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Advances in the application of amino acid nitrogen isotopic analysis in ecological and biogeochemical studies

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Abstract

Compound-specific isotopic analysis of amino acids (CSIA-AA) has emerged in the last decade as a powerful approach for tracing the origins and fate of nitrogen in ecological and biogeochemical studies. This approach is based on the empirical knowledge that source AAs (*i.e.*, phenylalanine), fractionate ^{15}N very little (<0.5‰) during trophic transfer, whereas trophic AAs (*i.e.*, glutamic acid), are greatly (~6-8‰) enriched in ^{15}N during each trophic step. The differential fractionation of these two AA groups can provide a valuable estimate of consumer trophic position that is internally indexed to the baseline $\delta^{15}N$ value of the integrated food web. In this paper, we critically review the analytical methods for determining the nitrogen isotopic composition of AAs by gas chromatography/isotope-ratio mass spectrometry. We also discuss methodological considerations for accurate trophic position assessment of organisms using CSIA-AA. We then discuss the advantages and challenges of the CSIA-AA approach by examining published studies including trophic position assessment in various ecosystems, reconstruction of ancient human diets, reconstruction of animal migration and environmental variability, and assessment of marine organic matter dynamics. It is clear that the CSIA-AA approach can provide unique insight into the sources, cycling, and trophic modification of organic nitrogen as it flows through systems. However, some uncertainty still exists in how biochemical, physiological, and ecological mechanisms affect isotopic fractionation of trophic AAs. We end this review with a call for continued exploration of the mechanisms of AA isotopic fractionation, through various studies to promote the evolution of the rapidly growing field of CSIA-AA.

Abbreviations

AA: amino acid, EAA: essential amino acid, SAA: source amino acids, TAA: trophic amino acids, Ala: alanine, Arg: arginine, Asn: asparagine, Asp: aspartic acid, Cys: cysteine, His: histidine, Glu: glutamic acid, Gly: glycine, Ile: isoleucine, Leu: leucine, Lys: lysine, Met: methionine, Phe: phenylalanine, Pro: proline, Ser: serine, Thr: threonine, Trp: tryptophan, Val: valine, CSIA: compound-specific isotope analysis, TFA: trifluoroacetic acid, TFAA: trifluoroacetic acid anhydride, Pv: pivaloyl, MOC: methoxycarbonyl, iPr: isopropyl, GC/IRMS: gas chromatography/isotope-ratio mass spectrometry, HPLC: high-performance liquid chromatography, TP: trophic position, TDF: trophic discrimination factor, OM: organic matter, POM: particulate organic matter, DOM: dissolved organic matter, THAA: total hydrolysable amino acid

Key words

amino acid, nitrogen isotopic composition, trophic discrimination factor, trophic position, ecology, biogeochemistry

1. Introduction

74
75 The stable nitrogen isotopic composition of organisms was first applied in the field of biogeoscience
76 more than half a century ago (*e.g.*, Parwel et al., 1957; Hoering and Ford, 1960; Cheng et al., 1964).
77 Miyake and Wada (1967) first reported that marine animals preferentially incorporate ^{15}N relative to
78 ^{14}N during metabolic processing of dietary nitrogen. These initial findings were later confirmed in
79 several seminal papers based on diet-controlled laboratory culture experiments and field studies that
80 provided further evidence of ^{15}N enrichment during heterotrophic processes (*e.g.*, DeNiro and
81 Epstein 1981; Minagawa and Wada 1984; Fry, 2006 and references therein). The stable nitrogen
82 isotopic composition provides unique insight into the dietary habits of animals, as well as
83 biogeochemical cycling of nitrogen because ^{15}N enrichment during trophic transfer integrates a
84 number of biochemical processes accompanying isotopic fractionation during nitrogen metabolism.
85 The nitrogen isotopic composition of organisms provides a unique approach for describing the
86 dietary habits of animals, a macroscale ecological phenomenon. Beyond ecological studies, this
87 approach has been widely applied to biogeochemical studies investigating the fate of nitrogen in
88 oceanographic, terrestrial and freshwater systems (*e.g.*, Cline and Kaplan, 1975; Wada et al., 1975;
89 Wada, 1980; Altabet and Francois, 1994).
90 These early stable nitrogen isotope studies were based on bulk isotope analysis, which
91 integrates across all nitrogen containing entities in a sample. While certainly informative for many
92 applications, interpretation of bulk $\delta^{15}N$ data can be challenging as multiple independent factors
93 including baseline isotope values, trophic transfer, and microbial degradation, all can influence bulk
94 $\delta^{15}N$ values. Compound-specific isotopic analysis of amino acids (CSIA-AA) has emerged as a
95 powerful approach in many ecological and biogeochemical applications (*e.g.*, Gaebler et al., 1963,
96 1966; Macko and Estep, 1984; Macko et al., 1986, 1987), because the differential fractionation of
97 individual amino acids can disentangle the relative influences of baseline and trophic variability on
98 consumer $\delta^{15}N$ values. The nitrogen in an organism is predominantly contained in proteins, which
99 are long chains of amino acids (AAs) linked by peptide bonds. Consequently, the CSIA-AA
100 approach is based on the fact that the nitrogen isotopic composition of individual AAs in organic
101 matter reflects isotopic fractionation associated with various biochemical reactions of different
102 individual AA involved in nitrogen metabolism. An organism's $\delta^{15}N$ value also inherently reflects
103 the isotopic composition of inorganic nitrogen sources (*e.g.*, nitrate, nitrite, ammonia, and urea)
104 assimilated by primary producers at the base of the food web. With appropriate calibrations,
105 CSIA-AA can therefore provide uniquely specific information about multiple aspects of nitrogen
106 metabolism in organisms and ecosystem properties. CSIA-AA now has a broad range of applications,
107 including the trophic position assessment of a broad range of consumers in aquatic (*e.g.,* McClelland
108 and Montoya, 2002; Chikaraishi et al., 2009; 2014; Hannides et al., 2009, 2013; Bradley et al., 2014;
109 Gutiérrez-Rodríguez et al. 2014) and terrestrial ecosystems (Chikaraishi et al., 2010, 2014; Steffan et

al. 2013), the identification of baseline isoscapes (the spatial pattern in isotopic signatures, Bowen, 2010) of nitrogen in marine systems, the assessment of the source and transformation of dissolved and detrital organic matter in marine waters and sediments (*e.g.,* Lorrain et al., 2009; McCarthy et al., 2007; Calleja et al., 2013; Hannides et al., 2013; Sherwood et al. 2014; Batista et al., 2014; Vokhshoori et al., 2014), tracing of animal migration (*e.g.*, Dale et al., 2011; Madigan et al., 2014, 2016), and the reconstruction of food resource consumption by ancient humans (*e.g.,* Hare et al., 1991; Fogel et al., 1997; Naito et al., 2013a; Styling et al., 2010). While these studies clearly demonstrated the potential of the CSIA-AA approach, they have also opened up many new questions that suggest a wide range of potential future applications, as well as areas that need further research to improve the interpretation of CSIA-AA data. Future work to address these case-specific problems and the associated overarching challenges will push the evolution of this rapidly growing field and improve CSIA-AA applications across a variety of scientific disciplines.

This paper reviews the most recent information about CSIA-AA analytical methods and their applications to ecology, biogeochemistry, and related fields. It is an outcome of the workshop "Technical Issues Integrating Advanced Isotope Analyses into Ecological Studies" organized in association with the 10th International Conference on the Applications of Stable Isotope Techniques to Ecological Studies (IsoEcol 10) held in Tokyo in April 2016. At the workshop, investigators with widely different expertise discussed a broad range of issues related to the CSIA-AA methods and reached the conclusion that it is now time to review both the analytical methods, as well as underlying theoretical grounding of CSIA-AA applications, as a guide for future research. The review covers many broad issues, but emphasis is placed on nitrogen isotopic composition of AAs where greatest consensus has been reached. We also discuss how carbon isotopic composition of AAs may also provide unique insights in ecological and biogeochemical studies and can be a complementary approach to nitrogen CSIA-AA. The paper first explores analytical methodologies and related issues (Sections 2 and 3), then follows with applications and case studies in various fields (Section 4), before concluding with remarks addressing future perspectives and directions (Section 5).

2. Analytical Considerations

2.1. Amino acid extraction and separation

AAs in sample material, such as an organism's tissue (*e.g.*, muscle), are extracted by a simple hydrolysis procedure that breaks the peptide bonds of the constituent proteins. The hydrolysis is generally conducted with 6 to 12 M HCl at 100° to 150°C for 1 h to 1 day. The AAs are hydrophilic because of their short carbon skeletons and zwitterionic functional groups, including -COOH, -NH_2, -SH, -OH, and imino groups (-NH-). Hydrophobic molecules produced by acid

hydrolysis (*e.g.,* lipids) should be eliminated, for example, with organic solvents by liquid/liquid extraction prior to derivatization procedures.

