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Stable Isotope Application in Animal Nutrition Science

Review Article

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ABSTRACT

The application of stable isotope analysis (SIA) has become a standard scientific approach in Agricultural and Ecological researches and, more in general, in several disciplines such as biology, botany, zoology, organic chemistry, climatology, and nutrition. The main objectives of this paper are (1) to provide a simple definition of stable isotopes and (2) to illustrate analytical measurement methods and general applications in animal nutrition. The stable isotopes of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) represent powerful tools to evaluate the trophic preferences of organisms and their trophic position. In association with Bayesian Mixing models, stable isotopes also enable the description of trophic links between species and, thus, of complex food webs. Stable isotope data should be complemented with additional dietary data on feeding behavior to provide information regarding the transfer of energy or nutrients. Nowadays, stable isotope analysis is employed to address animal and human diets around the world.

KEY WORDS animal nutrition, environment, food web, stable isotopes analysis, trophic position.

INTRODUCTION

Stable isotope analysis (SIA) is considered a helpful tool in a range of researches such as the study of climatic condition (Barnet *et al*. 2019), agricultural products, biological processes and biogeochemical cycles (Chaffee *et al*. 2007). In ecological studies, the measurement of stable isotopes in plants and animals is applied to the determination of animal feeding behavior, movement, and trophic position along food chains (Bouillon *et al*. 2011; Ben-David and Flaherty, 2012). Stable isotopes are safe (non-radioactive) and can be operated by humans. Even infants and pregnant women can be safely examined in medicine and nutrition studies. Naturally occurring stable isotopes are transferred from the physical environment to primary producers, as well as from a resource to its consumer, and emerge in hair, urine, feces, breath, and blood (Hagen, 1963; Rossi *et al*. 2018). Thus, they can be used to trace nutrient uptake in producers and consumers in both terrestrial and marine ecosystems (Madeira *et al*. 2019; Signa *et al*. 2019; Calizza *et al*. 2018). Many measurement techniques depend on natural differences in the way 'light' and 'heavy' isotopes react during metabolic processes through biological and chemical alterations. Other stable isotope techniques depend on adding trace amounts of compounds artificially enriched in the rare (heavy) isotope of the element of interest. These are called isotope tracer methods / techniques. About a century ago, Fredrick Soddy was the first to identify signs of the existence of isotopes (Wilkinson, 2018). Isotopes are classified into 'Stable' and 'Unstable' groups. The unstable isotopes, which are not the subject of this research, are radioactive. Here the question is 'What are the stable isotopes?'. To answer this question, we should start by focusing on the atomic nucleus. Indeed, a different number of neutrons

within the nucleus of the heavy and light stable isotopes leads to different atomic masses (Ben-David and Flaherty, 2012). Both light and heavy stable isotopes play a similar role in biological and chemical reactions, but with different response rates. The attractive forces and chemical bonds of the light stable isotope are weaker than the heavier isotope of an element. Thus, the lighter isotope reacts more quickly than the heavier one in both biological and chemical reactions. Even though oxygen (O), sulfur (S), and deuterium (D) are applied in some studies, nitrogen (N) and carbon (C) are the two main elements considered in the study of animal diet and food webs.

Nitrogen (^{14}N, ^{15}N)

Natural nitrogen includes two stable isotopes (^{14}N, ^{15}N). ^{14}N is the most common isotope, while ^{15}N is the rarest. Different nitrogen isotopes (^{14}N and ^{15}N) can be distinguished through thermal diffusion or chemical exchanges. Other isotopes of nitrogen can be found in nature, such as ^{12}N, ^{13}N, ^{16}N, and ^{17}N. However, these isotopes are radioactive. Living organisms through the 'nitrogen cycle' usually transform nitrogen. Microbes convert different nitrogen compounds (like ammonia, NH_3^+) to nitrates for green plants and algae (Finlay and Kendall, 2008). Animals get their required nitrogen by consuming other living organisms (Post, 2002). The measurement of the isotopic signature of nitrogen (δ^{15}N) plays an important role in biochemical, industrial and ecological applications such as food preservation, quantification of ecological processes and feeding interactions among organisms, medical and biomedical research (Schellekens *et al*. 2011; Calizza *et al*. 2018; Signa *et al*. 2019), and climate studies (Dotsika and Diamantopoulos, 2019).

Carbon (^{12}C, ^{13}C, ^{14}C)

One of the essential elements on earth is carbon, which forms the chemical basis of life. There are three natural isotopes of carbon, with atomic masses of 12, 13, and 14. ^{12}C and ^{13}C are stable and are used as tracers to understand nutrient cycling (Wang *et al*. 2019), food webs (Telsnig *et al*. 2019), and air-sea swapping of CO_2 (Lynch-Stieglitz *et al*. 1995).

Plants and phytoplankton have a preferential use of^{12}C to convert sunlight and carbon dioxide into biomass. The ocean surface is separated from the deeper water. However, when plankton dies, it sinks and removes ^{12}C from the surface (Flannery, 2006). The ^{14}C, or radiocarbon, is unstable. It is produced in the atmosphere and absorbed by living organisms (Marra, 2019). Carbon signatures can be used in agricultural and climate studies, authentication of foodstuff, description of nutrient fluxes in ecosystems, and in the determination of the age of archaeological specimens (Zeuner, 1958; Aitken, 2013; Signa *et al*. 2019).

Hydrogen (^{1}H, ^{2}H, ^{3}H, ^{4}H, ^{5}H, ^{6}H, ^{7}H)

Hydrogen has two naturally stable isotopes, ^{1}H and ^{2}H. The ^{2}H isotope is called deuterium (D), while ^{3}H is known as tritium (T), which is radioactive. Four other hydrogen isotopes, ^{4}H, ^{5}H, ^{6}H, and ^{7}H, are highly unstable and have been synthesized in the laboratory by bombarding tritium and by fast-moving deuterium or tritium nuclei (Golovkov *et al*. 2003).

Some applications of the hydrogen isotopes could be highlighted in the authentication of foodstuff, agricultural, ecological, geochemical studies, and medical applications (Finlay and Kendall, 2008; Boschetti *et al*. 2019).

Oxygen (^{16}O, ^{17}O, ^{18}O)

Oxygen isotopes include three stable forms. The most abundant is ^{16}O, while ^{17}O and ^{18}O are categorized as secondary stable isotopes. The ^{16}O is mostly produced by massive stars composed only of hydrogen. ^{17}O and ^{18}O nucleosynthesis needs seed nuclei. The ^{17}O is produced by hydrogen burning into helium in CNO (Carbon-Nitrogen-Oxygen) cycle, and ^{18}O is made when the^{14}N catches the^{4}He nucleus (Meyer, 2005; Emsley, 2011). Oxygen isotopes can be used in the authentication of foodstuff, agricultural, ecological, geochemical, climate, and medical studies (Finlay and Kendall, 2008; Boschetti *et al*. 2019; Duffy *et al*. 2019).

