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Biochemical and physiological bases for the use of carbon and nitrogen isotopes in environmental and ecological studies

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Abstract

We review the biochemical and physiological bases of the use of carbon and nitrogen isotopic compositions as an approach for environmental and ecological studies. Biochemical processes commonly observed in the biosphere, including the decarboxylation and deamination of amino acids, are the key factors in this isotopic approach. The principles drawn from the isotopic distributions disentangle the complex dynamics of the biosphere and allow the interactions between the geosphere and biosphere to be analyzed in detail. We also summarize two recently examined topics with new datasets: the isotopic compositions of individual biosynthetic products (chlorophylls and amino acids) and those of animal organs for further pursuing the basis of the methodology. As a tool for investigating complex systems, compound-specific isotopic analysis compensates the intrinsic disadvantages of bulk isotopic signatures. Chlorophylls provide information about the particular processes of various photoautotrophs, whereas amino acids provide a precise measure of the trophic positions of heterotrophs. The isotopic distributions of carbon and nitrogen in a single organism as well as in the whole biosphere are strongly regulated, so that their major components such as amino acids are coordinated appropriately rather than controlled separately.

Keywords: Carbon isotopic composition; Nitrogen isotopic composition; Ecosystem; Food web; Chlorophyll; Trophic position; Amino acid; Animal organ

Review

Introduction

The biological processes that significantly affect the Earth's surface environment generally lack rigorous formulations because they are intrinsically elastic. However, using the chemical signatures induced by molecular motion (vibrational, translational, and rotational), we can at least document them rigorously and accurately. In recent decades, the natural abundances of stable carbon and nitrogen isotopes (represented as $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios, respectively) have been widely used to probe biogeochemical and ecological processes. Thanks in part to such isotopic evidence, we have come to understand our living planet more clearly. Carbon and nitrogen are essential elemental constituents of organisms and form various chemical species on Earth. The isotopic compositions of these elements provide process-related and source information.

Historically, this field started as early as the 1930s, when a custom-made mass spectrometer was constructed to measure subtle differences in the isotopic ratios of natural carbon (Nier and Gulbransen 1939). Although the carbon and nitrogen isotopic compositions of diverse types of organic matter were measured and discussed by the pioneers of isotope geochemistry (e.g., Rankama 1948; Wickman 1952; Craig 1953; Hoering 1955; Parwel et al. 1957), the isotopic fractionation associated with various biochemical reactions was only determined precisely after some time (e.g., Park and Epstein 1960; DeNiro and Epstein 1977; Hoering and Ford 1960; Wada 1980). As our knowledge of the distributions of carbon and nitrogen isotopes in organic matter and the factors controlling them has accumulated, the scope of isotopic measurements has greatly expanded.

An important application of the analysis of carbon and nitrogen isotopic compositions is in ecological studies.

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In 1967, Y. Miyake and E. Wada first reported that marine animals incorporate dietary ^{15}N in preference to ^{14}N (Miyake and Wada 1967). Later, several seminal papers provided robust data on the ^{15}N-enrichment that occurs during heterotrophic processes, based on diet-controlled laboratory culture experiments (e.g., DeNiro and Epstein 1981; Minagawa and Wada 1984). This ^{15}N-enrichment is a reflection of biochemical processes that accompany significant isotopic fractionation. The carbon isotopic composition of an organism mainly reflects its dietary signature (DeNiro and Epstein 1978), and coordinated isotopic measurements of organisms provide a unique approach to describing the dietary habits of animals, a macroscale ecological phenomenon. Such macroscale functions in the biosphere strongly influence the Earth's surface environment via the population dynamics of ecosystems (Begon et al. 2006). In this review, we summarize our current knowledge on the theoretical background underlying this approach. To achieve this, we consider the processes sequentially, first describing the isotopic relationships between substrates and autotrophs. We also present original datasets to address two related topics, compound-specific and organ-level isotopic compositions, which are helpful in achieving this goal.

Isotopes and isotopic fractionation

In nature, organic matter contains substantial amounts of stable isotopes of the 'light elements', including hydrogen, carbon, and nitrogen. For example, a human body weighing 50 kg contains over 200 g of the heavier isotopes of these elements (Wada et al. 1995). Small variations in the isotopic compositions present in nature are generally described by comparing the ratios of the isotopes in the sample material with those of standard (or reference) materials. These are expressed in the conventional δ notation defined below:

$$\delta(‰) \equiv 10^3\left(R_{sample}/R_{standard} - 1\right) \quad (1)$$

where the term R denotes the $^{13}C/^{12}C$ ratio for carbon or the $^{15}N/^{14}N$ ratio for nitrogen. In general, Vienna Pee Dee belemnite (VPDB; $^{13}C/^{12}C \simeq 0.011179$; Coplen et al. 2002) and atmospheric nitrogen (AIR; $^{15}N/^{14}N \simeq 0.003677$; Junk and Svec 1958) are used as the standards for carbon and nitrogen, respectively. Because authentic standards such as NBS-19 are not largely available, the reference materials routinely used are prepared in each laboratory or community (e.g., Tayasu et al. 2011). When a sample contains less ^{13}C or ^{15}N than the standard, the δ value is negative. A δ value of −20‰ indicates that the isotopic ratio of the sample is 2% lower than that of the standard (in the case of carbon, $^{13}C/^{12}C \simeq 0.01096$).

All physicochemical processes cause isotopic fractionation, which can be observed by measuring the isotopic compositions of both the reactants and products. The magnitude of isotopic fractionation is described as either the 'isotopic fractionation factor' denoted by α, or the 'isotopic fractionation' denoted by ε, defined with the following equations:

$$\alpha_{r/p} \equiv R_r/R_p \quad (2)$$

$$\varepsilon_{r/p} \equiv 10^3\left(R_r/R_p - 1\right) = 10^3\left(\alpha_{r/p} - 1\right) \quad (3)$$

where the subscripts r and p designate the reactant and product of a reaction, respectively. The $\varepsilon_{r/p}$ values for enzymatic reactions are generally positive for both carbon and nitrogen.

There are two types of isotope effects: isotope (exchange) equilibrium and the kinetic isotope effect. Isotope equilibrium is observed in a closed, mixed system in which a bidirectional reaction occurs and chemical equilibrium is attained. A typical example of isotope equilibrium can be seen between gaseous CO_2 and dissolved bicarbonate (HCO_3^-):

$$^{13}CO_{2(gas)} + H^{12}CO_3^- \rightleftharpoons {}^{12}CO_{2(gas)} + H^{13}CO_3^- \quad (4)$$

In this case, the isotope effect occurs during the formation and destruction of the bonds involving a carbon atom. In this reaction, the equilibrium constant K is expressed as follows:

$$K = \left(\left[{}^{12}CO_{2(gas)}\right]\left[H^{13}CO_3\right]\right)/\left(\left[{}^{13}CO_{2(gas)}\right]\left[H^{12}CO_3^-\right]\right) \quad (5)$$

$$= \left({}^{13}C/{}^{12}C\right)_{HCO_3^-}/\left({}^{13}C/{}^{12}C\right)_{CO_{2(gas)}} \quad (6)$$

$$= \alpha_{HCO_3^-/CO_{2(gas)}} \quad (7)$$

In an exchange reaction containing only one exchangeable atom, the equilibrium constant K is equivalent to the isotopic fractionation factor α. At a water temperature of 25°C, the experimentally determined $\alpha_{HCO_3^-/CO_{2(gas)}}$ value for the above reaction is 1.0079 (Mook et al. 1974). It indicates that at equilibrium, the carbon isotopic composition of CO_2 in the atmosphere is −7.9‰ when that of HCO_3^- in the surface water is 0.0‰. Most of this fractionation occurs in the hydration stage ($CO_{2(aq)} + H_2O \rightleftharpoons H_2CO_3$) and not during the exchange across the air-water interface ($CO_{2(gas)} \rightleftharpoons CO_{2(aq)}$) or in the dissociation of carbonic acid ($H_2CO_3 \rightleftharpoons HCO_3^- + H^+$).

Although the isotopes of a particular element have rather similar chemical behaviors, their specific thermodynamic parameters and rate constants in chemical and biological reactions differ. The kinetic isotope effect is considered to best describe the isotopic fractionation that occurs in the metabolic processes within a cell. The kinetic isotope effect is observed in irreversible or unidirectional reactions, such as evaporation, diffusion, and

biologically mediated reactions. In such reactions, the isotopic compositions of the reactant (δ_r) and the cumulative product (δ_p) are conventionally described with the following equations:

$$\delta_r = \delta_0 - \varepsilon_{r/p} \ln f \quad (8)$$

$$\delta_p = \delta_0 + f/(1-f)\varepsilon_{r/p} \ln f \quad (9)$$

where f ($0 \le f \le 1$) is the fraction of unutilized substrate remaining and δ_0 is the isotopic composition when $f = 0$. It should be noted that these equations are approximate forms of the Rayleigh distillation model, and the error expands as f decreases. Figure 1 is a schematic representation of the isotopic compositions of the reactant(s) and product(s) in such a unidirectional reaction in a closed system.