In biological and most geochemical samples, AAs mostly exist as a "bound" form (*e.g.,* protein and peptide), with "free" AAs being a minor fraction. Some biological samples, such as calcareous and siliceous fossils, aggregated microbial samples, soils, sediments, and some biological tissue, contain large amounts of interfering materials. In such samples, solid phase extraction is required before derivatization. Cation-exchange chromatography is an effective method of removing interfering materials from the extracts with sufficient recovery (*e.g.*, Dowex WX-8, 200-400 mesh, Metges and Petzke, 1997; Biorad AG50 W-X8, 200-400 mesh, Hare et al., 1991; Takano et al., 2010). Alternatively, target AAs can be separated by high-performance liquid chromatography (HPLC) equipped with the fraction collector (Broek et al., 2013; Takano et al., 2015; Bour et al., 2016). Significant nitrogen isotopic fractionation or exchange may occur with some types of column resin (Macko et al., 1987; Hare et al., 1991; Styring et al., 2012) and therefore use of such a column resin (*e.g.*, C18) should be avoided unless the isotopic fractionation is carefully evaluated. Finally, for extremely complex geochemical sample matrixes, upstream HPLC isolation before derivatization (Broek et al., 2013) can be required to purify AA sufficiently for accurate CSIA-AA.

2.2. AA derivatization for precise determination of nitrogen isotopic composition

AAs require derivatization to reduce polarity and increase their volatility in order to be analyzed by GC/IRMS. The derivatization neutralizes polar carboxyl (-COOH), amino ($-NH_2$), and hydroxyl (-OH) groups in AAs by replacing active hydrogen atoms with nonpolar moieties, resulting in significant improvement in their chromatographic separation. Esterification of carboxyl groups with an alcohol under acidic conditions and subsequent acylation of the amino group (and simultaneous acetylation of hydroxyl group if AAs have a hydroxyl group) with an acid anhydride or acid chloride, is a common chemical reaction for the derivatization (Fig. 1a).

Although a variety of reagents have been used over the last two decades, to our knowledge, the following three derivatization reagents are most widely used in ecological and geochemical studies: trifluoroacyl-isopropyl ester (TFA/AA/iPr, Fig. 1b, *e.g.*, McCarthy et al., 2007; Popp et al., 2007), pivaloyl-isopropyl ester (Pv/AA/iPr, Fig. 1c, *e.g.*, Metges et al., 1996; Chikaraishi et al., 2007), and methoxycarbonyl (MOC) AA ester (Fig. 1d, *e.g.*, Walsh et al., 2014; Yarnes and Herszage, 2017). The first step of the derivatizations to TFA/AA/iPr and Pv/AA/iPr is the same esterification with isopropanol to form the isopropyl esters of AAs. A major advantage of the use of branched alcohol (*i.e.*, isopropanol) is that stable AA esters are obtained. The second step in the TFA/AA/iPr and Pv/AA/iPr derivatizations is acylation with trifluoroacetic acid anhydride (TFAA) or pivaloyl chloride (Pv-Cl), respectively. Because three atoms of fluorine, which is highly electrophilic, increase the nucleophilicity of the carboxyl carbon of TFAA, acylation with TFAA is

much faster than that with Pv-Cl. MOC AA ester requires a rapid one-step derivatization, which allows esterification of the carboxyl group and acylation of the amino group simultaneously at room temperature within 5 min, although the hydroxyl group is not acetylated in this derivatization. The TFA/AA/iPr and Pv/AA/iPr require strict hydrophobic conditions, whereas MOC AA ester works well in both hydrophobic and hydrophilic conditions. Detailed derivatization procedures using each reagent are described in the literature (*e.g.*, Silfer et al., 1991; Sacks and Brenna, 2005; Chikaraishi et al., 2007).

For all derivatizations, great care should be taken with respect to the chemical properties of the reagents and derivatives. First, because the ester groups in these derivatives are exchangeable with water, no alcohols or other ester compounds, including many polar solvents, can be used. For example, ethyl acetate, a convenient polar organic solvent, can exchange the isopropyl or methyl ester group in the AA derivatives with its ethyl ester group (Fig. 2a). In general, suitable solvents for the derivatives include ethers (*e.g.*, diethyl ether and tetrahydrofuran, although these solvents are highly flammable) or chlorinated methanes (*e.g.*, dichloromethane and chloroform, although these solvents are toxic). Second, most derivative reagents should be used in strict accordance with exposure controls. In particular, Pv-Cl is acutely toxic. Third, because esterified AAs are unstable in O_2 and water, even at 0°C, the derivatives must be stored at –20°C or lower (without O_2 and water, if possible) until isotope analysis. Although TFA/AA/iPr and Pv/AA/iPr esters (*i.e.*, branched alcohol esters) are relatively stable at low temperature (Fig. S1), they only survive for a few days to weeks at room temperature. Finally, these derivatizations are not equally applicable to the isotopic measurements of all 20 protein AAs. Arg, Asn, Cys, His, and Trp cannot be measured as TFA/AA/iPr and Pv/AA/iPr derivatives because of degradation (including conversion to other compounds) or less-quantitative reaction during derivatization. Although MOC AA esters can be useful for the isotope measurement of most of these AAs (Asn, Cys, His, and Trp, except for Arg), this derivatization is not appropriate for determining the isotope values of Glu, because two types of Glu derivatives are produced with distinct isotopic compositions (Fig. S2).

2.3. Nitrogen isotopic measurements of AAs

In GC/IRMS, the nitrogen isotopic compositions of AAs are determined by analyzing the ^{15}N:^{14}N ratios of N_2 molecules generated by combustion-reduction of the derivatives. The instrument consists of a conventional gas chromatograph (GC) connected to a chemical reaction interface including combustion and reduction furnaces (Merritt and Hayes, 1994). Individual AA derivatives are separately eluted by GC, and combusted mainly into N_2, NO_x, CO_2, and H_2O) in a combustion furnace with CuO and NiO with Pt at 950º-1050ºC. The NO_x generated by the combustion is subsequently reduced to N_2 in a reduction furnace with Cu at 550º-650ºC, and the H_2O and CO_2 generated during the combustion are eliminated using a liquid nitrogen trap. A countercurrent drier

can be used for H_2O elimination prior to the liquid nitrogen trap in some cases. To avoid isotopic fractionation, a nucleophilic stationary phase (*e.g.*, HP-5: phenyl-methyl polysiloxane; HP-INNOWAX: polyethylene glycols) is required for the GC separation of AA derivatives (Chikaraishi et al., 2010).

The nitrogen isotopic composition of AAs is expressed in the standard δ notation relative to atmospheric N_2 ($\delta^{15}N$, ‰ *vs*. AIR), which is calibrated to the internationally recognized scale through comparison of the $\delta^{15}N$ values of multiple reference AAs. In a typical sequence, derivatives of reference mixtures of 5-14 AAs with known $\delta^{15}N$ values, which should cover the $\delta^{15}N$ range of the samples, are analyzed every 4-8 sample runs. At the beginning and end of each chromatography run, 2-3 pulses of reference N_2 gas are discharged for all reference mixtures and samples (Fig. 3). The regression line between the known (‰, *vs*. AIR) and mean measured values (‰, *vs.* reference N_2 gas) represents the reproducibility of the isotope measurement (Fig. S3) and can be used to normalize the measured values (‰, *vs.* reference N_2 gas) to the internationally recognized scale (‰, *vs*. AIR) for both the reference mixtures and samples. In some laboratories norleucine and aminoadipic acid with known $\delta^{15}N$ values are co-injected with each sample and additional internal reference compounds that can be used for normalization (*e.g.*, Hannides et al., 2009; McCarthy et al., 2013). The average and standard deviation for the normalized values (1σ) and the difference in the normalized and known values ($\Delta_{normalized-known}$) for the reference AAs are frequently used as evidence of the precision and accuracy of the isotope measurement. Detection limits to achieve this level of precision and accuracy depend on various factors, but they are highly correlated with the signal/noise ratio of the GC/IRMS chromatogram (Fig. S4, Chan et al., 2016).

Baseline separation between the AA peaks on the GC/IRMS chromatogram is required to obtain accurate $\delta^{15}N$ values of the AAs. When an AA peak is co-eluted with other AAs or impurities, the isotopically heavy tail of the first peak underlies the isotopically light front of the second peak (Hayes et al., 1990). For example, in case of Pv/AA/iPr derivatives, Glu and Phe generally show good baseline separation, whereas Asp, Thr, Ser, and Met on the same chromatogram are sequentially eluted without baseline separation (Fig. 3).

We should note that, in addition to the analysis by GC/IRMS, off-line process (Broek et al., 2013) and HPLC-IRMS coupling may be useful in the future to determine nitrogen isotope ratios of AAs (Federherr et al., 2016).

3. Methodological considerations for trophic position assessment

3.1. Bulk *versus* CSIA-AA approach

As noted above, stable nitrogen isotope analysis of bulk organisms and their tissues has been used extensively for conventional estimation of the trophic positions of organisms in food

webs (*e.g.*, Post, 2002; Fry 2006; Ohkouchi et al., 2015). The trophic position (TP_{bulk}) is generally calculated using equation 1, based on the empirical observation that the ^{15}N content of bulk organisms tends to increase with each trophic transfer in food webs (*e.g.*, DeNiro and Epstein, 1981; Minagawa and Wada, 1984).

$$TP_{bulk} = (\delta^{15}N_{sample} - \delta^{15}N_{pp}) / TDF_{bulk} + 1 \quad (1)$$

where $\delta^{15}N_{sample}$ and $\delta^{15}N_{pp}$ are the $\delta^{15}N$ values of a target organism and the primary producers at the base of the food web, respectively. TDF_{bulk} is the trophic discrimination factor of $\delta^{15}N_{bulk}$ between prey and predator (DeNiro and Epstein, 1981; Minagawa and Wada, 1984). Many studies use a canonical TDF_{bulk} value of 3-4‰, however, a variety of TDF_{bulk} values are frequently used in studies focusing on specific tissues, such as collagen, or specific localized environments (*e.g.*, Vander Zanden and Rasmussen, 2001; McCutchan et al., 2003; Martinez del Rio et al., 2009). The 'bulk method' has been successfully applied to various ecological studies and has thus helped expand our knowledge of feeding ecology greatly over the last four decades (Fry, 2006). However, the method suffers from several problems that can cause large uncertainty in the estimated TP_{bulk} values. The most important problem is that the $\delta^{15}N$ values of bulk tissues intrinsically reflect i) the trophic changes in the $\delta^{15}N$ value in the food web and ii) temporal or spatial changes in the $\delta^{15}N$ value at the base of the food web (Fig. 4a). The former (~3-4‰) is often much smaller than the latter (in some cases >10‰) (*e.g.*, Hannides et al., 2009; Rolff, 2000; Dore et al., 2002; O'Reilly et al., 2002).

