relatively fatty, such as a gut and tissue, liquid nitrogen is required; otherwise, the fatty sample will turn into paste when milling. Fatty samples should be placed in a stainless-steel mixer-mill container and then immersed in liquid nitrogen for about five minutes (until the liquid nitrogen stops bubbling, approximately two minutes) and then placed into the mixer mill with protective gear (i.e. appropriate gloves, goggles, laboratory coat). Following homogenization, the samples should then be transferred into glass containers and stored until further use.

4.1.5. Preparation of sediment materials

To measure the elemental composition of organic carbon and $\delta^{13}\text{C}$ of the sedimentary organic matter, carbonates should be removed through acidification. About 0.5–1 grams of dry and homogenized sediment sample should be weighed and placed into a 10 mL teflon tube. Using a precombusted pipette, add 1M HCl (32% analysis grade) slowly to the sample one drop at a time. The sample will bubble due to the carbonates in the sediment reacting with the acid to release carbon dioxide. HCl should be added until the bubbling subsides, and the sediment mixture reaches a pH of 1–2. The uncapped tubes should then be placed in a sonicator, with the water bath temperature set to 40°C and left overnight. The next morning the pH needs to be checked again to ensure that the pH is still 1–2, if not more acid needs to be added.

Following acidification, the tubes should be filled with Milli-Q water and then centrifuged for five minutes at 5000 rpm. With a precombusted pipette, remove the water taking care not to disturb the sediment. Refill the tubes with Milli-Q water, shake and then repeat the washing procedure two times. After the third time, check the pH to ensure that the acid has been completely removed and that the sediment mixture has a pH of 5–6. If not, repeat the washing procedure until the pH of the aqueous phase is 5–6.

Freeze the wet sediment tubes in the freezer and then dry the samples in a freeze drier. If needed, homogenize the samples again using a mixer mill.

4.1.6. Lipid extraction in fatty tissues

The $\delta^{13}\text{C}$ in lipids are depleted relative to proteins and carbohydrates, and therefore tissue with high lipid content can affect the interpretation of the results when studying trophic levels [35, 36]. Therefore, lipids should be extracted from the bulk samples when the C/N ratio is higher than 3.5 [36].

Weigh approximately 0.2–0.5 grams of sample and place into a 10 mL teflon tube. Using a graduate cylinder, pour 3.5 mL of (hexane: methylene chloride) (4:1) into the tube and place on a shaker for 15 minutes at 700 OSC/min. Centrifuge the tubes at 5000 rpm for 5 minutes. With a precombusted pipette, remove the solvent being careful not to disturb the settled sample and put into a clean glass test tube. Fill the sample tubes with 3 mL of solvent mixture and repeat the process an additional two times.

Place the lipid extracted samples under N₂ gas, until all of the solvent evaporates and the sample is dry again, this is the lipid extracted biota (i.e. the lipid free fraction).

In order to calculate extractable organic matter (EOM), place the lipid fractions under N₂ gas until the volume reduces to 1 mL. Tare a tin capsule on a balance and then place on a hot plate. Place 10 µL of the lipid sample in the tin capsule for about 20 seconds until all the solvent evaporates. Weigh the tin capsules on a micro balance.

4.1.7. Preparation of Capsules

Homogenized samples and standards should be weighed into individual tin capsules, and then run on the EA-IRMS in an identical manner, according to the Principle of Identical Treatment (PIT) [37]. Throughout this final process, it is important to always use clean tools and wear gloves as oils from your hands can contaminate samples. Cover the benchtop with foil or lint free paper. This work surface can be easily replaced in the event of spillage.

After opening the balance door, use forceps to crimp the lip of the tin cup shut so that no sample is lost during folding. Then, using the flat tip forceps, fold the tin cup into a small tight cube on top of the work surface. Make sure there are no sharp pieces sticking out of the cube and that the tin capsule is flat so that the cube does not get caught up on the autosampler. Using forceps, place the sample in the appropriate cell in the well plate and move on to the next sample. Make sure to wipe down all of the tools along with the bench surface with lint free paper towelette between every sample, so as not to cross-contaminate samples. Repeat this process for all the samples and standards in the set. Store the well plates in the desiccator until they are ready to be loaded on the IRMS. When you are finished using the tools, thoroughly rinse them in solvent (e.g. ethanol) and store them away until the next time they are used. Always place the lid on the tin that the capsules are stored in between uses. The balance should always be left with the door shut between uses.

Weigh three separate capsules per sample using a micro balance (with a minimum resolution of 1 µg following the PIT). The weight of the sample should be enough in order for the CO₂ and N₂ signal intensity to be within the linearity range of the elemental composition. For the CO₂ measurements, bulk sediment samples are usually weighed between 1 and 20 mg and biota material ~0.6 mg depending on the configuration of the EA-IRMS reactors. Due to the low N₂ content in marine sediment samples, some sediment samples may need to be reweighed after measuring sediments for carbon values in order to achieve a higher N₂ intensity. If the N% is found to be greater than 0.07 weight % [38] then new capsules should be prepared with 10 000 mg of non-acidified sediment for determining more precisely the δ¹⁵N of the sediments. Appropriate isotopic reference materials, including primary, secondary and matrix should be measured in duplicates at the start and end of the sequence. Quality control standards, such as acetanilide certified in δ¹³C and δ¹⁵N should be measured in duplicates at the start middle and end of each sequence. One elemental composition reference material (e.g. acetanilide) should also be run at the beginning of each sequence as a calibration for elemental composition with weight of approximately 100, 200 and 300 µg. Blanks (i.e. empty capsules) are run in the beginning in triplicates for blank correction and throughout the sequence to monitor the background for drift.

4.2. ELEMENTAL ANALYSER ISOTOPE RATIO MASS SPECTROMETRY (EA-IRMS)

For bulk stable isotope analysis, EA-IRMS can be used on a wide range of materials. Solids and non volatile liquids can be run in the EA system packed into tin capsules (for C/N/S analysis), while liquids with low viscosity can be injected directly through a liquid inlet system. Before being analysed, the carbon and nitrogen of the samples should first be quantitatively transformed into simple analyte gases (CO_2 and N_2 for carbon and nitrogen respectively).

Figure 30 below shows a schematic diagram of an EA-IRMS system for the determination of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$.

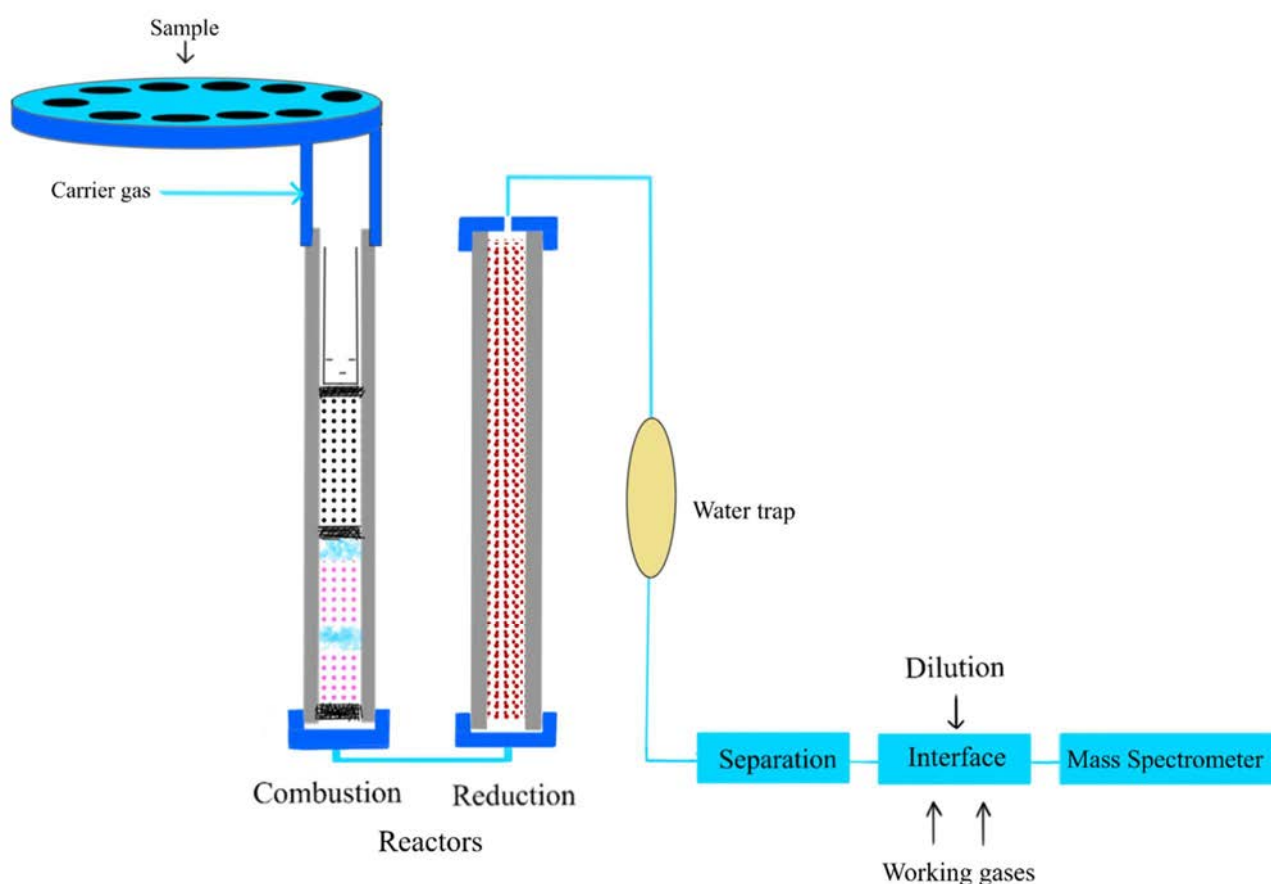


FIG. 30. Simple schematic diagram of an EA-IRMS system for the determination of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$.

The analyzer is usually made up of two reactors: a 'combustion' reactor and a 'reduction' reactor, or alternatively both reactors can be integrated into a single reactor tube. Following the complete oxidation/reduction in the reactors, a water separation unit and (usually) a packed GC column are used to separate the produced gases (N_2 and CO_2). Using an autosampler, each tin capsule falls into the reaction tube where the combustion takes place in an oxygen (O_2) environment, to produce N_2 , NO_x , CO_2 and H_2O . Although numerous variants are proposed for specific purposes, the reactor normally incorporates an oxidation catalyst [copper (II) or chromium (III) oxide] and a scavenger to

bind sulphur and halogens [cobalt (II, III) oxide and/or silver]. The temperature of the reactor is normally kept between 900 and 1050°C, but the reaction of oxygen with the tin capsules boosts the sample temperature up to 1800°C, ensuring a complete sample combustion. To collect the ash, sample residue, and tin capsules, the use of removable ash crucibles is recommended. The type of insert used will determine the number of samples that can be analysed without the need to remove the reactor, typically 50 to 250 samples. More details on the different types of combustion and reduction reactors are described in Ref. [30].