Sulfur (^{32}S, ^{33}S, ^{34}S, ^{36}S)

Sulfur has twenty-four isotopes. Among these, ^{32}S, ^{33}S, ^{34}S, and ^{36}S are stable. Understanding acidic deposition in the forest ecosystems is the major application of sulfur isotopes (Campbell *et al*. 2006). The ^{34}S values increase with pollution sources and gas emission, which makes sulfur a powerful detector (Mayer *et al*. 1993).

The sulfur input to marine systems mainly arises from seawater sulfate (δ^{34}S=21‰), whereas terrestrial inputs mainly depend on precipitations (δ^{34}S=2-8‰) (Michener and Kaufman, 2008).

Instrument

The abundance of stable isotopes in mineral and biological samples is measured as the heavy-to-light isotope ratio (R). For a given element (X), the isotopic signature of a sample (δ) is expressed as the per mill deviation (‰) from an international standard (Muccio and Jackson, 2009; Philp, 2015), according to the following equation:

$$\delta X = (R_{Sample}/R_{Standard} - 1) \times 1000 \quad (1)$$

The International Atomic Energy Agency (2004) and the National Institute of Standards and Technology (Muccio and Jackson, 2009) provided an accurate evaluation of the reference standard elements (Lynch-Stieglitz *et al.* 1995; Werner and Brand, 2001; Flannery, 2006; Brand *et al.* 2014). The ratios of $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, and $^{2}H/^{1}H$ have been used widely to measure stable isotopes in carbon, nitrogen, oxygen, and hydrogen (Muccio and Jackson, 2009). The analytical determination of δ values implies the use of few light gases such as CO_2, CO, N_2, O_2, and SO_2. Accordingly, this standard technology has been called Isotope Ratio Mass Spectrometry (IRMS) (Brand, 2004). This analytical method identifies the chemical substance by ionizing it, focusing the resulting ions into a beam, and by separating the light and heavy atoms according to their net electric charge (Finlay and Kendall, 2008). The classical method of analysis includes two gases that are stored in containers connected via capillaries to a switching unit, the changeover valve. An isotope ratio mass spectrometer uses one gas as its ion source, while other available gas flow to the waste vacuum line (Werner and Brand, 2001). Both gases are used and compared a few times and measured separately through the ion currents. The relative difference in the ratio of light and heavy ions is calculated according to an international relative isotope ratio scale (Paul *et al.* 2007). The instrument has six basic components (Figure 1) (Edmond de and Stroobant, 2013), which include: 1) a vacuum system; 2) an ion source; 3) a mechanism to concentrate ions into a narrower beam; 4) the speeding up of the beam; 5) a mass analyzer; 6) a detector. The material is initially present in the vacuum system, which produces the required low pressure to produce electrons and ions in the gas phase. Then, samples are transformed and concentrated into a narrower beam. (Brenna *et al.* 1997; Meier-Augenstein, 1999; Paul *et al.* 2007). Commonly, two connectors are used to introduce samples into isotope ratio mass spectrometry (IRMS): elemental analysers (EAIRMS) and gas chromatographers (GCIRMS). For many years, techniques such as gas chromatography (GC) and gas chromatography-mass spectrometer (GCMS) have been used to identify contamination sources (Philp, 2015). Similarly, the combined gas chromatography isotope ratio mass spectrometry (GCIRMS) technique can be used to determine individual compounds and soil contamination sources.

Sampling procedures

Before sampling, it is necessary to define what kind of information is needed. This information usually depends on research objectives and the type of samples to be collected. Otherwise, the risk is to waste time and resources in collecting either wrong or not enough data.

The sampling design is a tool that is utilized to infer how many data to collect, where, when, and how often they should be collected.

Plant sampling

Samples of vascular plants should be collected in the field and separately kept cold or frozen until processing. Samples of non-vascular plants are divided into two sections: lichens and marine algae. Lichens samples need to be collected directly into paper bags and dried once in the laboratory (Eldridge *et al.* 2003). If no oven is available, they can be spread out in a warm and well-ventilated place in packets and stored upright in a box. The samples of marine algae may be partially dried in the sun, but the small ones should be placed between sheets of paper, and the large ones should be placed in a box for further drying. Afterward, specimens should be pressed and stored in a dry and warm place (Steinitz and Kurle, 2014).

Animal sampling

As regards terrestrial animals, it is possible to collect a sample of muscle, skin, feathers, eggshell, egg albumen, fur hairs, bones, *etc*. The samples may reflect diets ingested months before sampling, e.g. during the moulting or laying phase, and they can be collected from live or dead animals. Bone growth rings and whole bone can reflect an annual diet trend, during the whole animal's lifetime. If bone samples are collected, soft tissues should be removed, and bones should be rinsed to remove impurities. When dry, bone samples can be placed in a paper bag. Feather samples should be cleaned to remove residual dirt and oil using a chloroform-methanol solution (Paritte and Kelly, 2009). Also, inorganic calcium carbonate from eggshell samples should be removed through a process of acidification (Finlay and Kendall, 2008).

As regards aquatic animals, small invertebrates can be collected by using kick nets, grabs, and litterbags, while large predators such as crabs, sea snails, and stomatopods, can be gathered by using traps baited with fresh fish flesh (Careddu *et al.* 2015).

For bigger organisms, the muscle tissue often provides enough biomass to perform stable isotope analyses (Abrantes *et al.* 2013), while for small invertebrates such as amphipods and polychaetes, the whole body can be used (Ng *et al.* 2007).

In order to study a consumer's diet, sampling should also include any dietary item that has likely been accessed by the consumer. All animal samples should be kept frozen at -20 ˚C and then lyophilized or dried at 60 ˚C overnight and kept in dry conditions until analysis (Finlay and Kendall, 2008).

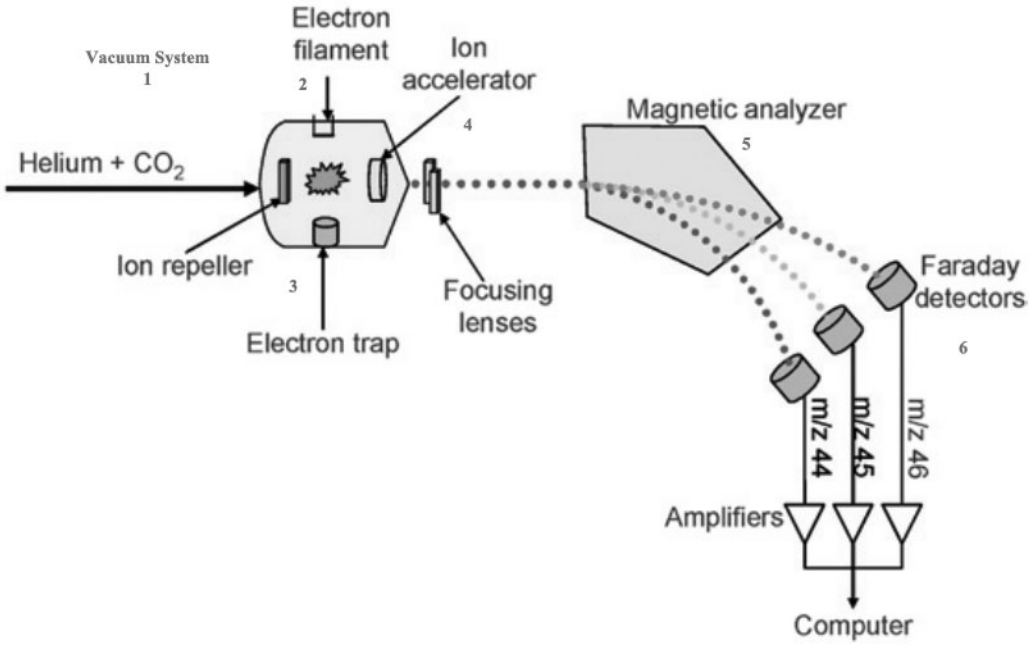

Figure 1 Schematic fundamental components of mass spectrometry (Edmond de and Stroobant, 2013; Muccio and Jackson, 2009)