In contrast, trophic position ($TP_{TAA/SAA}$) estimated from CSIA-AA using equation 2 can constrain both trophic changes in the $\delta^{15}N$ value and baseline variation within a single organism (*e.g.*, McClelland and Montoya, 2002; Chikaraishi et al., 2007; McCarthy et al., 2007; Popp et al., 2007).

$$TP_{TAA/SAA} = [(\delta^{15}N_{TAA} - \delta^{15}N_{SAA} + \beta_{TAA/SAA}) / \Delta_{TAA/SAA}] + 1 \quad (2)$$

where $\delta^{15}N_{TAA}$ and $\delta^{15}N_{SAA}$ are the $\delta^{15}N$ values of the trophic and source AAs, respectively, from a single organism; $\beta_{TAA/SAA}$ is the isotopic difference between these AAs in primary producers at the base of the food web; and $\Delta_{TAA/SAA}$ is the difference in the *TDF* of the TAAs and SAAs during each trophic transfer ($\Delta_{TAA/SAA} = TDF_{TAA} - TDF_{SAA}$). Trophic amino acids (TAAs) (*e.g.*, Ala, Asp, Glu, Ile, Leu, Pro, and Val) tend to show large ^{15}N enrichment (by ~3-8‰) relative to diet during trophic transfer, which likely reflects isotopic fractionation associated with deamination (a first step in transamination, Macko et al., 1986; Miura and Goto, 2012) as a dominant metabolic pathway for these AAs in consumers (Fig. 5a). Source amino acids (SAAs) (*e.g.*, Met, Lys, and Phe) show little ^{15}N enrichment (~0-1‰) relative to diet during trophic transfer, which probably reflects the fact that

the initial steps in their metabolism are generally dominated by reactions that neither form nor cleave C-N bonds (Fig. 5a) and thus directly provide an estimate of the $\delta^{15}N_{SAA}$ value of the base of the food web. Therefore, *CSIA-AA derived TP values are independent of temporal or spatial changes in the $\delta^{15}N$ value at the base of the food web* (Fig. 4b).

Chikaraishi et al. (2009, 2010) first suggested the utility of Glu and Phe as a TAA and a SAA, respectively, with $\beta_{Glu/Phe}$ values of −3.4‰ for aquatic and +8.4‰ for terrestrial C3 plant-based food webs, and with $\Delta_{Glu/Phe}$ values of 7.6‰ for both ecosystems. Later, it was found that the β value in vascular plants is increased by the deamination of Phe for lignin biosynthesis, a process specific to vascular plants (Fig. 5b; Ohkouchi and Takano, 2014; Naito et al., 2016a). Therefore, algal *vs.* vascular grouping is a better classification than aquatic *vs.* terrestrial (Chikaraishi et al., 2009, 2010). Indeed, the observed β values in seagrasses (vascular plants from coastal marine environments) are similar to those of terrestrial vascular plants (*e.g.*, Vander Zanden et al., 2013; Choi et al., 2017). However, for simplified nomenclature we use the terms aquatic and terrestrial throughout this paper.

$$[TP_{Glu/Phe}]_{aqua} = [(\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - 3.4)/7.6] + 1 \quad (3)$$

$$[TP_{Glu/Phe}]_{terr} = [(\delta^{15}N_{Glu} - \delta^{15}N_{Phe} + 8.4)/7.6] + 1 \quad (4)$$

Because of the large differences in $\beta_{TAA/SAA}$ values between aquatic and terrestrial producers, mixing models must be constructed so as to consider two potential food webs where both aquatic and terrestrial primary producers may serve as basal food resources. These environments include rivers (Ishikawa et al., 2014) and coastal marine ecosystems (Vander Zanden et al., 2013; Choi et al., 2017). In this paper, and many others, there has been a focus on glutamic acid and phenylalanine as the canonical trophic and source amino acids, However, in principle, any combination of trophic and source amino acids can be used in equation 2 (*e.g.*, Decima et al., 2013; Nielsen et al., 2015; Bradley et al., 2015) as long as $\beta_{TAA/SAA}$ and $\Delta_{TAA/SAA}$ values appropriate for the combination of trophic and source amino acids are used.

3.2. Uncertainties and errors in the *TP* assessment

3.2.1. Variability in trophic discrimination factors

Constant $\Delta_{Glu/Phe}$ (= $TDF_{Glu} - TDF_{Phe}$) or $\Delta_{TAA/SAA}$ values throughout the food web is prerequisite for estimating *TP* precisely. However, the stability of $\Delta_{Glu/Phe}$ has recently come under increasing scrutiny based on new laboratory and field studies (*e.g.*, Dale et al., 2011; Matthews and Ferguson, 2014; Chikaraishi et al., 2015; McMahon et al., 2015a). Comprehensive meta-analyses of CSIA-AA from wild animals with known *TP* values (Nielsen et al., 2015; Bradley et al., 2015)

and controlled feeding experiments (McMahon and McCarthy, 2016) that examine individual TDF_{AA} values have addressed the following primary questions: i) what are the magnitude and variability in $\Delta_{TAA/SAA}$ values across a wide range of consumer-resource relationships, and ii) are there systematic underlying mechanisms driving this variability in predictable ways that could be used to improve CSIA-AA-based estimates of consumer trophic dynamics.

These meta-analyses found large variability in $\Delta_{TAA/SAA}$ values. For example, McMahon and McCarthy (2016) found the overall mean $\Delta_{Glu/Phe}$ value was 6.2 ± 2.5‰ across a wide range of taxa, diet types, and modes of nitrogen excretion, consistent with other recent large scale analyses of field-collected data for wild-caught marine consumers (6.6 ± 1.7‰; Nielsen et al., 2015; 5.7 ± 0.3‰; Bradley et al. 2015). However, within this distribution there were also some very significant excursions, with $\Delta_{Glu/Phe}$ values from 0 to >10‰ across 70 species (317 individuals) and 88 distinct species-diet combinations. Some of the reported $\Delta_{Glu/Phe}$ values, particularly for animals with *TP* values of less than 3, were within a small range (6 to 8‰) that overlapped with the original $\Delta_{Glu/Phe}$ values of 7.0‰ (McClelland and Montoya, 2002) and 7.6‰ (Chikaraishi et al., 2007). However, simply focusing on the mean can inherently obscure large variation underlying that mean. The meta-analysis of controlled feeding studies by McMahon and McCarthy (2016) is also consistent with large scale studies of wild consumers by Nielsen et al. (2015) and Bradley et al. (2015), which together strongly suggest that the observed variability in $\Delta_{Glu/Phe}$ and $\Delta_{TAA/SAA}$ values is not simply noise, but rather is predictably linked to consumer biochemistry. Below, we discuss two possible underlying biochemical and physiological processes that influence $\Delta_{TAA/SAA}$: diet quality and metabolic flux (*e.g.*, mode of nitrogen excretion).

Diet quality: In the aquatic environment, there is a trend between *TP* and $\Delta_{Glu/Phe}$ across a wide range of (although not all) consumers (Bradley et al., 2015; Nielsen et al. 2015; McMahon and McCarthy, 2016). However, this trend was not observed in insects kept in ecologically realistic pure cultures, representing three distinct communities from the terrestrial environment (Steffan et al., 2013). Further, low variability in the *TDF* was observed among 15 consumer species, representing a phylogenetically diverse group of consumers, from freshwater crustaceans and fish, to terrestrial mammals, fungi, and bacteria (Steffan et al., 2015) Most primary consumers examined in the marine environment (*e.g.*, grazing teleost fishes, zooplankton, etc.) had $\Delta_{Glu/Phe}$ values between 6‰ and 8‰, often not substantially different from the value of ~7-8‰ originally reported by McClelland and Montoya (2002), and substantiated by Chikaraishi et al. (2007). In contrast, most marine consumers with *TP* higher than 3 showed lower $\Delta_{Glu/Phe}$ values (Bradley et al., 2015; Nielsen et al. 2015; McMahon and McCarthy, 2016). One hypothesis for the pattern of decreasing $\Delta_{Glu/Phe}$ value with increasing *TP* is the effect of diet quality (defined here as the relative AA composition of a food source relative to the needs of a consumer) on consumer $\delta^{15}N_{AA}$ values,

and thus on $\Delta_{Glu/Phe}$ values.

