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Stable isotope tracers: Enriching our perspectives and questions on sources, fates, rates, and pathways of major elements in aquatic systems

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Abstract

Stable isotope applications have evolved from simple characterizations of isotope composition in organisms and organic matter, to highly complex methodologies on scales ranging from individual compounds and cells, to broad ecosystem-level approaches. New techniques are rapidly evolving, allowing novel, difficult, and inconvenient questions to be addressed. This article aims to provide an overarching perspective on how the field has evolved and where it is going with regard to aquatic systems, some of the oceanographic and limnological concepts derived from these approaches, and important challenges. To this end, we highlight a selection of natural abundance and tracer enrichment studies that represent a wide range of stable isotope applications. These include studies of rate processes and biogeochemical cycling, source tracking, food webs, and paleoenvironments. Our coverage of stable isotope applications is by no means complete, but by highlighting a mixture of classic and new applications across a wide range of research areas, our goal is to convey the power of stable isotope tools and the excitement in this rapidly expanding field, while also encouraging the scrutiny and healthy respect for limitations and assumptions necessary to take full advantage of these powerful tools.

Stable isotope analysis is a tool. Applications of stable isotope methods—from hydrography to physiology, geochemistry, and ecosystem management—are as varied as the creativity of the users and the questions being asked. Applications of stable isotopes have contributed to our understanding of many of the major concepts in oceanography and limnology. In this article, we aim to provide a perspective on stable isotope applications, how the field has evolved and where it is going as it relates to the aquatic realm, some of the concepts derived from these approaches and important challenges. This article is not a review per se—that is a near-impossible task, but by highlighting some classic, as well new approaches and studies, across a wide range of applications, our goal is to convey excitement in this rapidly expanding field, while also encouraging the scrutiny and healthy respect for limitations and assumptions that is necessary to take full advantage of these powerful tools. Examples and studies included herein are not meant to be comprehensive, but rather to be illustrative of the breadth of applications.

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This article begins with a synopsis of the vocabulary and fundamental concepts of stable isotope applications. A brief review of the time line in tools and capabilities is then given. Next, focusing on the major stable isotopes of carbon, nitrogen, oxygen, silicon, and sulfur (C, N, O, Si, and S)—but biased toward our experience with C and N—examples of applications and lessons learned from these applications are highlighted. In general, the types of questions/applications fall into several major categories that we will emphasize, including biogeochemical rate processes, source tracking, food webs, and paleoecology/global change (Fig. 1). By painting with a broad, multi-scale, multi-ecosystem, multi-isotope brush, it is hoped that this effort will serve as both an overview and introduction to methodological approaches and may inspire new questions, new applications, new conceptual advances, and cross-fertilization of ideas. The perspective we aim to convey is that the field has advanced from focusing primarily on tool development and testing, to increasing use of isotopic abundance and labeling to address novel, difficult, and inconvenient questions.

Basic principles

Kinetic isotope effects underlie many of the differences in stable isotope ratios that we observe among ecosystem

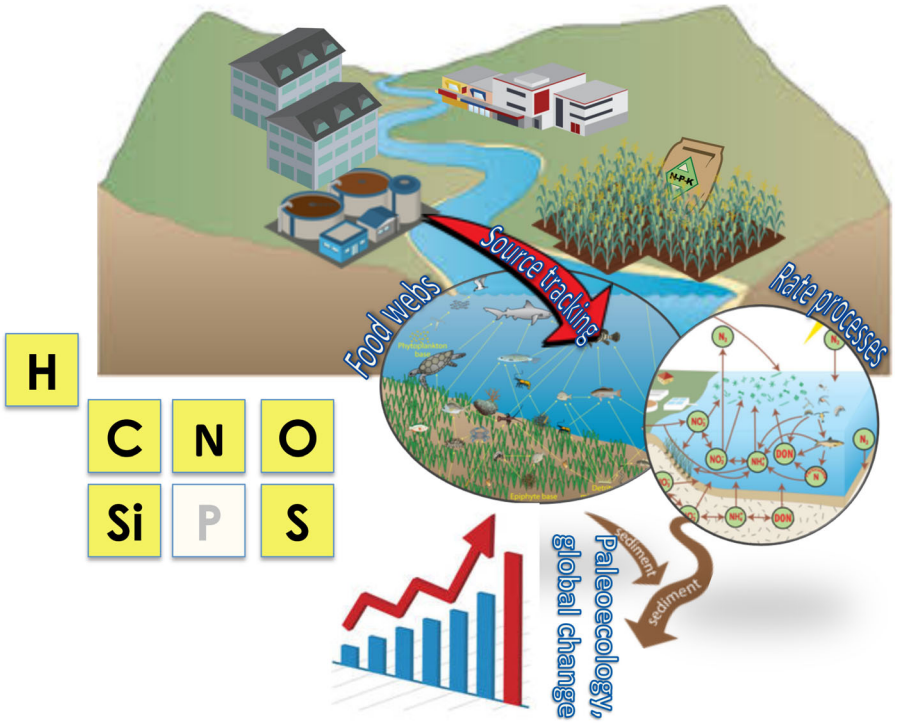

Fig. 1. Conceptual diagram of the elements for which the most common stable isotopes are used in ecology and the various applications emphasized herein.

components, and fractionation associated with specific biogeochemical reactions, different isotopes, and different physiological states of organisms are often distinct, allowing fundamental processes to be interpreted based on changes in stable isotope values. The nomenclature of stable isotopes is quite varied. Some of this variability relates to specific applications, but this does create potential for confusion. The vocabulary associated with enrichment studies is also different from that of the natural abundance stable isotope literature. Basic terms and relationships are defined and provided here; more detailed reviews of terminology and best practices based on publications of the Commission on Isotopic Abundances and Atomic Weights of the International Union of Pure and Applied Chemistry (IUPAC) can be found in Coplen (2011) and Bond and Hobson (2012).

In general, natural abundance stable isotope ratios are expressed using the lowercase delta (δ) symbol, which represents the deviation in isotopic ratio of a sample relative to a standard minus 1. Thus,

$$\delta = R_{\text{sample}}/R_{\text{standard}} - 1 \quad (1)$$

in which R is the isotopic ratio of sample or standard (e.g., $^{15}N/^{14}N$). The typical standards are atmospheric N for $\delta^{15}N$, the (Vienna) PeeDee Belemnite limestone for $\delta^{13}C$, and Vienna standard mean ocean water for ^{18}O or ^{17}O.

Many studies report $\delta \times 1000$ to amplify the small differences between samples and standards. In many such studies, the unit (parts per thousand; ‰, per mil) may be implied rather than given expressly (e.g., Fry 2006). However, δ values may also be reported as parts per hundred (% or percent), parts per million (ppm), or other units, depending on the specific element or application (Coplen 2011).

Differences in δ values between two substances are expressed with an uppercase delta, Δ.

Thus:

$$\Delta_{A-B} = \delta_A - \delta_B \quad (2)$$

Differences in δ or Δ, for example $\Delta\delta^{15}N$ or $\Delta\delta^{13}C$, may be between reactant and product, food source and consumer, or any other comparison between a measured value and a baseline, however that is defined. Values of Δ may reflect changes in isotope ratios associated with isolated processes or net effects of multiple factors influencing differences in isotope values between any two pools of interest. The Δ term is also used for mass-independent isotope variation (e.g., $\Delta^{17}O$; $\Delta^{33}S$; $\Delta^{36}S$) to express deviations from a specific mass-dependent fractionation law and for *clumped* isotopes (e.g., Δ^{47}) to quantify deviations from a stochastic distribution (Eiler 2007). Clumped isotopes represent a comparatively new field of isotope applications, in which variations in the isotopic composition of both C and O of CO_2 (representing the substitution of ^{13}C and/or ^{18}O in the CO_2 molecule—with a mass of 47) are measured in the same sample, with the advantage that inferences, such as those related to paleotemperatures, can be

made without assuming a value for the $\delta^{18}O$ value of seawater. It is therefore important for practitioners to define how Δ is being used and what it represents in any given study.

Differences in δ result from equilibrium or kinetic isotope effects. The fractionation factor associated with a particular isotope can be designated as α, the ratio of isotope ratios in product (P) and reactant (quotient Q), or:

$$\alpha = R_P / R_Q \quad (3)$$

More formally α is often defined as the ratio of $R({}^iE/{}^jE)_P$ and $R({}^iE/{}^jE)_Q$, where R is the isotope ratio of heavier isotope ${}^iE$ and lighter isotope ${}^jE$ of element E in substances P and Q; α thus represents the rate constants for the heavy and light isotopes. As with Δ, there are variations in the literature in how α is defined, specifically which substance, and therefore which isotope, the lighter or heavier, is in the numerator or denominator of each R (e.g., Hayes 2004; Fry 2006). Many geochemists prefer the definition above, with the lighter isotope in the denominator, while many chemists and biologists adopt the inverse formulation (Fry 2006). Where the lighter isotope reacts faster and α is defined with the lighter isotope in the denominator, $\alpha - 1$ is defined as ε, the kinetic isotope effect. Thus:

$$\varepsilon = \alpha - 1 \quad (4)$$

This kinetic isotope effect, or isotope discrimination factor, can be thought of as the ratio of rates with which isotopes are converted from reactant to product. This value, as with δ, may be multiplied by a factor, typically 1000 and expressed per mil (‰) to magnify small differences (Mariotti et al. 1981; Lajtha and Michener 1994). ε and Δ are approximately equal when δ values fall within the natural abundance range, but they diverge as δ values become increasingly positive or negative (Coplen 2011). As a rule of thumb, ε and Δ can be used interchangeably when δ values fall between +20‰ and −20‰ (Fry 2006). As discussed above for Δ, the isotopic fractionation factor α (as well as ε) can be used with more or less specificity, for example, as related to equilibrium reactions, reactants and products of nonreversible reactions, or net effects of multiple factors, and thus it is important to define what the calculated values specifically represent in a given study. Ultimately, Δ, α, and ε are contextually defined.

For unidirectional reactions in a closed system, kinetic isotopic fractionation follows that of a Rayleigh distillation model (reviewed by Sigman and Casciotti 2001; Sigman et al. 2009; Van Hale and Frew 2010; Fig. 2). As reactant is converted to product, instantaneous isotope fractionation (ε_{inst}; i.e., the fractionation inherent to the reaction) remains constant, but the observed fractionation (ε_{net}) between the residual reactant pool and the cumulative product changes. In general, the lighter isotope is incorporated into product at a faster rate than the heavier isotope, causing the residual reactant pool to become progressively more enriched in the heavier isotope with time. Newly formed product remains offset from the residual reactant pool by ε_{inst}, while the isotope ratio of the cumulative product converges on that of the original reactant pool. When all of the heavy and light isotopes in the reactant pool have been converted to product, there is no longer an observable isotope effect. However, even when kinetic fractionation effects are strong, resolving the different processes that contribute to these effects remains challenging.

Applications involving added isotope to trace enrichment during a process generally involve a different set of terms and assumptions (e.g., Dugdale and Goering 1967; Dugdale and Wilkerson 1986; Glibert and Capone 1993; Fry 2006). In this case, the atom fraction or atom% notation is more common than the δ notation. Atom fraction is an absolute measure of the number of atoms of the isotope relative to 100 atoms of the element, the mole fraction of heavy isotope relative to heavy plus light. When expressed as a percent, this value is known as atom percent (at %, or ${}^{15}AP$, in which the superscript represents the heavy isotope—in this example ${}^{15}N$). Atom fraction or at %, assuming no measurement error, are always precise, whereas Δ is a relative value. The difference between δ and at % are illustrated for N_2, for which natural atmosphere is the accepted standard. Its δ value is set to 0.0‰, but its at % is 0.366, as atmospheric N_2 has 0.366 atoms of ${}^{15}N_2$ in 100 atoms of N_2. Atom fraction excess (or in % terms, at % excess, APE) is that atom fraction in a sample minus the measured standard. At % enrichment is the amount of enrichment added in the experiment (e.g., ${}^{15}NO_3$) relative to the total NO_3 (added + ambient).