The excess of oxygen is consumed, and any nitrogen oxides (NO_x) are reduced to elemental nitrogen (N₂) at lower temperatures. This takes place, either in a cooler area of a single tube or in a separate reactor, which is normally kept at 650°C. High purity elemental copper is commonly used in the reduction process, but variations are indicated for certain applications. At the outlet of the reduction reactor, the gas mixture containing CO₂, N₂ and H₂O enters a 'water trap' generally comprising magnesium perchlorate (also known as Anhydrone®) or related desiccants, which absorbs the water produced by combustion. In the case that only nitrogen isotopes ratios are evaluated, the CO₂ can be extracted from the gas stream using a chemical trap comprising soda lime or sodium hydroxide on a silica substrate, such as Ascarite® or Carbo-sorb®. When these reagents absorb CO₂, they produce water and therefore, they should be placed between two water traps. To end, the gas mixture of N₂ and CO₂ flows through a gas chromatography (GC) column packed with stationary phases, such as Porapak® QS, Poraplot PQS, allowing the separation of CO₂, N₂ before the final detection. Alternatively to the chromatographic system, other equipment use the 'purge-trap' technique [39], where nitrogen travels through the system, and other generated gases (CO₂, etc.) are trapped on adsorption tubes, and then released into the MS by sequentially heating the traps.

To obtain precise isotope measurements, the IRMS system should be placed in an environment where temperature and humidity are precisely controlled, and the gases supplied to the instrument of high quality. Temperature variations can cause considerable shifts in the isotopic composition of the working gases, thus it's critical that the cylinders delivering the working gases to the IRMS device are also housed in a temperature-controlled environment. Also, the working gas cylinders should be kept as close to the instrument as possible for similar reasons, while this is not always practical due to safety concerns. The pressure in the gas cylinder should be checked regularly to ensure that enough gas is available for the analytical procedure. When the pressure drops below 48.3 bar (700 psi), the CO₂ working gas is replaced, notifying that the liquid in the cylinder has run out.

Helium gas with a purity higher than 99.9992% is the carrier gas recommended for the IRMS instruments. Filters should be included in the carrier gas supply to eliminate trace amounts of residual oxygen and moisture. Other than basic frits, filters should not be used in working gas supply since they can produce isotopic fractionation.

4.2.1. Use of the IRMS

It is essential to conduct regular testing and record the results during the entire life of the instrument and not just when it is malfunctioning. During the installation process, the normal working performance should be established and from that moment on, checking tests with predetermined acceptance criteria can be used to monitor the instrument's operability and ensure that action is taken if needed. It is important to always check the instrument logbook for maintenance needs and act consequently. When the reactors are replaced, the system should be left overnight to equilibrate before performing any background and air/water checks.

4.2.1.1. Preconditioning

About 12 to 24 hours before analysing the samples, the EA reactor(s) should be set to the operational temperature(s) with normal helium flow. It may be also recommended to do a preconditioning of the reactor system by analysing a set of capsules containing chemically comparable homogeneous material until the δ value is relatively constant. Preconditioning is required before analysing the reference materials used for the normalization, as the δ value does not need to be exact, but stable.

4.2.1.2. EA-IRMS Maintenance

Before you plan to run a set of samples on the IRMS, check the status of the instrument. Check how many samples have gone through the water trap, reactor, and ash tube. Replace the water trap after every 200 to 500 samples, according to your instrument configuration. Replace the reactor after 200 to 700 samples according to your reactor configuration (single vs. dual). Replace the ash tube. Check the levels on all of the gas tanks. Bake the GC column. Checking the background gases after the replacement of the reactor or trap reagents will guarantee that the system is leak free.

4.2.1.3. Running Elemental Analysis

System checks should start with a scan of the background gases in the instrument before running the sample set. If they're outside the normal range, an examination of the problem and necessary corrective actions will be required. If the background scan is satisfactory, the precision of the instrument is tested using a zero enrichment (on-off) test with the working gas. If the test results are not satisfactory, the root cause of the problem should be inspected. After passing the background and zero enrichment tests, the instrument's linearity for both carbon and nitrogen is checked, i.e., how the measured δ value changes with peak size. Depending on the manufacturer, the standard deviation for CO₂ on-offs and N₂ on-offs should be ≤ 0.06 and the slope ≤ 0.06 .

4.2.1.4. Background gases

Normally, background levels will differ from one laboratory to another, depending on the instrument configuration, carrier and working gas quality, and a variety of other factors. However, a key point is to inspect the background values every time the instrument is operated to establish those acceptable levels that help to detect any fluctuation for identifying the potential issues. Figure 31 shows a typical background scan for the EA/IRMS system. The intensity of m/z 18, 28, 32, 40, and 44 should be recorded and verified with the acceptable background values settled for the instrument configuration. Table 3 includes typical problems with the possible reasons that may cause background values greater than usual. Figure 31 depicts an example of background scan consisting of a plot of magnetic field strength and thus m/z against signal intensity.

Not only should the backgrounds be established for the mass spectrometer alone, but also with the EA coupled. The changes in background levels and performance between these two settings can assist in defining the cause of any trouble.

TABLE 3. TYPICAL PROBLEMS WITH BACKGROUND VALUES AND POSSIBLE CAUSES IN EA/IRMS

m/z	Species	Problems and possible cause
18	H ₂ O ⁺	Produces protonated species that may interfere with ions containing heavy isotope; too much water, change water traps
28	N ₂ ⁺	Leaks from atmosphere
30	NO	Copper is depleted
32	O ₂	Bleed from EA oxidation catalyst
40	Ar ⁺	Leaks from atmosphere
44	CO ₂ ⁺	Contamination of C/N analysers

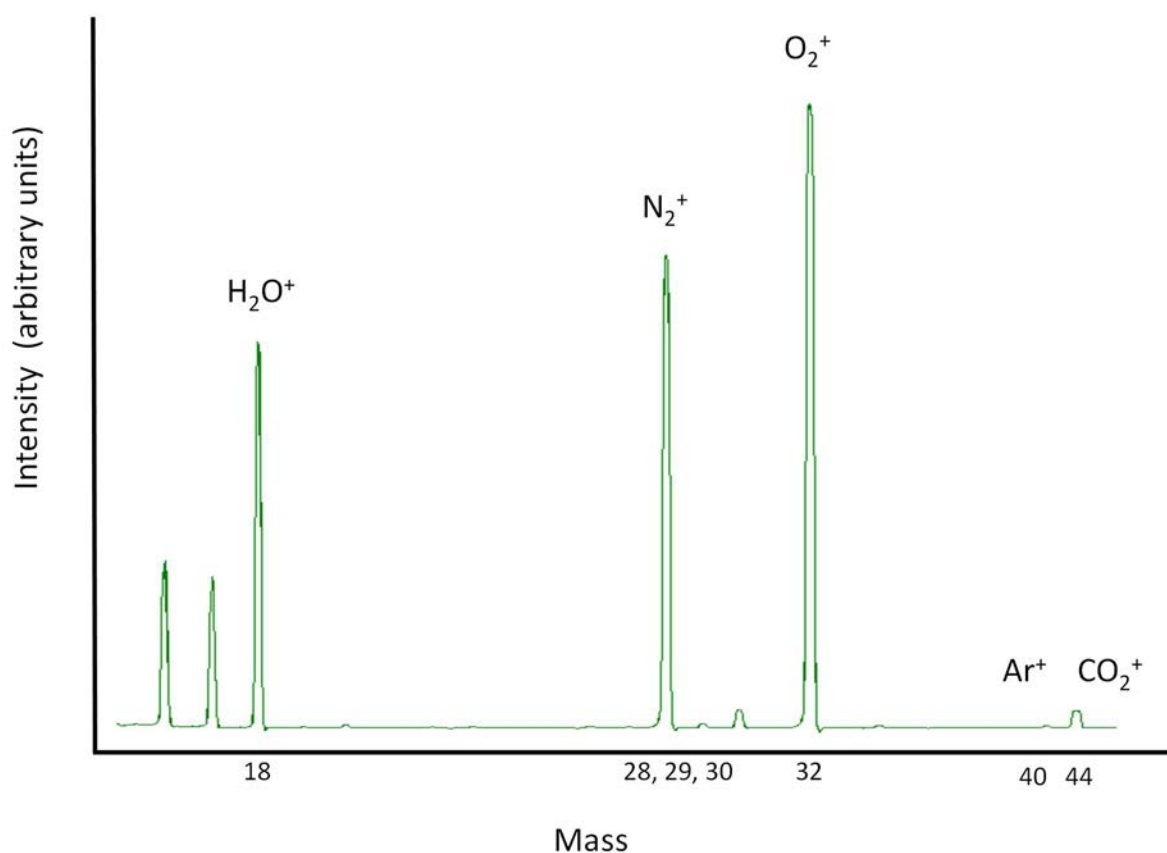


FIG. 31. A plot of magnetic field strength and thus m/z against signal intensity is an example of a background scan (courtesy of J. van Holst, ANSTO, Australia).

Zero Enrichment or on-off test (Stability)

With the EA connected to the IRMS instrument, daily stability checks should be performed. By injecting ten pulses of the working gas (CO₂ and N₂) and calculating the standard deviation of the delta values, zero enrichment monitors the stability of the measurement of the working standard (see Fig. 32). For that, the intensity of the gas pulses should be set within the expected operating range of sample peak abundances. Acceptance criteria for a given instrument, like with other performance tests, should be previously set. In most cases, the standard deviation for CO₂ and N₂ should be less than 0.1.