In general, the rate constant for the light molecule (the molecule containing the light isotope), which has higher vibrational zero-point energy, is greater than that for the heavy molecule (the molecule containing the heavy isotope) (Bigeleisen 1949). Therefore, the product of the reaction is depleted in heavy isotope (^{13}C or ^{15}N) relative to the reactant ($\alpha_{r/p} > 1$ and $\varepsilon_{r/p} > 0$). The magnitude of the isotopic fractionation depends on various factors, but in biochemical reactions, it is strongly related to the inherent characteristics of the enzyme catalyzing the reaction. The assimilation of CO_2 and nitrate by autotrophs exemplifies reactions of this kind, which will be discussed in the following "Carbon and nitrogen isotopic compositions of autotrophs: isotopic changes during anabolism" section.

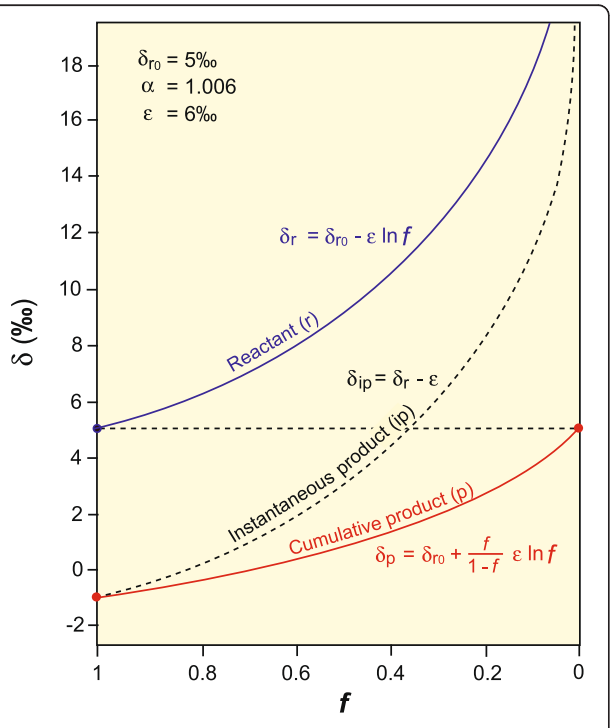

Figure 1 A schematic diagram showing the kinetic isotope effect. The isotopic compositions of unconsumed reactant (blue, solid line), instantaneous product (black, broken line), and cumulative product (red, solid line) are shown as a function of the fraction of unutilized substrate (f) for a unidirectional reaction. Mathematical formulae for these reactants and products are also shown. Subscripts r, ip, and p represent reactant, instantaneous product, and accumulated product, respectively. Modified from Mariotti et al. (1981).

Carbon and nitrogen isotopic compositions of autotrophs: isotopic changes during anabolism

Both the carbon and nitrogen that form autotrophic cells are originally derived from inorganic substrates, such as environmental CO_2 and nitrate, respectively (Figure 2). In addition to CO_2, methane (CH_4), acetate (CH_3COOH), amino acids, and other small carbon compounds are also used as carbon substrates by some specific autotrophs. Although we still do not know the quantitative importance of these substrates or the 'recycling' of organic matter in nature (Takano et al. 2010), here we assume that CO_2 is the overwhelmingly important substrate in both terrestrial and aquatic environments. The situation is more complicated for nitrogen. Although nitrate (NO_3^-) and ammonium (NH_4^+) are often considered to be the substrates of autotrophic processes, dinitrogen (N_2), nitrite (NO_2^-), urea (NH_2CONH_2), and some amino acids are also used as substrates by some autotrophs. For simplicity, we will mainly treat CO_2 and nitrate as the carbon and nitrogen substrates, respectively, in the discussion below.

When CO_2 is assimilated by an aquatic alga, it diffuses across the plasma membrane from the environment to the intracellular CO_2 pool, which is followed by carboxylation catalyzed by ribulose bisphosphate carboxylase/oxygenase (RubisCO). In contrast, nitrate is actively transported across the membrane to the intracellular nitrate pool, where it is subjected to sequential enzymatic reduction to nitrite and ammonium used in the synthesis of amino acids (summarized by Ohkouchi and Takano 2014). In both cases, isotopic fractionation occurs during the transport of the substrate across the plasma membrane and the enzymatic fixation of the substrate in the cell. The isotopic fractionation associated with the assimilations of CO_2 and nitrate by photoautotrophs has been successfully explained with a two-step model (Figure 2a,b; Park and Epstein 1960; Wada and Hattori 1978; O'Leary 1981; Farquhar et al. 1982; Hayes 1991). In this model, the diffusional process across the membrane is reversible, whereas enzymatic fixation is irreversible. The overall kinetic isotope fractionation ε_p is expressed with several types of formulae (e.g., Wada and Hattori 1978; Farquhar 1983; Hayes 1991; Chikaraishi 2014).

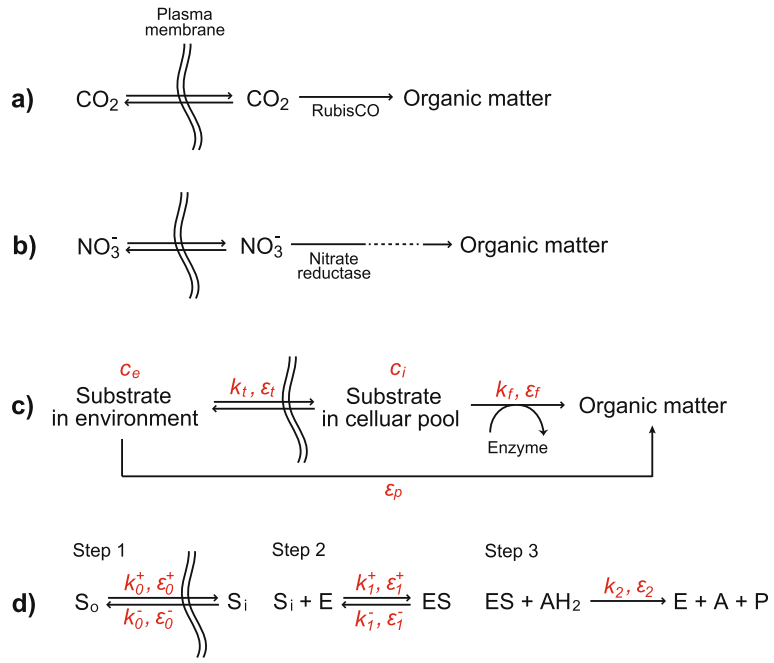

Figure 2 Schematic illustrations of the autotrophic assimilation of inorganic substrates. Diagrams **(a)** and **(b)** indicate assimilation schemes for CO_2 and nitrate, respectively. Diagram **(c)** is a scheme of the normal two-step model, and **(d)** is a more rigorous scheme based on Michaelis-Menten kinetics (Wada and Hattori 1978). The terms *c*, *k*, and ε indicate the concentration, rate constant, and isotopic fractionation, respectively.

The equation often seen in the literature is related to the substrate concentrations outside and inside the cell (specified as c_e and c_i, respectively).

$$\varepsilon_p = \varepsilon_t + \left(\varepsilon_f - \varepsilon_t\right) c_i / c_e \tag{10}$$

where ε_t is the isotopic fractionation associated with the diffusion or active transport of the substrate across the plasma membrane, and ε_f is the net fractionation caused by the enzymatic reaction that fixes the substrate (carboxylation or nitrate reduction; see Figure 2c). The values of ε_t during CO_2 assimilation were determined experimentally to be 2.9‰ to 4.4‰ for terrestrial plants (Craig 1957; Farquhar 1983) and 0.7‰ to 0.9‰ for aquatic plants (O'Leary 1984; Jähne et al. 1987). In contrast, the value of ε_f is far greater than that of ε_t, ranging from 18‰ to 30‰, which was estimated with pure RubisCO isolated from various plants and assayed under physiological conditions (summarized by Chikaraishi 2014). The equation given above has been applied when reconstructing the CO_2 concentrations in water or the atmosphere in the geological past (e.g., Pagani et al. 2005).