The velocity of the rate of uptake or transformation of an isotopic enrichment into a product (V, also termed specific uptake rate, with units of reciprocal time, i.e., h^{-1}), is the excess atom fraction of the target or product pool divided by that of the initial enrichment times the duration of the experiment (Sheppard 1962; Dugdale and Goering 1967):

$$V = \text{at\%excess of product} / [\text{at\%enrichment of substrate} \times \text{time}] \quad (5)$$

Tracer experiments are commonly used in determining the uptake rate of N by phytoplankton. Collos (1987) suggested a modification to Eq. 8 for conditions under which the phytoplankton may be assimilating more than one N compound; this may cause substantial artifacts due to the nature of the measurements of ratios of the isotopic compound. Glibert et al. (1982*b*) also modified this equation to account for the dilution of isotope that occurs due to remineralization of substrate (especially NH_4) during the incubation. The magnitude of errors associated with failure to apply such modifications depends on the type of sample (e.g., laboratory culture vs. field sample), length of incubation, amount of isotope added, and other factors. Kinetic isotope effects in understanding of enrichment studies are typically

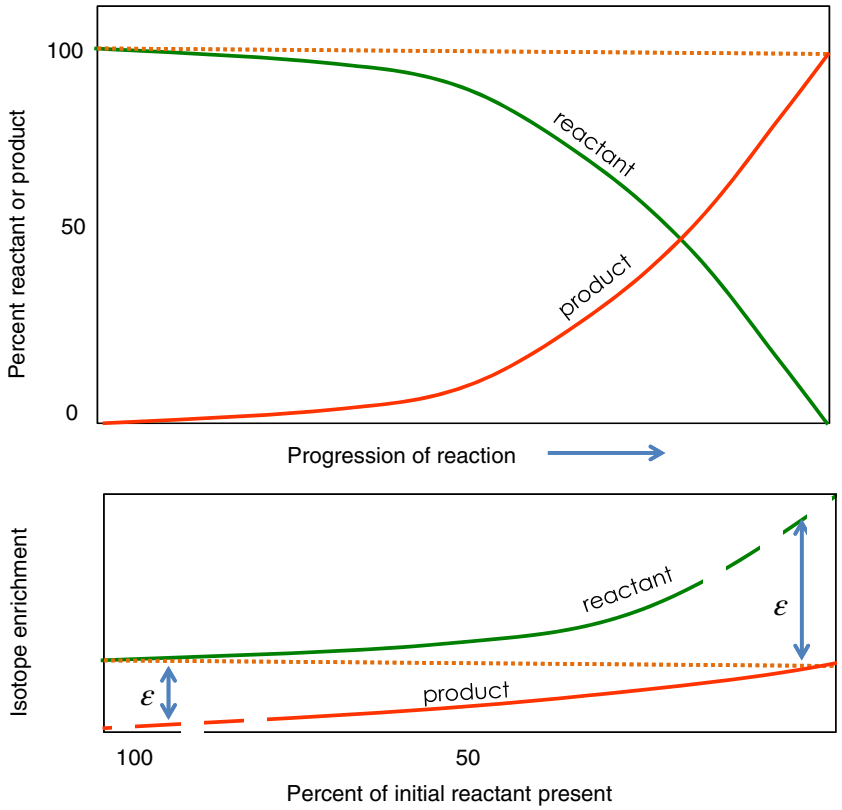

Fig. 2. Relationship between isotope fractionation of reactant and product and their consumption, Rayleigh distillation kinetics. The term ε denotes the difference in isotope enrichment between reactant and product. Note that at the initiation of the reaction and near completion of the reaction, this value is difficult to determine as there is either virtually no product at the start and if the reaction has gone to completion, no reactant at the end (ends of these curves have dashed lines).

ignored, as the magnitude of tracer addition generally overwhelms such effects and it is thought that introduced isotopes do not modify metabolic pathways. However, Andriukonis and Gorokhova (2017) have shown that there are profound differences in the growth of algae when provided ^{15}N enriched media. Such effects may need to be (re)considered when very high isotope enrichment levels are used, as in experiments involving DNA or proteome labeling.

To calculate an absolute flux (denoted as ρ), such as a rate of uptake or transport of a particular N compound, the specific uptake rate (V) is multiplied by the concentration of that element in the target pool. For example, for N uptake,

$$\rho = V \times \mathrm{PN} \tag{6}$$

where PN is the particulate N concentration of the sample, ideally measured at the end, rather than beginning of an incubation. In nitrification studies where the added isotope is NH_4, the velocity of the rate is calculated the same way, but the target pool is the concentration of NO_2 or NO_3.

In sum, isotope notation, regardless of preferred terms, is the consideration of ratios: ratios of the less abundant to the more abundant isotope, ratios in samples relative to standards, and ratios of enrichment relative to total compound availability. Many final calculated values are, in fact, ratios of ratios of ratios. Moreover, common notations and calculations are derived to amplify the often very small differences between samples and standards. This can make it difficult to resolve sensitivities and/or sources of error. The importance of recognizing sources of error and assumptions in isotopic approaches is a theme returned to repeatedly throughout this article.

The isotope time line

Mass spectrometers have been in use for about a century for isotope analysis, initially largely in laboratories of a few renowned physicists, but the sophistication of isotope applications is rapidly advancing, and tools are quickly evolving (Fig. 3). Pioneering work by Craig (1953) revealed that C isotope differences among organisms are primarily generated during primary production. Through seminal work by Goering et al. (1964, 1966), and then by Dugdale and Goering (1967), tracer studies of N were developed. By applying ^{15}N tracers, mostly in the form of $^{15}NO_3$ and $^{15}NH_4$, the understanding of environmental regulation of N uptake by phytoplankton was

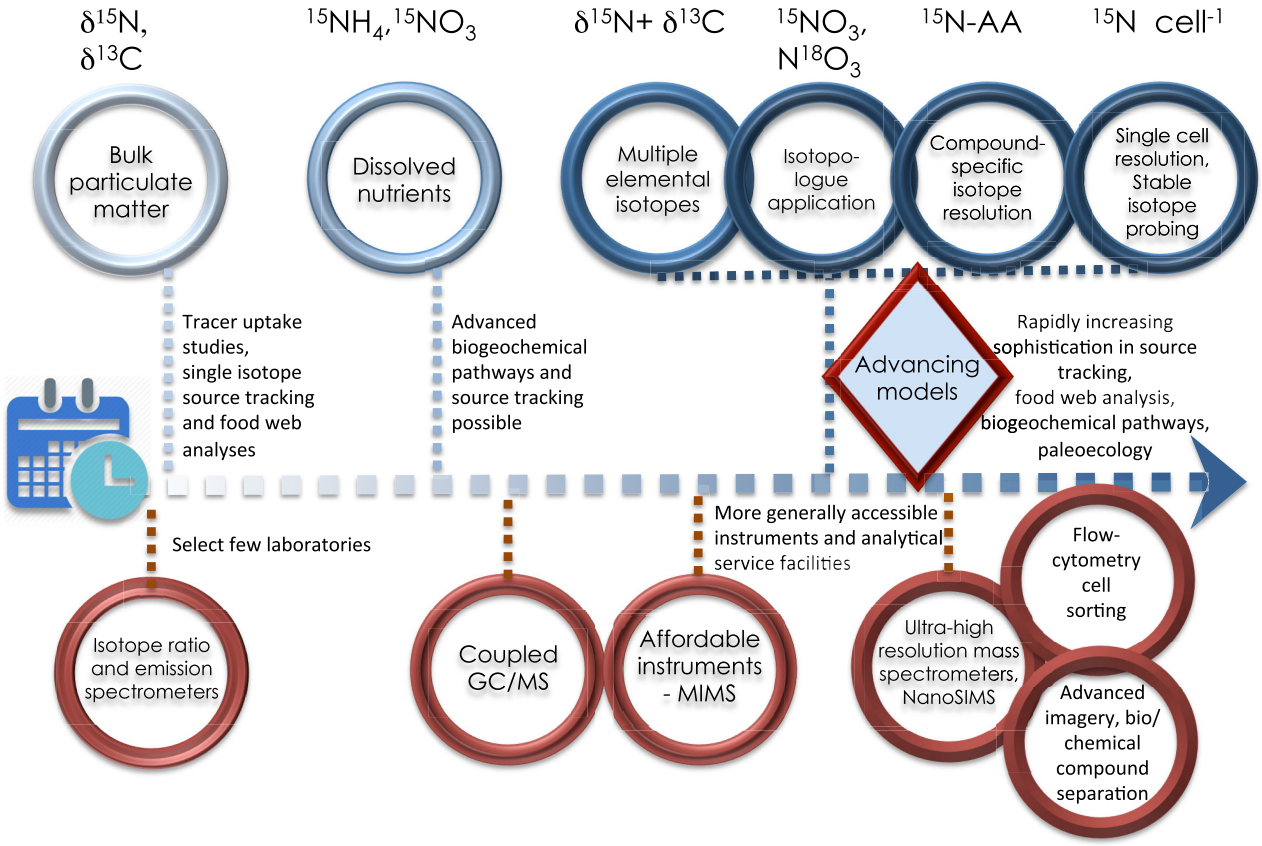

Fig. 3. Infographic depicting the generalized time line of isotope applications (top) and instrumental developments (bottom).

made possible. From these studies, the theoretical basis for the differentiation of "new" and "regenerated" N was developed (Dugdale and Goering 1967), and several decades of focused work on phytoplankton nitrogenous physiology was made possible. Early natural abundance work showed that C isotopes are transferred to consumers without fractionation (DeNiro and Epstein 1978), while N isotope ratios of consumers become enriched by a few per mil during trophic transfers (DeNiro and Epstein 1981; Minagawa and Wada 1984). The latter studies provided the basis and rationale for the use of N isotopes in trophic transfer studies.

Most measurements made during this era were on "bulk" particulate matter, whether algal suspensions from laboratory cultures or field-filtered participate matter, and analyzed using isotope ratio or emission spectrometers. Size fractionated material (e.g., Glibert et al. 1982*a*; Probyn 1985; Probyn and Painting 1985; Probyn et al. 1990), and that of isolated larger organisms, such as zooplankton and tissue of larger animals, was also possible (e.g., Montoya et al. 1990). These analyses generally required a relatively large sample size, precluding analysis of single microbes, protists, or individual nematodes or zooplankton. While organisms of a few 10 s of micrometers can be physically isolated, lumping of individuals was necessary to obtain the minimum μg of C or N necessary for isotopic measurements. This limited species-specific isotope ratio measurements and investigations of individual life stages of these small organisms. Based on work by Carman and Fry (2002), modifications to conventional elemental analysis and mass spectrometry approaches were developed that allowed for both ^{13}C and ^{15}N analysis in very small samples.

Techniques for isolation or extraction of dissolved nutrients, e.g., NH_4, NO_3, NO_2, were a major advance, furthering the ability to resolve rates of transformation of pathways such as denitrification, nitrification, and ammonification. Such methods were chemically and labor-intensive, and even more so in the presence of salt. The ability to resolve the $^{15}NH_4$ and its change over time in experiments in which tracer $^{15}NH_4$ was added led to an expansion in application of isotope dilution approaches in sediment and water-column studies (e.g., Harrison 1978; Blackburn 1979; Caperon et al. 1979; Glibert 1982; Glibert et al. 1982*b*; Paasche and Kristiansen 1982; Harrison and Harris 1986). Isotope dilution approaches allow the simultaneous resolution of two opposing processes, such as N incorporation and N regeneration in a natural sample. An enrichment of isotope is made, and its incorporation is measured in biomass. However, over time, the atom fraction of the isotope in dissolved form (i.e., the NH_4 in water) will decline because of substrate recycling that favors the lighter isotope (Glibert et al. 1982*b*). This is due to the fact that those organisms carrying out regeneration, such as microzooplankton, will release NH_4 that is not

isotopically labeled as the short time scale of the experiment precludes this. Sigman et al. (1997) developed an extraction method for NO_3 in seawater that soon found widespread use for assessment of the isotope effects associated with nitrification.

Mass spectrometers advanced and the coupling of gas chromatographs (GC) with mass spectrometers were especially important (Fig. 3). The development of the continuous flow interface between an elemental analyzer and isotope ratio mass spectrometer allowed for automation of sampling by the instrument (no longer did individual samples have to be prepped independently for injection into the mass spectrometer). Sample throughput increased many fold and environmental and ecological applications became routine. With more rapid and automated analysis, ecological and biogeochemical studies could now be replicated, and environmental gradients could be better resolved. The coupling of gas chromatographs and mass spectrometers also facilitated the dual analysis of C and N on the same sample.

More affordable mass spectrometers, which included quadrupole mass spectrometers, became available, mostly in the early 1990s (Fig. 3). Quadrupole mass spectrometers led the way to applications of membrane inlet mass spectrometry (e.g., MIMS, Kana et al. 1994), which can now resolve gasses in water with very high precision (e.g., N_2/Ar) and can be used for isotope discrimination as well. A MIMS instrument coupled to a quadrupole mass spectrometer is often used, for example, in applications of the isotope pairing method for denitrification and N_2 fixation (e.g., An et al. 2001; Lundstrum and Acki 2016).