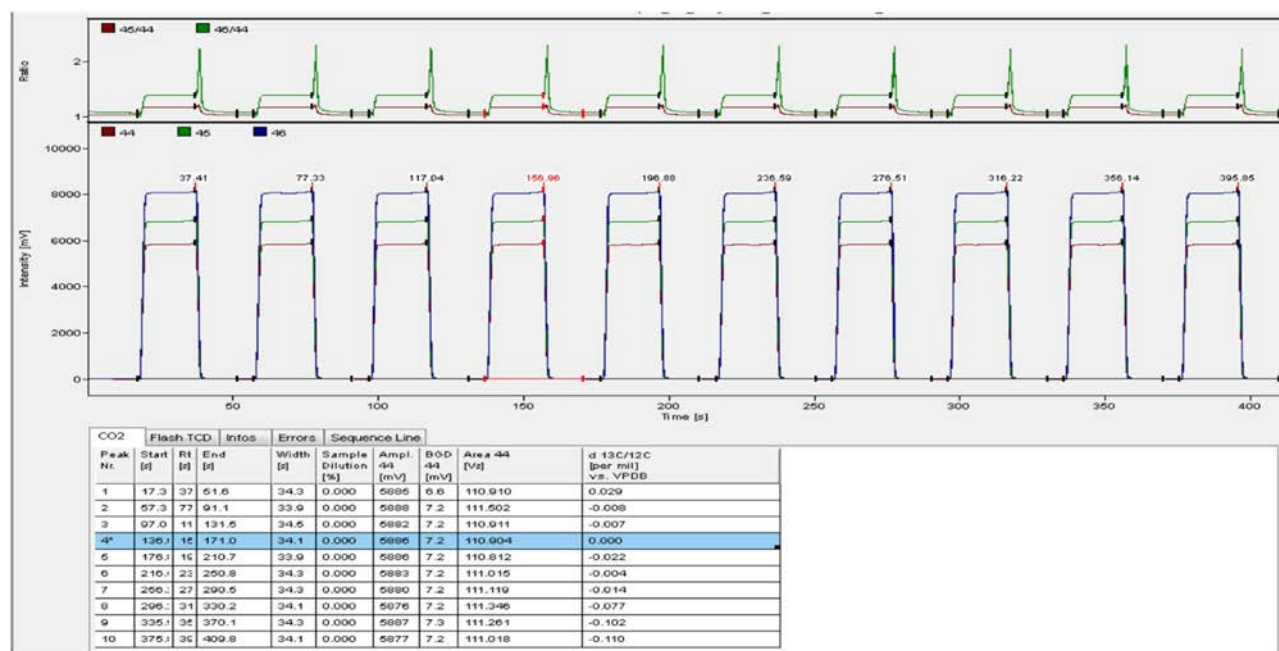


FIG. 32. Example of a zero-enrichment check or on-off test

Linearity (peak size)

Linearity should be preferably tested every analysis day with the EA connected to the IRMS instrument. For the linearity test the intensity of the working standard increases incrementally (see Fig. 33). The intensity of the working gas should encompass the intensity of the samples to be measured. Preferably, the δ values for N₂ and CO₂ working gases should not fluctuate by more than the standard deviation determined for the working gas zero-enrichment test across the usual range of sample intensity. If the δ value variation is larger, there is a factual effect that can be corrected.

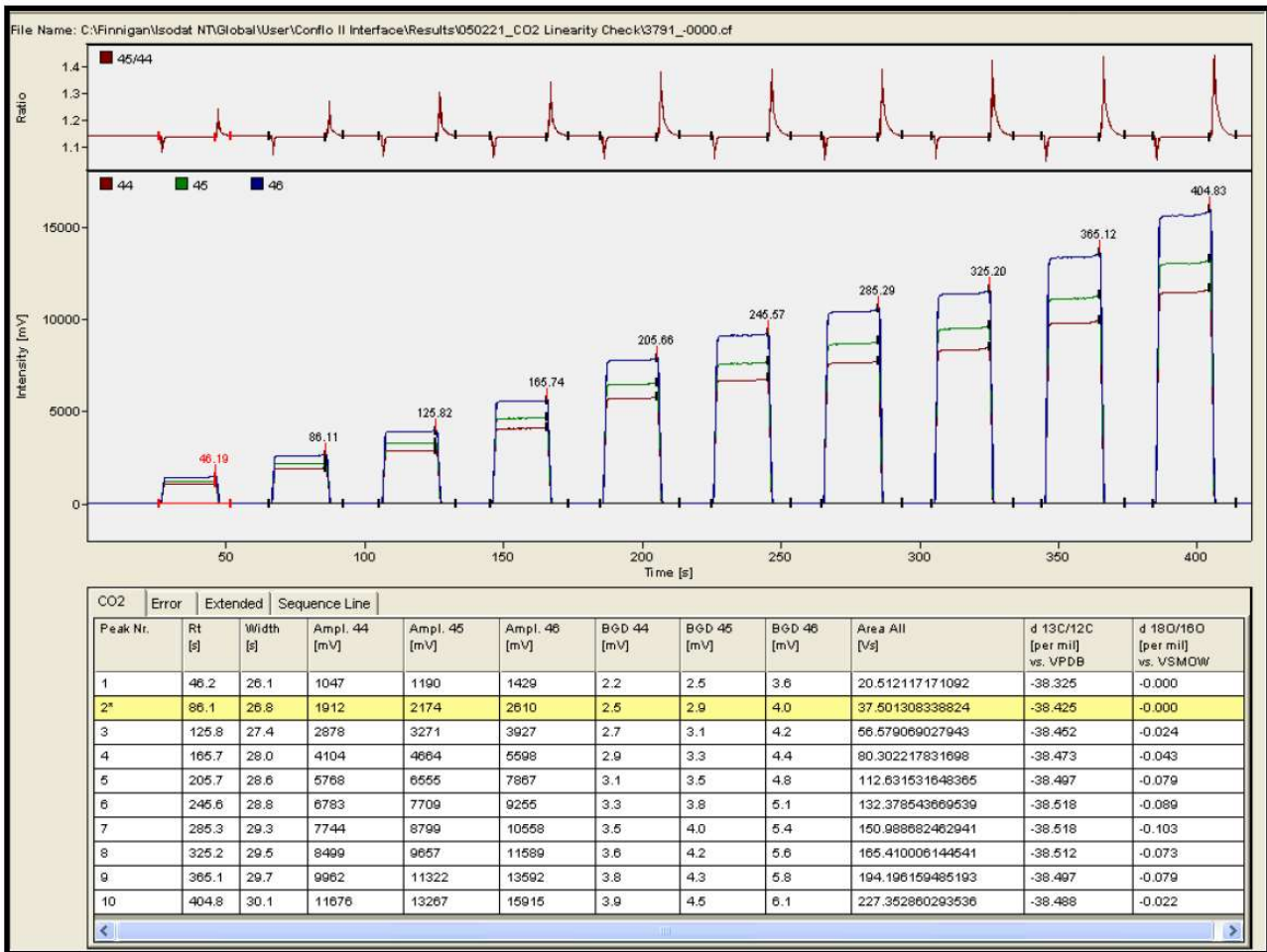


FIG. 33. Example of a linearity check using working gas.

4.3. ASSURING ACCURACY

4.3.1. QA/QC and data processing procedure

4.3.1.1. Method Blanks and Corrections

The method blanks for EA/IRMS analyses are tin cups without sample, that are analysed in the same way as the environmental samples. A signal in the blank analysis might also originate from the atmospheric nitrogen gas introduced by the autosampler or from impurities of the oxygen pulse. These blanks are used to subtract the traces of nitrogen or carbon particles in the tin cup from the $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values measured in the samples.

As indicated in the equation below, the blank contribution, which takes in account the average peak area and δ value of the blank measurement, are used for adjusting the δ value of the sample.

$$\text{Blank Corrected } \delta = \frac{(\delta_{\text{sample}} \times \text{area}_{\text{sample}}) - (\delta_{\text{blank}} \times \text{area}_{\text{blank}})}{\text{area}_{\text{sample}} - \text{area}_{\text{blank}}} \quad (3)$$

The correction for the blank capsule is recommended to be applied only if the δ value of the blank is repeatable, as the variability of the δ value might vary dramatically for the small peak areas. As a result, a laboratory may just specify a minimum permissible sample peak size based on the intensity of the average blank peak area, i.e., a small blank peak should barely affect a sample peak hundred times higher [30].

As a first recommendation, the peak amplitudes in the blank samples for N_2 (m/z 28) and CO_2 (m/z 44) should be lower than 50 mV, otherwise the source of contamination should be identified and corrected. An unacceptably high blank value usually might indicate contamination of the tweezers, capsules, benches, and so on. In these cases, the reason for the high blank measurements should first be identified and eliminated. At the start of each batch, at least three blanks should be measured [33].

4.3.1.2. Drift Correction

Within an analytical sequence, changes in the isotopic composition of the working gas or changes in the ion source due to fluctuations in the background gases (e.g. water), can cause a drift of the δ values measured as a function of time. Isobaric interferences, such as CO_2H^+ (m/z 45), can be caused by traces of water or other protonating species. The occurrence of drift in the measured δ values over time can be calculated by analysing QC materials on a frequent basis during the analytical sequence (every 5 to 10 samples). If sufficient drift is identified, the data from the QC materials can be used to make the appropriate drift correction. If the QC materials show a linear drift with time, then Eq. (4) can be used to take in account the drift correction for all materials analysed in the same sequence [30, 40]:

$$\delta_{\text{drift corr}} = \delta_{\text{meas}} - m \times \text{position} \quad (4)$$

where

$\delta_{\text{drift corr}}$ drift corrected δ value of the sample;
 δ_{meas} determined (raw) δ value of the sample;

m slope of linear drift curve (plot of δ value of the standards versus autosampler position);

and *position* is given by the autosampler position within the sequence (assuming this is a proxy for time).

This procedure implies that the δ values of the QC materials and samples drift in the same direction, and the accuracy of the drift correction should be validated by analysing a second QC material throughout the sequence. Drift can also be identified comparing the δ values of the reference materials (RMs) analysed at the start of the sequence with those measured for the same RMs at the end of the sequence [30].

4.3.1.3. Sample Measurement

For each sample, at least two individual analytical results should be obtained. However, additional measurements will provide a better confidence on the experimental uncertainty. At least two RMs for each isotope ratio are analysed at the beginning and at the end of the sequence. These measurements will be used to normalize the data collected throughout the sequence. For quality control, an in-house RM is analysed at regular intervals during the sequence. Figure 34 below illustrates a typical sequence.