It must be remembered that ε_p is not only controlled by c_e but also by other factors. In particular, in low-CO_2 environments such as the present atmosphere, the growth rates of autotrophs have been shown to strongly control ε_p. The equation below was developed by Takahashi et al. (1991) to explain the CO_2 assimilation by the freshwater green alga *Chlamydomonas reinhardtii*:

$$\varepsilon_p = \varepsilon_f + \left(\varepsilon_t - \varepsilon_f\right) k_f / k_t \tag{11}$$

where k_t and k_f are the rate constants for the transport of the substrate across the membrane and from the intracellular pool to the organic matter, respectively (Figure 2c). Because $0 \le k_f/k_t \le 1$, the value of ε_p is expected to vary between ε_t and ε_f. Because ε_t is much smaller than ε_f, it is clear that the overall isotopic fractionation (ε_p) correlates negatively with the growth rate (k_f), which is controlled by environmental factors such as light intensity and substrate supply. When the growth rate increases, the mass transport tends to be a rate-limiting step. This relationship was clearly observed in laboratory culture experiments of not only *C. reinhardtii* (Figure 3a; Takahashi et al. 1991) but also the cyanobacterium *Agmenellum quandruplicatum* (Wada et al. 2012).

In contrast, Wada and Hattori (1978) derived a more rigorous formula based on Michaelis-Menten kinetics to explain the nitrogen isotopic fractionation that occurs during nitrate assimilation by the marine diatom *Phaeodactylum tricornutum* (Figure 2d):

$$\varepsilon_p = {\varepsilon_0}^+ + \varepsilon_2 XY - {\varepsilon_0}^- X \tag{12}$$

$$\left(X = 1 - k_2[ES]/{k_0}^+[S_0],\ Y = {k_1}^-/({k_1}^- + k_2)\right)$$

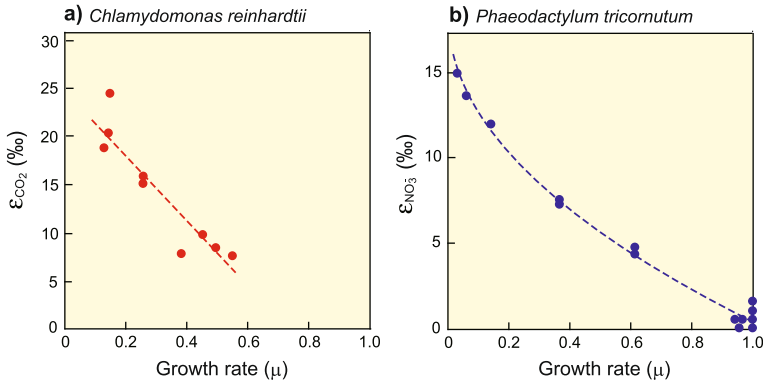

Figure 3 Experimental results indicating the relationship between isotopic fractionation and growth rate (μ) of an alga. Variations in **(a)** the carbon isotopic fractionation associated with CO_2 assimilation (ε_{CO_2}) by the freshwater green alga *Chlamydomonas reinhardtii* (modified from Takahashi et al. 1991) and **(b)** the nitrogen isotopic fractionation associated with nitrate assimilation (ε_{NO_3}) by the marine diatom *Phaeodactylum tricornutum* (modified from Wada and Hattori 1978).

where subscripts 0, 1, and 2 represent the sequential reactions from substrate transport across the membrane to the formation of organic matter (Figure 2d), and the superscripts + and − represent the forward and backward reactions, respectively. In this equation, the overall isotopic fractionation (ε_p) correlates negatively but not linearly with the growth rate (k_2) (Figure 3b).

In natural environments, the isotopic compositions of photoautotrophs occur across wide ranges: from −38‰ to −10‰ for carbon (Figure 4a) and from −9‰ to +10‰ for nitrogen (Figure 4b). The approximately 10‰ depletion of ^{13}C in terrestrial plants relative to that in aquatic plants may be explained by the fast diffusion of CO_2 in air relative to that in water. These wide ranges are partially ascribed to fluctuations in the isotopic compositions of the substrates in the aquatic and terrestrial environments. For example, in a modern cultivated field, nitrogen is heavily supplied as chemically synthesized fertilizers whose $\delta^{15}N$ values generally range from −6‰ to +7‰ (Heaton 1986; Vitòria et al. 2004). Therefore, fertilizer significantly overwrites the isotopic signature of the nitrogen substrate in the soil. The processes and dynamics of substrate assimilation also affect the isotopic compositions of biosynthetic products (Hayes 1991; Fogel and Cifuentes 1993; Raven 1997; Chikaraishi 2014; Ohkouchi and Takano 2014). A well-known example is the carbon isotopic compositions of C_3 and C_4 plants. The former assimilate CO_2 simply via RubisCO, whereas the latter assimilate CO_2 through phosphoenolpyruvate carboxylase (PEPC) after CO_2 is concentrated in the cell as a form of phosphoenolpyruvate. Because most CO_2 transported into the C_4 plant cell is used for the synthesis of organic matter, the carbon isotopic composition of C_4 plants is relatively close to that of the substrate (atmospheric CO_2) (Figure 4a). In all cases, the substrate assimilation processes are controlled by Rayleigh-distillation-type kinetics, and the isotopic composition of the product is not constant but intrinsically variable.

During nitrate assimilation, the isotopic fractionation of the enzymatic reaction catalyzed by nitrate reductase ranges from 2‰ to 10‰ (summarized by Ohkouchi and Takano 2014). Because the nitrate concentration in the pelagic ocean is generally up to several micromolar (approximately 3 orders of magnitude lower than the concentration of dissolved CO_2) except at high latitudes, nitrate is actively transported into the cell (Falkowski 1975). This may cause little isotopic fractionation during the transport of nitrate into the cell. Dissolved N_2 is slightly enriched in ^{15}N relative to that in the atmosphere (Figure 4b), reflecting the slight isotopic fractionation associated with the equilibration of N_2 between the gaseous and aqueous phases ($\varepsilon_{N_{2(aq)}/N_{2(gas)}} \simeq 0.7‰$ at 25°C; Klots and Benson 1963). Except for regions where N_2-fixation and denitrification are dominant, the nitrogen isotopic composition of oceanic nitrate usually ranges from 5‰ to 10‰. The nitrogen isotopic composition of nitrate from the surface photic zone is generally greater than 5‰ the mean value for oceanic nitrate (Sigman et al. 2009) because of the biological uptake of nitrate. When nitrogen is supplied via biological N_2-fixation, the bulk cells display a narrow $\delta^{15}N$ range, predominantly between −2‰ and 0‰ (Figure 4b; Hoering and Ford 1960; Minagawa and Wada 1986). The small isotopic fractionation associated with this process suggests that the cleavage of the strong triple bond of N_2 takes place under irreversible conditions (Wada et al. 2012) with high utilization (i.e., $f \simeq 0$; Figure 1) of the N_2 incorporated into the cell. In aquatic environments, most cyanobacteria and photosynthetic bacteria have an ability to conduct N_2-fixation, and in the terrestrial environment, some plants such as legumes contain symbiotic bacteria that fix N_2 in their root nodules. In contrast,