Compound-specific isotope analysis (CSIA) has also been rapidly advancing (Hayes et al. 1990; Boschker and Middelburg 2002; Middelburg 2014; Figs. 3–4). Here, rather than bulk isotopic analysis of particulate or aqueous samples, various biomarkers, such as lipids, carbohydrates, or amino acids (AAs), are extracted and their isotope composition compared (Boschker and Middelburg 2002). Biomarkers are compounds that ideally are specific to a species, or more usually a group of organisms. The CSIA approach can be used at natural abundance as well as for tracer applications. When coupled with other advancing methods, such as those of molecular biology, this approach can be very powerful in the analysis of microbial communities, and also of the diet of animals (e.g., McClelland and Montoya 2002; de Kluijver et al. 2012). In food web studies, the ability to resolve AAs aids dietary reconstruction in that the ^{13}C and ^{15}N enrichment of individual AAs between food source and consumer varies widely, but essential AAs have distinct isotope fingerprints which may be passed through food webs without modification, giving clues of source identity. In addition to food web studies, CSIA is finding increasing application in studies of compound biodegradation where contaminants are of concern.

The ability to resolve isotopes of microbes has advanced with the ability to physically isolate microbes by techniques such as flow-cytometry coupled to cell sorting and subsequent isotope analysis. Flow cytometric sorting of phytoplankton or bacterioplankton cells followed by isotope ratio mass spectrometry has been done both for natural abundance studies (e.g., Pel et al. 2003; Bontes et al. 2006 in lakes; Fawcett et al. 2011 in ocean) and tracer applications (e.g., Lipschultz 1995; Bontes et al. 2006). Recently, van Roij et al. (2017) reported a laser ablation combustion isotope ratio mass spectrometer allowing for measurement of ng of particulate organic C. Such a technique was also used to measure $\delta^{13}C$ in single-species dinoflagellate cysts (Sluijs et al. 2017). This technique can, in principle, bridge the gap between traditional isotope data for larger organisms and compound-specific isotope ratio mass spectrometry for microbes (Fig. 4). Resonance Raman microspectrometry in combination with ^{13}C-bicarbonate labeling has been introduced to probe single-cell growth rates of phytoplankton (Li et al. 2012; Taylor et al. 2017). Cavity ring-down spectroscopy is also emerging as a new high-throughput, easy-to-use technique providing high-quality isotope ratio data for C in dissolved and particulate organic and inorganic C and methane, for N in nitrous oxide, and H and O in water (Becker et al. 2012; Maher et al. 2013; Erler et al. 2015). The relatively modest investment and robustness of this method will further stimulate the use of stable isotopes in experimental and field studies.

Nanometer-scale secondary ion mass spectrometry (nanoSIMS) provides isotope ratio imaging with a spatial resolution of < 100 nm and has been used together with ^{15}N, ^{13}C, or ^{2}H labeling to study dissolved inorganic C uptake, N_2 fixation, NH_4 and NO_3 assimilation by phytoplankton communities (Ploug et al. 2010; Musat et al. 2012), mixotrophy (Terrado et al. 2017), symbioses (Thompson et al. 2012), C processing by cable bacteria (Vasquez-Cardenas et al. 2015) and consortia of microbes in sediments (Hatzenpichler et al. 2016). When coupled with other highly sensitive tools such as molecular approaches, information on microbial associations and other difficult to resolve pathways is now possible. An additional method gaining rapid application within microbial ecology is that of stable isotope probing (SIP, e.g., Radajewski et al. 2000; Neufeld et al. 2007). In these methods, an enrichment is made with an isotopic compound, e.g., ^{13}C or ^{15}N, and after uptake by natural or cultured organisms, separation of nucleic acids is undertaken using a density gradient ultracentrifugation. Nucleic acids with different densities can thus be separated and their differential incorporation of the label can be measured. Various molecular analyses of these labeled nucleic acids can be undertaken in tandem, using fingerprinting, clone libraries, metagenomics, and so on. By linking function and taxonomic identity, the responses and interactions of microorganisms to ecological conditions can be resolved at very fine scales.

Collectively, these tools are rapidly increasing sophistication in biogeochemical studies, source tracking, food web analysis, and paleoecological studies. As will be shown in the

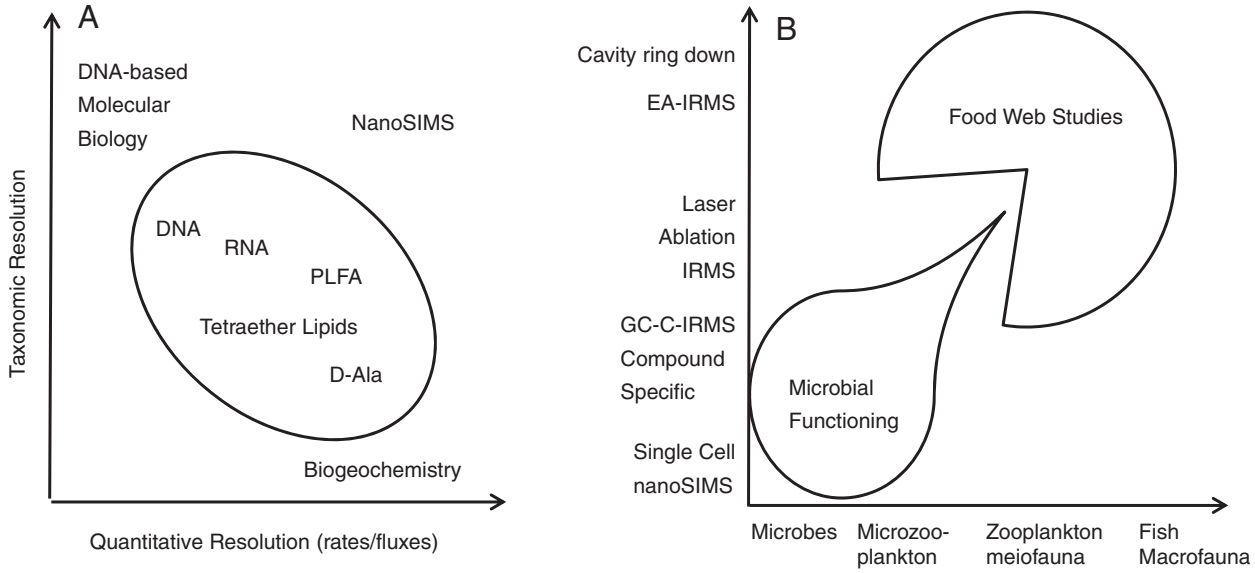

Fig. 4. Two conceptual depictions of the relationship between organismal insight and the quantitative resolution or instrumental approach necessary. Panel **A** highlights that biogeochemical rate measurements (often based on isotope techniques) provide accurate quantification but no information on the identity of organisms involved. Molecular biology tools provide high-quality information on the presence and potential capabilities of organisms, but limited information on their rates. Compound-specific isotope characterization of biomarkers (DNA, RNA, PLFA, tetraether lipids, D-alanine) in combination with tracer techniques provide rates as well as identity information but with low-to-moderate taxonomic resolution and intermediate accuracy. NanoSIMS can provide individual cell isotope ratio measurements and thus provides high accuracy and high-taxonomic resolution, as well as spatial context. Modified and redrawn from Middelburg (2014) under a creative commons license. Panel **B** highlights how different isotope analysis can be related to either microbial functioning or food web studies and the scale of the organisms for which they are most applicable.

next section, the examples of applications are wide and stable isotopes have become a critical part of a larger toolkit by which ecological, oceanographic, limnological, and paleoecological questions are addressed.

A deeper dive: Insights from classic to cutting-edge isotope applications

Examples that are highlighted here were selected to provide a breadth of approaches, ecosystems, questions addressed, or insights gained. These examples paint the field with a broad brush, a glimpse into this enormous diversity of applications, and are broadly divided in studies related to rate processes and biogeochemical cycling, source tracking based on isotopic values, food web studies, and paleoecological/paleoceanographic studies. These categories are for convenience only, as many approaches and insights span multiple disciplines.

Rate processes and biogeochemistry

Stable isotope applications for the study of rate processes and biogeochemical cycling are so extensive that justice cannot be done to all the insights that have been so gained; only a few snapshots are captured here. Quite simply, isotopes—and especially isotope enrichment studies—provide the foundation for our understanding of the biogeochemical pathways of nutrient cycling and the physiological rate processes associated with primary production and nutrient assimilation.

Nitrogen

An enormous literature exists with respect to applications of stable isotopes in studies of oceanic N cycles (see reviews by Sigman and Casciotti 2001; Sigman et al. 2009; Casciotti and Buchwald 2012; Ryabenko 2013). Much is known regarding the isotopic characteristics of the predominant sources and sinks of N, and processes such as N_2 fixation, nitrification, and denitrification.

The fixation of N_2, which has been extensively studied in the ocean (with a particular focus on *Trichodesmium*), introduces bioavailable N at isotopic values near atmosphere; i.e., there is little isotopic discrimination ($\delta^{15}N \sim 0‰$; Minagawa and Wada 1986; Macko et al. 1987; Montoya et al. 2002). Not only does this "light" N_2 (compared to oceanic NO_3, for example) produce a characteristic organic matter isotopic signature, but it can also be traced through the food web (Montoya et al. 2002). Thus, using a mass balance approach, Montoya et al. (2002) were able to estimate the N fixed by diazotrophs according to:

$$\%\,\text{diazotrophs} = 100\left(\left(\delta^{15}N_m - \delta^{15}N_{ref}\right)/\left(\delta^{15}N_d - \delta^{15}N_{ref}\right)\right) \quad (7)$$

where $\delta^{15}N_m$ is that measured in the sample, $\delta^{15}N_{ref}$ is the reference value for nondiazotophic plankton, and $\delta^{15}N_d$ is that of the diazotrophs. This approach was applied in an assessment of the influence of *Trichodesmium* in the stable isotopic composition of different size fractions along in a study of the

subtropical North Atlantic (Mompeán et al. 2013). This study showed that the contribution of atmospheric N was larger than anticipated, and there was a major dependence of the planktonic food web on this N derived by fixation, revealed by the calculated contribution of N from diazotrophs to four different size classes of plankton.

Denitrification, in contrast to N_2 fixation, is a process with strong isotopic discrimination. Denitrifiers, in consuming NO_3 in the water column, use the lighter form of N at a faster rate than the heaver form of N, leaving NO_3 in the dissolved pool that can be substantially enriched with ^{15}N. Isotope discrimination factors are on the order of 20–30‰ (Cline and Kaplan 1975; Altabet et al. 1999; Voss et al. 2001). In the sediment, however, denitrifiers are more often limited by available NO_3, and consume most (all) of it, and thus do not leave a residual enriched pool of NO_3 and isotopic discrimination is not resolvable (Brandes and Devol 1997; Sigman et al. 2003, 2009). To overcome this problem in resolving sediment denitrification rates, the isotope pairing method may be applied, which is based on measurements of the changes in N_2 gas with different isotope compositions ($^{29}N_2 = {}^{14}N + {}^{15}N, {}^{30}N_2 = {}^{15}N + {}^{15}N$) after enriching the overlying water with $^{15}NO_3$ (e.g., Nielsen 1992; Middelburg et al. 1996; Steingruber et al. 2001).

Although the processes of NO_3 assimilation and denitrification have been shown to have fairly strong isotopic effects using N isotope analysis, unraveling those associated with the process of nitrification has proven to be more challenging. This is largely due to the fact that nitrification is carried out through NH_4 oxidation to NO_2 and subsequent NO_2 oxidation to NO_3. The former is due to chemoautotrophic processes of NH_4-oxidizing bacteria and archaea (AOB and AOA), while NO_2 oxidation is due to the chemoautotrophic process of NO_2-oxidizing bacteria (Casciotti and Buchwald 2012). Nitrification also processes N_2O as an intermediate. By using multiple isotopes within the same compound, isotopologues, different processes could be revealed. By resolving both $\delta^{18}O$ and $\delta^{15}N$ in N_2O, it was shown that the N_2O produced by AOA incorporates O from O_2, not H_2O, and the $\delta^{15}N$ and $\delta^{18}O$ produced by AOA is more enriched than that produced by AOB (Santoro et al. 2011). Moreover, dual isotope measurements of both NO_2 and NO_3 have revealed NO_3 regeneration in the euphotic zone and in O_2 deficient zones (e.g., Sigman et al. 2005), and thus nitrification is now recognized to play a significant role in the oceanic N cycle (e.g., Casciotti and Buchwald 2012). Recently, triple isotopes ($\delta^{15}N$, $\delta^{18}O$, and $\Delta^{17}O$ of NO_3) have been applied to the study of rates of nitrification and the assimilation of NO_3 in streams (Rose et al. 2015) and lakes (Tsunogai et al. 2018). Due to the fact that atmospheric NO_3 generally has positive $\Delta^{17}O$ values, while microbially derived NO_3 has $\Delta^{17}O$ values ≤ 0, measurements of $\Delta^{17}O$ of NO_3 were found to reliably resolve atmospheric NO_3^- and collectively, the isotope analyses yielded gross nitrification and metabolic rates of NO_3^- where there were detectable quantities of NO_3 in the water column.