The diet quality hypothesis suggests that nitrogen isotope discrimination decreases as dietary protein quality (degree of AA similarity between diet and consumer) increases (Hobson and Clark, 1992; Roth and Hobson, 2000; Robbins et al., 2005, 2010; Mill et al., 2007; Florin et al., 2011). McMahon et al. (2015a) showed that diet quality had a large and systematic effect on the isotopic fractionation of individual AAs in an estuarine fish (*Fundulus heteroclitus*) fed compositionally distinct diets. This study found a strong relationship between the *TDF* value of most TAAs and protein quality between diet and consumer, and no change in TDF_{Phe} across diet types. Furthermore, Chikaraishi et al. (2015) recently showed that with extreme manipulation of dietary composition (*i.e.*, the relative composition of protein/fat/carbohydrates), vastly different $\Delta_{Glu/Phe}$ values can be obtained in a single consumer. However, these two studies found opposite trends in the $\Delta_{Glu/Phe}$ *vs*. diet quality relationship, defined as relative AA composition of a food source relative to the needs of a consumer. McMahon et al. (2015a), showed that as the diet AA composition converged on that of the consumers, the $\Delta_{Glu/Phe}$ values tended to decrease. In contrast, Chikaraishi et al. (2015) indicated that the $\Delta_{Glu/Phe}$ values decreased as diet quality declined. While both of these studies indicate that diet composition strongly affects individual AA isotopic fractionation, more work is necessary to resolve the full relationship between diet quality and $\Delta_{Glu/Phe}$ value.

The reason why $\Delta_{Glu/Phe}$ often varies with *TP* might reflect differences in diet quality across different consumer-resource relationships within a food web. Generally, lower *TP* consumers often feed on diets that are more compositionally distinct relative to their own tissues (*e.g.*, zooplankton feeding on phytoplankton) than higher *TP* consumers (*e.g.*, fish feeding on other fish). When feeding on low-quality diets, defined as having highly imbalanced AA composition compared with consumer requirements, the consumer synthesizes scarce AAs *de novo* from surplus AAs. Because TAAs enriched in ^{15}N relative to SAAs tend to be abundant in the organisms, synthesis leads to the apparent increase in $\delta^{15}N_{AA}$ (Krueger and Sullivan, 1984; Roth and Hobson, 2000; Clements et al., 2009). Conversely, carnivores feeding on high-quality diets can meet more of their AA requirements *via* direct isotopic routing of dietary AAs, which should reduce ^{15}N enrichment of heavily transaminating AAs (*e.g.*, Glu) compared with consumers feeding on low-quality diets (Schwarcz, 1991; Ambrose and Norr, 1993). It should be noted that Ishikawa et al. (2017) recently showed that satiated and starved dobsonfly (*Protohermes grandis*) larvae had similar $\Delta_{Glu/Phe}$ values (7.1‰ and 7.3‰, respectively), suggesting that the $\Delta_{Glu/Phe}$ value was independent from starvation.

Mode of nitrogen excretion: There is also a clear pattern of lower $\Delta_{Glu/Phe}$ values for some urea/uric acid-producing organisms relative to ammonia-producing organisms, largely driven by differences

in TDF_{Glu} but not TDF_{Phe} (Dale et al., 2011; Germain et al., 2013; Nielsen et al., 2015; McMahon and McCarthy, 2016). The typically low $\Delta_{Glu/Phe}$ values for urea/uric acid producers may be explained by the nitrogen storage and cycling capabilities of animals (Wilkie, 2002), or by the way urea is produced in the liver (Dale et al., 2011). Key nitrogen-transferring enzymes preferentially remove ^{14}N-amines during metabolism, resulting in the subsequent ^{15}N enrichment of residual animal tissue and the excretion of ^{15}N-depleted nitrogenous waste (DeNiro and Epstein, 1981). The final isotope value of a biochemical reaction depends not only on the number of steps and associated ε values (*i.e.*, the maximal potential isotopic fractionation), but also on the relative nitrogen fluxes through branch points in the reaction chain (*e.g.*, reviewed by Hayes et al., 2001; Koch et al., 2007). Germain et al. (2013) proposed that this concept of variable nitrogen flux through additional branch points in the ornithine-to-urea pathway probably underlies the offset in $\Delta_{Glu/Phe}$ values for urea *vs.* ammonia-excreting organisms. In elasmobranchs, which have reduced hepatic glutamate catabolism relative to ureotelic organisms, a lower ε value may be related to their unique glutamate-glutamine-urea pathway (Dale et al., 2011). In addition, the recycling of ^{15}N-depleted urea by gut microbes for subsequent AA synthesis is another possible explanation for low $\Delta_{Glu/Phe}$ values in urea/uric acid-producing consumers (Davidson et al., 2003; Fouillet et al., 2008).

Summary: Independent meta-analyses of controlled feeding studies (McMahon and McCarthy, 2016) and wild consumers (Bradley et al. 2015; Nielsen et al. (2015) have shown that both diet quality and metabolic flux (*e.g.*, mode of nitrogen excretion) affect $\Delta_{Glu/Phe}$ values considerably. These processes are not mutually exclusive, and both appear to impact TDF_{Glu} by affecting the flux of nitrogen through transamination and deamination isotopic branch points. There are many systems that appear to be well characterized by a single $\Delta_{Glu/Phe}$ value, where there are minimal changes in diet quality and/or mode of nitrogen excretion within a food web (*e.g.*, Chikaraishi et al., 2009, 2011; Ishikawa et al., 2014; Kruse et al., 2015; Miyachi et al., 2015). However, the accuracy of $TP_{Glu/Phe}$ estimates may be improved by directly incorporating $\Delta_{Glu/Phe}$ variability into $TP_{Glu/Phe}$ estimates in systems where such changes do occur (*e.g.*, Lorrain et al., 2009; Dale et al., 2011; Choy et al., 2012; Germain et al., 2013; Ruiz-Cooley et al., 2013, 2014; Matthews and Ferguson, 2014; McMahon et al., 2015b). This probably requires moving toward multi-Δ equations (*e.g.*, Hoen et al., 2014), potentially averaging across multiple AAs (*e.g.*, Decima et al., 2013; Nielsen et al., 2015; Bradley et al., 2014), although averaging across multiple AAs has been shown to profoundly increase variability surrounding the TDF in terrestrial and freshwater systems (*e.g.*, Table 1 in Steffan et al., 2015). While accounting for key transitions in diet quality and mode of nitrogen excretion with multi-Δ equations improves *TP* estimates in many cases (*e.g.*, McMahon et al., 2015a,b), diet quality and metabolic flux are likely not the only drivers of variability in

$\Delta_{TAA/SAA}$ values. Continued exploration of the underlying mechanisms controlling AA $\delta^{15}N$ fractionation is critical to improve our ability to accurately estimate consumer *TP* with the CSIA-AA approach.

3.2.2. Propagation of error calculations for trophic position determination

For both ecological and geochemical / paleoceanographic applications, interpreting CSIA-AA based *TP* data requires a rigorous estimation of uncertainty in values being compared. However, uncertainty in *TP* based on nitrogen isotopic composition of AAs is more complex than standard uncertainties in measured isotopic values, because it must take into account analytical uncertainty in source and trophic AA isotopic measurements, as well as environmental uncertainty in β and Δ values. The combination of these uncertainties can be calculated using propagation of errors. The variability in the parameters used for *TP* determination can be modeled using Monte Carlo simulations, however it is also straightforward to propagate errors using a first-order Taylor series expansion (Ku, 1966), resulting in a formula easily solved in a spreadsheet or programmed into an algorithmic language (*e.g.*, Matlab, R).

In general, for any result w that is a function of two or more experimentally determined independent variables, variance in w can be calculated by Taylor series expansion if the variance in the variables is known (*e.g.*, Gelwicks and Hayes, 1990; Phillips and Gregg, 2001). In the case where $w = f(x, y, z)$, variance in w can be determined using the analytical solution of

$$\sigma_w^2 = (\partial w/\partial x)^2 \sigma_x^2 + (\partial w/\partial y)^2 \sigma_y^2 + (\partial w/\partial z)^2 \sigma_z^2 \quad (5)$$

The measured values of $\delta^{15}N_{TAA}$ and $\delta^{15}N_{SAA}$ have inherent analytical uncertainty and there is uncertainty in the values of β and Δ compiled in the literature. If we assume a general formulation of the equation used for calculation of *TP* as equation 2, uncertainty in *TP* can be determined by propagation of errors (*e.g.*, Blum et al., 2013; Bradley et al., 2015) using the analytical solution of

$$\sigma_{TP}^2 = (\partial TP/\partial \delta^{15}N_{TAA})^2 \sigma_{\delta 15N(TAA)}^2 + (\partial TP/\partial \delta^{15}N_{SAA})^2 \sigma_{\delta 15N(SAA)}^2 + (\partial TP/\partial \beta_{TAA/SAA})^2 \sigma_{\beta(TAA/SAA)}^2 + (\partial TP/\partial \Delta_{TAA/SAA})^2 \sigma_{\Delta(TAA/SAA)}^2 \quad (6)$$

The exact solution to equation 6 has been published elsewhere (Bradley et al., 2015) and an equation for calculating the propagated variance in *TP* is summarized in equation 7.

$$\sigma_{TP}^2 = (1/\Delta_{TAA/SAA})^2 \sigma_{\delta 15N(TAA)}^2 + (-1/\Delta_{TAA/SAA})^2 \sigma_{\delta 15N(SAA)}^2 + (1/\Delta_{TAA/SAA})^2 \sigma_{\beta(TAA/SAA)}^2 + \{-1/\Delta_{TAA/SAA}^2 (\delta^{15}N_{TAA} - \delta^{15}N_{SAA} + \beta)\}^2 \sigma_{\Delta(TAA/SAA)}^2 \quad (7)$$

The analytical uncertainty in isotopic measurements of trophic and source AAs in samples must be determined. Because the AA distribution in samples is more complex than that of artificial mixtures of AAs, we suggest replicate analysis of each sample following the recommendations of Hayes et al. (1990).