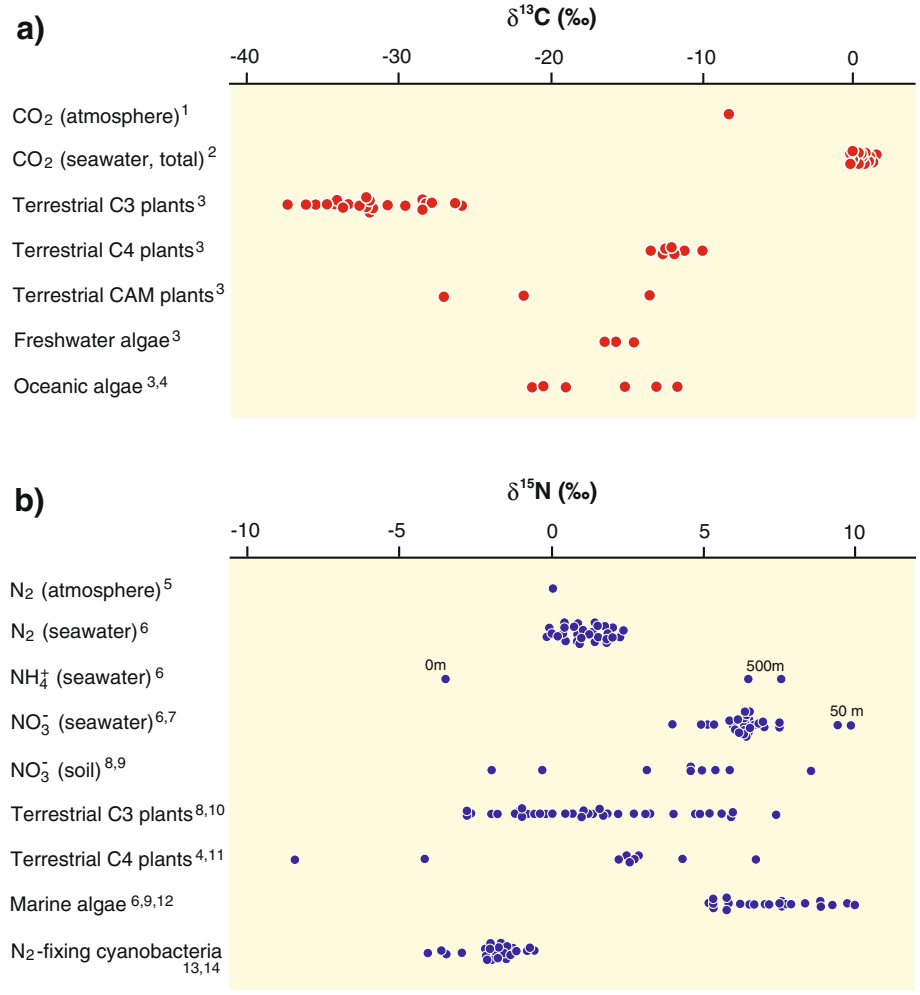

Figure 4 Compilation of a) carbon and b) nitrogen isotopic compositions of inorganic substrates and autotrophs from natural environments. All the samples shown here were collected from Japan or the northwest Pacific Ocean, except the atmospheric CO_2 and N_2. Note that most terrestrial plants were collected from cultivated areas, so their nitrogen isotopic compositions are more or less affected by chemical fertilizers. Sources: [1]Cunz (2011), [2]Kroopnick (1985), GEOSECS Station 224, [3]Chikaraishi and Naraoka (2003), [4]Wada et al. (1993), [5]Mariotti (1983), [6]Miyake and Wada (1967), [7]Minagawa et al. (2001), [8]Wada et al. (1975), [9]Wada et al. (1986), [10]Chikaraishi et al. (2005), [11]Chikaraishi et al. (2010), [12]Chikaraishi et al. (2009), [13]Minagawa and Wada (1986), [14]Wada et al. (2012).

when nitrogen is abundantly supplied to the soil via precipitation as nitrate, the isotopic signatures of plants are generally consistent with that of the nitrate.

Figure 5 summarizes the $\delta^{13}C$ and $\delta^{15}N$ values for autotrophs from various environments, many of which have been investigated by one of the authors (EW) over 40 years of his career. In natural environments, the isotopic compositions of autotrophs range widely, depending upon the substrate, the assimilation process, and the substrate utilization. To our knowledge, an algal felt from a penguin rookery in Antarctica displayed the highest autotrophic $\delta^{15}N$ value (approximately +31‰), which was attributed to the evaporation of ammonia from the soil, whereas the lowest value (−49‰) was observed in algae collected from a pond in the Dry Valley area in Antarctica (Wada et al. 1981). Such ^{15}N-depletion is probably attributable to the large isotopic fractionation during assimilation with a slow growth rate and the elevated nitrate concentration in the pond. In methane seeps, some archaea and bacteria assimilate methane, causing strong ^{13}C-depletion in the cellular components (approximately −130‰; e.g., Hinrichs et al. 1999). The biochemical processes related to the ^{13}C-depletion in these microorganisms are not well understood.

In aquatic environments, it is technically difficult to rigorously separate autotrophs (i.e., algae and cyanobacteria) from heterotrophs and detritus. Consequently, the carbon and nitrogen isotopic compositions of particulate organic matter (POM) have long been substituted for those of photoautotrophs in surface water. However,

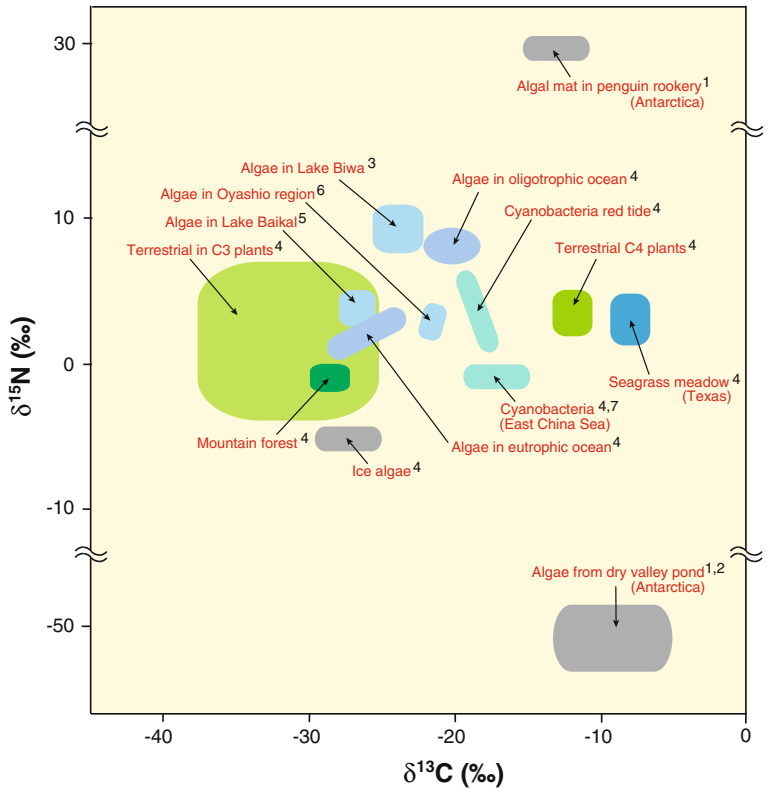

Figure 5 Summary of the $\delta^{13}C$ and $\delta^{15}N$ values for autotrophs from various environments. Superscripts indicate the sources: [1]Wada et al. (1981), [2]Wada et al. (2012), [3]Yamada et al. (1998), [4]Wada and Hattori (1991), [5]Yoshii et al. (1999), [6]Aita et al. (2011), [7]Minagawa and Wada (1986)".

these records must be more or less overprinted by the isotopic signatures of the detritus from other organisms. Therefore, the isotopic compositions of chlorophylls are useful for more accurately characterizing the isotopic signatures of primary photosynthates. Chlorophylls are abundantly produced only by photoautotrophs and contain four nitrogen atoms. The biochemical processes of chlorophyll synthesis in the photoautotrophic cell have been studied intensively (e.g., Beale 1995). This strong research background is an advantage when interpreting the isotopic signatures of chlorophylls (Katase and Wada 1990; Ohkouchi et al. 2006, 2008), although the number of applications of this technique is still limited.

This approach is particularly useful for understanding complex ecosystems. Lake Kaiike is a meromictic lake located on the coast of Kamikoshiki Island, southwest Japan. Because of its locality, seawater leaks into the deeper part of the lake through a gravel bar. In contrast, the surface water of the lake is covered by freshwater flowing from the watershed. Therefore, the lake is permanently stratified with a strong density gradient, generating an anaerobic water body below the chemocline at a depth of around 5 m. Figure 6 illustrates both the carbon and nitrogen isotopic compositions of three types of chloropigments isolated from the water column of the lake, chlorophyll *a*, bacteriochlorophyll *a*, and bacteriochlorophylls *e* (Nakajima et al. 2004). In this lake, these chloropigments were derived from cyanobacteria, purple sulfur bacteria, and brown-colored green sulfur bacteria, respectively. The three types of chloropigments have unique isotopic signatures, attributable to the distinctive ecologies and physiologies of these photoautotrophs. At the redox boundary of the water column, bacteriochlorophylls *e* are nearly 10‰ depleted in ^{13}C relative to bacteriochlorophyll *a,* even though both the green and purple sulfur bacteria assimilate CO_2 (Figure 6a; Ohkouchi et al. 2005). This large variation in ^{13}C is ascribed to the different assimilation pathways used by these photoautotrophs in synthesizing these pigments: purple sulfur bacteria fix CO_2 via the C_3 carboxylation pathway catalyzed by RubisCO, whereas green sulfur bacteria use the reverse tricarboxylic acid cycle catalyzed by α-ketoglutarate synthase. The latter process fractionates ^{13}C far less than the former process (Sirevåg et al. 1977; Quandt et al. 1977). Bacteriochlorophylls *e* are far more depleted in ^{15}N relative to either chlorophyll *a* or bacteriochlorophyll *a* (Figure 6b). This suggests that the green sulfur bacteria either fix N_2 or grow very slowly by assimilating ammonium. In the aquatic environment where various processes occur simultaneously, compound-specific isotope analysis