Isotopes, together with molecular approaches, have also been used to detect and measure anammox in sediments and water, and these methods have been reviewed by Song and Tobias (2011). It is clear that isotope effects of O or N associated with NO_3 reduction in field studies is variable, complicated by variability in microbial communities. Isotopic effects associated with O may be decoupled from those of N, driven by NO_2 reoxidation where denitrification dominates, or net NO_3 production where anammox dominates (Dähnke and Thamdrup 2015).

Algal assimilation of N also discriminates, with uptake of inorganic nutrients containing lighter isotopes occurring faster than uptake of nutrients containing heavier isotopes. Discrimination factors vary considerably depending on environmental conditions and specific N form, but range between ~ 0‰ and 30‰ (Altabet et al. 1991, 1999; Sigman et al. 1999; Altabet and Francois 2001; Armstrong et al. 2018). Early studies suggested isotope discrimination during the process of NO_3 assimilation (Wada and Hattori 1976), but various subsequent studies attempted to resolve whether the isotopic discrimination was at the transport step (e.g., Montoya and McCarthy 1995) or the enzymatic reduction of NO_3 (Granger et al. 2004). As regeneration also discriminates, with production of the lighter isotope, measured values of dissolved and particulate N represent the net effect of N_2 fixation, denitrification, assimilation, and regeneration, and distinguishing these different effects and contributions remains a daunting challenge.

The processes of algal N assimilation have been extensively studied with tracer enrichments. Tracer enrichment studies, in which isotope is often added at a level of ~ 10% of the ambient source (e.g., Dugdale and Goering 1967; Dugdale and Wilkerson 1986), have contributed extensively to our understanding of new and regenerated production, oceanic *f* ratios, i.e., that fraction of primary production fueled by NO_3 (e.g., Eppley and Peterson 1979), understanding of relative preferences (e.g., McCarthy et al. 1977; Glibert et al. 1982*a*; Smith and Nelson 1990), and relationships between the uptake of different N forms and phytoplankton community composition (e.g., Lomas and Glibert 1999; McCarthy 2002; Berg et al. 2003; Glibert et al. 2004). Tracer enrichment studies have also contributed substantially to characterization of physiological parameters such as nutrient kinetic relationships (e.g., Goldman and Glibert 1983; McCarthy et al. 1992; Collos et al. 1997; Lomas and Glibert 1999; Kudela and Cochlan 2000; Fan et al. 2003; Cochlan et al. 2008; Li et al. 2010; Lindehoff et al. 2011), and in the understanding and quantification of processes such as NH_4 repression of NO_3 assimilation (e.g., Wheeler and Kokkinakis 1990; Dugdale et al. 2007; L'Helguen et al. 2008; Glibert et al. 2014, 2016).

In addition to isotope tracer studies carried out with isotope compounds readily purchased from chemical suppliers, much work has been done in using isotopes to understand and quantify the sources and rates of cycling of complex organic

material. As one example, Glibert and Bronk (1994) enriched colonies of the diazotroph *Trichodesmium* with N_2 gas, and after a period of incubation, measured the isotopically labeled dissolved organic N (DON) released and compared those rates with rates of N_2 fixation determined for the same assemblages at the same times of day. These experiments documented that on average, rates of DON release were ca. 50% the rates of N_2 fixation, and that 60–80% of the total organic release was comparatively small molecules. These data showed that release of these organic compounds could be a significant source of new N for the associated bacteria or the non-N_2-fixing filaments of the *Trichodesmium* colonies. The study of the contribution of DON to phytoplankton nutrition has also been advanced through application of labeled complex organic material. Using ^{15}N dissolved organic material produced in the laboratory (through growth of phytoplankton, bacteria, and plants such as *Spartina alterniflora* enriched with ^{15}N media and subsequent extraction of the organics from biomass), and then fed to various algal cultures, plankton communities, and seagrasses, the use of natural DON has been quantified (e.g., Gagnon et al. 2005; See et al. 2006; Bronk et al. 2007; Van Engeland et al. 2013 and references therein).

These and other related studies collectively demonstrate that much of our understanding and insight into biogeochemical processing of N has come from applications of stable isotopes. Whether it is the physiological processes of uptake on the time scale of minutes to hours, to the multiday scales of whole stream studies, to studies integrating over long time scales, our understanding of N cycling pathways is largely a result of creative applications of natural abundance and enrichment stable isotope studies.

Carbon and oxygen

Plankton photosynthesis and primary production are routinely measured using tracer techniques, although application of stable isotopes is not as common. Among the stable isotope tracer methods are ^{13}C and ^{18}O. The ^{13}C method, analogous to that of the radiotracer ^{14}C, is relatively straightforward, as $H^{13}CO_3$ is added as a tracer and its incorporation into particulate material (and sometimes dissolved organic material) over time is measured (Hama et al. 1993) and yields estimates similar to those determined by ^{14}C (e.g., Cullen 2001; Pimenov et al. 2008). There are basic differences, however. With ^{14}C, an absolute amount of isotope incorporation is measured, whereas with ^{13}C, a ratio is determined. Thus, if C sources other than the $H^{13}CO_3$ label are incorporated (e.g., organic C substrates), there can be biases leading to underestimates of productivity, but these can be corrected if the final particulate C concentrations is considered (Collos and Slawyk 1985). The ^{13}C method can be applied in situ without the concerns associated with use of radioactive isotopes and can, in combination with CSIA, be used to quantify group-specific primary production via measurement of ^{13}C incorporation into biomarkers such as alkenones (Popp et al. 2006) or phospholipid-derived fatty acids (Dijkman et al. 2009; de Kluijver et al. 2010, 2013). Moreover, it does not only provide information on C fixation, but also on the transfer of recently fixed C to heterotrophic bacteria and other consumers in benthic ecosystems (Middelburg et al. 2000; Oakes et al. 2012) as well as in the plankton (Norrman et al. 1995; Van den Meersche et al. 2004; Pace et al. 2007), including its dependence on ocean acidification (de Kluijver et al. 2010, 2013). Using CSIA coupled with total ^{13}C incorporation, Grosse et al. (2015) followed the incorporation of fixed C into neutral carbohydrates, AAs, and fatty acids for several sites in the North Sea and found distinctly different allocations of C under P and N limiting conditions. The application of ^{13}C tracers coupled with metabolomics studies as a means to understand functional pathways is rapidly advancing (You et al. 2014 and references therein).

The measurement of productivity with ^{18}O begins with enrichment of sample water with $H_2{}^{18}O$ (Bender et al. 1987, 1999, 2000) and the enrichment of $^{18}O_2$ over time is determined, reflecting the product of the splitting of water during photosynthesis. This method, which is much more labor intensive and technically difficult than that of ^{13}C incorporation, is thought to be a better measure of true gross primary production, but there are uncertainties. Key questions are the associated cycling of O_2 in the cells via respiration through the cytochrome oxidase pathway, respiration by the alternative oxidase pathway, photorespiration, and the Mehler reaction (Kana 1992; Robinson et al. 2009; Regaudie-de-Gioux et al. 2014). A recent comparison of estimates of marine primary productivity derived by these and other methods confirms that the highest reported rates of primary production are, in fact, derived from ^{18}O enrichment methods (Regaudie-de-Gioux et al. 2014). The ^{18}O method has also been used to estimate gross primary production, community metabolism, and net metabolism in a productive agricultural stream in the Midwestern US (Tobias et al. 2007).

An alternative ^{18}O method for measuring productivity was introduced using a modified MIMS dissolved gas analyzer (Kana et al. 2006). This method overcomes the tedious extraction process used in traditional ^{18}O mass spectrometry, giving a much more simple method, with quick turnaround, ~ 5 min total, and which cross-calibrates well with the traditional approach (Ferrón et al. 2016). With shipboard processing possible, this allows many more samples to be run, more questions to be addressed. For example, at the Pacific Ocean long-term time series station, where gross primary production ranged from 0.2 μmol O_2 L^{-1} d^{-1} to 1.1 μmol O_2 L^{-1} d^{-1}, a standard primary production depth profile with six depths and triplicate samples for each depth could be analyzed in approximately 3–4 h. A recent review (Mader et al. 2017) explored recent developments in the use of stable isotope ratios of O ($^{18}O/^{16}O$ and $^{17}O/^{16}O$) and how isotopic shifts have provided insights into sources and sinks of O, rates of productivity, and other processes such as gas–water exchange.

Hydrogen

The heavy isotope of hydrogen, deuterium, δ^2H has been used for tracing the water cycle. Transitions between water vapor and liquid water, evaporation and condensation, involve fractionation (reviewed by Fry 2006). During evaporation, the vapor phase is enriched in the lighter isotope, yielding δH values typically in the range of −10 to −20. With condensation (rain, snow), the isotopically heavy water falls out. Gradients in δH are thus seen as water vapor moves inland and upland, and larger fractionation patterns are seen in cold regions, i.e., polar areas. Isotope maps of δH have been created for large regions, and plant and animal δH reflect these patterns.

Phosphorus

Phosphorus (P) does not have a convenient stable isotope, so studies of P rates and cycling have been hampered by a lack of this tool. Recently, the application of isotopic ratios of O within the PO_4 molecule has advanced biogeochemical studies of the cycling of P (e.g., Jaisi et al. 2011; Payton and McLaughlin 2011; Davies et al. 2014 and references therein). The application of $\delta^{18}O_P$ (e.g., $P^{18}O_4$) has allowed a means to identify biotic and abiotic interactions of P in biogeochemical cycling and metabolism (e.g., McLaughlin et al. 2004, 2006*c*; Elsbury et al. 2009; Goldhammer et al. 2011). The P—O bonds in inorganic P are resistant to hydrolysis, so in abiotic reactions there is no exchange of O between PO_4 and H_2O, and no fractionation. In contrast, enzymatic reactions cleave the P—O bond, leading to fractionation or kinetic equilibrium of O isotopes either in intracellular fluids or in the external environment (Blake et al. 2005; Jaisi et al. 2011; Davies et al. 2014).

Applications of $\delta^{18}O_P$ have been rapidly advancing. Using $\delta^{18}O_P$ in concert with sediment chemistry, X-ray diffraction, and other approaches, the sediments from an organic-rich, sulfidic (hypoxic) site in the mesohaline portion of the mid Chesapeake Bay were studied to identify sources and pathway of sedimentary P cycling (Josji et al. 2015). These isotopic data led to the conclusion that regeneration of inorganic P from organic matter degradation (remineralization) is the predominant, if not sole, pathway for authigenic P precipitation in the midbay sediments, with implications for diffusion of dissolved P, an important regulator of eutrophication and hypoxia. In Monterey Bay, California, McLaughlin et al. (2006*b*) found $\delta^{18}O_P$ values in the upper water column to be influenced by both terrestrially derived freshwater and by deeper water. In a similar study Elkhorn Slough, California, samples collected toward the mouth of the estuary suggested that the $\delta^{18}O_P$ values were dominated by oceanic-derived P_i, but further upstream values were reflective of a chemical fertilizer input (McLaughlin et al. (2006*a*). In a study of wastewater treatment plants in France, Gruau et al. (2005) found that the $\delta^{18}O_P$ for fertilizer and for the wastewater PO_4 were statistically different, but small. On the other hand, Li et al. (2011) were able to show that anthropogenic PO_4 from fertilizers could be traced within freshwater wetlands of the Everglades National Park, Florida. Blake et al. (2001) were able to attribute inputs of PO_4 to groundwater from septic systems based on $\delta^{18}O_P$.

Clearly, the application of $\delta^{18}O_P$ provides many opportunities for advancement, but such studies are at a comparatively early stage (Davies et al. 2014). Studies that couple this tool with those of stable isotope applications of C and N will greatly advance our understanding of the biogeochemical cycling of nutrients and have broad implications for eutrophication studies.