Project Name: _____
LIMS Batch: _____

SampleType _____
Target Isotopes _____
Instrument Dilution % _____

Animal/Sediment/Plant _____
C N or C/N _____

	A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12
Sample ID	Blank	Blank	Blank	%RM	%RM	%RM	Isotope RM1	Isotope RM2	Isotope RM1	Isotope RM2	GC 1	GC 2
Set Weight (mg)				0.100	0.200	0.300						
Actual Weigh (mg)												
Date												
Weighed by												

	B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B12
Sample ID	Sample 1a	Sample 1b	Sample 2a	Sample 2b	Sample 3a	Sample 3b	Sample 4a	Sample 4b	Sample 5a	Sample 5b	Sample 6a	Sample 6b
Set Weight (mg)												
Actual Weigh (mg)												
Date												
Weighed by												

	C01	C02	C03	C04	C05	C06	C07	C08	C09	C10	C11	C12
Sample ID	GC 1	GC 2	Sample 7a	Sample 7b	Sample 13a	Sample 13b	Sample 3a	Sample 3b	Sample 10a	Sample 10b	Sample 11a	Sample 11b
Set Weight (mg)												
Actual Weigh (mg)												
Date												
Weighed by												

	D01	D02	D03	D04	D05	D06	D07	D08	D09	D10	D11	D12
Sample ID	Sample 12a	Sample 12b	GC 1	GC 2	Sample 13a	Sample 13b	Sample 14a	Sample 14b	Sample 15a	Sample 15b	Sample 16a	Sample 16b
Set Weight (mg)												
Actual Weigh (mg)												
Date												
Weighed by												

	E01	E02	E03	E04	E05	E06	E07	E08	E09	E10	E11	E12
Sample ID	Sample 17a	Sample 17b	Sample 18a	Sample 18b	Blank	%RM	%RM	%RM	Isotope RM1	Isotope RM2	Sample 19a	Sample 19b
Set Weight (mg)						0.100	0.200	0.300				
Actual Weigh (mg)												
Date												
Weighed by												

	F01	F02	F03	F04	F05	F06	F07	F08	F09	F10	F11	F12
Sample ID	Sample 20a	Sample 20b	Sample 21a	Sample 21b	Sample 22a	Sample 22b	Sample 23a	Sample 23b	Sample 24a	Sample 24b	GC 1	GC 2
Set Weight (mg)												
Actual Weigh (mg)												
Date												
Weighed by												

	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
Sample ID	Sample 25a	Sample 25b	Sample 26a	Sample 26b	Sample 27a	Sample 27b	Sample 28a	Sample 28b	Sample 29a	Sample 29b	Sample 30a	Sample 30b
Set Weight (mg)												
Actual Weigh (mg)												
Date												
Weighed by												

	H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12
Sample ID	Blank	%RM	%RM	%RM	Isotope RM1	Isotope RM2	Isotope RM1	Isotope RM2				
Set Weight (mg)		0.100	0.200	0.300								
Actual Weigh (mg)												
Date												
Weighed by												

FIG. 34. Template for IRMS analysis illustrating a typical measurement sequence (courtesy of J. van Holst, ANSTO, Australia).

4.3.2. Reference material and traceability

Reference Materials (RMs) for isotope ratio measurements may be classified as:

- Primary RMs;
- Secondary RMs;
- Tertiary RMs;
- In-house (laboratory) RMs. In some cases, the in-house RMs can also be considered as secondary or tertiary RMs depending on which RMs are used during their calibration [30].

The current internationally agreed zero points for the most commonly used isotope delta scales are:

- Carbon ($^{13}\text{C}/^{12}\text{C}$) VPDB (Vienna Pee Dee Belemnite);
- Nitrogen ($^{15}\text{N}/^{14}\text{N}$) Air- N_2 (atmospheric nitrogen).

Calcium carbonate from the Cretaceous Pee Dee Belemnite formation (PDB) in South Carolina was used as the original carbon isotopic anchor. The CO_2 produced by phosphoric acid treatment of PDB was set as the zero point for carbon isotopic measurements. When the PDB was exhausted, it was replaced by assigning accurate $\delta^{13}\text{C}$ to another carbonate (NBS 19 or ‘TS-limestone’) against a hypothetical Vienna PDB, resulting in a new isotope delta scale (VPDB) for carbon.

NBS 19 is no longer available as it has been exhausted. IAEA-603 was recently released as a replacement for NBS 19 [41] and it will serve as the highest metrological realization for the carbon VPDB scales. NBS 19 remains the primary RM for the VPDB scale, but IAEA-603, 610, 611 and 612 should be used by laboratories that do not have access to NBS 19.

Because the isotopic composition of atmospheric nitrogen (Air- N_2) has been shown not to vary measurably around the world or over time [42, 43], it has been adopted as the zero point for all nitrogen isotope ratio analyses. However, to be used as a practical RM, N_2 should be isolated from the atmosphere without fractionation. In this context, for practical reasons, the IAEA, NIST, and USGS have prepared a number of RMs (mostly ammonium and nitrate salts). In addition to the atmospheric nitrogen zero point, the Air- N_2 scale has now a second defined point, which is realized by a potassium nitrate salt (USGS32) with an assigned $\delta^{15}\text{N}$ value of +180‰. Because Air- N_2 is difficult to produce free of argon, which can interfere with isotopic analysis, the IUPAC's CIAAW recommends using the RM IAEA-N-1 (ammonium sulphate) as a scale anchor for samples that require combustion as a means of sample preparation [44].

TABLE 4. INTERNATIONALLY AGREED ZERO-POINTS, PRIMARY RMS AND CURRENTLY AVAILABLE HIGHEST METROLOGICAL REALIZATIONS FOR CARBON AND NITROGEN ISOTOPE RATIO δ SCALES.

Ratio	Zero-point material	Primary RMS	Highest metrological realizations	
			Name	δ value (‰)
$^{13}\text{C}/^{12}\text{C}$	VPDB	NBS 19	IAEA-603 (calcite)	+2.46 ± 0.01
			IAEA-610 (calcite)	-9.11 ± 0.03
			IAEA-611 (calcite)	-30.79 ± 0.04
			IAEA-612 (calcite)	-36.72 ± 0.03
$^{15}\text{N}/^{14}\text{N}$	Atmospheric nitrogen	USGS32	IAEA-N-1 (sulfate)	+0.43 ± 0.07
			USGS32 (nitrate)	+180

Natural or synthetic chemical substances that have been precisely calibrated versus the primary RM(s) are defined as secondary RMs (s). Assigned δ values are agreed upon and adopted internationally for most of the commercially available secondary RMs. In contrast to primary RMs, $\delta^{13}\text{C}$ values of all secondary RMs' are assigned with a defined uncertainty. The related IUPAC Technical Report [44] contains a summary of the commercially available secondary (and primary) RMs for all isotope δ scales. This report covers RMs mostly distributed by the International Atomic Energy Agency (IAEA), the National Institute of Standards and Technology (NIST), and the United States Geological Survey (USGS).

TABLE 5. SECONDARY RMS FOR $\delta^{13}\text{C}$ MEASUREMENTS COMPILED from Ref. [44], IAEA^a and USGS^b

Description	Nature	$\delta^{13}\text{C}$ (‰) \pm uncertainty (sd)
USGS-41a	L-glutamic acid	+36.55 \pm 0.08
USGS-40	L-glutamic acid	-26.39 \pm 0.04
IAEA-CH-6	Sucrose	-10.45 \pm 0.03
USGS-24	Graphite	-16.049 \pm 0.035
IAEA-CH-3	Cellulose	-24.724 \pm 0.041
IAEA-600	Caffeine	-27.771 \pm 0.043
NBS-22	Oil	-30.031 \pm 0.043
IAEA-CH-7	Polyethylene	-32.15 \pm 0.05
NBS-18	Carbonatite	-5.014 \pm 0.035
IAEA-601	Benzoic acid	-28.81 \pm 0.04
IRMM-BCR 657	Glucose	-10.76 \pm 0.04

^a<https://nucleus.iaea.org/sites/ReferenceMaterials/Pages/Stable-Isotopes.aspx>

^b<https://isotopes.usgs.gov/lab/referencematerials.html>

TABLE 6. SECONDARY RMS FOR $\delta^{15}\text{N}$ MEASUREMENTS COMPILED from Ref. [44], IAEA^a and USGS^b

Description	Nature	$\delta^{15}\text{N}$ (‰) \pm uncertainty (sd)
USGS-41a	L-glutamic acid	+47.55 \pm 0.15
USGS-40	L-glutamic acid	-4.52 \pm 0.06
USGS-26	Ammonium sulphate	+53.7 \pm 0.4
IAEA-N-2	Ammonium sulphate	+20.3 \pm 0.2
USGS-35	Sodium nitrate	+2.7 \pm 0.1
USGS-34	Potassium nitrate	-1.8 \pm 0.1
USGS-25	Ammonium sulphate	-30.4 \pm 0.4

^a <https://nucleus.iaea.org/sites/ReferenceMaterials/Pages/Stable-Isotopes.aspx>

^b <https://isotopes.usgs.gov/lab/referencematerials.html>

Secondary RMS have been used to calibrate tertiary RMS, which give traceability to the scale's zero point. Materials from commercial organizations and universities can be included in this third category. Their δ assigned values have usually been set-up by internal calibration or consensus values attained through interlaboratory comparisons (ILCs). Although, these materials generally lack the international agreement ascribed to the IAEA, NIST, and USGS materials included in the IUPAC Technical Report [44], they may be beneficial in situations where no other RMS are available.

End users of commercially supplied tertiary RMS should inspect the supplier's certificates about traceability and measurement uncertainty.

To report the isotope ratios on the agreed international scale, the laboratories should own the appropriate reference materials for calibration and normalization. Because primary and secondary RMS are in scarce supply, they are not advised for everyday use. Instead, in-house RMS calibrated using primary and/or secondary RMS, might be daily used for the normalization and quality assurance (QA). Other certified organic sediment/soils reference materials (high and low) for elemental composition (%C, %N and %S) and $\delta^{15}\text{N}$, $\delta^{13}\text{C}$ from commercial providers (Elemental Microanalysis and IVA) are also good QC samples for analysing sediment samples as they are matrix certified reference materials.

Up to date, there is no certified tissue RM for carbon and nitrogen stable isotopes, but the SRM 1946 (fish muscle tissue) from NIST can be used as an isotope RM. Their assigned reference values ($\delta^{15}\text{N}$, $\delta^{13}\text{C}$, %N, and %C) are derived from the average of numerous in-house replicate analyses.

Other recommended isotope standards for animal tissues are Chitin (B2160) and Casein (B2155) from Elemental Microanalysis. Methionine is also a good standard for %C and %N in animal tissue and plants, as its elemental composition is within the same range.

4.3.3. Instrument Calibration

For accurate isotope ratio measurements, the EA/IRMS method requires at least two delta levels of calibration standards. Primary/secondary reference materials, such as IAEA CH-7, IAEA-CH-6, NBS-22, USGS 24, and USGS 41, should be used to assign $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values for in-house calibration standards. The $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ should be determined using linear calibration and a minimum of five replicate analyses of each calibration standard, with at least one at the start and end of the sequence. If the $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values of the replicate analyses of the calibration standards vs the corresponding assigned δ values do not show a correlation of $r > 0.9900$, the samples in the sequence should be reanalysed [33].