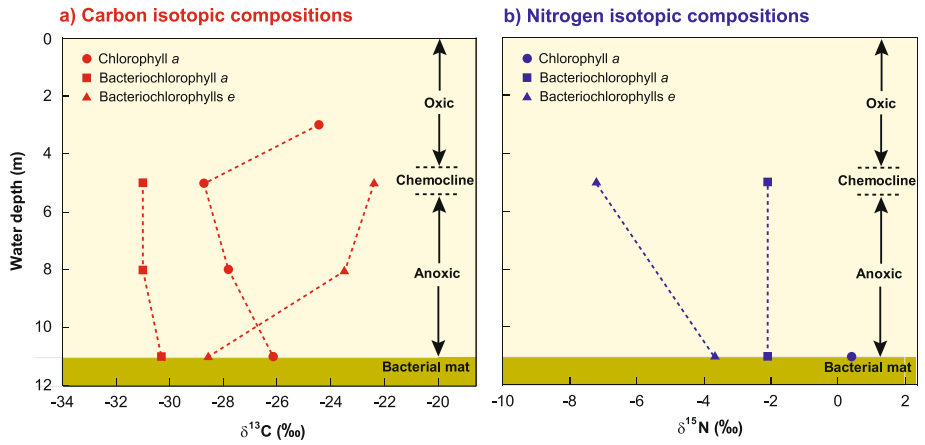

Figure 6 Carbon and nitrogen isotopic compositions of three types of chloropigments from Lake Kaiike, Japan. Carbon **(a)** and nitrogen **(b)** isotopic compositions of chloropigments (i.e., chlorophyll *a*, bacteriochlorophyll *a*, and bacteriochlorophylls *e*) from the water column of meromictic Lake Kaiike, Japan. The water column of the lake is strongly stratified with a chemocline at a depth of around 5 m. Below the chemocline, oxygen is depleted but hydrogen sulfide is abundant. The isotopic compositions of bacteriochlorophylls *e* are the mean values for those of bacteriochlorophylls e_1, e_2, and e_3. Redrawn from Ohkouchi et al. (2005).

is a useful tool for disentangling multiple coexisting signals. However, it must be kept in mind that isotopic discrimination occurs during chlorophyll biosynthesis, which produces substantial isotopic differences between the chlorophylls and the whole algal cells (chlorophylls are approximately 0.5‰ depleted in ^{13}C and approximately 5‰ depleted in ^{15}N relative to the cell; Hayes et al. 1989; Sachs et al. 1999; Ohkouchi et al. 2006). Nevertheless, the interpretation of the isotopic records of complex ecosystems can advance by separating processes, which is made possible by compound-specific isotope analyses.

Food chain analysis with bulk isotopic compositions: isotopic changes during catabolism

Once produced by autotrophs, organic matter is utilized by heterotrophs as an energy source. The heterotrophic processes generally involve breaking down organic matter to smaller compounds. In these catabolic processes, ^{12}C and ^{14}N tend to be preferentially metabolized, so that the body tissues of higher animals including muscle are generally enriched in ^{13}C and ^{15}N relative to their diets. These isotopic discriminations provide a useful approach for estimating prey-predator relationships.

During the last several decades, the analysis of grazing food chains has been a topic greatly advanced by the analysis of stable carbon and nitrogen isotopes (e.g., Peterson and Fry 1987; Wada and Yoshioka 1995; Tayasu et al. 1997; Fry 2006). As theoretically expected, diet-controlled experiments have clearly demonstrated that a predator is markedly enriched in both ^{13}C and ^{15}N relative to its prey (DeNiro and Epstein 1978, 1981; Minagawa and Wada 1984). The principles are generalized in the following equations:

$$\delta^{13}C_{TP=n} = {}^{13}\Delta(n-1) + \delta^{13}C_{TP=1} \quad (13)$$

$$\delta^{15}N_{TP=n} = {}^{15}\Delta(n-1) + \delta^{15}N_{TP=1} \quad (14)$$

where TP represents the trophic position, and TP = 1, 2, and 3 correspond to the trophic positions of plants, i.e., autotrophs, herbivores, and primary carnivores, respectively. The terms $^{13}\Delta$ and $^{15}\Delta$ are the magnitudes of the trophic enrichment for ^{13}C and ^{15}N, respectively. A number of studies have demonstrated that $^{13}\Delta$ generally ranges from 0‰ to 1.5‰ with a mean value of 0.8‰, whereas $^{15}\Delta$ ranges from 0.5‰ and 5.5‰ with a mean value of 3.4‰ (McConnaughey and McRoy 1979; Fry and Sherr 1984; Rau et al. 1983; Post 2002; Vander Zanden and Rasmussen 2001). Empirically, the $^{13}\Delta$ value tends to be smaller in lakes and coastal oceans than in the pelagic ocean (Fry and Sherr 1984; Post 2002; Wada et al. 1987, 1993).

The diversity in the isotopic compositions of autotrophs is integrated and averaged by herbivorous and carnivorous processes. Most heterotrophs forage over wide areas and depths within varying environments. For example, zooplankton such as the copepod is known to migrate by day and night in the photic zone to feed. This migration of heterotrophs must homogenize the isotopic signatures in time and space.

Why and how do predators become enriched in heavy isotopes? Because amino acids in the form of proteins are the major analytes investigated in food web studies, they are logically the key compounds with which to answer this question. Among the processes that catabolize amino acids, enzymatic decarboxylation and deamination potentially induce the isotopic fractionation of carbon and nitrogen, respectively (O'Leary 1977; Abell and O'Leary 1988). These enzymatic reactions are multistep

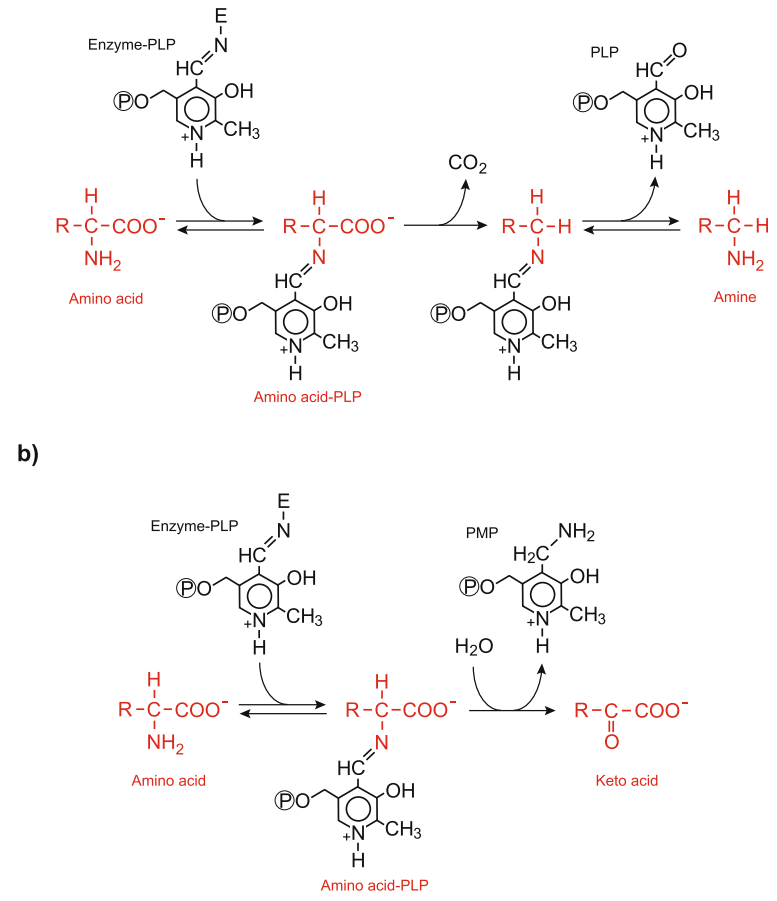

Figure 7 Chemical schemes of the biologically mediated (a) decarboxylation and (b) deamination of amino acids.

processes (Figure 7). In both enzymatic decarboxylation and deamination, the amino acid is first bound to the enzyme pyridoxal phosphate (PLP, a derivative of vitamin B_6) to form amino acid-PLP (Figure 7). Simply put, after the substrate combines with the enzyme, CO_2 or NH_3 drops off. The first step (formation of the amino acid-enzyme complex) is reversible, whereas the second step is irreversible. Therefore, the magnitude of the isotopic fractionations associated with enzymatic decarboxylation and deamination is *dynamically* controlled. The carbon isotopic fractionation that occurs during the *in vitro* decarboxylation of glutamic acid varies between 1.014 and 1.022 (at 37°C, $3.6 \leq p\mathrm{H} \leq 5.5$; O'Leary 1977). The small apparent carbon isotopic fractionation associated with decarboxylation *in vivo* (0.8‰ on average) suggests that the first reversible step (e.g., the supply rate of amino acids) may be a major limiting factor in this process.