Sulfur

Sulfur has four stable isotopes: ^{32}S, ^{33}S, ^{34}S, and ^{36}S with abundances of 96%, 0.76%, 4.22%, and 0.0136%. Traditionally, S isotopes have been reported as $\delta^{34}S$, differences in the ratio of the two most abundant isotopes ($^{34}S/^{32}S$) of a sample relative to the standard (Vienna) Canyon Diablo troilite. Although isotope fractionation usually declines with mass, $\delta^{34}S$ values show a wide range (about 100‰). This is related to the prominent role of redox reactions within the biogeochemical sulfur cycle (Harrison and Thode 1958; Kaplan et al. 1963; Canfield 2001). While fractionation of S during assimilation is rather limited (a few ‰), isotope fractionation during sulfate reduction can be more than 55‰, with pure cultures showing lower values than natural populations (Canfield 2001). Fractionation during sulfate reduction depends primarily on the rate of sulfate reduction, the electron donor (H_2 vs. organics), and sulfate concentrations with low activities, high concentration, and organic substrates leading to highest isotope discrimination (Canfield 2001). Most sulfides produced in anoxic waters and sediments is microbially reoxidized to regenerate the energy in reduced sulfur compounds (Jørgensen 1977). Although S isotope fractionation is rather limited during reoxidation (Kaplan and Rittenberg 1964; Fry et al. 1986), reoxidation of sulfide normally results in the formation of intermediate compounds such as elemental sulfur, thiosulfate, sulfite, and so on. These compounds disproportionate, i.e., split into a more reduced (e.g., sulfide) and more oxidized form (e.g., sulfate), with further isotope fractionation as a result (Canfield and Thamdrup 1994; Canfield 2001). As a consequence, isotope differences between sulfide (and sulfide minerals) and sulfate in nature are larger than those expected if only sulfate reduction would be occurring. The combined information of $\delta^{34}S$ and $\delta^{18}O$ of seawater and pore-water sulfate has shown useful to constrain the cryptic S cycle in ocean oxygen minimum zones (Johnston et al. 2014) and to evaluate the relative contribution of anaerobic methane oxidation to sulfate reduction (Antler and Pellerin 2018).

The very negative $\delta^{34}S$ values of reduced sulfur compounds are transferred to autotrophs assimilating these substrates, in particular benthic algae and rooted macrophytes (Fry et al. 1982; Trust and Fry 1992). Intertidal cordgrass as well as

seagrasses obtain part of their sulfur via the roots and consequently often has a distinct sulfur isotope composition relative to other coastal and estuarine organic matter sources (Stribling et al. 1998; Holmer and Hasler-Sheetal 2014). Isotopes of S can complement C isotopes to elucidate the contribution of benthic vs. pelagic resources in lacustrine, coastal, and marine systems (Peterson and Howarth 1987; Croisetière et al. 2009). Strong changes in S isotopes along estuarine gradients have also been found, with rapid increases in $\delta^{34}S$ as salinities increase from < 1 to ~ 5, but more constant values at higher salinities and this knowledge, together with measures of $\delta^{34}S$ in fish muscle, have been used to track the extent to which they remain resident in a habitat or become mobile if and when habitat conditions are less favorable (Fry and Chumchal 2011).

Silica

There has been substantial application of Si isotopes in understanding global Si biogeochemistry and rates of Si incorporation, and a recent review (Sutton et al. 2018) has compiled much of this literature for both marine and freshwater systems. The natural isotopes of Si are ^{28}Si, at 92.23%, ^{29}Si at 4.67%, and ^{30}Si at 3.10%; ^{30}Si is the most commonly used and reported isotope. The major processes fractionating Si are weathering and organismal incorporation. Weathering releases the lighter isotope so the dissolved Si in water becomes lighter. Si assimilation by organisms leaves behind a pool of dissolved Si that is enriched. Most freshwater studies have shown that dissolved $\delta^{30}Si$ values are generally derived from chemical weathering and secondary mineral formation, with seasonal biomineralization processes, mainly the uptake of DSi by diatoms, adding a secondary signal (Sutton et al. 2018 and references therein). This general pattern has been shown, for example, for the Nile River (Cockerton et al. 2013), the Yangtze River, where uptake by wetland grasses and rice paddies contribute seasonally (Ding et al. 2004), rivers in Switzerland (Georg et al. 2006), and in Kenya (Hughes et al. 2012). Distributions of $\delta^{30}Si$ in marine waters are similarly driven, with active fractionation by diatoms in surface waters being the dominant process in oligotrophic waters. Consequently, productive upwelling systems have among the most highly fractionated $\delta^{30}Si$ values reported (Reynolds 2011; Sutton et al. 2018).

There has also been substantial use of ^{30}Si as an enrichment tracer in marine studies (Nelson and Goering 1977), but less so in freshwater. Early studies by Banahan and Goering (1986) showed that uptake rates in coastal, middle, and outer domains of the southern Bering Sea shelf were comparable. Uptake rates from ^{30}Si tracer studies have been reported for midocean gyre studies (Brzezinski and Nelson 1995, 1996; Nelson and Brzezinski 1997) and upwelling regions (Nelson et al. 1981) and the Southern Ocean (Nelson and Gordon 1982). Brzezinski and Kosman (1996) measured Si uptake rates in the Sargasso Sea in spring and found that a substantial portion of the diatom production occurred near the base of the euphotic zone, in the nitracline.

Source tracking

Natural abundance stable isotope ratios have been widely used to help identify and track biogeochemical sources in the environment (Kendall 1998; Kendall et al. 2008). Different sources of inorganic nutrients or organic matter often have distinct isotopic signatures, and various biological and/or physical processes alter isotope ratios in expected ways (Kendall et al. 2008; Fig. 5). Source tracking has been successful through analysis of dissolved nutrient pools, detrital mixtures, and organisms such as macrophytes (e.g., McClelland et al. 1997; Cole et al. 2004; Cohen and Fong 2006; Benson et al. 2008) mollusks (Fila et al. 2001; McKinney et al. 2001, 2002), or some combination (Gartner et al. 2002; Fry and Allen 2003) whose biomass may reflect source contributions.

Stable isotope ratios of C are often used to distinguish between autochthonous and allochthonous sources of organic matter in freshwater systems (Cole et al. 2011; Cole and Solomon 2012), between pelagic and benthic primary producers (France 1995; Vadeboncoeur et al. 2003; Christianen et al. 2017), and between terrestrial- and marine-derived organic matter in estuaries (Salomons and Mook 1981; Peterson and Fry 1987; Cifuentes et al. 1988; Peterson et al. 1994; Middelburg and Herman 2007). Tank et al. (2011) applied stable isotopes to identify sources of dissolved organic matter (DOM) in lakes of the Mackenzie Delta (western Canadian Arctic) by coupling various measurements of DOM and dissolved organic C (DOC) with the $\delta^{13}C$ and $\delta^{15}N$ values of DOM and their precursor materials, and found that distinct sources of DOM to adjacent lakes may contribute to differences in the fate and cycling of various C sources.

Stable isotopes are also frequently used to track anthropogenic N in aquatic systems (e.g., Owens 1987; Tucker et al. 1999; Costanzo et al. 2001; Lapointe et al. 2011; Loomer et al. 2014). In particular, increases in $\delta^{15}N$ (relative to a defined baseline or reference site) are often associated with contributions of sewage-derived N (Kendall 1998). The $\delta^{15}N$ of inorganic N derived from manure or sewage is often enriched (> 10 ‰) due to isotopic fractionation that occurs at either the sewage treatment facility or downstream thereof. The $\delta^{15}N$ values of N in raw sewage are not necessarily enriched, but processes such as NH_3 volatilization and denitrification drive the $\delta^{15}N$ values of the residual DIN up during treatment and/or processing within the environment. This, in turn, imparts a ^{15}N-enriched signal to primary producers that take up the sewage-derived N (McClelland et al. 1997; McClelland and Valiela 1998).

In one classic example, Savage and Elmgren (2004) used $\delta^{15}N$ values in the macroalgae *Fucus vesiculosus* to track sewage-derived N in an embayment of the Baltic Sea and quantify effects of reductions in N inputs following implementation of tertiary sewage treatment. They sampled *Fucus*

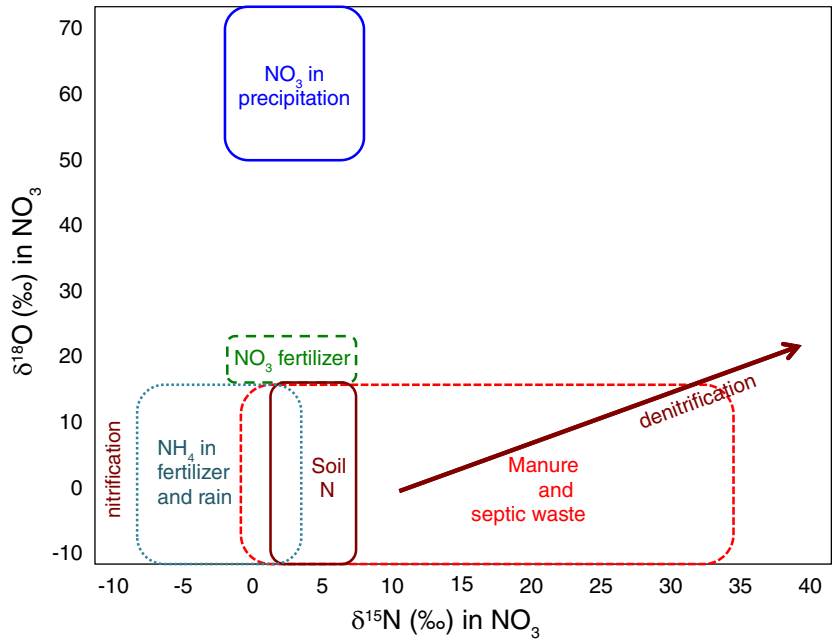

Fig. 5. Typical δ^{18}O-NO_3 and δ^{15}N-NO_3 ranges for nitrate sources and the processes that alter these values. Modified and redrawn from Kendall (1998) and Kendall et al. (2008)).

along a 36 km transect and documented a gradient of elevated δ^{15}N that extended from peak values near the sewage outfall to ~ 25 km seaward of the outfall. Sewage N was traceable in *Fucus* before and after tertiary treatment was implemented, but δ^{15}N values decreased more rapidly with distance from the outfall and were less enriched along much of the transect after tertiary treatment began, demonstrating a marked decline in the contribution of sewage-derived N to *Fucus*.

A variation of this approach for N source tracking is the deployment of specific organisms for a set length of time over which the isotopic signature of their biomass will change, reflecting the local environment. Costanzo et al. (2001) deployed macroalgae in porous containers for several days, during which time their biomass incorporated the δ^{15}N signature of dissolved N, and were thus able to map a sewage plume in Moreton Bay, Australia. Oysters represent another appropriate organism for such deployment studies, and field trials have been conducted to resolve appropriate length of time for adequate resolution and how different tissues (mantle or gill) vary in isotope incorporation after exposure to different source nutrients/foods (Fertig et al. 2009, 2010).

Stable isotopes are also used to identify and track water itself. For example, differences in δ^{18}O and δ^{2}H between groundwater and surface water are frequently used to characterize interactions between these two sources. As but one example, Fan et al. (2016) examined spatial variation in δ^{18}O and δ^{2}H of river water and their sources in the Tarim River Basin, an inland river system of Central Asia. They found that headstreams were composed of water originating as precipitation, ice-melt water and groundwater, and using these isotopic ratios together with other measures of particulate material, the various proportions of each of these components were calculated by season. Stable O isotope ratios have also been essential for quantifying freshwater contributions from sea-ice melt vs. meteoric water (i.e., river inputs) in the Arctic Ocean and surrounding seas (e.g., Bauch et al. 1995; Benetti et al. 2016).

Successful source tracking applications depend on substantive isotopic differences between sources of interest, and are most successful when system-specific end-member information is available. However, distinguishing between changes in end members and changes in isotope values that occur along environmental gradients is a perennial challenge (Fry 2002; Middelburg and Herman 2007). For example, resolving δ^{13}C values of organic matter as a consequence of end-member mixing vs. changes in δ^{13}C values of in situ production along estuarine gradients (that mirror changes in DIC-δ^{13}C) is often difficult (Deegan and Garritt 1997).

Aside from the deployment of specific organisms for source tracking that can yield relatively straightforward results, source tracking generally involves a mixing model, but which all too often can turn into a "mixing muddle" (Fry 2006). Applications of multiple isotopes and new approaches to data analysis are accounting for this variability in some studies, but it is still extremely common to apply average source values and treat them as constants.

Whole ecosystem studies

In the past two decades, there have been multiple mesocosm and whole-ecosystem labeling studies designed to measure integrated biogeochemical pathways or flows of C and N through

food webs. Examples of whole system tracer enrichment that have lasted up to months can be found in streams, rivers, lakes, estuaries, wetlands, and marshes (e.g., Middelburg et al. 2000; Hamilton et al. 2004; Pace et al. 2004; Gribsholt et al. 2005, 2009; Oakes et al. 2010, 2012; de Goeij et al. 2013). Whole-lake labeling experiments have been instrumental in identifying the role of allochthonous subsidies for lacustrine consumers (e.g., Cole et al. 2002; Pace et al. 2004; Carpenter et al. 2005). Sánchez-Carrillo and Álvarez-Cobelas (2018) have recently reviewed 78 publications in which ^{13}C and/or ^{15}N additions were used at the whole-aquatic-ecosystem scale and insights achieved, as well as constraints and future prospects for such studies.