As a recommendation, each calibration standard should be analysed at least two times, at the start and at the end of each batch of samples, and every 15 field samples or less.

Two-point calibration, such as NBS-22 (oil) and IAEA-C-6 sucrose and one QC, such as USGS24 can be used for the calibration and QC.

Two quality controls such as? USGS24 graphite and IAEA N2 ammonium sulphate should be measured in duplicates at the start, middle and end of each sequence. One elemental composition reference material (e.g. acetanilide) should be run at the beginning of each sequence as a calibration for elemental composition with weights of about 100, 200, 300 μg . Blanks (i.e. empty capsules) should be run in the beginning in triplicates for blank correction and throughout the sequence to monitor the background.

4.3.4. Normalization

The analysis of RMs in the same batch of the samples provides the link of the measured δ values in the samples to the international δ scale. When scale contraction effects occur, they should be compensated for by using RMs with widely varying isotope ratios, a procedure known as 'normalization'.

4.3.4.1. Single-point anchoring

The isotopic values from the commercial IRMS equipment are usually automatically normalized using a single-point anchoring with the working calibrated gas (CO_2 for $\delta^{13}\text{C}$ and N_2 for $\delta^{15}\text{N}$) [45]. The true (corrected) δ -value of the sample can be standardized using the following formula when one certified reference standard (std) is analysed alongside the unknown samples using the same treatment principle:

$$\delta_{\text{Spl}}^{\text{T}} = \left[\frac{(\delta_{\text{Spl}}^{\text{M}} + 1000)(\delta_{\text{Std}}^{\text{T}} + 1000)}{(\delta_{\text{Std}}^{\text{M}} + 1000)} \right] - 1000 \quad (5)$$

where

$\delta_{\text{Spl}}^{\text{M}}$ is the measured (relative to the working gas) δ -value of the unknown sample (Spl);
and $\delta_{\text{Std}}^{\text{T}}$ is the true (in the isotope reference scale) or accepted δ -value of the certified reference standard (Std).

4.3.4.2. Two-point linear normalization

The normalization uncertainty can be reduced by using a normalization factor determined from the measured δ values of two RMs with δ values that are far apart, providing that systematic errors are linear in the overall method's dynamic range.

The values are normalized using the following equation:

$$\delta_{\text{reference}} = m \times \delta_{\text{measured}} + b \quad (6)$$

where

$$m = \frac{(\delta_{\text{Std 1 Reference}} - \delta_{\text{Std 2 Reference}})}{(\delta_{\text{Std 1 Measured}} - \delta_{\text{Std 2 Measured}})}$$

b is calculated by using the measure and true value of one of the standards [45].

$$b = \delta_{\text{reference}} - m \times \delta_{\text{measured}}$$

As an example, Fig. 35 below shows how the $\delta^{13}\text{C}$ values are normalized using a linear regression using two certified reference materials.

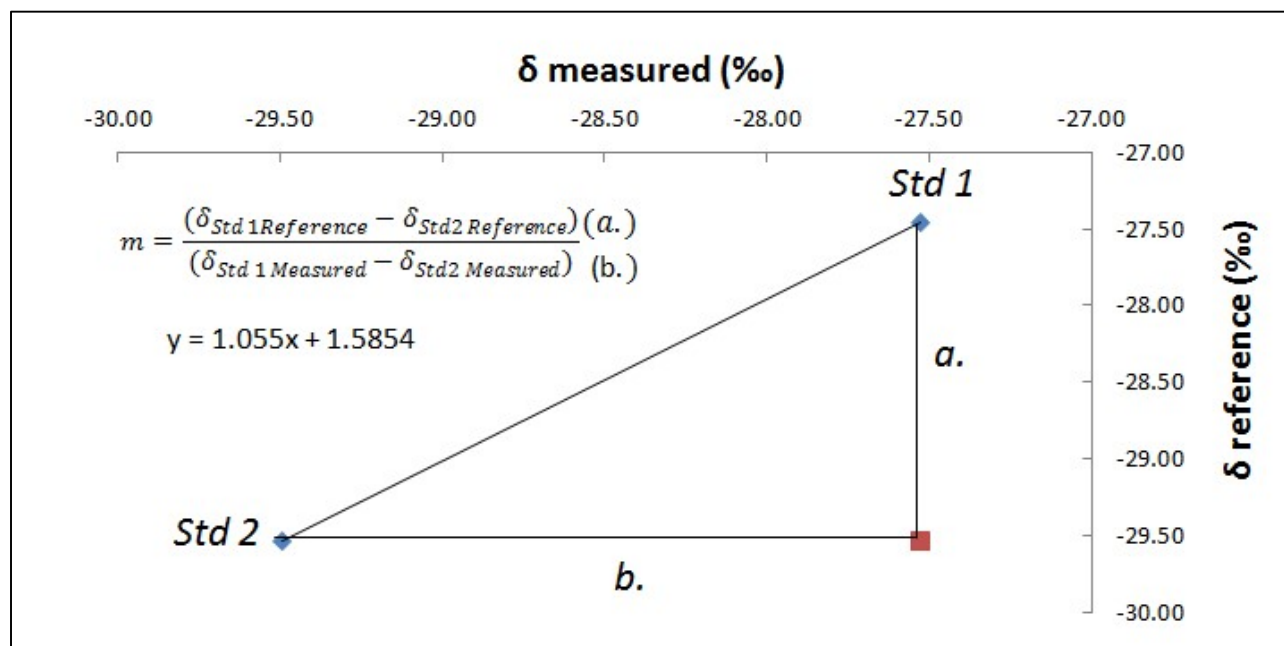


FIG. 35. A two-point normalization plot measured versus reference $\delta^{13}\text{C}$ value for Std1 ($\delta^{13}\text{C} -27.46 \pm 0.11\text{‰}$) and Std2 ($\delta^{13}\text{C} -29.53 \pm 0.01\text{‰}$) reference material. The difference between the reference values over the difference between the two measured values of the two standards is the slope between the two points.

4.3.5. Quantitation Range

If the responses for CO_2 and N_2 peaks in the EA-IRMS equipment are either too small or too large, the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values can be biased. Therefore, only results from peak amplitudes within the

linearity range (usually 500 and 8000 mV) should be reported. Reanalysis is performed on samples that do not fit these requirements. If the sample's peak amplitude for N₂ (mass 28 or 29) or CO₂ (mass 44 or 46) fell outside of the linearity range, the accuracy of the result is questionable; hence the corresponding delta and weight percentage (Wt%) measurements will not be reported.

4.3.6. Quality Assurance/Quality Control (QA/QC)

Quality control (QC) is the process of verifying that the procedures are in control and working properly to produce reliable data. Measurements of control samples are part of the control process to verify the established performance of the system. Hence, QC can be as basic as running control samples. QC, in a broader sense, can be defined as detection, which can include testing, inspection, and gathering feedback from stakeholders, in addition to measurement. Control charts are used to monitor performance. This is a QC activity. QC samples, such as reference materials that were not used for calibration, should be analysed as part of the sample analysis sequence.

Quality assurance (QA) involves a set of procedures to ensure high quality results for preventing nonconformities with the stakeholder standards. This comprises, among other things, qualified team members, a proper working environment, verified processes, and traceable measures. The IRMS Guide contains more detailed information on QC/QA [30].

As a guide for acceptable performance using the EA/IRMS method, the $\delta^{15}\text{N}$ values of a minimum of three isotope RM per batch should have a standard deviation $\leq 0.3\%$, while the $\delta^{13}\text{C}$ values should have a standard deviation $\leq 0.2\%$.

4.3.6.1. Control charts

A Quality Control chart is a graphical representation to study the laboratory performance based on the measurements of control materials, including appropriate in-house RMs. Control charts can also be used to assess if a candidate in-house RM might be suitable for long-term use.

Materials used as in-house RMs should meet the following criteria:

- Be sufficiently isotopic homogeneous to the smallest sample intake;
- Stable and constant isotopic composition over a long time period;
- The isotope composition should be within the working range of the samples to be measured.

To obtain consistent results and following the Principle of Identical Treatment (PIT), it is strongly advised to use 'matrix-matched' in-house material, as the uncertainties associated with sample processing should be similar [37].

In order to calibrate an in-house RM, a number of analytical sequences with primary and/or secondary RMs in addition to the candidate in-house RM(s) is required. The isotopic composition of the in-house RMs can be linked to the zero point of the reporting scale by using an unbroken chain of comparisons, usually international RM, establishing traceability.

Although matching the primary and secondary chemical materials to the in-house matrix RMs is not always achievable, efforts should be pursued to guarantee that the in-house RMs values are accurate for the purpose of the analytical method. In some circumstances, it may be worth to validate the isotope ratio data using other distinct and/or complementary techniques. For instance, an in-house RM for EA/IRMS, may be combusted off-line and the CO₂ analysed by dual inlet/IRMS.

Also, the in-house RMs may be analysed in other analytical laboratories to assess the accuracy of the values. When assigning an isotope reference value to a new in-house RM, the most critical step is to ensure the quantitative conversion of the candidate in-house RM to the analyte gas, in relation to the favorable conversion of the primary or secondary RM [30].

Warning limits ($\text{mean} \pm 2\sigma$) and control limits ($\text{mean} \pm 3\sigma$) are commonly used in mean value control charts. These boundaries are usually established using previous historical data, with roughly 20 results obtained on at least six distinct days, preferably by multiple analysts. These data were most likely collected as part of the validation procedure.

The ‘Westgard rules’ adapted from [46] are shown in Fig. 36 as a flow chart for the assessment of an in-house QC material. Figure 37 illustrates several violations of the Westgard principles using the data from Fig. 36.