It has been suggested (Hoen et al., 2014) that the *TP* of a carnivore might best be determined using separate Δ-values for herbivores and carnivores:

$$TP = \{(\delta^{15}N_{TAA} - \delta^{15}N_{SAA} + \beta_{TAA/SAA} - \Delta_{herbivore}) / \Delta_{carnivore}\} + 2 \quad (8)$$

where $\Delta_{herbivore}$ is the ^{15}N enrichment in a TAAs relative to a SAA of a grazing herbivore and $\Delta_{carnivore}$ is the ^{15}N enrichment in a TAAs relative to a SAA associated with each trophic transfer for an omnivore or carnivore (Hoen et al., 2014). An expression for the variance in *TP* based on two different Δ values is

$$\sigma_{TP}^2 = (1/\Delta_{carnivore})^2 \sigma_{\delta 15N(TAA)}^2 + (-1/\Delta_{carnivore})^2 \sigma_{\delta 15N(SAA)}^2 + (1/\Delta_{carnivore})^2 \sigma_{\beta(TAA/SAA)}^2 + (-1/\Delta_{carnivore})^2 \sigma_{\Delta carnivore}^2 + \{-1/\Delta_{carnivore}^2 (\delta^{15}N_{TAA} - \delta^{15}N_{SAA} + \beta - \Delta_{herbivore})\}^2 \sigma_{hervibore}^2 \quad (9)$$

TP for animals feeding in aquatic and terrestrial environments can be calculated using the nitrogen isotopic composition of AAs if the fraction of one of the binary components is independently determined (*e.g.*, Jarman et al., 2017):

$$TP = \{(\delta^{15}N_{TAA} - \delta^{15}N_{SAA} + f_1 \beta_1 + (1 - f_1) \beta_2) / \Delta_{TAA/SAA}\} + 1 \quad (10)$$

where β_1 and β_2 are the C_3, C_4, or aquatic plant ^{15}N enrichment in the same trophic and source AAs measured in the sample, and f_1 is the fractional contribution of one of those plant types. The propagated variance in *TP* when there is a binary mixture of feeding is given by

$$\sigma_{TP}^2 = (1/\Delta_{TAA/SAA})^2 \sigma_{\delta 15N(TAA)}^2 + (-1/\Delta_{TAA/SAA})^2 \sigma_{\delta 15N(SAA)}^2 + \{(\beta_1 - \beta_2)/\Delta_{TAA/SAA}\}^2 \sigma_{f1}^2 + \{(1 - f_1) / \Delta_{TAA/SAA}\}^2 \sigma_{\beta 2}^2 + (f_1 / \Delta_{TAA/SAA})^2 \sigma_{\beta 1}^2 + \{-1/\Delta_{TAA/SAA}^2 (\delta^{15}N_{TAA} - \delta^{15}N_{SAA} + (1 - f_1) \beta_2 + f_1 \beta_1)\}^2 \sigma_{\Delta(TAA/SAA)}^2 \quad (11)$$

Propagated uncertainty in f_1 and f_2 must be input into equation 11 and can be determined using Phillips and Gregg (2001) or a similar approach.

4. Applications of the CSIA-AA approach to ecological and biogeochemical studies

4.1. Food web analyses in aquatic ecosystems

Several recent studies have used $\delta^{15}N_{SAA}$ variation to understand the baseline of food webs in the North Pacific Subtropical Gyre ecosystem. Hannides et al. (2013) used differences in $\delta^{15}N_{SAA}$ between zooplankton and suspended particles to demonstrate that deep water zooplankton in the subtropical gyre probably depend on surface rather than *in situ* particulate food, either through sinking of surface particles or vertical migrations. Choy et al. (2015) further showed that surface productivity also fuels higher-order consumers in the North Pacific Subtropical Gyre food web. A large range of $\delta^{15}N_{Phe}$ values in the tissues of both large and small pelagic micronekton suggested that some components of the food web instead are fueled by slowly settling particles that are highly modified by microbes. In contrast, there was no relationship between depth and $\delta^{15}N_{SAA}$ for large predatory fish (Choy et al., 2015), demonstrating how CSIA-AA may also be used to infer species movement and foraging across large depth gradients in oceanic ecosystems, and thus the dependence on a range of nutrient sources.

The *TP* of consumers can be estimated based only on consumer tissue $\delta^{15}N_{AA}$ (dashed trophoclines in Fig. 6), with food chain length inferred from the *TP* of apex predators. Chikaraishi et al. (2014) used these trophoclines to describe the food web of a coastal rocky shoreline community in Japan. Based on 39 species (n = 100) covering macroalgae, gastropods, echinoderms, bivalves, crustaceans, fish, and a cephalopod to document the food web structure, the study documented a food web covering 4.5 trophic levels (Fig. 7a). Probably supported by macroalgae at *TP* 1, the top predator in the system was the Kidako moray eel (*Gymnothorax kidako*) with an average *TP* of 4.6. Despite a large variation in baseline $\delta^{15}N$ values, demonstrated by $\delta^{15}N_{Phe}$ values varying between 3.5‰ and 8.7‰, all algal samples had *TP* close to 1 with known herbivores all close to *TP* 2, demonstrating the importance in knowing the baseline $\delta^{15}N$ value across appropriate time and space scales. In Lake Baikal, Ohkouchi et al. (2015) reported analytical results for seven species (n = 53) covering diatoms, amphipods, sculpins, and seals (Fig. 7b). The *TP* of seals, a top predator in the lake, was as high as 5.1, suggesting that the trophic length of the lake was one unit longer than that calculated based on the $\delta^{15}N_{bulk}$ record. Furthermore, the potential for baseline variation to confound analysis of spatial changes in *TP* based on bulk $\delta^{15}N$ values has recently been highlighted by a study on Lake Superior food webs by Kruger et al. (2016). TP_{bulk} suggested that the top predator (lake trout) spatially varied by up to *TP* of 1; however, the $\delta^{15}N_{AA}$ values confirmed a common *TP* and the likelihood that baseline $\delta^{15}N$ variation confounded TP_{bulk} estimates. A recent study by Papastamatiou et al. (2015) is one of the few to demonstrate variation in bulk isotopic composition due to trophic differences rather than

baseline differences. The authors combined acoustic tracking with CSIA-AA to demonstrate trophic flexibility in giant trevally from deep water reefs on a Pacific atoll. Individuals showed variability in their diel migration and feeding behavior that was mirrored in the wide range of *TP* determined by CSIA-AA (*TP* 3.5 to 4.6).

In addition to demonstrating the utility of the $\delta^{15}N_{AA}$ approach, these studies highlight the potential variability in food webs, such that higher-order consumers do not occupy a single *TP*. Indeed, the capacity for intraspecific variation in *TP* has long been known based on theory and empirical work (Polis, 1991; Polis and Strong, 1996). Conceptual examples of trophic omnivory associated with ontogeny are provided in Fig. 6. Adults and juveniles of an anchovy prey species are shown with varying *TP* of around 2, whereas the increase in the size classes of an apex consumer (tuna) leads to increased *TP*, likely reaching a *TP* close to 4.5-5.0 at their maximum size (Choy et al., 2015; Estrada et al., 2005). The degree of trophic omnivory within a food web could be quantified based on the deviation of consumers from integer values (*e.g.*, 2 for strict herbivores, 3 and 4 for strict carnivores) for *TP* derived from $\delta^{15}N_{AA}$. Recognition of intra-individual variability in *TP*, as observed by Papastamatiou et al. (2015) in deep reef giant trevally, will be an important outcome of future $\delta^{15}N_{AA}$ studies.

The trophic role of apex consumers will probably ultimately be elucidated by applying CSIA-AA to *TP* estimates in aquatic systems. Several recent studies have highlighted the importance of accurate *TP* estimates for apex consumers, and raised questions about the trophic role of sharks as apex predators in coral reef ecosystems. Hussey et al. (2014) highlighted the dependence of TP_{bulk} estimates on TDF_{bulk} values and suggested that the *TP* of apex shark species may have been underestimated using $\delta^{15}N_{bulk}$. Hussey et al. (2015) later demonstrated an expanded trophic complexity among large sharks using CSIA-AA. Hilting et al. (2013) used bulk stable isotope analysis to suggest that apex predators in central Pacific reefs might be predominately supported by benthic productivity and N_2-fixation. However, strong conclusions could not be drawn because of spatial variability in primary producer bulk isotopes (see also the CSIA-AA findings of O'Malley et al. (2012) on two species of lobster in the Northwest Hawaiian Islands). Conversely, based on limited bulk isotope analyses, Frisch et al. (2016) assigned Great Barrier Reef tiger sharks (*Galeocerdo cuvier*) to pelagic productivity, whereas whitetip and blacktip reef sharks were assigned to predominately benthic sources (65% and 72%, respectively). Coupling $\delta^{15}N_{bulk}$ values with stomach contents analyses suggested these reef sharks occupy similar trophic positions to large predatory fish, such as snapper, rather than acting as apex predators (Frisch et al., 2016).