One such large experiment was carried under the umbrella of the Lotic Intersite Nitrogen eXperiments (LINX; Peterson et al. 2001). The first LINX experiments involved a continuous drip of $^{15}NH_4$ into streams across the US. Water samples, organic matter, and various types of biota—from phytoplankton to insects and riparian vegetation—were collected over time. The second suite of LINX experiments involved $^{15}NO_3$ additions to 72 streams across the US. In addition to the biota, N_2 and N_2O were also quantified. Key findings were that $^{15}NH_4$ was rapidly removed by the biota, while comparatively more $^{15}NO_3$ was transported downstream. The cross-site analysis also showed that small streams were particularly effective at efficiently removing N because they have a higher ratio of streambed to water volume compared to larger streams (e.g., Peterson et al. 2001; Merriam et al. 2002; Mulholland et al. 2002, 2008; Webster et al. 2003; Ashkenas et al. 2004). Böhlke et al. (2004) enriched an agricultural stream with $^{15}NO_3$ to determine reach-averaged rates of denitrification and other N processes. Applying $^{15}NO_3$ labeling to a mountain stream in Idaho, Hall et al. (2009) compared uptake, storage, and export during periods of snowmelt and baseflow. They found snowmelt floods had higher than expected demand for NO_3 because of hyporheic exchange. A more recent study involving NH_4 additions to 13 streams showed the effect of canopy cover on the efficiency with which N was transferred from heterotrophic microorganisms and primary producers to primary consumers (Norman et al. 2017). Whole-estuary experiments, e.g., NISOTREX and NISOTREX II, have been performed with $^{15}NO_3$ enrichment to characterize estuarine N processing under both low- and high-flushing conditions (Holmes et al. 2000; Hughes et al. 2000; Tobias et al. 2003*a*,*b*). Whole-stream ^{13}C additions have also been conducted and allowed resolution of the fate of newly fixed C as well as longer term storage and export (Hotchkiss and Hall 2015). Whole-lake enrichments of ^{13}C-labeled HCO_3 have also been made and results revealed that terrestrial C did support a portion of higher trophic level production in these systems (Cole et al. 2002, 2011; Carpenter et al. 2005). The interpretation and broader implications of these findings have since been the subject of debate (Brett et al. 2009), though these labeling studies have highlighted the potential role of terrestrial-aquatic linkages to supporting aquatic food webs.

Food webs

The movement of matter, energy, and elements to higher trophic levels through the food web is a central aspect of ecology, leading to interest in the possibility of using stable isotopes to elucidate food web relationships in aquatic systems, including estimation of consumer trophic position (TP) and determination of the importance of basal energy sources and dietary relationships (e.g., Peterson and Fry 1987; Vander Zanden and Rasmussen 2001; Middelburg 2014 and references therein). Most commonly, ^{13}C and ^{15}N are applied in such studies, and the common axiom is that for C, "you are what you eat" (i.e., isotope ratios in consumers reflect those of their food), while for N, "you are what you eat +3‰" (i.e., consumers are typically enriched by 2–4‰ relative to their diet; Smith and Epstein 1970; DeNiro and Epstein 1978, DeNiro and Epstein 1981; Minagawa and Wada 1984; Fig. 6A). Studies using natural abundance stable isotopes to make inferences about energy sources (^{13}C) and TP (^{15}N) have become commonplace, and have been applied to a wide range of aquatic ecosystem types (Middelburg 2014 and references therein). While ^{13}C and ^{15}N have been the dominant stable isotope tracers, studies have used ^{34}S to differentiate food sources in marine (Peterson and Fry 1987) and freshwater systems (Croisetière et al. 2009), and H isotopes to track aquatic-terrestrial linkages (Doucett et al. 2007; Cole et al. 2011; Solomon et al. 2011; Cole and Solomon 2012; Karlsson et al. 2012). Advances in food web studies have also been made through application of δ^2H, an emerging tool for trophic positioning studies (Vander Zanden et al. 2016). Stable isotope applications to food web studies can generally be binned into those in which TP is characterized, and those in which food sources are identified. The latter can be probed through characterization of isotopic composition of individual foods, or from isoscapes that are derived from substantial records of the isotopic composition of organisms or inorganic nutrients and other materials over broad locations.

Trophic position

Early laboratory experiments showed that animal tissues tend to be more enriched in ^{15}N than that of their food (DeNiro and Epstein 1981; Minagawa and Wada 1984). This provided the conceptual basis for the use of N isotopes to estimate the number of trophic steps between the base of a food web (i.e., primary producers, trophic level 1) and a consumer of interest. Building on this, field studies began to make qualitative yet powerful inferences about trophic structure of an ecosystem from ^{15}N measurements across the food web (Hobson and Welch 1992; Kling et al. 1992). Fundamental questions about food chain length, highlighting relationships with ecosystem size, ecosystem productivity, and species diversity were subsequently addressed (Vander Zanden et al. 1999; Post et al. 2000). Such studies have provided evidence for food chain biomagnification of contaminants (Cabana and Rasmussen 1994; Lavoie et al. 2013), and how anthropogenic drivers such

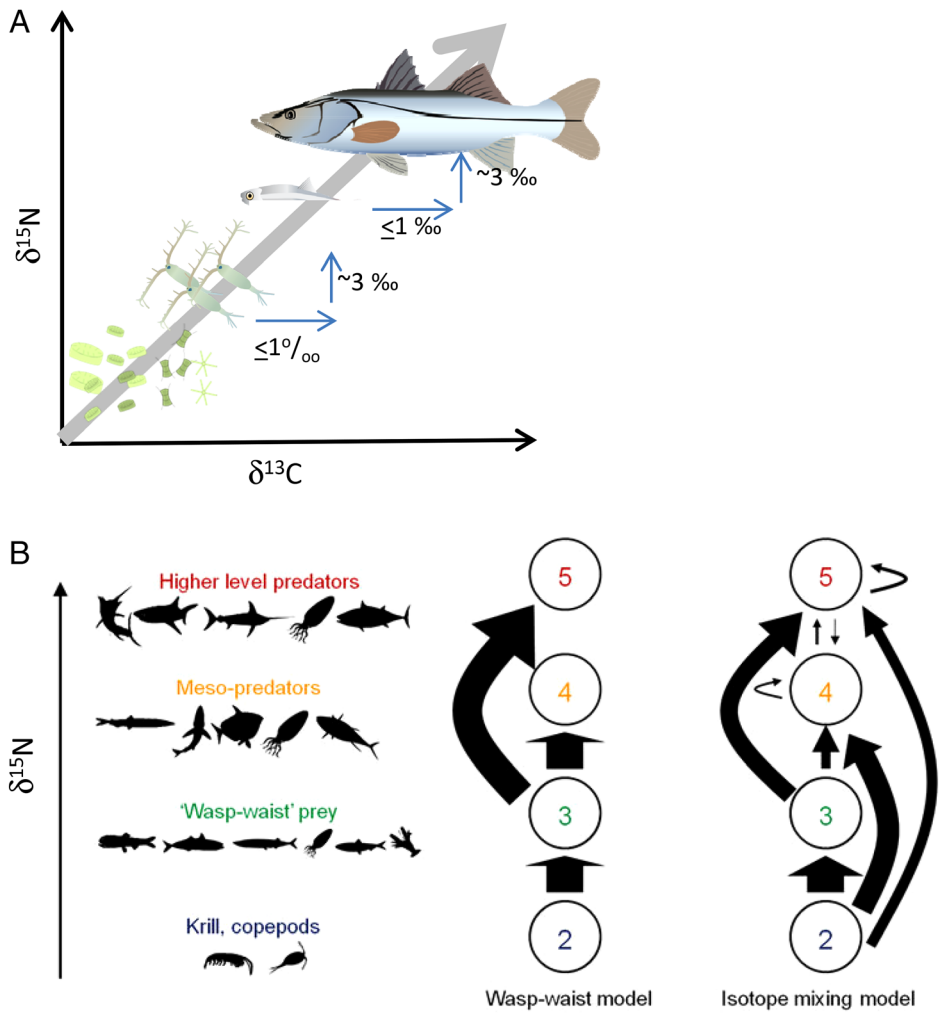

Fig. 6. (**A**) Classic conceptual food web relationships based on $\delta^{13}C$ and $\delta^{15}N$. (**B**) Schematic showing different trophic levels of the California Current system, the expected proportional prey inputs expected under a wasp-waist model, and far right, the proportional prey to various trophic groups based on isotopic measurements and mixing model results. Panel **B** reproduced from Madigan et al. (2012) under a creative commons license.

as climate change and introduced species affect food webs (Vander Zanden et al. 1999; Tunney et al. 2014).

While the potential for this approach is apparent, the ability to compare food webs in a quantitative manner is hindered by variation in ^{15}N at the base of the food chain, analogous to the variation in end-member resolution in source tracking studies. Cabana and Rasmussen (1996) found that the ^{15}N of long-lived primary consumers such as unionid mussels varied by ~ 13‰ among lakes, with much of the variation explained by anthropogenic nutrient inputs. To address this, Cabana and Rasmussen (1996) expressed the ^{15}N of higher consumers relative to that of long-lived primary consumers such as mussels, which show little temporal variation in isotopic values, and thus provide a desirable baseline indicator organism. They determined TP as:

$$TP = {}^{15}N_{consumer} - {}^{15}N_{baseline}/TDF + 2 \quad (8)$$

in which TDF is the trophic discrimination factor, the estimated per trophic level isotopic enrichment, which is commonly assumed to be 3.0 or 3.4 for ^{15}N. Subsequent studies found that the isotopic baseline in lakes can vary among habitats within an ecosystem, and proposed the establishment of a baseline curve whereby consumer ^{13}C values were used to determine the appropriate ^{15}N baseline (Vander Zanden and Rasmussen 1999; Post 2002). Expressing consumer ^{15}N relative to that of an isotopic baseline provided ecologists with a relatively simple way to estimate the TP of a consumer, defined as a continuous measure of the number of trophic steps leading to a consumer that is reflective of food web omnivory and actual feeding pathways. Stable isotopes allowed this fundamental ecological concept to be operationalized in a way that has stimulated research interest in trophic relationships and energy flow pathways.

Increasingly, however, it has been recognized that the common axioms of little to no fractionation with trophic transfer for C and approximately +3‰ for N are too simplistic. There is considerable variability associated with these values, and

applying them in a generic way can lead to misunderstanding. Middelburg (2014) summarized key factors that affect the accuracy of TP estimates as follows: (1) the assumption that C and N flow through the food web similarly (i.e., no concentration effect) and that per trophic level enrichment of C is limited, (2) variation in the TDF for N, (3) uncharacterized isotopic variation among tissues, and (4) difficulty in estimating appropriate C and N isotopic baselines (Lorrain et al. 2002; Phillips and Koch 2002; Fry 2006; Phillips 2012). Variation in the TDF has received significant attention. While 3.0 is a reasonable approximation of the overall average trophic enrichment factor, the degree of variation is substantial and depends on factors such as the form of excretion, diet, tissue type, taxon, and habitat type (e.g., Vander Zanden and Rasmussen 2001; Post 2002; Vanderklift and Ponsard 2003; Caut et al. 2009; Auerswald et al. 2010; Bunn et al. 2013). Moreover, the variability for herbivores is far greater than that for carnivores (Vander Zanden and Rasmussen 2001). In recent years, Bayesian mixing models have been applied that incorporate many of the uncertainty issues: concentration dependence, variable numbers of sources, uncertainty in sources, and so on (reviewed by Phillips et al. 2014).

Food webs often deviate strongly from simple linear food chains and discrete trophic levels, and stable isotope applications have been used to resolve these complexities. For example, a multiyear stable isotope food web study of the California Current found separation of species into trophic groups based on $\delta^{13}C$ and $\delta^{15}N$, and that a wide diversity of prey were incorporated into upper level predators (Madigan et al. 2012; Fig. 6B). Predators did not cluster together as expected if they were uniformly feeding on lower trophic level prey. Bayesian mixing models revealed a high degree of omnivory and more complex trophic linkages, a finding that could have implications for ecosystem stability and resilience to perturbations.