Before isotopic analysis, all samples should be reduced to a fine homogeneous powder with a ball mill (Rossi *et al.* 2018). Then, powder of animal tissues (0.20±0.05 dry-mg) and vegetal tissues (3.0±0.05 dry-mg) should be weighed into tin capsules and analysed with an isotope ratio mass spectrometer. Thus, based on $\delta^{13}C$ and $\delta^{15}N$ values of consumers and their potential food sources, the animal diet can be determined through the R software (R Core Team, 2013), and Bayesian stable isotope mixing model (Rossi *et al.* 2018). Specifically, Bayesian mixing models allow the estimation of the proportion of each resource in the consumer's diet. The model requires three inputs: the isotopic signatures of the target consumer, the isotopic signatures of potential food sources, and the trophic enrichment factor, which represents the expected isotopic increase from a resource to its consumer due to metabolic processes (McCutchan *et al.* 2003; Careddu *et al.* 2015).

Stable isotope applications in animal nutrition

As stated before, SIA is considered a helpful tool to be used in many disciplines. Among these, stable isotope-based environmental studies have recently flourished. Stable isotope signatures can be used to measure environmental stressors by monitoring plant uptake of carbon dioxide (Zheng *et al.* 2019) and greenhouse gas emissions (International Atomic Energy Agency, 2004; Popa *et al.* 2014) as well as by tracing the source of water in catchments (Philp, 2015; Fiorentino *et al.* 2017; Barbieri, 2019) and organic and mineral compounds during biogeochemical processes (Finlay and Kendall, 2008) and cycles (Lichtfouse, 2000). The study of past climatic conditions is essential (Barnet *et al.* 2019; Jafari and Jafari, 2019) because it enables to modelling climate variability and make predictions of future conditions (Noorollahi *et al.* 2011). When dealing with animal nutrition, climate variability, ecological transitions, temporal and spatial scales, and individual choices can all affect variation and adaptation in the diet of organisms across trophic levels (Careddu *et al.* 2015; Bentivoglio *et al.* 2016; Calizza *et al.* 2018; Jafari and Jafari, 2019). Therefore, the study of temporal and spatial patterns of animal foraging through stable isotope analysis can provide useful information to predict future variations in feeding preferences according to climate change scenarios (Finlay and Kendall, 2008; Calizza *et al.* 2018; Rossi et al. 2019). In this perspective, oxygen isotope values can be used to indicate hotter and drier climate (^{18}O enrichment) versus colder and wetter conditions (^{18}O depletion). As an example, results published by Noorollahi *et al.* (2011) showed that increasing temperature has a positive correlation with rising $\delta^{18}O$ values. Also, enrichment in ^{15}N has been reported as an indication of arid conditions (Pate and Anson, 2007). The $\delta^{18}O$ and $\delta^{2}H$ values have been shown to vary across geographic regions (Bowen and Revenaugh, 2003) or along environmental gradients (Lee *et al.* 2019), being thus useful to infer the geographic origin of samples.

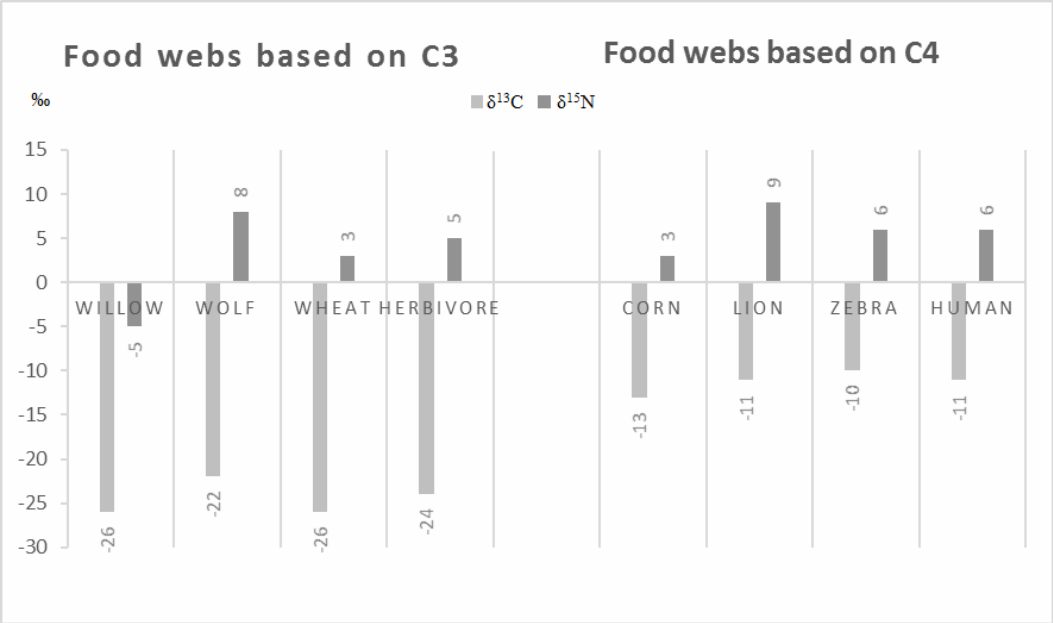

Figure 2 Differences in δ13C (‰) and δ15N (‰) between food webs based on C3 and C4 plants (Schulting, 1998)