The *in vivo* isotopic fractionation associated with the deamination of amino acids has been investigated more extensively than the isotopic fractionation associated with their decarboxylation (e.g., Chikaraishi et al. 2007). During these metabolic processes, most amino acids display strong (2.1‰ to 8.0‰) ^{15}N-enrichment. In contrast, phenylalanine and methionine display little ^{15}N-enrichment (Macko and Estep 1984; Chikaraishi et al. 2007). We have ascribed this difference in ^{15}N-enrichment between the two groups of amino acids to differences in their metabolic routes (Ohkouchi and Takano 2014). In the first step of metabolism, most amino acids are deaminated to form the corresponding keto acids. However, neither phenylalanine nor methionine is deaminated; instead, phenylalanine forms tyrosine by hydration and methionine forms S-adenosylmethionine by reacting with ATP. This evidence and theoretical considerations confirm that deamination (cleavage of the α-carbon-nitrogen bond) is responsible for the ^{15}N-enrichment in bulk organisms along trophic steps. The variation in $^{15}\Delta$ may also be partly ascribed to differences in the amino acid compositions of organisms.

Lake Baikal, located in southern Siberia, is one of the aquatic environments in which this method has been most successfully applied (Yoshii et al. 1999; Wada 2009; Ogawa 1999). The structure of its grazing food

chain has been extensively studied with field observations and is suggested to be composed of five major ecological groups: phytoplankton, mesozooplankton, macrozooplanktonic amphipods, fish, and seals. Because the ecological diversity is low and the dietary options for each species are consequently limited, the food chain in this pelagic lake is clearly demonstrated in the $\delta^{13}C$-$\delta^{15}N$ diagram shown in Figure 8 (modified from Yoshii et al. 1999). The $\delta^{13}C$ values for these organisms are similar to those in pelagic phytoplankton (−27‰ to −25‰), suggesting that neither coastal phytoplankton nor terrestrial organic matter is an important carbon source (or energy source) for the pelagic food web. From the phytoplankton at the base of the food chain to the seals as the top predator, these organisms show a clear trend in stepwise ^{15}N-enrichment, beautifully demonstrating the simple prey-predator relationships in this ecosystem.

Another case study introduced here was undertaken in Lake Biwa, the watershed of which has a human population density much higher than that of Lake Baikal. We have been particularly interested in this industrialized and urbanized watershed because it displays significant eutrophication as a consequence of elevated nutrient loading (e.g., Tezuka 1992; Nakanishi and Sekino 1996). Figure 9 summarizes the isotopic compositions of various organisms collected from Lake Biwa in the 1990s (Yamada et al. 1998). The $\delta^{15}N$–$\delta^{13}C$ diagram not only provides information about the grazing food chain, but also a diagnosis of the effects of human activity on the watershed. The nitrogen supplied from the surrounding mountains to the lake (approximately 0‰) is substantially depleted in ^{15}N relative to the phytoplankton (Wada and Hattori 1991; Wada et al. 1998), suggesting the presence of either a source(s) or a process (es) that causes ^{15}N enrichment. A heavy loading of domestic sewage substantially disturbed the natural nitrogen cycle in both the lake and the watershed. In particular, it induced denitrification, leading to the enrichment of ^{15}N in the nitrogen pool of the lake (Miyajima 1994; Ogawa et al. 2001; A. Makabe and K. Koba, personal communication). A recent microbiological study found abundant denitrifying methane-oxidizing bacteria in the surface layer of the sediment, supporting this view (Kojima et al. 2012). Isotopic analyses of sediment cores and formalin-fixed fish archives revealed that the denitrification was enhanced from the early 1960s to the 1980s, the period of severe nutrient loading in the lake (Ogawa et al. 2001, 2013).

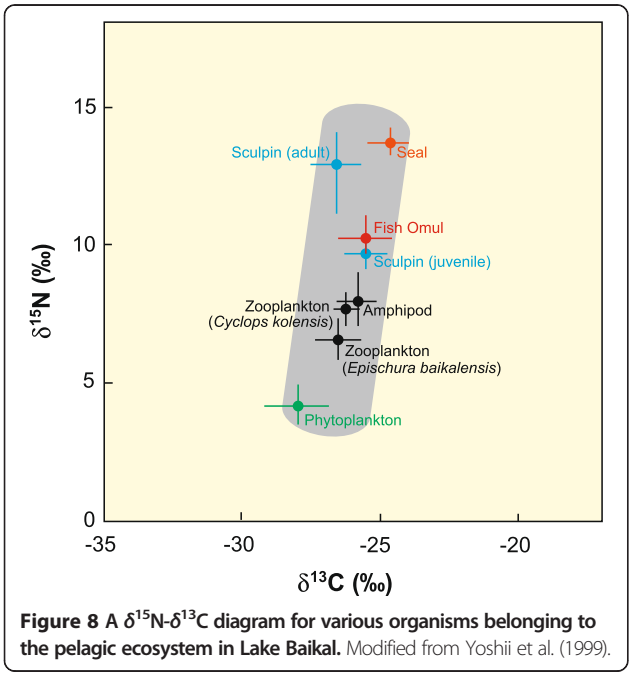

Figure 8 A $\delta^{15}N$-$\delta^{13}C$ diagram for various organisms belonging to the pelagic ecosystem in Lake Baikal. Modified from Yoshii et al. (1999).

The accumulation of such case studies over decades has provided great insight into the isotopic signatures of carbon and nitrogen. Except for lagoons and sea-grass meadows, where no or little carbon isotopic fractionation has been observed (i.e., $^{13}\Delta \simeq 0$; Fry and Sherr 1984; Wada et al. 1993), linear relationships have often been observed between ^{13}C- and ^{15}N-enrichment factors. In the cases of Lake Baikal and Lake Biwa shown in Figures 8 and 9, respectively, the slopes of the plots in the $\delta^{13}C$-$\delta^{15}N$ diagrams were calculated to be *ca.* 2.1 and 1.5, respectively, using analysis of covariance (Wada et al. 2013). In many ecosystems, the magnitudes of ^{13}C- and ^{15}N-enrichment at each trophic step are cross-linked with the following equations (Aita et al. 2011; Wada et al. 2013):

$$\text{Terrestrial:} \quad {}^{15}\Delta/{}^{13}\Delta = 1.61 \pm 0.41 \tag{15}$$

$$\text{Marine:} \quad {}^{15}\Delta/{}^{13}\Delta = 1.24 \pm 0.24 \tag{16}$$

Such empirical relationships may be explained from a kinetic perspective. The $^{15}\Delta/^{13}\Delta$ ratio will be constant if the limiting steps in the intrinsic metabolic processes have adapted to the external substrate conditions, whereas the ratio will vary if the balance between the available nitrogen and carbon varies. The relationships between $^{15}\Delta$ and $^{13}\Delta$ can also be interpreted from a physiological perspective. Primarily, the equations should reflect the well-regulated dynamics between enzymatic decarboxylation and deamination, the key processes involved in isotopic enrichment (i.e., $^{15}\Delta$ and $^{13}\Delta$) and shared by the whole biota. In the cell, deamination mainly occurs in amino acids, whereas decarboxylation occurs not only in amino acids but in various carboxylic acids including citric acid and oxalic acid, which are intermediates of the citric acid cycle, and in fatty acids, the components of membranes and energy storage materials. The differences in the compound groups subjected to deamination and decarboxylation potentially produces the variations observed in the balance between the ^{13}C and ^{15}N distributions among heterotrophs.

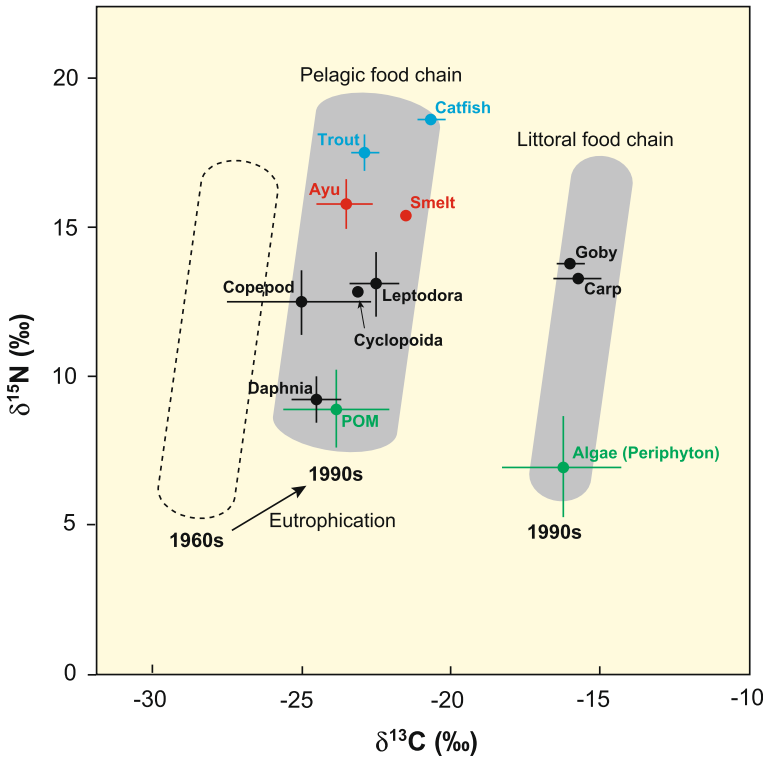

Figure 9 A δ^{15}N-δ^{13}C diagram for the ecosystem in Lake Biwa. Both pelagic and littoral food chains are shown. The estimated isotopic range of Lake Biwa before substantial eutrophication (*ca.* 1960s to 1980s) is shown as the area surrounded by a broken line. POM, particulate organic matter. Colors indicate the food web groups estimated by Yamada et al. (1998): green, primary producers; black, primary consumers; red, secondary consumers; blue, tertiary consumers. Modified from Yamada et al. (1998) and Wada (2009).