With increasing recognition of the challenges and limitations of the bulk stable isotope approach for estimating TP, analyses of stable N isotope ratios of individual AAs have emerged as a promising technique (McClelland and Montoya 2002; McClelland et al. 2003). McClelland and Montoya (2002) found that the stable N isotope ratios of some AAs exhibit high-trophic enrichment, while other showed virtually none. Thus, they proposed that the difference between enriching (i.e., glutamic acid, glu) and nonenriching (phenylalanine, phe) AAs could provide an indicator of consumer TP. This approach cleverly provides a baseline ^{15}N correction internal to the organism of interest, thereby addressing the issue of accurately defining an external isotopic baseline signature in many ecosystems. Subsequent studies largely confirmed the TDFs reported by McClelland and Montoya (2002). Chikaraishi et al. (2009) reported TDF_{AA} to be $7.6 \pm 1.3‰$ from controlled feeding studies at the time, and proposed the following equation to estimate consumer TP based on AAs (TP_{AA}):

$$TP_{AA} = \frac{\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - \beta}{TDF_{AA}} + 1 \quad (9)$$

where $\delta^{15}N_{glu}$ and $\delta^{15}N_{phe}$ are ^{15}N for glutamic acid and phenylalanine, respectively, β is the isotopic difference between the two AAs in primary producers, and TDF_{AA} is the difference in TDF between the two AAs. While it appears that the AA-specific approach may provide higher precision and accuracy than traditional bulk methods for estimating consumer TP (Chikaraishi et al. 2009), a meta-analysis by McMahon and McCarthy (2016) highlighted substantial variability in TDF_{AA} values, and suggest diet quality and mode of N excretion to be factors influencing this variation. Although a truly universal TDF_{AA} is unlikely to exist, efforts to understand the patterns of variation will underpin future advances in the application of this approach for elucidating food webs.

For microbial food webs, it is far more challenging to resolve the isotopic composition among bacteria, archaea, eukaryotic microbes, and detritus (dead organic matter), in particular for N, as there are few biomarkers for tracing N through the microbial domain (Boschker and Middelburg 2002). A biomarker for bacterial peptidoglycan, D-alanine, has been used to trace N uptake and flow through bacteria in coastal waters, seagrass beds, and sediments (Veuger et al. 2006, 2012; Eyre et al. 2016). Stable N isotope analyses of diaminopimelic acid have also been used to track bacterial N (Tobias et al. 2003*a*,*b*), although small yields can make this approach challenging. Assimilation of N by heterotrophic microbes feeding on detrital resources leads to elevated $\delta^{15}N$ (Caraco et al. 1998) with consequences for the isotope composition, hence trophic level inference, of top consumers (Steffan et al. 2017). However, Guiterrez-Rodriguez et al. (2014) have shown that protozoan trophic steps do not necessarily lead to an increase in $\delta^{15}N$. Polar-lipid derived fatty acids (PLFA) are another suitable biomarker, in this case for ^{13}C, for microbial food web studies because of their relatively easy isotope analysis with GC-C-IRMS and because these cell membrane constituents occur in bacteria and eukaryotes and are present in relatively constant amounts in organisms of interest (Boschker and Middelburg 2002; Middelburg 2014). However, specificity of PLFA (for identification of microorganisms) is rather low, and thus bacteria and eukaryotes can be distinguished quite well from each other, but PLFA spectra of many phytoplankton groups overlap (Dijkman et al. 2009). This implies that resolving among phytoplankton groups should be done with utmost care and requires compositional estimators (Van den Meersche et al. 2008; Dijkman et al. 2009).

McCarthy et al. (2007) measured AA distributions in plankton tows, sinking particulate organic matter (POM), and ultrafiltered dissolved organic matter in the central Pacific Ocean and observed differences in $\delta^{15}N$ of individual AA indicative of trophic transfers. Their finding underscored that the AAs associated with trophic transfer were also clearly preserved in sinking POM, but with additional changes indicative of

subsequent microbial reworking after incorporation into particles. There is much scope for future work on isotope fractionation in microbial biogeochemical cycling (McMahon and McCarthy 2016).

Pathways in food webs

An important aspect of aquatic food web studies involves identifying the basal resources supporting higher trophic levels. Stable isotopes of C have been widely used to track energy sources and pathways into and through aquatic food webs. However, it is important to consider system-specific end-member values when using this tool. For example, C stable isotope ratios are useful for distinguishing between contributions of C4 plants and phytoplankton to higher trophic levels in aquatic systems, whereas it is more difficult to distinguish between C3 plant and phytoplankton contributions. This is particularly true for freshwater systems, where the $\delta^{13}C$ values of phytoplankton frequently overlap with $\delta^{13}C$ values of C3 plants.

While limnology has traditionally focused on planktonic production and processes, C isotope studies have demonstrated a surprisingly important role of periphyton-based production in supporting higher trophic levels in lakes. Periphyton (benthic) and phytoplankton (pelagic) have distinct ^{13}C values due to differential fractionation during photosynthesis related to turbulence and boundary layer conditions. In summarizing data from a suite of Canadian lakes, Hecky and Hesslein (1995) found that fish species often have a strongly benthic ^{13}C signal. This has been found to be a general pattern, with benthic pathways often comprising the dominant C source supporting fish (Vander Zanden and Vadeboncoeur 2002). Stable isotope studies showing the importance of benthic C to higher trophic levels are part of a broader body of literature highlighting benthic-pelagic coupling in lakes (Schindler and Scheuerell 2002; Vadeboncoeur et al. 2002).

A central question in riverine food web ecology pertains to the importance of autochthonous and allochthonous C sources to riverine consumers (Rounick and Winterbourn 1986). From the earliest studies, it has been recognized that differentiating the role of different habitats and C sources supporting stream food webs can be complicated by a variety of factors: high variability in algal ^{13}C related to productivity and flow, the ^{13}C of the underlying DIC source, and the difficultly of isolating algae from heterogeneous epilithon (Rosenfeld and Roff 1992). Stable isotope studies in a wide range of riverine systems have demonstrated an important and often outsized role of autochthonous algal C in supporting higher trophic levels. Finlay et al. (1999) reported strong differences in ^{13}C between pool and riffle habitats in productive streams, but no difference in unproductive streams. Subsequent studies exploited these differences and highlight previously unrecognized food web linkages, and the role of shallow pools in supporting a range of consumers in streams (Finlay et al. 2002).

Stable isotope studies have also elucidated aquatic to terrestrial linkages in streams. Organic matter, invertebrates, and fish were enriched in ^{15}N and ^{13}C in streams with spawning coho salmon (*Oncorhynchus kisutch*) in Pacific Northwest streams, demonstrating the role of salmon carcasses, which are isotopically distinct, in fueling the food web and increasing productivity (Bilby et al. 1996). Studies have used natural abundance ^{13}C and ^{15}N (Paetzold et al. 2005), as well as ^{15}N tracer additions (Sanzone et al. 2003) to evaluate the importance of aquatic insects as a resource for riparian arthropods.

Isoscapes

Isoscapes, or spatially explicit maps of isotopic composition (West et al. 2008; Bowen et al. 2010), are also beginning to be developed in marine systems as a tool for reconstruction of broad scale animal movements which have important implications for understanding diet sources. Isoscapes are derived from substantial records of the isotopic composition of organisms or inorganic nutrients and other materials in known locations. In terrestrial systems, large spatial interpolations of isotopic measurements have been made for at least a decade, providing a foundation for research on migration patterns of insects, birds, and mammals (e.g., Flockhart et al. 2013).

Broad marine applications of this approach have been limited by data availability, but there has been recent development of isoscapes of ^{13}C and ^{15}N in coastal, shelf, and lake regions. In Florida Bay, US, the C and N isotopic composition of the seagrass *Thalassiosira testudinum* was measured across the expanse of the bay (10,000 km^2) and over multiple years (Fourqueran et al. 2005). Clear seasonal trends were seen, with $\delta^{13}C$ and $\delta^{15}N$ values showing maxima in the summer and minima in the winter. Coupled with elemental ratios of the plant material, underlying the seasonal changes in nutrient supply and processes that fractionate the DIN pool. In the Dutch Wadden Sea, the C isotopic composition of 178 species was measured across the entire intertidal area (1460 km^2) with high-spatial resolution (Christianen et al. 2017). Benthic microalgae were identified to be the dominant energy source for the majority of consumers at higher trophic levels (from worms and molluscs to fish and birds). Moreover, large spatial heterogeneity in C isotope composition of benthic algae and their consumers was observed, implicating the need for spatially explicit sampling of tidal food webs. Hobson et al. (2012) applied a $\delta^{13}C$ and $\delta^{15}N$ isoscape approach to the study of fish community structure and large-scale dietary nutrient sources in Lake Winnipeg.

An open ocean isoscape of ^{13}C, with one degree and monthly resolution, was developed based on the coupling of a physical-biogeochemical model and predictions of isotopic fractionation of phytoplankton (Magozzi et al. 2017; Fig. 7A). In this model, values of $\delta^{13}C_{PLK}$ (phytoplankton) were predicted from the isotopic composition of dissolved CO_2 ($\delta^{13}C_{CO2}$) and ε_p, which was estimated from the concentration of dissolved CO_2, phytoplankton growth rates, size, and

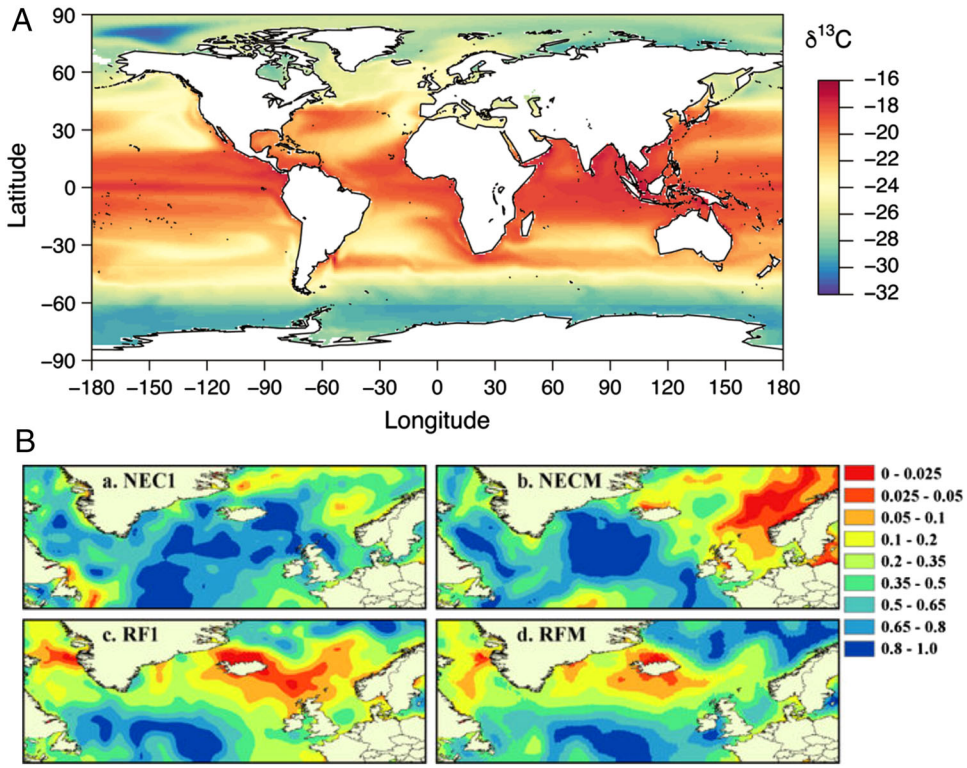

Fig. 7. (**A**) Modeled annually averaged surface-water distribution of the carbon isotope composition of phytoplankton ($\delta^{13}C_{PLK}$, ‰). Annual average $\delta^{13}C_{PLK}$ values are calculated using a monthly climatology for the period 2001–2010. (**B**) Proposed feeding areas for two Atlantic salmon populations indicated by the strength of correlation between temporal records of sea surface temperature and scale collagen $\delta^{13}C$ values. Each map represents feeding areas for a specific population and cohort: (a) North East Coast 1SW, (b) North East Coast MSW, (c) River Frome 1SW, and (d) River Frome MSW. Panel **A** reproduced from Magozzi et al. 2017) and panel **B** from MacKenzie et al. (2011) under creative commons licenses.

community composition (Magozzi et al. 2017). Such a model may be useful in interpreting foraging range and diet sources in further food web models and overcoming the limitation of poorly resolved spatio-temporal distributions of baseline isotope compositions (Magozzi et al. 2017). An isoscape approach was applied to the study of populations of Atlantic salmon and their feeding grounds (MacKenzie et al. 2011; Fig. 7B). Using the relationship between fractionation of C during photosynthesis and temperature, these variations were applied by correlating the temporal records in tissue isotopes and temperature over the possible spatial range of the species; importantly this analysis did not depend on a priori knowledge of distribution of baseline isotope values.