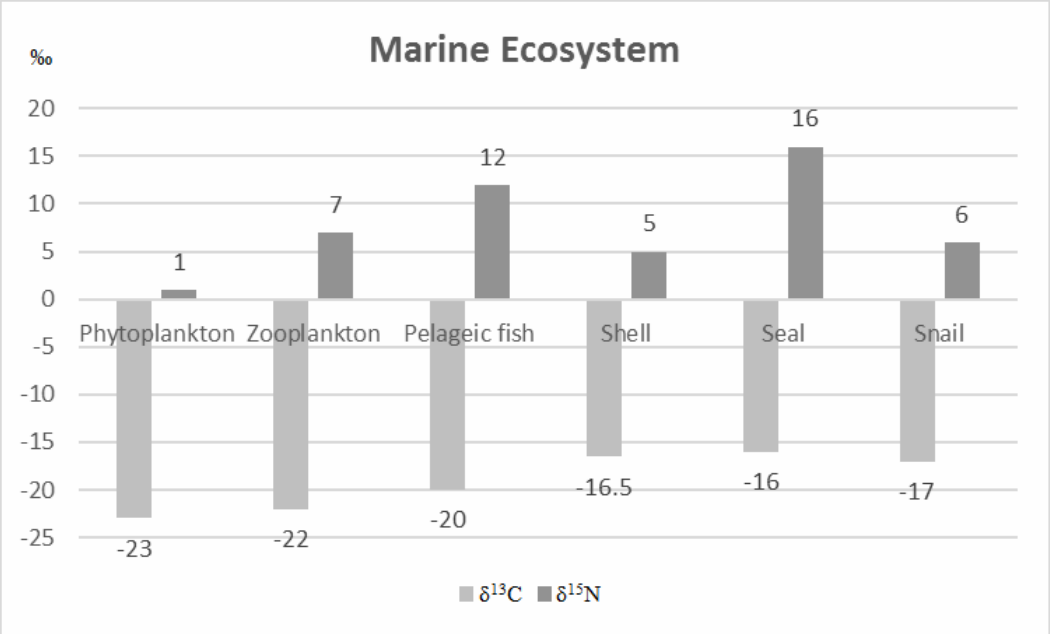

Figure 3 Differences in $\delta^{13}C$ (‰) and $\delta^{15}N$ (‰) values in Marine ecosystem (Schulting, 1998)

SIA has proven to be a beneficial tool for: (1) studying nutrient uptake by humans, nutrient body reserve and nutrient metabolism paths (Schoeller, 2002), (2) describing contaminant flows (Signa *et al*. 2019), trophic relationships and food web structures (Careddu *et al*. 2015; Rossi *et al*. 2015; Calizza *et al*. 2018; Signa *et al*. 2019), as well as nutrient status (Calizza *et al*. 2016), (3) examining animal movement and migration (Di Lascio *et al*. 2016; Cicala *et al*. 2019; Madeira *et al*. 2019), (4) clarifying patterns of resourceallocation (Stachowicz *et al*. 2007; Di Lascio *et al*. 2013), (5) identifying primary and secondary food sources (Komorita *et al*. 2014), and (6) detecting nutrient and mineral uptake by plants (Clewlow *et al*. 2019). The principal aspects of animal nutrition generally investigated through SIA are diet patterns and trophic position of organisms along food chains (Boecklen *et al*. 2011; Bentivoglio *et al*. 2016). As mentioned, stable isotope analysis enables the evaluation of the trophic position of organisms and popula-

tions in food webs. However, the isotopic values in the consumer alone do not trace its trophic position (Bentivoglio *et al*. 2016). Indeed, there is a stepwise increase between the corresponding isotope signature of food consumed and the consumers' tissue. Such expected isotopic increase between consumers and resources is referred to as isotopic discrimination or trophic enrichment (McCutchan *et al*. 2003). As an example, when an animal, such as a cow or a sheep, eats a specific plant, it will express the plant isotopic value in its muscles, bones or teeth, but the heavy isotope will be relatively more retained in the consumer's tissues than the light one (Careddu *et al*. 2015; Cassano *et al*. 2016; Reid and Koch, 2017). However, the plant energy and nutrition values also vary through growth stages (Jafari and Torbatinejad, 2015). Thus, it is essential to consider potential differences in environmental conditions and diet components when studying animal diet (Jafari and Torbatinejad, 2015).

Isotopic fractionation is particularly marked for nitrogen, while the carbon and sulfur isotopic composition of consumers closely reflects that in their diet. Studies with cows, fish, and zooplankton show that animal's feces are enriched in ^{15}N versus the diet, but urinary nitrogen (both NH_3^+ and urea) is depleted in ^{15}N. For example, cow urine can be 1‰ to 4‰ depleted in ^{15}N versus diet, while feces (2‰), and milk and blood (4‰ both) are enriched in ^{15}N. The ratio of sulfur isotopes ($\delta^{34}S$) varies substantially among salt marsh and marine primary producers from -9.6‰ to +12.9‰ (Currin *et al*. 1995). Thus, the $\delta^{34}S$ can be used to identify resource pools in these ecosystems. Similarly, the $\delta^{34}S$ values have been measured in transitional water ecosystems and marine ecotones to distinguish between marine and freshwater inputs (Peterson and Howarth, 1987; Currin *et al*. 1995; Martinetto *et al*. 2006; Finlay and Kendall, 2008).

$\delta^{13}C$ values in phytoplankton can vary markedly with latitude and longitude. Indeed, $\delta^{13}C$ values may vary with temperature, location, and growth rates that can affect the carbon uptake rate by phytoplankton (Zheng *et al*. 2019). Significant differences in carbon isotopes between animals indicate that consumers rely on different food sources or that their respective food webs are based on primary producers characterised by different isotopic signatures (Michener and Kaufman, 2008). Differences in the processing of carbon, nitrogen, and sulfur isotopes by animals stand out even more clearly, when the whole food web is examined. In many food webs, nitrogen isotope values increase by 10‰ to 15‰ from basal resources to top predators due to 3‰ to 5‰ stepwise increase among subsequent trophic levels. The opposite effect – no change with increasing trophic level – is observed for sulphur (Saggar *et al*. 1981).

The isotopic differences in consumer $\delta^{13}C$ may arise also by the consumption of C_3 or C_4 plants. The C_3 plants are linked with a wetter and colder climate, while C_4 plants are related to more arid and warmer conditions. As a consequence of metabolic adaptation by plants to such different climatic conditions, C_4 plants generally show markedly higher $\delta^{13}C$ values (from -10‰ to -18‰) than C_3 plants (from -22‰ to -30‰) (Philp, 2015) (Figure 4). In addition, C_3 plants may also show lower $\delta^{15}N$ values than C_4 plants (Figure 4).

DeNiro and Epstein (1981) mentioned that $\delta^{13}C$ values in C_3 plants averaged around -25.5‰, while values around -9.0‰ were reported for C_4 plants (Figure 2). The $\delta^{13}C$ value of meat was around -18.0‰, suggesting that the animal's diet was composed by a mix of C_3 and C_4 plants (Deniro and Epstein, 1981). Differences in $\delta^{13}C$ values have also been reported between terrestrial plants and aquatic algae (Rossi *et al*. 2010). The latter generally show higher $\delta^{15}N$ values than the former (Figure 3) (Schulting, 1998). This mainly depends on differences between the sources of carbon used for primary production in the two systems (Schulting, 1998). As shown in Figure 4, the $\delta^{13}C$ of terrestrial C_3 primary producers is generally lower than that of marine producers (Vinagre *et al*. 2011). In addition, $\delta^{13}C$ signatures in plants can be affected by plant development and water management (Schulting, 1998; Barbieri, 2019). Given such expected differences among marine and terrestrial ecosystems, isotopic differences among aquatic consumers can inform on the benthic or terrigenous origin of nutrient inputs at the base of coastal or littoral food webs. Furthermore, dissolved inorganic carbon in estuaries commonly derives from different sources, either CO_2 from the atmosphere or the dissolution of carbonate with approximately zero per mill value of $\delta^{13}C$ (Finlay and Kendall, 2008; Bouillon *et al*. 2011). Given the predominance of C_3 metabolism in coastal and aquatic vegetation, the $\delta^{13}C$ in aquatic consumers usually display values around -28‰. Chanton and Lewis (1999) showed that the $\delta^{13}C$ values in estuaries are closely related to the soluble inorganic carbon and water salinity.