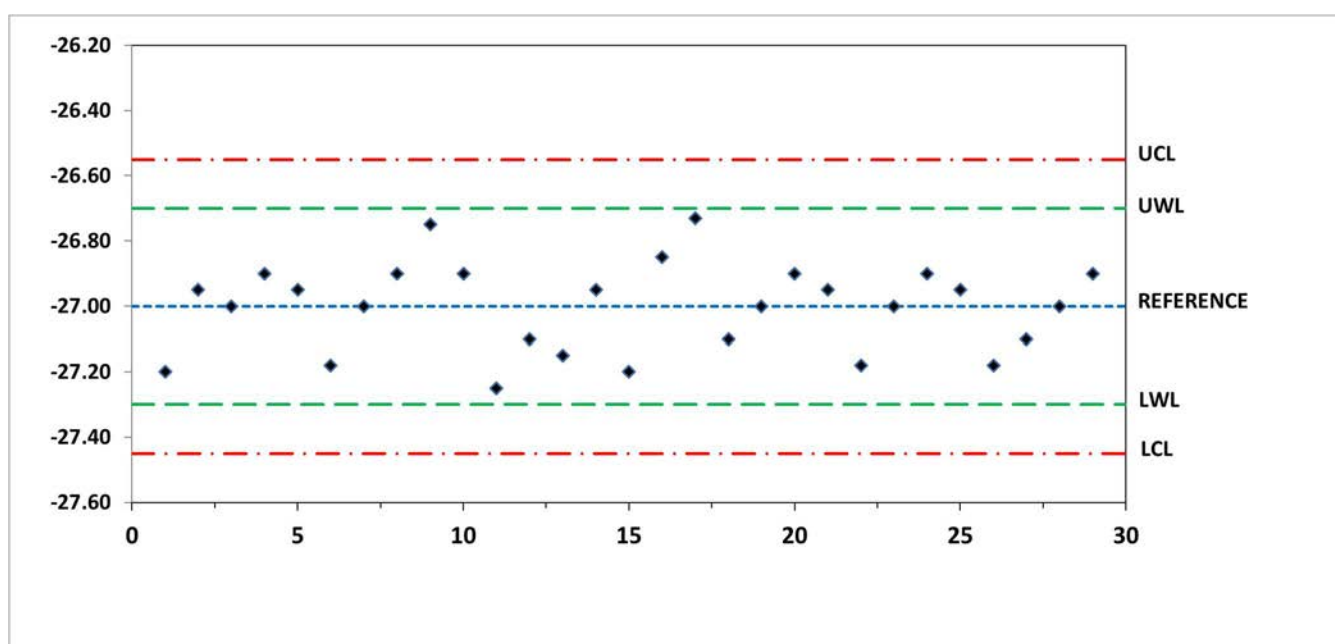


FIG. 36. Control chart for $\delta^{13}\text{C}$ measurements of a QC material; centre line (REFERENCE), UCL = upper control limit (3σ), UWL = upper warning limit (2σ), LWL = lower warning limit (2σ), LCL = lower control limit (3σ). In this example all of the measurements fall within the control limits and comply with the Westgard rules (from Ref. [30]).

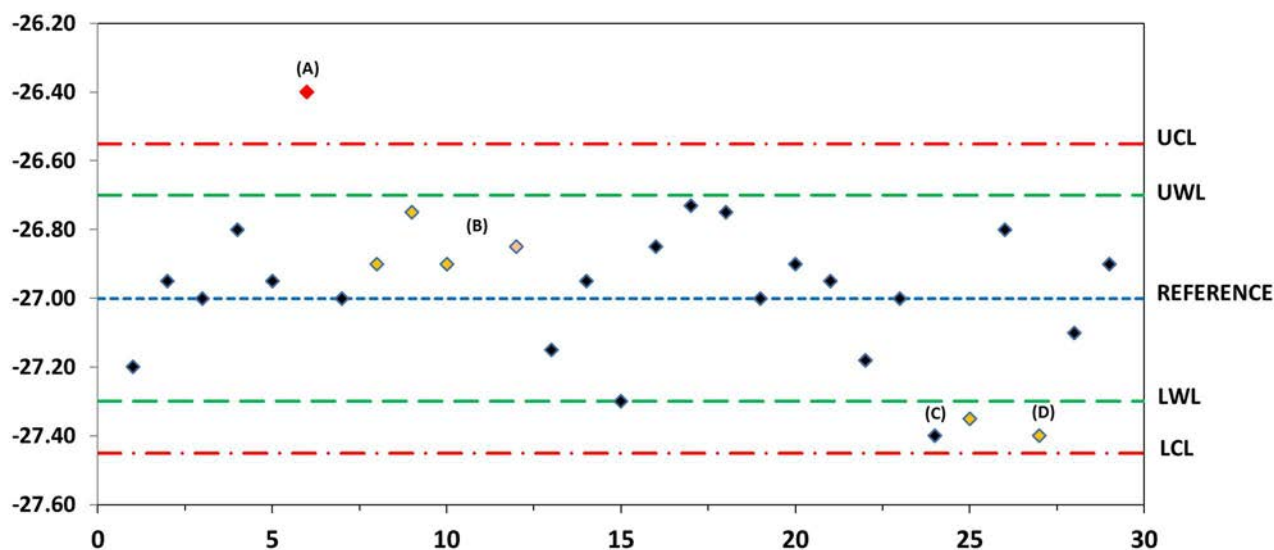


FIG. 37. An example of a control chart showing violations of the Westgard rules; (A) result greater than 3σ from mean, (B) 10 results on the same side of mean (also four results greater than 1σ from mean), (C) two consecutive results greater than 2σ from mean and, (D) two consecutive results differ by more than 4σ (from Ref. [30]).

4.3.6.2. Sample Replicates

To assess the performance of the EA/IRMS system, replicate samples of the RM are analysed every 15 or less field samples, with a minimum of three per batch. Although not used for quality control, it is also recommended to run duplicate or triplicate field samples at least every 10 field samples. Generally, there are no acceptance criteria for the repeated field samples due to the numerous factors that may affect the variability, such as sample processing issues, or sample homogeneity. However, the researcher may find useful the within-sample heterogeneity for the replicate field samples [33]. Recommended accepted criteria for the standard deviation of δ values in replicate analyses of the standards is ≤ 0.25 per mil (‰) for $\delta^{15}\text{N}$ and ≤ 0.35 ‰ for $\delta^{13}\text{C}$.

4.3.7. Reporting results

Usually, the IRMS instrument software calculates raw isotope-delta values automatically, which can then be used for data processing. Integration of the sample and working gas peak signals from the Faraday collectors, calculation of ratios of these integrated ion currents, correction for isobaric interferences ($^{13}\text{C}^{16}\text{O}_2$ and $^{12}\text{C}^{17}\text{O}^{16}\text{O}$ both nominally $m/z = 45$) and conversion of the corrected ratios to raw isotope delta values will all be part of this process. Other parameters, such as known/assigned isotope delta value(s) of the working gas, may be specified by the operator [30]. At least two RMs of known isotope ratio should be used to normalize results.

The $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values are reported for stable isotope ratios, where $\delta^{13}\text{C}$ is the difference in ratio of carbon isotope ^{13}C to carbon isotope ^{12}C in a sample, relative to the Pee Dee Belemnite international scale, and $\delta^{15}\text{N}$ is the difference in ratio of nitrogen isotope ^{15}N to nitrogen isotope ^{14}N

in a sample relative to atmospheric nitrogen used as the standard (Eqs (7,8)). Analysts are recommended to report their isotope sample results along with the isotope values obtained for the internationally reference materials used within the measurement method [44]. Also, the elemental composition of carbon, and nitrogen is a relevant parameter to be reported.

$$\delta^{13}\text{C} = \left(\frac{\left(\frac{^{13}\text{C}}{^{12}\text{C}} \right)_{\text{sample}}}{\left(\frac{^{13}\text{C}}{^{12}\text{C}} \right)_{\text{standard}}} \right) - 1 \quad (7)$$

$$\delta^{15}\text{N} = \left(\frac{\left(\frac{^{15}\text{N}}{^{14}\text{N}} \right)_{\text{sample}}}{\left(\frac{^{15}\text{N}}{^{14}\text{N}} \right)_{\text{standard}}} \right) - 1 \quad (8)$$

4.3.8. Measuring Uncertainty

Following the ISO guide to the Expression of Uncertainty in Measurement [47], uncertainty (U) is defined as “a parameter associated with the result of a measurement, that characterises the dispersion of the values that could reasonably be attributed to the measurand”. Its measurement uncertainty is basically quantified by combining all sources of uncertainty components, typically expressed as a standard deviation in the same units as the measurement result. The basic approach for combining uncertainties is the “root of the sum of the squares” method, using the law of propagation of uncertainties, assuming an additive measurement model, as shown in Eq. (9) and that the uncertainty of the components are independent.

$$u_c(y) = \sqrt{u(x_1)^2 + u(x_2)^2 + \dots + u(x_n)^2} \quad (9)$$

However, in EA-IRMS analyses with the process of normalization many of the individual uncertainties are correlated and therefore not independent. For that, the Kragten spreadsheet approach can be used to calculate the uncertainty budget and allows identifying the individual contributing factors responsible for a high proportion in the uncertainty budget. More details and examples of uncertainty calculations by the Kragten approach can be found in the Ref. [30].

Another simplified approach for estimating uncertainty can be derived by the long-term reproducibility of the method validation data. Such estimate can be easily obtained by evaluating a quality control sample, which is analysed every day multiple times to derive its standard deviation. Its average long term standard deviation can be used as a proxy for the long-term reproducibility of the samples if the control sample is similar enough to the majority of the analysed samples, and follows the Principle of Identical Treatment (PIT).

5. INTERPRETATION OF STABLE ISOTOPE DATA

5.1. TROPHIC FRACTIONATION. RULES OF THUMB, VARIABILITY AND CAUSES

We have noted in Section 2 how the isotopic signature of a consumer will reflect the isotopic signature of food sources. These isotopes are incorporated into the tissue of the consumer. However, in the process of assimilation small changes occur in the isotope ratios, due to metabolic and excretion processes which may prefer either the lighter or heavier isotope [48]. Once the isotopic composition of a consumer has equilibrated with its diet, the saying is true that “you are what you eat, plus a few per mill [49]”.

How many parts per mill (‰) of fractionation occurs between diet and assimilation in the consumer is termed the ‘diet-tissue discrimination factor’ (DTDF) [3], and this needs to be accounted for in any mixing model linking consumers to diets. As a general ‘rule of thumb’, the DTDF is around 1‰ for the isotopes of Carbon, ~3‰ for the isotopes of Nitrogen [50], and negligible for the isotopes of Sulfur [51]. However, a range of environmental and metabolic processes may cause the DTDF to vary. These may include the trophic level being considered, the quality of the diet, and whether the organism is feeding in a freshwater or marine environment [52]. In general, isotopic discrimination is fairly consistent in $\delta^{13}\text{C}$ between birds, fish, and invertebrates, but fish and invertebrates may show lower fractionation in $\delta^{15}\text{N}$ than mammals and birds [53].

Laboratory studies in which diet is controlled have helped define DTDFs, and several of these have considered organisms important to wetland ecosystems [54, 55]. However, it will not always be possible to gain specific information on the DTDF associated with the system under consideration, and a decision will need to be made about the most likely value. Some of the Bayesian mixing models (MixSIR[56]; SIAR [57]) allow a likely DTDF input range to be specified.