Paleoenvironments

Fossil foraminifera shells have routinely been analyzed for their C and O isotopic compositions for the purpose of reconstructing past climate conditions; some of this work dates back many decades (e.g., Urey et al. 1951; Epstein et al. 1953; Shackleton 1967). These early studies established that the $\delta^{18}O$ and $\delta^{13}C$ signals of the shell reflect those of the seawater during calcification. It is generally assumed that the $\delta^{18}O$ of the tests primarily reflects the seawater temperature (and paleosalinity) while the $\delta^{13}C$ reflects primarily the state of paleoproductivity and water mass age (e.g., Shackleton and Opdyke 1973). If the shell $\delta^{18}O$ in calcite is in equilibrium with seawater, the isotopic discrimination factor between calcite and seawater ($\varepsilon \sim \delta^{18}O_{CaCO3} - \delta^{18}O_{H20}$) is related to the temperature of calcification and therefore, if the $\delta^{18}O$ of seawater is known, the $\delta^{18}O$ of calcite can be used to reconstruct paleotemperatures (Ravelo and Hillaire-Marcel 2007). The $\delta^{18}O$ of foraminifera have been instrumental to reconstruct the cooling of the ocean and climate during the last 50 Ma (e.g., Zachos 2001; Lisiecki and Raymo 2005). However, the assumption that that these isotopic signatures "faithfully record relative changes in isotopic conditions…can be problematic" (D'Hondt and Zachos 1993). These isotope values can be offset due to factors such as shell size, microhabitat differences and/or species or individual variabilities, pore-water chemistry, pH, and other "vital effects" (e.g., McCorkle et al. 1997; Schmiedl et al. 2004; Fontanier et al. 2006; Ishimura et al. 2012). Recently, the isotopic composition of shells of pteropods have been analyzed, and as they secrete aragonite close to the $CaCO_3$-seawater $\delta^{18}O$ equilibrium, their $\delta^{18}O$ is suggested to be a good oceanographic proxy of seawater temperature and their $\delta^{13}C$ a good proxy of carbonate ion concentration (Keul et al. 2017). Moreover, given their longer life span relative to foraminifera, they may provide a more integrative measure.

Isotopic measurements of sediments have also proven to be useful in understanding paleoceanography, paleolimnology, and past biogeochemical changes, as well as relationships to climate change. Measurements of the bulk $\delta^{15}N$ of sediments, while relatively straightforward to obtain, can be complicated to interpret, as this material reflects three components, marine organic matter, potentially terrestrial organic matter, and inorganic N (Robinson et al. 2012; Tesdal et al. 2013). The latter can be significant in organic poor sediments and is due to NH_4 adsorption onto clay materials. The isotopic signal of N in sediments reflects alteration in the isotope signal of the organic matter during sinking, but especially during early burial and early diagenesis (Altabet and Francois 1994). During sinking and diagenesis, organic N becomes increasingly enriched in ^{15}N relative to that of the surface layer (Robinson et al. 2012). Isolating the source of the isotopic signature—sinking particle alteration or sediment diagenesis—is not easy, but specific biomarkers for C and H analysis have been shown to be useful, whereas analogous biomarkers for N are less useful for various reasons (Altabet 2006).

Based on a compilation of available surface marine sediment $\delta^{15}N$ samples in the published literature (http://www.ncdc.noaa.gov/paleo/pubs/nicopp/nicopp.html), values were found to fall in a range from 2.5‰ to 16.6‰, with an average of 6.7‰ (Tesdal et al. 2013). This global data set shows, and as previously described (e.g., Galbraith et al. 2008), lower $\delta^{15}N$ values in sediments are found in upwelling regions, while in anoxic regions higher values are found—a result of differential biogeochemical processing of NO_3. Where subseafloor data exist, mostly along the coast, the data are biased toward the more recent Holocene and Late Pleistocene timescales (Tesdal et al. 2013). Moreover, patterns are suggestive of an accelerating N cycling in the late Holocene, indicative of greater rates of denitrification and/or N_2 fixation in the water column.

Past changes in denitrification have been constructed from application of downcore $\delta^{15}N$ analysis. Such data are now available for a number of oceanic denitrification zones. In the Arabian Sea record, transitions from $\delta^{15}N$ minima (more denitrification) to maxima (less denitrification) appear to occur abruptly, a few hundred years or less, with minima corresponding to cooler temperature changes (Altabet 2006). Accordingly, climate changes are associated with the strength of the summer monsoon and upwelling favorable winds. This, in turn, influences productivity, altering levels of dissolved O_2 and thus denitrification. Using $\delta^{13}C$ in conjunction with measures of carbonate, organic C, and N contents of cores of Lake Kinneret, Israel, the lake's productivity and regional climate conditions were reconstructed for the late Holocene and five distinct stages were identified which may be related to changes in geo-political settlements over the past 3000 yr (Dubinski et al. 2003).

Other isotopic signals have also been used to interpret paleoceanographic changes. Diatom-bound organic N has been used to reconstruct past N utilization, with a number of studies focused on the Southern Ocean (e.g., Sigman et al. 1999; Crosta and Shemesh 2002; Robinson et al. 2004). In contrast to bulk $\delta^{15}N$ measurements of sedimentary material, measuring that of just the diatom-bound fraction, while more labor intensive, does not appear to influenced by diagenetic processes (Robinson et al. 2012; Sutton et al. 2018 and references therein).

Diatom ^{18}O is another useful isotopic signal for paleo studies. It is a reflection of sea surface temperature and this isotopic signal is retained on very long time scales, at least 430 ka (Shemesh et al. 1995). There also appear to be few species effects. This tool was used in the Southern Ocean and showed a ^{18}O enrichment during the transitions from the last glacial maximum to the Holocene, as well as a post glacial maximum spike, suggestive of melting icebergs.

Another signal in diatoms, that of its Si isotopes ($\delta^{30}Si$), has also been applied in paleo-reconstructions in oceans and in lakes (Panizzo et al. 2016). During biomineralization, diatoms preferentially incorporate ^{28}Si in their frustules, making the ambient water progressively enriched in ^{30}Si (e.g., De La Rocha et al. 1997; De La Rocha 2006), but during dissolution there appears to be an absence of Si fractionation. This isotope signal appears to be faithfully transferred as diatoms sink through the water column, a conclusion based on sediment trap comparisons from Lake Baikal, Siberia (Panizzo et al. 2016). The $\delta^{30}Si$ of sponge spicules and radiolarian tests have also been recently used to reconstruct DSi concentrations in deep waters of the Equatorial Pacific (Fontorbe et al. 2017). Both signals indicate a reduction in value about 37 Ma, a likely consequence of changes in the $\delta^{30}Si$ of source DSi and a transition to higher DSi concentrations overall, related to the shift in the deep-water source of the Pacific during the Paleogene.

Analytical challenges remain, however. For example, in applications of $\delta^{30}Si$, $\delta^{18}O$, $\delta^{13}C$, and $\delta^{15}N$ studies of diatoms, especially in paleo-reconstruction studies, analytical contamination is a significant problem (Sutton et al. 2018). Inadequate cleaning of the biogenic SiO_2 can introduce substantial isotopic offsets (De La Rocha 2006). Moreover, there is much work to be done to further understand fractionation associated with biogenic Si and water, and disparate fractionation factors have been reported, limiting the usefulness of $\delta^{18}O$ as a paleoceanographic proxy (Sutton et al. 2018 and references therein).

Reinforcing challenges, conundrums, and caveats

As repeatedly noted throughout this article, stable isotope applications are based on ratios, ratios of lighter to heavier isotopes (or alternatively heavier to lighter isotopes), ratios of sample to standard, and ratios of these ratios. These ratios may be further manipulated (subtracted, multiplied by arbitrary factors or meaningful environmental parameters) to yield final fractionation values or rate measurements that are subsequently interpreted. The power of isotopes is that small

differences allow great insight—into rate processes, sources of nutrients and organic matter to ecosystems, food web structure, and paleoecological or oceanographic conditions. Increasingly sensitive analytical tools are allowing resolution of isotopic differences at finer scales, including smaller and/or individual organisms, discrete compounds and molecules. Yet, by virtue of the fact that isotopic abundances are ratios, they are also very sensitive to errors, and errors magnify, especially when that error is associated with the denominator. When ratios of ratios (of ratios) produce a synthetic value, the origin of errors can be lost. Thus, it is worth underscoring three perennial problems that have been touched on above, but which are often overlooked by both practitioners and consumers of the isotope literature.

1. Rate measurements involving isotopic enrichment are particularly sensitive to the isotopic enrichment. This value is in the denominator of the estimate of rate velocity (*see* Eq. 5). For rate studies, the amount of tracer added—especially relative to the biomass that will react with this tracer and the time allowed for incubation—will have major effects on final rates estimated. As noted by Glibert and Capone (1993), the decision of how much tracer to add is not always straightforward. While 10% is nominally accepted as a value that will not perturb rates substantially (Dugdale and Goering 1967), this amount may become rapidly depleted if concentrations of N are low and biomass is high (as in the case of a phytoplankton bloom). Using a Monte-Carlo error analysis, Aufdenkampe et al. (2002) estimated how errors in measurement can propagate to a calculated uptake rate. They found that uncertainties in uptake rates could be 6–20%, with higher uncertainties associated with lower uptake rates. Moreover, at higher uptake rates (ρ_{max}) uncertainties are most sensitive to PN concentrations, whereas at lower rates, uncertainties in ^{15}N enrichment have a larger effect (Aufdenkampe et al. 2002). In a meticulous analysis, Tang and Maggi (2012) have explored/studied the effects of ^{15}N content on rates of nitrification, denitrification, and dissimilatory NO_3 reduction to NH_4 (DNRA). Specifically, they showed increasing $\delta^{15}N$-NO_3 values generally resulted in a decrease of the rates of the reactions immediately that used NO_3 as a substrate and that this effect propagated to the secondary reaction that consumed the first successor to NO_3, such as NH_4 and N_2O. The magnitudes of such effects were large across the gradient of enrichment levels tested, leading to 50% reduction in nitrification rates and up to 60% reductions in rates of denitrification and DNRA. Collectively these results underscore that the quantity of isotopic enrichment can have profound effects on the reported rate of reaction.
2. In applications of source tracking of nutrients or organic matter, variability in end member values is often ignored, and average (assumed constants) are applied instead. Overlapping ranges of source/end-member values need to be acknowledged, as do effects of biological transformations along environmental gradients. Open, complex, biologically active systems are too often fit with closed system equilibrium equations. Unless source δ values (for whatever element is being compared) are thoroughly characterized, Δ, α, ε cannot be established with any accuracy (*see* Eqs. 2–4). Similarly, in whole system studies, another potentially major source of bias in tracer rate measurements is related to the heterogeneity of tracer distribution within the environment after tracer addition. This may be important in complex, spatially structured communities such as corals and in situ studies of stratified lakes, heterogeneous wetlands and marshes, river beds, and sediment, e.g., in the particular rhizosphere and bioturbated sediments.
3. In food web studies, the extent of variability in fractionation with trophic transfer must be better recognized, and assumptions of no fractionation for C and approximately +3‰ for N cannot or should not be blindly accepted (*see* Eqs. 8, 9). Variability in isotopic abundance of food resources must be recognized, even if sampling necessitates a composite or pooled sample. And, as cautioned by Fry (2006, p. 133–134), "a disconcerting possibility is that animals might be consuming most of the important foods, keeping those foods at such low abundances that they are hard to sample… your sampling may have missed something important." Bayesian mixing models and other advanced data assimilation techniques are partially addressing the variability issue, but sophisticated analyses cannot overcome underlying faulty assumptions or resolve the underdeterminancy of many food web studies.

Conclusions

Stable isotope applications have played a starring role in the advancement of modern limnology and oceanography. Advancements in instrumentation have gone hand in hand, and currently available tools, such as nanoSIMS, SIP, resonance Raman microspectrometry, cavity ring down spectroscopy among others, are launching isotope aficionados into a new era of isotope resolution and associated new applications and questions. Finer definition of food webs is possible with CSIA relative to classic bulk measurements. Better resolution of biogeochemical processes is possible, not only with better, faster, more automated analysis, but also with applications of isotopologues and integration of molecular information with isotope resolution. Even as technology improves, successful application of stable isotope techniques will continue to depend as much on the savviness of users as the robustness of the tool itself. However, when treated with a healthy respect, and when resulting data are placed in context with other biological, chemical, or ecological understanding, stable isotope applications will remain pivotal for addressing the many challenging questions that limnologists and oceanographers are tackling.

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