At the community level, the range of $\delta^{13}C$ values (Carbon Range) can provide a useful indication of the diversity of basal resources consumed by animals (Wilkinson, 2018) and both $\delta^{13}C$and $\delta^{15}N$ describe the niche space occupied by all the organisms. To move from the isotopic description of organisms to the quantification of trophic interactions within the food web, Phillips (2012) proposed the use of mixing model equations. By explicitly taking into account uncertainties in consumer and resource isotopic signatures, the development of Bayesian approaches has enabled a more robust description of trophic links between species (Careddu *et al*. 2015; Rossi *et al*. 2019).

Some tissues, such as the dentine of teeth, hairs, and feathers are metabolically inert.

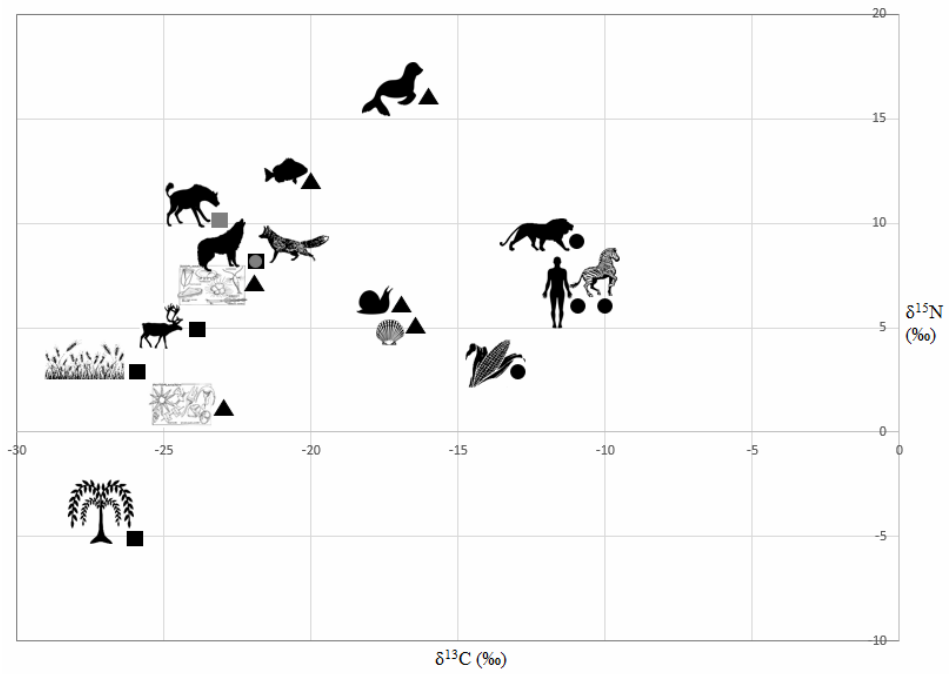

Figure 4 Trophic enrichment in stable carbon and nitrogen values from primary producers, to terrestrial herbivores and predators (circles and squares) and marine ecosystems (black triangles)
Differences in δ13C between food webs based on C3 (black square symbols) and C4 plants (black circles symbols), omnivore/carnivore (grey circles), carnivore (grey squares) are also shown (Reid and Koch, 2017; Schulting, 1998)

Therefore, the study of these tissues can inform on the isotopic signature of a consumer's diet at the time of tissue deposition. If the rate of tissue deposition is known, these tissues can provide a timeline of the consumer's dietary history (Layman *et al*. 2012). For example, Hobson and Sease (1998) recorded ontogenetic isotopic shifts in Steller sea lions from tooth annuli (Hobson and Sease, 1998). Newsome *et al*. (2009) documented temporal changes in resource use by the California sea otter *Enhydra lutris nereis* by using regular sections of whiskers (Newsome *et al*. 2009). In these cases, information on the inert tissue deposition processes is necessary. Indeed, the process can be continuous over time (e.g., for whiskers of some mammal species), or discontinuous (e.g., for feathers) (Layman *et al*. 2012). In addition, it must be considered that different tissues are characterised by different turnover rates, thus providing dietary information over different time scales. Therefore, turnover rate data in the distinct tissues are required to conclude the degree of dietary proficiency (Layman *et al*. 2012). For instance, in some vertebrates, blood plasma integrates the diet over days to weeks, whereas turnover in muscle tissue is on the scale of months (Dalerum and Angerbjörn, 2005; Phillips and Eldridge, 2006).

Nowadays, SIA is utilized to address questions about human diets around the world, and it has been said that 'we are what we eat'(Deniro and Epstein, 1981).

SIA provides quantitative data that complete floral, faunal, and other information about dietary habits of individuals. This passage through human metabolism is specifically valuable to the quantitative study of human nutrition (Cooper *et al*. 2019). Humans express different isotope signatures according to the consumption of C_3 and C_4 plants (Figure 2), terrestrial animal proteins like cow, sheep, and goat meat, or aquatic animal resources such as fish and shellfish (Figure 3) (Schulting, 1998). Interestingly, there are diverse plant groups in human nutrition that can be differentiated through $\delta^{13}C$ values in human tissues (such as hair), including C_3 plants such as wheat, barley, soy, potatoes, fruits, vegetables versus C_4 plants such as corn, sorghum, millet, sugar cane. This difference is also reflected in animal-derived food products such as milk carbon signatures ranging from -14‰ (diet-based C_4 plants) to -27‰ (diet-based C_3 plants) (Petzke *et al*. 2005).

CONCLUSION

The present paper has highlighted the stable isotope concept, applications, measurement method, and its relationships with animal nutrition. The many examples cited allow us to conclude that the analysis of stable isotopes of nitrogen and carbon is a powerful tool for evaluating animal feeding choices and trophic position in food webs, as well as the trophic sources supporting aquatic and terrestrial

consumers. In addition, coupled with isotopic Bayesian mixing models, stable isotopes are a valuable tool that can provide insights into the structure and the complexity of food webs, as well as into the pathways of nutrient and energy transfer among ecosystem compartments and trophic levels. Nevertheless, it must be noted that many of the ecological questions addressed through the analysis of stable isotopes are reliant on the assumption that source pools have distinguished isotope values. When sources cannot be distinguished, stable isotopes may have little performance in answering questions about trophic relationships. In this case, stable isotope analysis should be complemented with additional information, such as stomach content and/or feces analysis, as well as other data on feeding behaviour including direct observation of feeding preferences in the field. In any case, both source and consumer pools must be sampled on suitable spatial and temporal scales to provide reliable information on diet composition. Isotope signature differences in samples depend on the climate, the isotopic baseline of the food web the consumer is part of, organisms' dietary habits, and body conditions. Therefore, all these aspects should be considered in isotopic studies in order to achieve accurate results. Besides its broad application in environmental and ecological studies, SIA is increasingly used in thestudy of human diet, and ithas the potential to resolve many ambiguities in nutritional and medical studies.