Food chain analysis based on the nitrogen isotopic compositions of amino acids

The analysis of the nitrogen isotopic compositions of individual amino acids has recently emerged as a tool in food web studies. The nitrogen in an organism is predominantly contained in proteins (i.e., mixtures of amino acids), and as a logical consequence, individual amino acids can provide more accurate information on prey-predator relationships than the bulk isotopic method described above.

Careful observation both in the field and in the laboratory has shown that predators are enriched in ^{15}N to various extents in most amino acids including alanine, valine, and glutamate (a mixture of glutamine and glutamic acid). In contrast, phenylalanine and methionine from predators are only slightly enriched in ^{15}N relative to their prey (McClelland and Montoya 2002; Chikaraishi et al. 2007). The former group of amino acids is often referred to as 'trophic amino acids' and the latter as 'source amino acids.' Enzymatic deamination (i.e., cleavage of the covalent α-carbon-nitrogen bond) to form the corresponding keto acid is the dominant metabolic route for the trophic amino acids. This biochemical reaction is responsible for fractionating the isotopes of nitrogen bonded to the α-carbon (Macko et al. 1986; Chikaraishi et al. 2007). In contrast, phenylalanine and methionine, which display little ^{15}N-enrichment, are metabolized to tyrosine and S-adenosylmethionine, respectively, during which the α-carbon-nitrogen bond is not cleaved. If the ^{15}N-enrichment factors for most amino acids were reasonably constant among various organisms, it would indicate a well-controlled common reaction network in terms of amino acid metabolism over a wide range of organisms. Because both phenylalanine and methionine are indispensable (essential) amino acids, the nitrogen isotopic compositions of these amino acids in any heterotroph are inherited from autotrophs, the ultimate nitrogen source in the food chain. Consequently, the nitrogen isotopic difference between the source and the trophic amino acids is a first-order function of the trophic position of a given organism in the food web. The balanced nature of amino acids is in strong contrast to the nature of the secondary metabolites (e.g., most lipids, alkaloids, etc.), whose production and degradation are mainly controlled by the physical environment rather than by the physiology of the cell.

Diet-controlled culture experiments in various organisms have shown that the differences in the nitrogen

isotopic compositions of glutamic acid and phenylalanine are most useful for the accurate quantification of trophic position (Chikaraishi et al. 2009). Trophic position is expressed with an equation in the following form:

$$[TP] = \left(\delta^{15}N_{Glu} - \delta^{15}N_{Phe} + \beta\right)/7.6 + 1 \qquad (17)$$

where β denotes the isotopic difference between glutamic acid and phenylalanine in autotrophs ($[TP] = 1$). The analysis of a number of plants has shown that the mean value of β is −3.4 for the aquatic plants with a 1σ error of 0.9 (Figure 10a; Chikaraishi et al. 2009). In terrestrial plants, the value of β was estimated to be 8.4 ± 1.6 (Figure 10b; Chikaraishi et al. 2011; Steffan et al. 2013). The difference in the β values of aquatic and terrestrial plants may be attributable to the differences in their metabolism of phenylalanine. In terrestrial plants, a significant fraction of phenylalanine is metabolized for the synthesis of lignin phenol, making it a branching point in the metabolic pathway (Ohkouchi and Takano 2014). The advantage of amino acid isotopes over bulk isotopes for reconstructing food chains is twofold. First, the isotopic composition of the autotroph ($[TP] = 1$) is not required to estimate the trophic position. Second, the error in the estimated trophic position can be minimized.

With this approach, we reexamined the pelagic ecosystem of Lake Baikal (Figure 11). The results confirmed not only the simple food chain but also revealed new aspects of it. The estimated trophic position of the zooplankton *Episula baikalensis* collected in different years was distributed in a narrow range from 1.9 to 2.1, confirming it as an herbivore rather than an omnivore. The seals were in the highest trophic position with an average value of 5.1. These results basically confirm those of the bulk isotope data described in the previous "Food chain analysis with bulk isotopic compositions: isotopic changes during catabolism" section (Figure 8). However, an important aspect is newly revealed. The trophic length of this food web estimated with the amino acid method is approximately 1 unit longer than that estimated with the bulk method (Yoshii et al. 1999). Assuming that the amino acid method provides the correct information, the $^{15}\Delta$ value in Lake Baikal was recalculated to be approximately 2.5‰ rather than 3.3‰. The mean trophic position of the endemic fish omul (*Coregonus migratorius*) was 3.6 rather than 3.0, suggesting that it feeds not only on herbivorous zooplankton but also on carnivores or omnivores. Sculpin, whose mean trophic position was 4.3, may be the bridge between the omul (and probably other fish species with similar trophic positions) and the seal. This view is consistent with the bulk isotope results, which showed approximately 3‰ enrichment in ^{15}N for the adult sculpin relative to the omul (Figure 8). However, these conclusions are inconsistent with the microscopic observation of the sculpin's stomach contents, which included few fish fragments (Miyasaka et al. 2006). A confident solution has yet to be reached, but the 'snapshot effect' of the stomach contents might explain the inconsistency among the other two approaches.

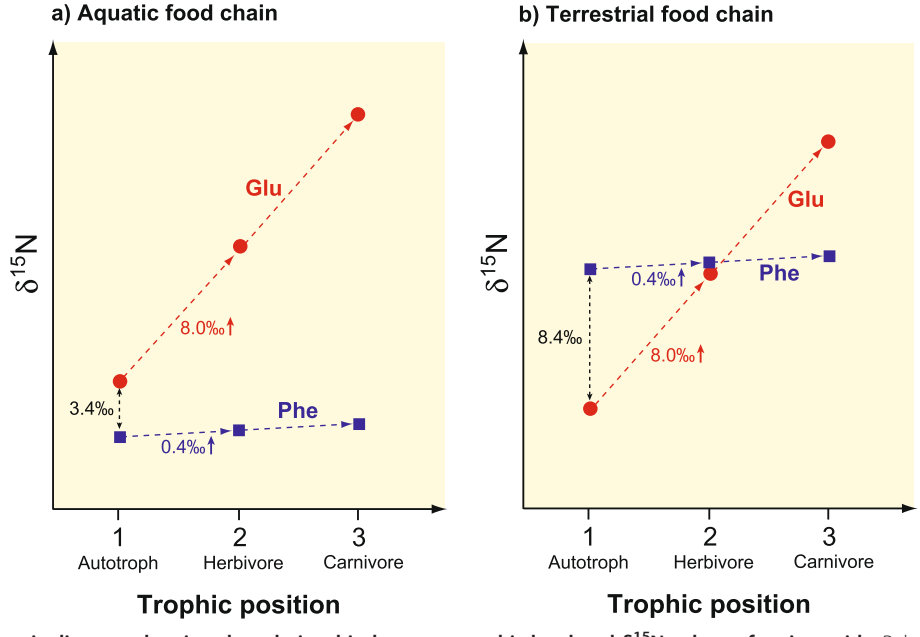

Figure 10 A schematic diagram showing the relationship between trophic level and $\delta^{15}N$ values of amino acids. Relationships between trophic positions and nitrogen isotopic compositions of phenylalanine and glutamate in **(a)** aquatic and **(b)** terrestrial C_3 and C_4 plants. Vertical axis indicates the relative $\delta^{15}N$ values of the amino acids. The mathematical formula for these relationships are given in the text as equation 17.