In addition to comprehensive studies at ecosystem scales, several studies have used CSIA-AA tools to understand the habitat of cryptic species. One example is Miller et al. (2012), who measured the $\delta^{15}N_{AA}$ values from leptocephali, the larvae of the Japanese eel (*Anguilla*

578 *japonica*), whose food source is unknown. The estimated mean *TP* of the eel larvae was 2.4, which
579 in that ecosystem was most consistent with a diet based on particulate organic matter (POM)
580 composed of detritus from multiple sources. Ohkouchi et al. (2013) reported the *TP* of deep-water
581 ram's horn squid (*Spirula spirula*), one of the most enigmatic cephalopods found commonly all
582 over the world. Such information is useful for conserving endangered species through developing
583 artificial diets for aquafarming.
584 Finally, it is important to note that CSIA-AA is also applicable to laboratory and
585 museum specimens. A laboratory experiment conducted over the period of one year indicated that
586 formalin-fixation does not affect $\delta^{15}N_{AA}$ values derived from an aquatic consumer (Ogawa et al.,
587 2013). Ogawa et al. (2013) used formalin-fixed samples to reconstruct historical variation (1916 to
588 1992 CE) in *TP* of isaza (*Gymnogobius isaza*), a pelagic gobiid fish from the eutrophic Lake Biwa,
589 Japan. The $\delta^{15}N_{bulk}$ value of isaza has increased greatly during the 20th century (Ogawa et al., 2001;
590 Nakazawa et al., 2010), which can be explained either by an increase in *TP* with the reorganization
591 of bio-communities because of eutrophication, or by the increase of $\delta^{15}N$ in the nitrogen pool
592 owing to denitrification. The CSIA-AA results strongly suggested that eutrophication did not affect
593 the *TP* of the fish in the lake, and that the $\delta^{15}N_{AA}$ value of the formalin-fixed fish reflected the $\delta^{15}N$
594 of the nitrogen pool of the lake accurately (Fig. 8). A large global archive of formalin-fixed
595 samples would offer a tool for reconstructing paleo-limnological changes, and for constraining the
596 ecological consequences of environmental change with CSIA-AA.
597
598 **4.2. Food web analyses in terrestrial ecosystems**
599 CSIA-AA has provided new insights into the trophic roles of terrestrial organisms and, as
600 in aquatic ecosystems, distinguished itself from traditional bulk isotopic approaches (Chikaraishi et
601 al., 2011; Steffan et al., 2013). Chikaraishi et al. (2011) showed that equation 2 is equally valid for
602 terrestrial systems and Steffan et al. (2013) demonstrated that accurate and precise *TP* could be
603 derived for higher-order carnivores, using the CSIA-AA method to measure *TP* across four trophic
604 levels in terrestrial insect communities. Recent work in regards to carbon CSIA-AA shows great
605 promise in filling gaps currently left open by nitrogen CSIA-AA approaches because carbon
606 isotopic composition among essential AAs, also called $\delta^{13}C_{EAA}$ fingerprints, can provide
607 information about trophic pathways from plant sources and gut/soil microbes to consumers in
608 terrestrial ecosystems (Larsen et al., 2016a, 2016b). Carbon and nitrogen CSIA-AA has also
609 revealed novel aspects of animal and microbial biology, proving CSIA-AA to be a powerful new
610 tool for examining modern and ancient biological communities (O'Brien et al., 2002, 2004;
611 Chikaraishi et al., 2014; Steffan et al., 2015a).
612
613 4.2.1. Trophic position estimation

The first evidence that CSIA-AA is a feasible method for *TP* analysis among terrestrial organisms was obtained by using the δ^{15}N values of Glu and Phe of primarily herbivorous organisms and their plant host material collected from a farm in Japan (Chikaraishi et al., 2011). Because aphids are strict herbivores, they were ideal subjects for testing the accuracy of this tool, and the estimated *TP* of the aphids was shown to be the expected value of ~2.0. Carnivorous insect specimens (*e.g.*, lady beetles, wasps, and hornets) were also analyzed *via* CSIA to estimate their *TP* values. The data provided interesting insights into the trophic ecology of these animals; however, because most carnivores are free-roaming generalists, their actual *TP* values were not known and they were not suitable for testing the accuracy of this tool. These early studies revealed that insect *TP* remained constant through major ontogenetic shifts, including insect pupation (Chikaraishi et al., 2011). During such metamorphoses, there is much synthesis of new tissues and organs, so it was expected that there would be significant fractionation or routing of ^{15}N within the pupating insect. The finding that arthropod metamorphosis left the δ^{15}N values largely unchanged was critical to further applications of $\delta^{15}N_{AA}$ to insect food web ecology (Chikaraishi et al., 2011).

Steffan et al. (2013) used terrestrial insect populations in axenic culture to test whether top carnivore *TP* could be reliably determined using $\delta^{15}N_{AA}$. In this study, two different insect communities were maintained, each spanning four trophic levels, and each consuming an ecologically realistic component of their diet. Steffan et al. (2013) showed that the $TP_{Glu/Phe}$ of higher-order consumers (carnivorous insects) could be measured with high accuracy, and that the $\Delta_{Glu/Phe}$ value was consistent between herbivores and tertiary carnivores. The $\Delta_{Glu/Phe}$ value for herbivorous and carnivorous arthropods was similar to that found by Chikaraishi et al. (2009) for marine fish and gastropods, showing that the *TP* formula using a $\Delta_{Glu/Phe}$ value ~7.6‰ and a $\beta_{Glu/Phe}$ value appropriate for each environment in equation 2 was applicable to a wide variety of ecosystems. The consistency in the CSIA-AA findings across animal taxa and ecosystem types observed on land (Chikaraishi et al., 2010; Steffan et al., 2013) provided a foundation to begin investigating consumer *TP* in the field, at larger spatial scales and in more diverse communities. Further CSIA-AA of directly sampled terrestrial organisms in the wild revealed a high degree of trophic omnivory among 38 consumer species, providing some of the strongest empirical evidence of the predominance of omnivory in food webs (Chikaraishi et al., 2014).

Novel contributions of CSIA-AA to terrestrial ecology have centered around the microbiome and mainly the inclusion of microbes in trophic hierarchies. Studies involving multiple phyla of fungi and bacteria, plus vertebrate and invertebrate animals, showed that the CSIA-AA approach provides a new way to probe trophic ecology of the three domains of life (Steffan et al., 2015a). Fungi are particularly important consumers and symbionts in many terrestrial systems (Bardgett and Cook, 1998; Moore and de Ruiter, 2012). Showing that these organisms can be integrated into food-chains has allowed more refined interpretations of animal trophic identity.

However, this also raises questions of how to interpret the *TP* values of detritivores. Recent work has shown that microbes increase the *TP* values of detrital complexes, and when animals eat such microbe-colonized complexes, the consumer *TP* values increase to the same degree (Steffan et al., 2017). Given that detritivory is the dominant trophic paradigm on land (Coleman, 1996; Hagen et al., 2012) and that microbes are the dominant consumers among the detritivores (Peterson and Luxton, 1982; van der Heijden et al., 2008; Moore and de Ruiter, 2012), the ability to explicitly integrate microbes into trophic hierarchies represents a major advance in trophic ecology. Common detritivorous animals, such as earthworms, fruit flies, and springtails, exhibit *TP* values (2.4-2.8), providing evidence of the degree to which they mix microbivory with herbivory (Steffan et al., 2017). Detritivores form an immense prey base for predators in terrestrial systems (Haraguchi et al., 2013; Hyodo et al., 2015), and this prey base tends to shape the trophic identity of most carnivores (Coleman, 1996; Bardgett and Cook, 1998).

4.3.2. Recent discoveries in terrestrial biology and ecology

$\delta^{15}N_{AA}$ was used to reveal that leafcutter ants (*Acromyrmex*) in Neotropical rainforests are trophically carnivorous (Steffan et al., 2015a). The ants feed almost exclusively on the fruiting bodies of their fungal symbiont, *Leucoagaricus*. Since this fungus feeds solely on plant material, the fungus is a strict herbivore, and the ants are strict carnivores. This finding implies that fungi, not ants, are the dominant herbivores of the Neotropics. Interestingly, there is a third symbiont, a bacterium, in the leafcutter ant fungus gardens that gathers in powdery white masses on the ant exoskeleton (Currie et al., 2006). It was unclear whether this bacterium fed on the ants or some other resource. CSIA-AA showed that the bacteria were feeding on ant tissues; thus, a bacterium was the apex carnivore within the fungus-garden community (Fig. 9, Steffan et al., 2015a). Fungi can also be predators, and $\delta^{15}N_{AA}$ values were used to demonstrate that an entomopathogenic fungus, *Beauveria bassiana*, registered a *TP* of 3.0 after killing and consuming its prey, a herbivorous caterpillar. At the other end of the trophic spectrum, Asiatic black bears (*Ursus thibetanus*) were shown to feed remarkably low in the food-chain, registering near *TP* 2.0. Thus, there are now multiple examples in the literature where the trophic tendencies of terrestrial mammals (*e.g.*, mice, bears) have been measured using $\delta^{15}N_{AA}$ (Nakashita et al., 2011; Steffan et al., 2015a).