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REFERENCES

Abrantes K.G., Barnett A., Marwick T.R. and Bouillon S. (2013). Importance of terrestrial subsidies for estuarine food webs in contrasting East African catchments. *Ecosphere*. **4,** 1-33.

Aitken M.J. (2013). *Science-based Dating in Archaeology*. Longman, London, United Kingdom.

Barbieri M. (2019). Isotopes in hydrology and hydrogeology. *Water*. **11(2),** 291-297.

Barnet J.S.K., Littler K., Westerhold T., Kroon D., Leng M.J., Bailey I., Röhl U. and Zachos J.C. (2019). A high-fidelity benthic stable isotope record of late cretaceous–early eocene climate change and carbon-cycling. *Paleoceanogr. Paleoclimatol.* **34,** 672-691.

Ben-David M. and Flaherty E.A. (2012). Stable isotopes in mammalian research: A beginner's guide. *J. Mammal*. **93,** 312-328.

Bentivoglio F., Calizza E., Rossi D., Carlino P., Careddu G., Rossi L. and Costantini M.L. (2016). Site-scale isotopic variations along a river course help localize drainage basin influence on river food webs. *Hydrobiologia*. **770,** 257-272.

Boecklen W.J., Yarnes C.T., Cook B.A. and James A.C. (2011). On the use of stable isotopes in trophic ecology. *Annu. Rev. Ecol. Evol. Syst*. **42,** 411-440.

Boschetti T., Cifuentes J., Iacumin P. and Selmo E. (2019). Local Meteoric water line of Northern Chile (18 °S-30 °S): An application of error-in-variables regression to the oxygen and hydrogen stable isotope ratio of precipitation. *Water*. **11,** 791807.

Bouillon S., Connolly R.M. and Gillikin D.P. (2011). Use of stable isotopes to understand food webs and ecosystem functioning in estuaries. Pp. 143-173 in Treatise on Estuarine and Coastal Science. E Wolanski and D.S. McKusky, Eds. Elsevier, Amsterdam, Netherlands.

Bowen G.J. and Revenaugh J. (2003). Interpolating the isotopic composition of modern meteoric precipitation. *Water Resour. Res.* **39(10)**, 1-9.

Brand W.A. (2004). Spectrometer hardware for analyzing stable isotope ratios. Pp. 835-856 in P.A. de Groot, Ed. Handbook of Stable Isotope Analytical Techniques. Elsevier, Amsterdam, Netherlands.

Brand W.A., Coplen T.B., Vogl J., Rosner M. and Prohaska T. (2014). Assessment of international reference materials for isotope-ratio analysis (IUPAC Technical Report). *Pure Appl. Chem.* **86(3),** 425-467.

Brenna J.T., Corso T.N., Tobias H.J. and Caimi R.J. (1997). High-precision continuous-flow isotope ratio mass spectrometry. *Mass Spectrom. Rev.* **16,** 227-258.

Calizza E., Careddu G., Sporta Caputi S., Rossi L. and Costantini M.L. (2018). Time- and depth-wise trophic niche shifts in Antarctic benthos. *PLoS One*. **13,** e0194796.

Calizza E., Costantini M.L., Rossi D., Pasquali V., Careddu G., and Rossi L. (2016). Stable isotopes and digital elevation models to study nutrient inputs in high-arctic lakes. *Rend. Lincei.* **27,** 191-199.

Campbell J.L., Mitchell M.J. and Mayer B. (2006). Isotopic assessment of NO3− and SO42− mobility during winter in two adjacent watersheds in the Adirondack Mountains, New York. *J. Geophys. Res. Biogeo.* **111,** 1-15.

Careddu G., Costantini M.L., Calizza E., Carlino P., Bentivoglio F., Orlandi L. and Rossi L. (2015). Effects of terrestrial input on macrobenthic food webs of coastal sea are detected by stable isotope analysis in Gaeta Gulf. *Estuar. Coast. Shelf S.* **154,** 158-168.

Cassano J.J., Seefeldt M.W., Palo S., Knuth S.L., Bradley A.C., Herrman P.D., Kernebone P.A. and Logan N.J. (2016). Observations of the atmosphere and surface state over Terra Nova Bay, Antarctica, using unmanned aerial systems. *Earth Syst. Sci. Data.* **8,** 115-126.

Chaffee M., Shanks W., Rye R., Shwartz C., Adams M., Carlson R., Crock J., Gemery-Hill P., Gunther K., Kester C., King H. and Podruzny S. (2007). Applications of Trace-Element and Stable-Isotope Geochemistry to Wildlife Issues, Yellowstone National Park and Vicinity. Publications of the US Geological Survey, Nebraska,USA.

Chanton J.P. and Lewis F.G. (1999). Plankton and dissolved inorganic carbon isotopic composition in a river-dominated estu-

ary: Apalachicola bay, Florida. *Estuaries.* **22,** 575-583.

Cicala D., Calizza E., Careddu G., Fiorentino F., Sporta Caputi S., Rossi L. and Costantini M.L. (2019). Spatial variation in the feeding strategies of Mediterranean fish: Flatfish and mullet in the Gulf of Gaeta (Italy). *Aquat. Ecol.* **53(4),** 529-541.

Clewlow H.L., Takahashi A., Watanabe S., Votier S.C., Downie R. and Ratcliffe N. (2019). Niche partitioning of sympatric penguins by leapfrog foraging appears to be resilient to climate change. *J. Anim. Ecol.* **88,** 223-235.

Cooper C.G., Lupo K.D., Zena A.G., Schmitt D.N. and Richards M.P. (2019). Stable isotope ratio analysis (C, N, S) of hair from modern humans in Ethiopia shows clear differences related to subsistence regimes. *Archaeol. Anthropol. Sci.* **11,** 3213-3223.

Currin C.A., Newell S.Y. and Paerl H.W. (1995). The role of standing dead *Spartina alterniflora* and benthic microalgae in salt marsh food webs: Considerations based on multiple stable isotope analysis. *Mar. Ecol. Prog. Ser.* **121,** 99-116.

Dalerum F. and Angerbjörn A. (2005). Resolving temporal variation in vertebrate diets using naturally occurring stable isotopes. *Oecologia*. **144,** 647-658.

Deniro M.J. and Epstein S. (1981). Influence of diet on the distribution of nitrogen isotopes in animals. *Geochim. Cosmochim. Ac.* **45,** 341-351.

Di Lascio A., Madeira F., Costantini M.L., Rossi L. and Pons X. (2016). Movement of three aphidophagous ladybird species between alfalfa and maize revealed by carbon and nitrogen stable isotope analysis. *Biol. Control*. **61,** 35-46.

Di Lascio A., Rossi L., Carlino P., Calizza E., Rossi D. and Costantini M.L. (2013). Stable isotope variation in macroinvertebrates indicates anthropogenic disturbance along an urban stretch of the river Tiber (Rome, Italy). *Ecolo. Indic.* **28,** 107-114.

Dotsika E. and Diamantopoulos G. (2019). Influence of climate on stable nitrogen isotopic values of contemporary greek samples: Implications for isotopic studies of human remains from neolithic to late bronze age Greece. *Geoscience.* **9**, 217.