5.1.1. Plotting your data

Before the application of any mixing model, it is a good idea to plot your data. Usually, this will take the form of a Carbon-Nitrogen biplot, as illustrated in Fig. 38. To return to the example of crabs, fish and sediment organic matter in two east coast Australian estuaries, the example below illustrates the influence of DTDFs in a system within which grazing herbivores (crabs) are clearly dependent on sediment organic matter. Here, the DTDFs have not been applied prior to plotting, and all individuals are plotted, along with the means and standard deviation. Crabs are slightly enriched in $\delta^{13}\text{C}$, well within the range for which SOM could be the primary, even exclusive dietary source (remembering the typical 1‰ enrichment between diet and consumer for $\delta^{13}\text{C}$). Note that this remains true despite the different $\delta^{13}\text{C}$ SOM signatures between the two estuaries (~4‰ more depleted in the Parramatta River). The crabs are enriched ~2–3‰ in relation to the $\delta^{15}\text{N}$ of the SOM, which may represent the actual level of fractionation. Fish are enriched in both ^{13}C and ^{15}N in relation to the crabs, and in the Brisbane water site, sufficiently enriched in both Carbon and Nitrogen to suggest a trophic intermediary between crabs and fish that has not been sampled.

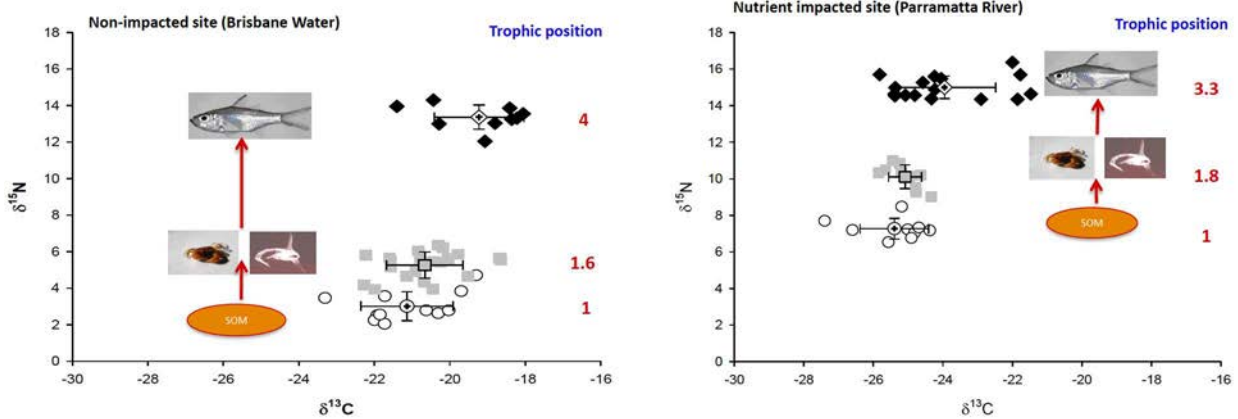


FIG. 38. Example of a simple Stable Carbon-Nitrogen Isotope biplot: (reproduced from Ref. [1] with permission courtesy of Elsevier).

This biplot gives the impression that we are ‘on the right track’ in sampling the components of the estuarine ecosystem in the Parramatta River and Brisbane Water. A more complex biplot is presented in Fig. 39 in which a greater range of organisms have been sampled. As a result of plotting the means and standard deviations in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of all organisms sampled in the Towra Point wetland, groups of organisms can be clustered together. For example, the C_3 plants (two species of mangrove and two species of saltmarsh) group together (on the depleted end of the range), as do benthic grazers (on the enriched end of the range). Omnivores and carnivores align on an axis with a slight enrichment in $\delta^{13}\text{C}$, suggesting a series of trophic dependencies.

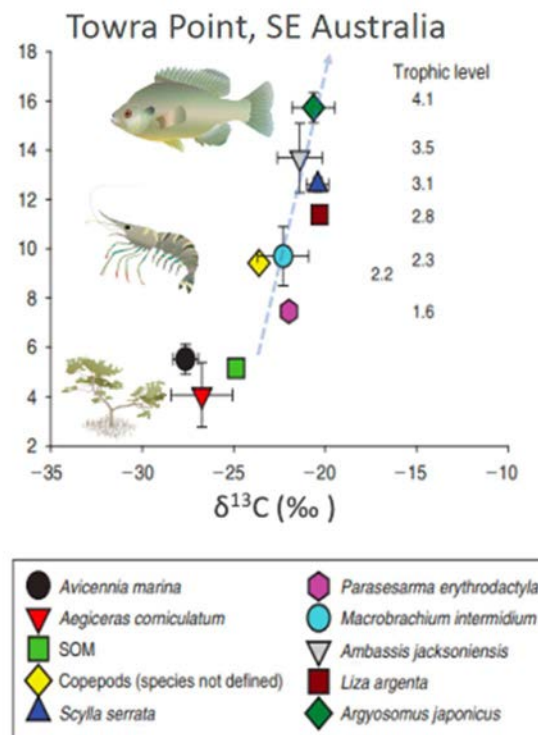


FIG. 39. Stable carbon and nitrogen isotope biplot of the Towra Point wetland ecosystem (reproduced from Ref. [16] with permission).

The advantage of plotting the data in this way is that it allows us to form several inferences prior to mixing model application. First, the C₃ plants are too depleted to be contributing alone to the diet of herbivores and omnivores in the wetland. Either these species are grazing on both C₃ plants and the more enriched C₄ plants and seagrass, or they are feeding on fine benthic organic matter (FBOM), a more likely outcome, both in terms of the tight positioning of the consumer axis from this base (the green square in Fig. 39), and the difficulty in imagining so many consumers feeding on the C₃ and C₄ plants in equal measure. If there were no autotrophs in the above example other than the C₃ plants, there would be no possible solution to the problem of autotrophic sources, because the C₃ plants are of themselves all too depleted in $\delta^{13}\text{C}$ to explain the signatures of the consumers. For this reason, Mazumder and Saintilan [58] were able to exclude the possibility that crab diets could be explained by mangrove leaves in temperate coastal wetlands.

Secondly, the plot allows us to group diet sources, an important consideration in mixing models given the constraints on the number of dietary sources which may be considered. In the above example, it would be wise to cluster the mangroves *Avicennia marina* and *Aegiceras corniculatum*, and the saltmarsh plants *Juncus kraussii* and *Sarcocornia quinqueflora* together as ‘C₃ autotrophs’ in any subsequent mixing model analysis, comparing these to FBOM and perhaps a ‘C₄’ or ‘seagrass’ category.

5.2. MIXING MODELS

The representation of trophic linkages in biplots, as shown above, can contribute important insights into the structure of wetland ecosystems. In these examples, potential food sources can be discounted where they could not possibly be contributing to the diet of a consumer. They allow organisms to be grouped in relation to common position within a foodweb and likely diet, and provide some strong indication of diet sources, and combinations thereof. However, the conclusions drawn from these assessments are usually qualitative, and the push to quantification of dietary contributions led to the development of mixing models.

The website of US Environmental Protection Agency or EPA provides an outline to important mixing models and their application, along with links to spreadsheets for model calculations.⁸ In the following sections, we provide a guide to the appropriate context for the application of three of these mixing models: IsoSource, IsoConc, and IsoError.

5.2.1. How mixing models work: assumptions data requirements

It is important to appreciate the assumptions upon which mixing models rely, and the implications of these not being met. Mixing models assume that the most important components of the diet of an organism have been sampled. A mixing model will estimate the proportional contribution of the candidate dietary sources, such that the combination sums to 100 percent. If an important dietary source is missing, the estimated contribution of the remaining sources will be inflated.

The following example from Ref. [3] illustrates this well. Imagine a consumer which is feeding on four food items (labelled A, B, C and D in Fig. 40 below). In this graph, the discrimination factors are already accounted for, and so the position of the consumer will be a function of the contributions and position for each of the candidate sources. The estimated contribution of each of these items

⁸ <https://www.epa.gov/eco-research/stable-isotope-mixing-models-estimating-source-proportions>

depends on which are sampled, as illustrated in Table 7. There are a number of combinations of diet contributions which might explain the isotopic signature of the consumer. For example, the consumer might be feeding only on items A and C in equal measure, or only on B and D with a preference for B.

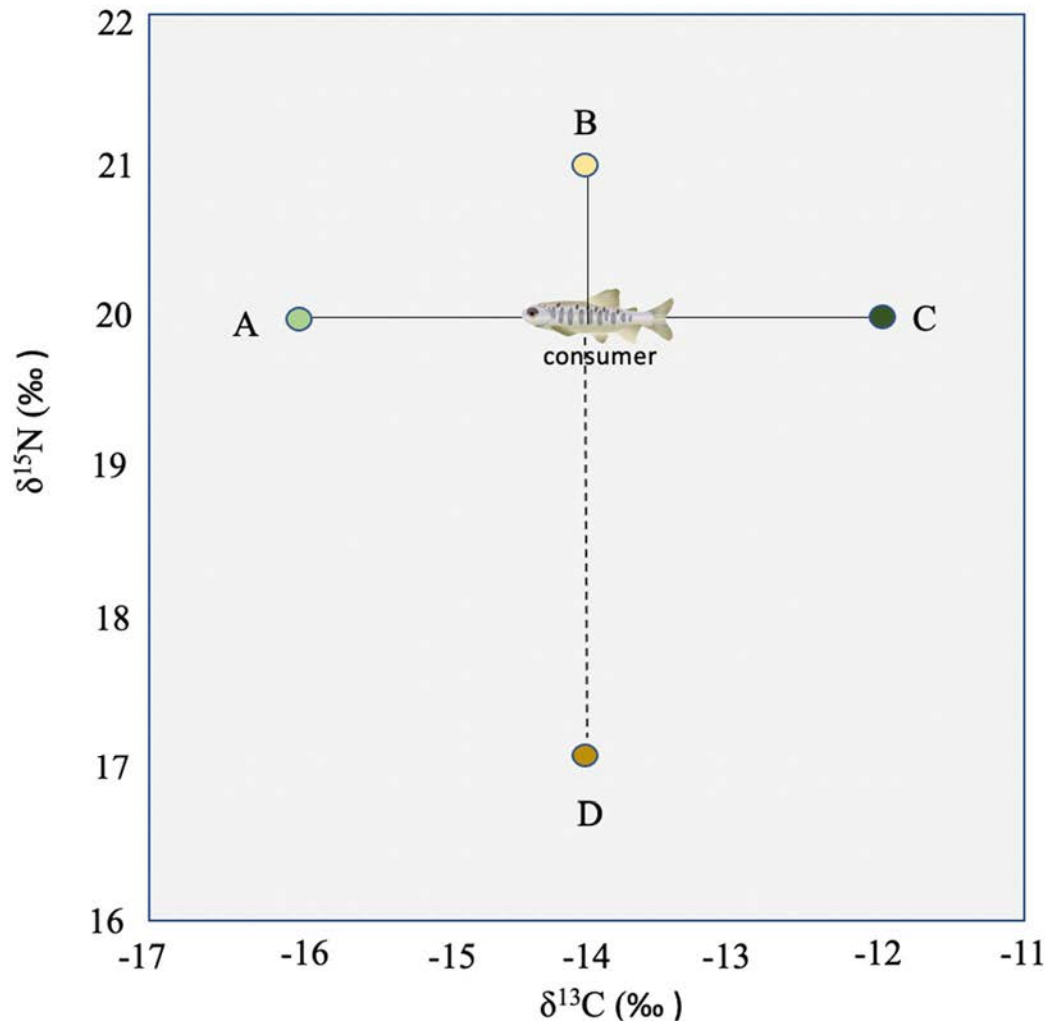


FIG. 40. The potential dietary items of a consumer. (Courtesy of N. Saintilan, Macquarie University, Australia.)