In agricultural contexts, CSIA-AA has been used to characterize the trophic roles of organisms thought to be beneficial to crop protection (Steffan et al., 2015b). Carnivorous arthropods are generally assumed to be helpful in suppressing herbivorous pest species, but CSIA-AA has shown that only certain predator species contribute substantially to pest control. Some carnivores are beneficial for crop protection, and some are neutral, and other species may undermine crop protection efforts by feeding on the beneficial carnivores (Fig. 10). Knowledge of

686 which predator communities are likely to help or harm crop protection is useful for the ecological
687 management of crop fields.
688
689 **4.3. Applications to ancient humans and extinct mammals**
690 CSIA-AA has been used to study tissues, like bone collagen and scalp hair, from
691 archaeological and contemporary humans, and ancient soils in archaeological and anthropological
692 studies. These studies span various fields, including paleodiet, nutrition, paleopathology, and
693 ancient land use (*e.g.*, Hare et al., 1991; Fogel, 1997; Petzke et al., 2005). Studies of paleodiet
694 mainly revolve around investigating i) marine protein consumption (Naito et al., 2010a, 2010b;
695 Styring et al., 2010), ii) the importance of animal proteins relative to plant proteins in terrestrial
696 ecosystems (Naito et al., 2013b, 2016b), and iii) the importance of proteins from freshwater
697 resources relative to proteins from terrestrial resources (Naito et al., 2013a). Goal iii) is challenging
698 because distinguishing terrestrial and freshwater food consumption is difficult since these two
699 environments may share the same nitrogen sources (*e.g.*, contributions of terrestrial primary
700 production to a stream ecosystem, Naito et al., 2016a).
701 $\delta^{15}N_{Phe}$ values in some archaeological contexts in animals mirrors their nitrogen source
702 owing to little trophic ^{15}N enrichment. For example, Naito et al. (2010, 2013b) examined coastal
703 and inland archaeological sites from the Jomon period in Japan (*ca.* 15,000 to 2,300 years BP). The
704 $\delta^{15}N_{Phe}$ values of animals in these contrasting ecosystems, including humans, were consistent
705 within each ecosystem, although there were differences between ecosystems (Fig. 11). The coastal
706 population showed $\delta^{15}N_{Phe}$ values between those of marine and terrestrial ecosystems, with values
707 closer to marine ecosystems, indicating heavier reliance of the humans on marine food resources.
708 However, the inland population had $\delta^{15}N_{Phe}$ values in the terrestrial ecosystem indicating purely
709 terrestrial food habits. In both cases, tracing the nitrogen source for humans was facile because
710 each ecosystem showed marked differences in $\delta^{15}N_{Phe}$ values. However, this is not the case in other
711 archaeological contexts where $\delta^{15}N_{Phe}$ values vary substantially within each ecosystem. $\delta^{15}N_{Phe}$
712 values in terrestrial prey animals can vary widely (>6‰), even for a single species from a single
713 site, which makes it difficult to trace the nitrogen source (Fig. 12). Nevertheless, this technique is
714 still useful for examining *TP* values of animals. Neanderthals from this site exhibited *TP* values of
715 2.7 to 2.8, similar to those of wolves (*TP* 2.9), suggesting that the Neanderthals ate meat-based
716 diets, with the possible addition of plant foods (Fig. 11).
717 CSIA-AA can also be used to investigate diets of extinct mammals, including wooly
718 mammoths (*Mammuthus primigenius*) (Naito et al., 2016b; Schwartz-Narbonne et al., 2015), cave
719 bears (*Ursus spelaeus*) (Naito et al., 2016c), scimitar-toothed cats (*Homotherium serum*), and
720 short-faced bears (*Arctodus* spp.) (Schwartz-Narbonne et al., 2015). Based on the high $\delta^{15}N_{Phe}$
721 value of mammoths, it has been hypothesized that the mammoth occupied a distinct foraging niche

or habitat compared with other coeval herbivores, owing to the high δ^{15}N values of bulk collagen arising from ^{15}N-enriched food sources (Naito et al., 2016b). This finding demonstrates the separation of mixtures of environmental signals (*e.g.*, aridity may elevate the δ^{15}N values of animal body tissues: Heaton et al., 1986; Schwarcz et al., 1999) and dietary signals in δ^{15}N values of collagen. However, the δ^{15}N values of body tissues, and probably the $\delta^{15}N_{AA}$ values, may also encode physiological states, illness, and quality of diet (Fogel et al., 1989; Fuller et al., 2004, 2006; Reitsema and Muir, 2015; Reitsema, 2013; Chikaraishi et al., 2015). In combination with studies on contemporary humans and archaeological human remains, the number of study fields for CSIA-AA, such as paleopathology, may expand (Fogel, 1997; Metges and Petzke, 1997; Petzke et al., 2006, 2010; Romek et al., 2013).

Lastly, CSIA-AA has also been used to investigate past land use by humans. Preliminary results suggest that the δ^{15}N values of Phe and Thr in the soil may be useful for distinguishing the soil under grassland from that under cereal (Bol et al., 1998; Simpson et al., 1997, 1999). Although the underlying mechanisms controlling the δ^{15}N dynamics of soil AAs are not well understood, some AAs may provide clues for understanding past human activities like cultivation (Styring et al., 2013), which is important because cultivars are rarely preserved in the archaeological record.

4.4. CSIA-AA and isoscapes: application to ecogeochemistry and the detection of animal migration

Ecogeochemistry is the application of geochemical techniques to fundamental questions in population and community ecology, and is inherently spatial (*e.g.*, Bowen, 2010; Graham et al., 2009; Ramos and Gonzalez-Solis, 2012; McMahon et al., 2013a). Consequently, accurate interpretation of stable isotopic compositions in ecological or environmental studies requires knowledge of the geospatial and temporal variability in isotope values at the base of the food web, often referred to as isotope baselines (Post, 2002; McMahon et al., 2013b). Spatiotemporally explicit maps of isotopic variability, termed isoscapes, have emerged as important tools for addressing interrelated ecological questions about animal movement, habitat use, biogeochemical cycling, and forensic science (*e.g.*, West et al., 2010).

Effective application of isoscapes to ecological questions requires specific information (Hobson et al., 2010). First, an isoscape must be established that characterizes systematic geospatial variability in isotopic compositions across environmental gradients. Second, tissue turnover rates that determine the period of spatial integration of isotopic composition for a particular animal tissue must be constrained. Finally, the isotope fractionation factors between the consumer and diet, or between animals and the ambient environment that offset geochemical values in animal tissues from baseline isoscape values, must be estimated or quantified.

Bulk tissue or whole animal isotope analyses have been the primary tools in applications

of terrestrial and marine ecosystem isoscapes (Bowen et al., 2009; Jaeger et al., 2010; MacKenzie et al., 2011; Hobson et al., 2012; Trueman et al., 2012; Clementz et al., 2014). However, in addition to characterizing the geospatial structure of isotope data within a system, we also must account accurately for how baseline isotope values are modified as they propagate through food webs to upper trophic level consumers (Hobson et al., 2010). Thus, a major obstacle to interpreting bulk tissue isotope values of consumers accurately is separating the relative effects of variability at the base of the food web from trophic dynamics within the food web that links consumers to those baselines (Post, 2002).

CSIA-AA can disentangle the relative effects of geographic and trophic dynamics on consumer isotopic compositions (Chikaraishi et al., 2007, 2009; Popp et al., 2007; Lorrain et al., 2009, 2015; Olson et al., 2010). The differential isotopic fractionation of individual AAs provides direct access to information about integrated ecosystem isotopic baselines without the confounding issue of trophic fractionation, and without the need to analyze and characterize all the trophic linkages between the baseline isoscapes and upper trophic level consumers *a priori*. Below we highlight several unique but complementary examples of how CSIA-AA, in the context of geospatial isotopic variations, provides unprecedented links between animal ecology and biogeochemistry in complex ecosystems.

4.4.1. Case study 1: Mussel isoscapes of the California coast

One promising CSIA-AA isoscape application for monitoring coastal biogeochemical change is the creation of detailed maps of coastal isotopic baselines, based on CSIA-AA measurements in filter feeding mollusks. Coastal system isoscapes are inherently challenging owing to high temporal and spatial variability in primary production and biogeochemical cycles. Many coastal regions are characterized by large seasonal swings in temperature, salinity, nutrient availability, and terrestrial inputs, while high spatial variability in oceanographic conditions is driven by coupled local winds, upwelling, and current patterns (*e.g.*, Walker and McCarthy, 2012; Walker et al., 2014). The isotopic compositions of consumers can often integrate this environmental variation. However, on the spatial scales of coastal processes, assigning mobile consumers to specific locations can be difficult. Tissues of sessile filter-feeders, such as mussels, offer a solution to this problem: they do not move, and specific tissues/organism sizes can be chosen to provide additional control over the integrated time scales represented by the samples. Early work coupling CSIA-AA proxies for isotopic baseline (*i.e.*, $\delta^{15}N_{Phe}$), coupled with high resolution sampling of filter feeding consumers, has allowed the creation of isoscapes of baseline coastal primary production, based on precisely known and replicable sampling locations (Vokhshoori et al., 2014; Vokhshoori and McCarthy, 2014).

However, the reconstruction of baseline isoscapes based on $\delta^{15}N_{AA}$ also poses a number of

challenges, primarily with the interpretation of $\delta^{15}N_{Phe}$ values in mollusk bioarchives. The challenges include clarifying the mix of littoral food sources using CSIA-AA proxy records, understanding temporal/seasonal effects in this signal, and the requirement to understand the influence of bulk algal $\delta^{15}N$ isoscapes on mollusk AA isotopic values. In two recent papers, Vokhshoori and coauthors explored these problems using littoral *Mytilus californianus* collected from 28 sites on the California coast, spanning ~10 degrees of latitude (32° to 42°N) within the California current system (Vokhshoori et al., 2014; Vokhshoori and McCarthy, 2014). CSIA-AA values of adductor muscle tissue from individuals of a similar size class were selected to represent approximately annual integration timescales.