Duffy J.E., McCarroll D., Loader N.J., Young G.H.F., Davies D., Miles D. and Ramsey C.B. (2019). Absence of age-related trends in stable oxygen isotope ratios from oak tree rings. *Global Biogeochem. Cycles.* **33,** 841-848.

Edmond de H. and Stroobant V. (2013). Mass Spectrometry: Principles and Applications. John Wiley and sons, New Jersey, United States.

Eldridge D., Skinner S. and Entwisle T.J. (2003). Survey Guidelines for Non-Vascular Plants. Botanic Gardens Trust, Sydney, Australia.

Emsley J. (2011). Nature's Building Blocks: An A-Z Guide to the Elements. Oxford University Press, Oxford, United Kingdom.

Finlay J.C. and Kendall C. (2008). Stable isotope tracing of temporal and spatial variability in organic matter sources to freshwater ecosystems Pp. 283-333 in Stable Isotopes in Ecology and Environmental Science. R. Michener and K. Lajtha, Eds. Blackwell Publishing, New Jersey, United States.

Fiorentino F., Cicala D., Careddu G., Calizza E., Jona-Lasinio G., Rossi L. and Costantini M.L. (2017). Epilithon $\delta^{15}N$ signatures indicate the origins of nitrogen loading and its seasonal dy

namics in a volcanic Lake. *Ecol. Indic.* **79,** 19-27.

Flannery T.F. (2006). The Weather Makers: The History and Future Impact of Climate Change. Atlantic Monthly Press, New York.

Golovkov M.S., Oganessian Y.T., Bogdanov D.D., Fomichev A.S., Rodin A.M., Sidorchuk S.I., Slepnev R.S., Stepantsov S.V., Ter-Akopian G.M., Wolski R., Gorshkov V.A., Chelnokov M.L., Itkis M.G., Kozulin E.M., Bogatchev A.A., Kondratiev N.A., Korzyukov I.V., Yukhimchuk A.A., Perevozchikov V.V., Vinogradov Y.I., Grishechkin S.K., Demin A.M., Zlatoustovskiy S.V., Kuryakin A.V., Fil'Chagin S.V., Il'Kayev R.I., Hanappe F., Materna T., Stuttge L., Ninane A.H., Korsheninnikov A.A., Nikolskii E.Y., Tanihata I., Roussel-Chomaz P., Mittig W., Alamanos N., Lapoux V., Pollacco E.C. and Nalpas L. (2003). Evidences for resonance states in 5H. *Phys. Lett. B.* **566,** 70-75.

Hagen J.H. (1963). The turnover of glycerol in plasma. *Life Sci.* **3,** 170-174.

Hobson K.A. and Sease J.L. (1998). Stable isotope analyses of tooth annuli reveal temporal dietary records: An example using Steller sea lions. *Mar. Mamm. Sci.* **14,** 116-129.

International Atomic Energy Agency. (2004). Isotope Hydrology and Integrated Water Resources Management. Vienna, Austria.

Jafari V. and Jafari M. (2019). Reverse impact of temperature as climate factor on milk production in ChaharMahal and Bakhtiari. *World Acad. Sci. Engin. Technol. Int. J. Anim. Vet. Sci.* **13,** 29-33.

Jafari V. and Torbatinejad N.M. (2015). Nutritive value of range *Frankenia hirsuta* as fodder resource for ruminant. *Am. Adv. J. Biol. Sci.* **1,** 54-62.

Komorita T., Kajihara R., Tsutsumi H., Shibanuma S., Yamada T., and Montani S. (2014). Food sources for *Ruditapes philippinarum* in a coastal lagoon determined by mass balance and stable isotope approaches. *PLoS One.* **9,** e86732.

Layman C.A., Araujo M.S., Boucek R., Hammerschlag-Peyer C.M., Harrison E., Jud Z.R., Matich P., Rosenblatt A.E., Vaudo J.J., Yeager L.A., Post D.M. and Bearhop S. (2012). Applying stable isotopes to examine food-web structure: An overview of analytical tools. *Biol. Rev.* **87,** 545-562.

Lee J., Cho J., Cho Y.J., Cho A., Woo J., Lee J., Hong S.G., Sul W.J. and Kin O.S. (2019). The latitudinal gradient in rock-inhabiting bacterial community compositions in Victoria Land, Antarctica. *Sci. Total Environ.* **657,** 731-738.

Lichtfouse E. (2000). Compound-specific isotope analysis. Application to archaeology, biomedical sciences, biosynthesis, environment, extraterrestrial chemistry, food science, forensic science, humic substances, microbiology, organic geochemistry, soil science and sport. *Rapid Commun. Mass Spectrom.* **14,** 1337-1344.

Lynch-Stieglitz J., Stocker T.F., Broecker W.S. and Fairbanks R.G. (1995). The influence of air-sea exchange on the isotopic composition of oceanic carbon: Observations and modeling. *Global Biogeochem. Cycles.* **9,** 653-665.

Madeira F., di Lascio A., Costantini M.L., Rossi L., Rösch V. and Pons X. (2019). Intercrop movement of heteropteran predators between alfalfa and maize examined by stable isotope analy

sis. *J. Pest Sci.* **92,** 757-767.

Marra J.F. (2019). Hot Carbon: Carbon-14 and a Revolution in Science. Columbia University Press, New York, United States.

Martinetto P., Teichberg M. and Valiela I. (2006). Coupling of estuarine benthic and pelagic food webs to land-derived nitrogen sources in Waquoit Bay, Massachusetts, USA. *Mar. Ecol. Prog. Ser.* **307,** 37-48.

Mayer B., Krouse H.R., Fritz P., Prietzel J. and Rehfuess K.E. (1993). Evaluation of biogeochemical sulfur transformations in forest soils by chemical and isotope data. Pp. 65-72 in Proc. Yokohama Symp., Yokohama, Japan.

McCutchan J.H., Lewis W.M., Kendall C. and McGrath C.C. (2003). Variation in trophic shift for stable isotope ratios of carbon, nitrogen, and sulfur. *Oikos.* **102,** 378-390.

Meier-Augenstein W. (1999). Applied gas chromatography coupled to isotope ratio mass spectrometry. *J. Chromatogr. A.* **842,** 351-371.

Meyer B.S. (2005). Nucleosynthesis and galactic chemical evolution of the isotopes of oxygen. *Oxygen Earl. Solar Syst.* **1278,** 32-38.

Michener R.H. and Kaufman L. (2008). Stable isotope ratios as tracers in marine food webs: An update. Pp. 238-282 in Stable Isotopes in Ecology and Environmental Science. R. Michener and K. Lajtha, Eds. Blackwell Publishing, New Jersey, United States.

Muccio Z. and Jackson G.P. (2009). Isotope ratio mass spectrometry. *Analyst.* **134,** 213-222.

Newsome S.D., Tinker M.T., Monson D.H., Oftedal O.T., Ralls K., Staedler M.M., Fogel,M.L. and Estes J.A. (2009). Using stable isotopes to investigate individual diet specialization in California sea otters (*Enhydra lutris nereis*). *Ecology.* **90,** 961-974.