TABLE 7. POSSIBLE SOLUTIONS WITH AND WITHOUT SAMPLING D IN FIG. 40 (from Ref. [3])

Hypothetic actual diets (%)						
Source	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Model estimate
A	0	10	20	30	40	50
B	75	60	45	30	15	0
C	0	10	20	30	40	50
D	25	20	15	10	5	-

Note that if food item D is not sampled, the mixing model estimate departs significantly from the range of actual diets. This is because the model determines that in the absence of food item D, neither could food item B be contributing (given the nitrogen isotope enrichment of B in relation to the consumer).

5.2.2. Interpretation of outputs: Isosource

A mixing model seeks to quantify the relative contributions of dietary sources using a mathematical model. Initially, these provided an estimate of the contributions of food sources to the diet of a consumer, being limited in considering only one more potential food sources than the number of isotopes measured ($n+1$). In a two-element analysis (for example, using both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, only three potential food sources could be explored). This may become an important limitation in wetlands, less so for herbivores with limited mobility, but certainly for omnivorous fish which provide an important intermediary trophic link to top order predators.

IsoSource version 1.3⁹, developed in Microsoft Visual Basic™, interprets the diet of a predator species when there is a greater number of candidate source items than the number of isotopes being considered (i.e. $n+1$ described above). The approach is to describe the proportional contribution of each potential food source to the mixture. It does this by providing a distribution of feasible solutions rather than suggesting a unique solution to the contribution of the candidate food items [59, 60].

This software provides the proportion of sources contributions which are consistent with the balance of isotopes being considered. Examples of possible application include:

- Dietary sources (e.g. benthic animals in wetlands);
- Water sources being taken up by a wetland plant;
- The source of carbon in soil organic matter (e.g. blue carbon);
- Sources of pollution contributing to high pollutant loads in a river.

⁹ Download IsoSource Version 1.3.1 (zip) in the following link: https://www.epa.gov/sites/default/files/2015-11/isosourcev1_3_1.zip [N.B: IsoSource software in a zip file - Unzip the file and run Setup.exe]

To use Isosource, the following information is required. First, the isotopic signatures for the target species and each possible source. Then the source increment (or how much increase of percentage for each possible food source during the iterative calculation) needs to be specified, for example, a source increment of 1% means that possible combinations of sources between 0% and 100% are considered in increments of 1 percent. Finally, the mass balance tolerance needs to be specified. How close can possible solutions be to the observed signature to be accepted? If 0.5‰ is specified here, it means that potential solutions can be within 0.5‰ of the measured signature to be accepted as a possible solution.

There are a range of output files provided by IsoSource. Each possible solution is described in terms of range of each source as a percentile, and histograms are provided. These can be exported into a spreadsheet for further examination if required.

5.2.3. Concentration dependence with IsoConc

One of the assumptions of a standard mixing model is that a food item will contribute $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in equal proportion. That is, if a food item is contributing 50% to the consumer nitrogen isotope composition, it will also be contributing 50% to the carbon isotope composition. However, this assumption is not always met. One reason for this is that different dietary items might have different concentrations of carbon and nitrogen. For example, in a mangrove forest, the leaves of the mangrove trees generally have a very low concentrations of nitrogen (high C: N). If a herbivore is consuming these leaves, along with epiphytic algae with a much higher Nitrogen concentration, the algae might make a low contribution to the consumer $\delta^{13}\text{C}$ signature but a disproportionately higher contribution to the $\delta^{15}\text{N}$ signature.

Some isotope mixing models allow for differences in the C and N concentrations in food sources, and these are termed concentration dependent models. For these, the concentration of the elements of Carbon and Nitrogen need to be measured. Where this is a concern, the IsoConc model¹⁰ is a useful way of calculating a concentration-weighted mixing model using stable isotopes [61]. In IsoConc, the elemental concentrations are included as input data, along with the isotope signatures of each source. Outputs include estimates of the contributions of various sources both to total mass and each isotopic element.

The IsoConc model can incorporate three potential sources in a two-element isotope mixing model. Several more recent models are capable of incorporating a greater range of potential food sources incorporating concentration dependence, including IsotopeR [62] and food reconstruction using isotopic transferred signals (FRUITS) [63].

5.2.4. Acknowledging uncertainty using IsoError

Not only should the isotopic variability of dietary sources be reported, along with the variability in consumer isotopes, but the uncertainty of proportional contributions should be represented also, rather than relying on simple point estimates of these contributions. The 95% confidence intervals provided by IsoError, described below are a good example.

¹⁰ Download Isoconcl_01.xls (Excel 2000 spreadsheet).xls in the following link:
https://www.epa.gov/sites/default/files/2015-11/isoconcl_01.xls

“To avoid misrepresenting the uniqueness of the results, users should report the distribution of feasible solutions rather than focussing on a single value such as the mean” [59].

Early mixing models were unable to provide estimates of uncertainty in the proportion of dietary contributions, even though variability in both the isotopic composition of the source and the consumer is reported. The mixing model *IsoError* [64] uses formula for the calculation of standard errors and confidence intervals in source proportion estimates. The proportion contribution standard errors is smaller if sources are more distinct isotopically: for example, doubling the source difference (from 2‰ to 4‰) reduced the standard error by half. Also, proportion contribution standard errors are lower if the source and consumer standard errors are lower.

Finally, ISOERROR1_04 is an Excel spreadsheet which can be used where the number of candidate sources is one more than the number of stable isotopes being used (that is, a more constrained number than in IsoSource). Use the link below to download IsoError.¹¹

In the case of IsoError, mixing models partition n+1 sources (n being n is the number of isotopes measured). For example, there might be two possible sources being considered in explaining the stable isotope composition in $\delta^{13}\text{C}$, or three possible sources where another isotope is added to the analysis (such as $\delta^{15}\text{N}$). Numerous examples are outlined in Section 2 of the Manual, including, for example, the proportions of:

- wetland plants contributing to a diet of a wetland herbivore;
- C₃ vs. C₄ source plant inputs to mangrove soil organic carbon;
- different pollution sources contributing to nitrogen loading in a stream.

The input data required include the number of samples and the mean, and standard deviation for each stable isotope. The output includes an estimate of the proportional contribution of each source (from 0 to 1), and the estimates of error for each source.

IsoSource, described above, can extend the number of potential dietary sources beyond the n+1 constraint of earlier models (underdetermined systems). In doing so it provides distributions of possible sets of dietary sources. However, IsoSource treats diet-tissue discrimination factors as fixed, and does not represent the extent of fractionation as a source of uncertainty.

5.3.LIMITATIONS OF MIXING MODELS

Stable isotopes are one of several tools that can be used to determine the diet of organisms, or the contribution of autotrophs to carbon accumulation in wetlands. The technique is not the only tool available, and careful consideration should be given to whether clarification of linkages between components of the environment might be provided by the application of complementary approaches, such as gut content analysis or elemental analyses. No tool has the precision of breath of application to provide the unequivocal answer.

¹¹ Download isoerror1_04.xls [Excel 2000 spreadsheet] (xls) in the following link:
https://www.epa.gov/sites/default/files/2015-11/isoerror1_04.xls

Stable isotope mixing models rely on core assumptions likely to be violated to varying degrees. Amongst these are:

- The isotopic composition of the food sources is constant through space and time. This may be violated if an organism switches diets during the time taken to assimilate dietary signatures, thereby obscuring results;
- Diet-tissue discrimination factors (DTDFs), the amount of fractionation which occurs between the diet and the consumer, is relatively constant;
- The potential dietary sources can be distinguished from one another. Overlaps between identified potential food sources limit the ability to discriminate between their contribution (between, for example, two species of C₃ plants);
- The key dietary sources have been sampled. Overlaps between sampled and important unsampled dietary sources will lead to incorrect attribution (as would occur, for example, if fish were assumed to be feeding on saltmarsh when in fact there were feeding on a type of phytoplankton with the same isotopic signature);
- Individuals of a sampled population are consuming the same diet. This may often be the case in wetland environments in which invertebrates may have highly specialized feeding habits, and fish swim and feed in schools. However, samples of individuals with a more solitary habits (top order predators) may show a degree of individual dietary history.

One key limitation of mixing models is that the number of potential food sources that can be considered is constrained by the number of isotopes being examined. A model is said to be 'underdetermined' if the number of important dietary sources is greater than the number of isotopic tracers being applied. It is difficult to precisely define the proportionate contribution of each dietary component if an organism is feeding across a wide spectrum of dietary sources. In these cases, stable isotope analyses may be more useful in verifying certain dietary sources rather than eliminate potential food sources [65].

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LIST OF ABBREVIATIONS

ANSTO	Australian Nuclear Science and Technology Organization
CAM	Crassulacean Acid Metabolism
CIAAW	Commission on Isotopic Abundances and Atomic Weights
DTDF	Diet-tissue discrimination factor
EA-IRMS	Elemental Analyser Isotope Ratio Mass Spectrometry
EOM	Extractable organic matter
FBOM	Fine benthic organic matter
IAEA	International Atomic Energy Agency
ILC	Interlaboratory comparison
IRMS	Isotope Ratio Mass Spectrometry
IUPAC	International Union of Pure and Applied Chemistry
NIST	National Institute of Standards and Technology
PDB	Pee Dee Belemnite formation
PIT	Principle of Identical Treatment
POM	Particulate organic matter
QA	Quality assurance
QC	Quality control
RCA	Regional Co-operative Agreement
RM	Reference material
SOM	Sediment organic matter
SPOM	Suspended Particulate Organic Matter
USGS	United States Geological Survey

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