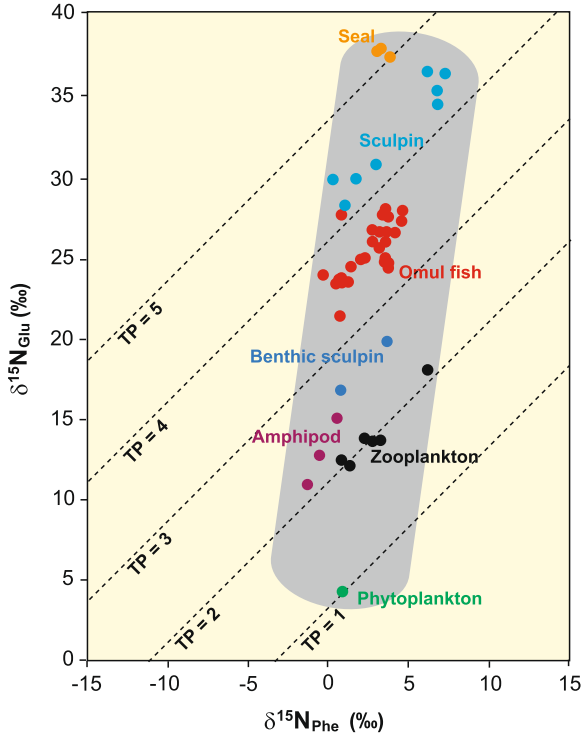

Figure 11 A $\delta^{15}N_{Phe}$-$\delta^{15}N_{Glu}$ diagram for various organisms belonging to the pelagic ecosystem in Lake Baikal. The broken lines indicate the isopleths of trophic positions 1 to 5 based on the formula described in Equation 17 and Figure 10a.

The amino acid method has been used to analyze not only ecological samples but also samples from related fields including anthropology, paleontology, and oceanography to investigate issues related to diet (e.g., Naito et al. 2010; Kashiyama et al. 2010; Popp et al. 2007; Miller et al. 2012; Ohkouchi et al. 2013). The nitrogen in the amino group of amino acids forms the peptide backbone $(-C-N-C-C-N-C-)_n$, so it is not readily exchangeable with ambient nitrogen (e.g., NH_4^+) unless it is hydrolyzed (Ohkouchi and Takano 2014). Consequently, the nitrogen isotopic signatures of the α-amino groups of amino acids are potentially preserved for a long time. Bone collagen and proteins encrusted in carbonate shell are the two major examples of situations in which this preservation occurs. Analysis of the bone collagen of ancient humans (approximately 5,000 years BP) from several archaeological sites in Japan has clearly demonstrated that this method is useful for reconstructing the diets of our ancestors (Naito et al. 2010, 2013). However, it must be kept in mind that the amino acid method provides information only on the protein source and not on the *total* diet. The carbon isotopic compositions of individual amino acids potentially offer a different perspective on food chains and more insight into $^{13}\Delta$ values (e.g., Macko et al. 1987; Minagawa et al. 1992; McCarthy et al. 2004; Choy and Richards 2009; McMahon et al. 2011; Larsen et al. 2013). This perspective includes not only the sources of diets (e.g., terrestrial vs. aquatic, C_3 plants vs. C_4 plants), but also the dynamics of amino acids in the cell and during diagenesis.

Isotopic variations in animal organs and the compartmentalization effect

Higher animals are compartmentalized systems in which numbers of organs work together systematically to perform metabolic activities. Not surprisingly, individual organs and tissues from a single organism have distinctive isotopic signatures (DeNiro and Epstein 1978, 1981; Mizutani et al. 1991). In higher animals such as humans, the isotopic composition of the hair or nails has proven useful in dietary analyses e.g., (Hare et al. 1991; O'Connell et al. 2001; Petzke et al. 2005). Here, we summarize the available information on the distinctive isotopic signatures of individual organs and tissues from a single organism.

The isotopic compositions of the individual organs and tissues of the salmon, globefish, cormorant, Mongolian sheep, rat, and three primates are illustrated in Figure 12 as deviations from those of muscle. In animals such as the rat, salmon, globefish, and Japanese macaque, the brain is enriched in ^{15}N and depleted in ^{13}C relative to most other organs and tissues (DeNiro and Epstein 1981). In contrast, the cormorant brain is substantially depleted in ^{15}N (Mizutani et al. 1991). However, this dataset is still limited, and some organs appear to show isotopic signatures inconsistent with those of similar organisms. For example, the liver of the salmon displays a similar $\delta^{15}N$ value to that of muscle, whereas the livers of the globefish and rainbow trout are approximately 1‰ depleted in ^{15}N relative to their muscle (Pinneger and Polunin 1999). Isotopic variations of a few permil among organs may be explained by the differences in the turnover rates of proteins. As explained in the last section, the carbon and nitrogen isotopic compositions vary quite markedly, even among the 20 essential amino acids. In an organism with $[TP] = 4$, for example, glutamic acid is over 25‰ enriched in ^{15}N relative to phenylalanine. The heterogeneous distribution of amino acids with distinctive isotopic compositions may partly explain the isotopic heterogeneity among organs.

In the guinea pig, the turnover times for carbon and nitrogen in the liver are several months because of the regular mitosis of hepatocytes, whereas the turnover of carbon and nitrogen is much slower in the cerebellum than in the liver. The isotopic signatures of cerebellar DNA acquired *in utero* are retained for at least the first 6 months of life (Strable et al. 2011). It has also been demonstrated that the neuronal DNA in the human cerebellum turns over little after maturity (Slatkin et al. 1985). This slowness or absence of protein turnover in the brain could explain why its isotopic signatures differ

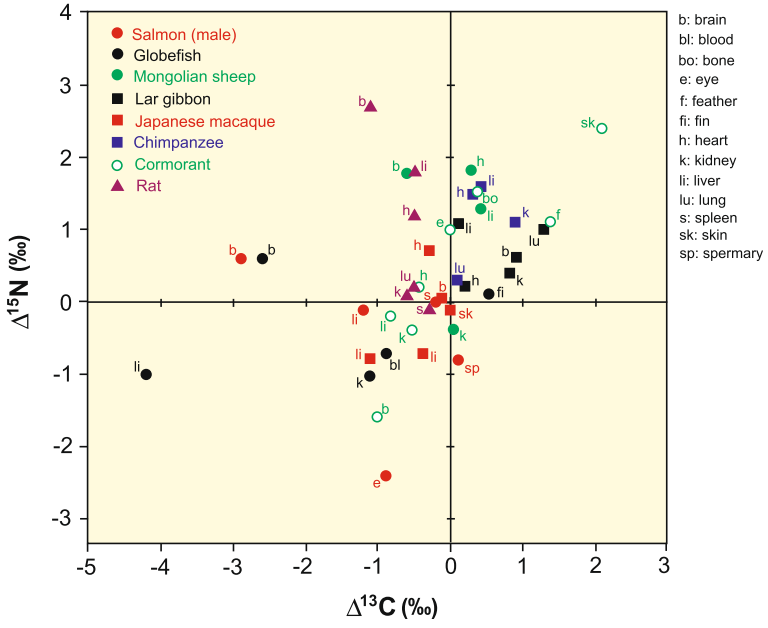

Figure 12 Carbon and nitrogen isotopic compositions of individual organs from various animal samples. These are shown as differences relative to the isotopic composition of muscle (i.e., $\Delta^{13}C = \delta^{13}C_{tissue} - \delta^{13}C_{muscle}$, and $\Delta^{15}N = \delta^{15}N_{tissue} - \delta^{15}N_{muscle}$). The letters next to the symbols are abbreviations of the organs or tissues. All institutional and national guidelines for the care and use of laboratory animals were followed.

from those of other organs with normal cellular turnover. If tissues are not metabolized, their isotopic signatures should be fixed at the point of production or equilibrated with the chemical substances around them. It is still too early to draw a clear picture, but the compartmentalization effect complicates the isotopic signatures of higher animals. Such isotopic variations potentially confound the interpretation of isotopic signatures, and caution is required when construing them, especially when the bulk isotopic method is applied to higher animals.

Conclusions

Here, we have reviewed the rules for and the theoretical background of the isotopic approach to environmental and ecological problems and have described the principles regulating the distribution of isotopes in the biosphere, which is inhabited by millions of species. Although we still have limited evidence, we stress that this approach relies strongly on the biochemical and physiological processes common to the whole biota. Even during biochemical reactions, isotopic fractionation originates solely from the physicochemical properties of molecules, the differences in molecular motion. Generalities can be drawn from comprehensive enzymatic processes with equivalent thermodynamic properties in the animal community. Therefore, a deep understanding of the isotopic fractionation that occurs through metabolic pathways is crucial to extend our knowledge of the physiological ecology of animals. Compound-specific isotopic analysis is an effective tool for this purpose.

In this review, we have focused on the grazing food chain, but clarifying the detritus food chain, the other side of the energy flow in ecosystems is essential for understanding the surface environment of this planet. Fragmentary evidence has suggested that compound-specific isotopic analysis is also effective in analyzing the detritus food chain (Macko and Estep 1984; Kohzu et al. 1999; Yamaguchi, 2012; Maki et al. 2014). If true, this approach will demonstrate our 'isotopically ordered world' even more vividly.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NO and EW proposed the topic and conceived and designed the study. NOO, YC, and HT carried out the experimental study. All authors have read and approved the final manuscript.

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