Ng J.S.S., Wai T.C. and Williams G.A. (2007). The effects of acidification on the stable isotope signatures of marine algae and molluscs. *Mar. Chem.* **103,** 97-102.

Noorollahi D., Lashkari H., Amirzade M., Azizi G. and Sharafi S. (2011). Climatic and environmental reconstruction based on stable isotopes of Parishan lake (Iran). *J. Rangel. Sci.* **1,** 203-216.

Paritte J.M. and Kelly J.F. (2009). Effect of cleaning regime on stable-isotope ratios of feathers in Japanese quail (*Coturnix japonica*). *Auk.* **126,** 165-174.

Pate F.D. and Anson T.J. (2007). Stable nitrogen isotope values in arid-land kangaroos correlated with mean annual rainfall: Potential as a palaeoclimatic indicator. *Int. J. Osteoarchaeol.* **18,** 317-326.

Paul D., Skrzypek G. and Fórizs I. (2007). Normalization of measured stable isotopic compositions to isotope reference scales: A review. *Rapid Commun. Mass Spectrom.* **21,** 3006-3014.

Peterson B.J. and Howarth R.W. (1987). Sulfur, carbon, and nitrogen isotopes used to trace organic matter flow in the salt-marsh estuaries of Sapelo island, Georgia1. *Limnol. Oceanogr.* **32,** 1195-1213.

Petzke K.J., Boeing H., Klaus S. and Metges C.C. (2005). Carbon and nitrogen stable isotopic composition of hair protein and amino acids can be used as biomarkers for animal-derived dietary protein intake in humans. *J. Nutr.* **135,** 1515-1520.

Philp R.P. (2015). Application of stable isotopes and radioisotopes in environmental forensics. Pp. 395-455 in Introduction to Environmental Forensics, B.L. Murphy and R.D. Morrison Eds. Elsevier, Amsterdam, Netherlands.

Phillips D.L. (2012). Converting isotope values to diet composition: the use of mixing models. *J. Mammal*. **93(2),** 342-352.

Phillips D.L. and Eldridge P.M. (2006). Estimating the timing of diet shifts using stable isotopes. *Oecologia.* **147,** 195-203.

Popa M.E., Vollmer M.K., Jordan A., Brand W.A., Pathirana S.L. and Rothe M. (2014). Vehicle emissions of greenhouse gases and related tracers from a tunnel study: CO: CO_2, N_2O: CO_2: CH_4: CO_2: O_2: CO_2 ratios, and the stable isotopes ^{13}C and ^{18}O in CO_2 and CO. *Atmos. Chem. Phys.* **14,** 2105-2123.

Post D.M. (2002). Using stable isotopes to estimate trophic position: Models, methods, and assumptions. *Ecology.* **83,** 703-718.

R Core Team. (2013). R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna.

Reid R.E.B. and Koch P.L. (2017). Isotopic ecology of coyotes from scat and road kill carcasses: A complementary approach to feeding experiments. *PloS One.* **12,** e0174897.

Rossi L., Calizza E., Careddu G., Rossi D., Orlandi L., Jona-Lasinio G., Aguzzi L. and Costantini M.L. (2018). Space-time monitoring of coastal pollution in the Gulf of Gaeta, Italy, using $\delta^{15}N$ values of *Ulva lactuca*, landscape hydromorphology, and Bayesian Kriging modelling. *Mar. Pollut. Bull.* **126,** 479-487.

Rossi L., Caputi S.S., Calizza E., Careddu G., Oliverio M., Schiaparelli S. and Costantini M.L. (2019). Antarctic food web architecture under varying dynamics of sea ice cover. *Sci. Rep*. **9,** 1-13.

Rossi L., Costantini M.L., Carlino P., di Lascio A. and Rossi D. (2010). Autochthonous and allochthonous plant contributions to coastal benthic detritus deposits: A dual-stable isotope study in a volcanic lake. *Aquat. Sci.* **72,** 227-236.

Rossi L., di Lascio A., Carlino P., Calizza E. and Costantini M.L. (2015). Predator and detritivore niche width helps to explain biocomplexity of experimental detritus-based food webs in four aquatic and terrestrial ecosystems. *Ecol. Complex.* **23,** 14–24.

Saggar S., Bettany J.R. and Stewart J.W.B. (1981). Measurement of microbial sulfur in soil. *Soil Biol. Biochem.* **13,** 493-498.

Schellekens R.C.A., Stellaard F., Woerdenbag H.J., Frijlink H.W. and Kosterink J.G.W. (2011). Applications of stable isotopes in clinical pharmacology. *British J. Clin. Pharmacol.* **72,** 879-897.

Schoeller D.A. (2002). Uses of stable isotopes in the assessment of nutrient status and metabolism. *Food Nutr. Bull.* **23,** 17-20.

Schulting R.J. (1998). Slighting the sea: Stable isotope evidence for the transition to farming in northwestern Europe. *Doc. Praehis.* **25,** 18-23.

Signa G., Calizza E., Costantini M.L., Tramati C., Sporta Caputi S., Mazzola A., Rossi L. and Vizzini S. (2019). Horizontal and vertical food web structure drives trace element trophic transfer

in Terra Nova Bay, Antarctica. *Environ. Pollut.* **246,** 772-781.

Stachowicz J.J., Bruno J.F. and Duffy J.E. (2007). Understanding the effects of marine biodiversity on communities and ecosystems. *Annu. Rev. Ecol. Evol. Syst.* **38,** 739-766.

Steinitz, R., and Kurle, C. (2014). Sample Collection Protocol for Stable Isotopes Analysis. International Union for Conservation of Nature – Iguana Specialist Group. Available at: http://www.iucn-isg.org.

Telsnig J.I.D., Jennings S., Mill A.C., Walker N.D., Parnell A.C. and Polunin N.V.C. (2019). Estimating contributions of pelagic and benthic pathways to consumer production in coupled marine food webs. *J. Anim. Ecol.* **88,** 405-415.

Vinagre C., Salgado J., Cabral H.N. and Costa M.J. (2011). Food web structure and habitat connectivity in fish estuarine nurseries-impact of river flow. *Estuar. Coast. Shelf Sci.* **34,** 663-674.

Wang J., Chen G., Kang W., Hu K. and Wang L. (2019). Impoundment intensity determines temporal patterns of hydrological fluctuation, carbon cycling and algal succession in a dammed lake of Southwest China. *Water Res.* **148,** 162-175.

Werner R.A. and Brand W.A. (2001). Referencing strategies and techniques in stable isotope ratio analysis. *Rapid Commun. Mass Spectrom.* **15,** 501-519.

Wilkinson D.J. (2018). Historical and contemporary stable isotope tracer approaches to studying mammalian protein metabolism. *Mass Spectrom. Rev.* **37,** 57-80.

Zeuner F.E. (1958). Dating the Past: An Introduction to Geochronology. Methuen, Massachusetts, United States.

Zheng Z., Xu Y., Wang J., Li Y. and Gu B. (2019). Environmental stress and eutrophication in freshwater wetlands: Evidence from carbon and nitrogen stable isotopes in cattail (*Typha domingensis* Pers.). *Ecol. Proc.* **8,** 31-37.