$\delta^{15}N_{bulk}$ values in mussels showed a strong linear trend with latitude (Fig. 13). Although there were clear site-specific and region-specific offsets in $\delta^{15}N$ values, the overall data indicated a strongly linear progressive change in $\delta^{15}N$ values, averaging 0.4‰ per degree of latitude, across the coastal California current system. This change reflects the relative geographical influence of water upwelled from the California undercurrent, which transports ^{15}N-rich nitrate poleward (*e.g.*, Altabet et al., 1999). The $\delta^{15}N_{Phe}$ values also tracked the $\delta^{15}N_{bulk}$ values, confirming the nutrient baseline as the underlying driver for changes in bulk mussel tissue. Prior studies had indicated generally lower $\delta^{15}N_{nitrate}$ values in more northern regions of this system (Altabet et al., 1999; Kienast et al., 2002; Sigman et al., 2009), however the strength of the linear trend revealed by high resolution mussel sampling was surprising. This result suggests that such mollusk-derived isoscapes can be used to precisely define changes in the effects of coastal oceanography on baseline Isoscapes, as well as to identify local regions of variation linked for example to upwelling patterns (Walker and McCarthy, 2012).

However, $\delta^{15}N_{bulk}$ records are inherently unable to reconstruct baseline $\delta^{15}N$ values directly. $\delta^{15}N_{bulk}$ values were 2 to 4‰ higher than the expected range of $\delta^{15}N_{nitrate}$ values in this system, probably because of a combination of trophic transfer and tissue-specific offsets. Vokhshoori and McCarthy (2014) found that $\delta^{15}N_{Phe}$ values corresponded most closely to the range of previously measured $\delta^{15}N_{nitrate}$, suggesting that $\delta^{15}N_{Phe}$ in mussels is a direct proxy for annual average $\delta^{15}N_{nitrate}$. Finally, a constant $\delta^{15}N_{bulk}$ *vs.* $\delta^{15}N_{Phe}$ offset observed for all samples allowed the construction of predicted "coastal nitrate" $\delta^{15}N$ values. The resulting isoscape was grounded in high-resolution bulk sampling, but then calibrated to baseline $\delta^{15}N$ values based on CSIA-AA data (Fig. 12). It is unclear how far isoscapes based on littoral species can be extrapolated. However, for Monterey Bay, a direct comparison of mussel $\delta^{15}N$ data with a greater variety of more offshore sample types (*e.g.*, sinking POM, plankton tows, and surface sediments) suggested that, at least on the timescales sampled, mussel $\delta^{15}N_{Phe}$ values reflect baseline $\delta^{15}N$ values in local coastal waters.

These results demonstrate the potential of CSIA-AA in sessile filter feeders to create the first true baseline isoscapes of coastal production, with the potential for an extraordinary degree of

geographic and temporal resolution. Although bulk tissue analysis can indicate geographic trends, coupling $\delta^{13}C_{EAA}$ fingerprinting with $\delta^{15}N_{AA}$ allows the fundamental ambiguity of organic matter sources to be addressed, and can quantify the relative balance of sources underlying CSIA-AA signals. $\delta^{15}N_{Phe}$ values can track baseline $\delta^{15}N$ values, and in systems with full nitrate utilization this should also allow direct assembly of $\delta^{15}N_{nitrate}$ isoscapes. Therefore, it may be possible to monitor fine-scale shifts in coastal nitrogen biogeochemical cycles linked to short- or longer-term fluctuations in climate and physical forcing. However, several challenges remain, including understanding in more detail the calibrations required to link measured $\delta^{15}N_{AA}$ values to average primary production (or nitrate) isotope values, and investigating integration timescales, such as potential seasonal bias and the effects of tissue type, organism size, and growth stage.

4.4.2. Case study 2: Detecting animal migration

Systematic variations in nitrogen isotopic compositions in the ocean, such as the mussel isoscapes in Section 4.4.1 or those in the eastern tropical North Pacific (Olson et al., 2010), create ecoregions with distinctive isotope ratios in baseline organisms (*e.g.*, phytoplankton). These regional differences allow the results of CSIA-AA to be used to recognize animal migrations. This approach principally relies on certain AA isotopic compositions in animals having reached a steady state with the $\delta^{15}N$ value at the base of the food web.

Marine animal migrations can be identified with CSIA-AA by two approaches. The first is a chronological reconstruction of isotopic compositions of AAs in an archival tissue (*e.g.*, otoliths, fin spines) that represent the animal's environment at different stages of ontogeny. Older tissues can represent an isotopic steady state with an environment different from an animal's current location, which can then be compared with recently synthesized tissue that has AA isotopic compositions in a steady state with the current location. The second approach is to compare isotopic compositions of AAs in a non-archival tissue (*e.g.*, muscle) across individuals that are a suspected mix of residents and recent migrants to a particular environment. With this approach, the timeframe for distinguishing residents from migrants is defined by the turnover time of nitrogen in the tissue analyzed. The $\delta^{15}N_{SAA}$ values in animals record the isotopic composition at the base of the food web. In addition, the difference in $\delta^{15}N_{SAA}$ and $\delta^{15}N_{TAA}$ values constrains potential *TP* variations between residents and suspected migrants (*e.g.*, Madigan et al., 2012b; Seminoff et al., 2012).

For example, $\delta^{15}N_{AA}$ values were used to study the foraging ecology and habitat use of the brown stingray (*Dasyatis lata*) near Kaneohe Bay, Oahu, Hawaii (Dale et al., 2011). Although quantitative stomach content analysis of *D. lata* indicated an ontogenetic shift to a higher *TP* in larger, older specimens, the largest stingrays had the lowest $\delta^{15}N_{bulk}$ values. Lower $\delta^{15}N_{bulk}$ values would indicate a decreased *TP* in the largest stingrays, contradicting stomach content analyses, if all analyzed individuals were feeding in environments with similar baseline $\delta^{15}N$ values. However, Dale

et al. (2011) used differences in $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ to show that *TP* of *D. lata* increased with size and that $\delta^{15}N_{bulk}$ values were independent of *TP*. These findings clearly indicated that the largest *D. lata* were feeding in habitats that had distinctly lower $\delta^{15}N$ values at the base of the food web than the environments where smaller stingrays foraged. One implication of this finding was that stingray $\delta^{13}C_{bulk}$ and $\delta^{15}N_{bulk}$ values reflected migration patterns better than *TP*. Both $\delta^{15}N$ and $\delta^{13}C$ values, examined as a function of size and stingray sex, revealed that changes in bulk isotopic compositions closely coincided with the onset of sexual maturity, confirming Kaneohe Bay as a nursery habitat for *D. lata* (Dale et al. 2011).

$\delta^{15}N_{AA}$ values have been used to recognize marine fish undergoing trans-Pacific migrations (Madigan et al., 2014, 2016). Pacific bluefin tuna (*Thunnus orientalis*) inhabit the western and eastern Pacific Ocean. All bluefin tuna spawn in the western Pacific and an unknown proportion of these tuna migrate to the eastern Pacific early in their life. Once in the eastern Pacific, these bluefin tuna migrants reside in the California Current ecosystem for several years and then return to the western Pacific to spawn. Tracking these transoceanic migrations has been challenging; however, large differences in baseline $\delta^{15}N$ values between the eastern and western Pacific Ocean (*e.g.*, Navarro et al., 2013) can be used to understand the timing and numbers of individuals undergoing trans-Pacific migration better.

Recently, Madigan et al. (2012a, 2013) showed that the short-lived Fukushima-derived radiocesium (^{134}Cs) content of bluefin tuna caught in the eastern Pacific unequivocally identified bluefin tuna that had fed off the coast of Japan and migrated from the western Pacific. Madigan et al. (2014) combined nitrogen isotope analyses of AAs with bluefin tuna containing Fukushima-derived ^{134}Cs to evaluate the migration history of different year class bluefin tuna caught in the eastern Pacific. Bluefin tuna in the eastern Pacific had a bimodal distribution of $\delta^{15}N_{bulk}$, with lower values consistently found in bluefin tuna specimens with Fukushima-derived ^{134}Cs. Bluefin tuna with Fukushima-derived ^{134}Cs had $\delta^{15}N$ values even lower than baseline organisms (krill, copepods) found in the eastern Pacific Ocean. Madigan et al. (2014) also found that the $\delta^{15}N_{SAA}$ in eastern Pacific bluefin tuna with Fukushima-derived ^{134}Cs were 7.7 to 8.7‰ lower than in fish that lacked ^{134}Cs, including resident bluefin tuna, yellowfin tuna, and prey (Pacific saury and jack mackerel). This indicated that $\delta^{15}N_{SAA}$ values were robust markers for distinguishing resident bluefin tuna from recent migrants. In addition, the results of CSIA-AA indicated that differences in $\delta^{15}N_{bulk}$ values were not due to trophic variability among bluefin tuna. Recently, Madigan et al. (2016) used the CSIA-AA results for giant bluefin tuna caught in the western Pacific Ocean to validate the westward trans-Pacific migration of sexually mature individuals from the eastern Pacific Ocean to spawning grounds off the coast of Taiwan.

The findings of Madigan et al. (2014) have important implications for the sustainable management of the bluefin tuna fishery in the eastern Pacific Ocean. The results of their study

902 indicated that the eastern Pacific bluefin tuna population was subsidized by a substantial number of
903 older individuals (*i.e.*, year class 2 to 3) from the western Pacific, which was not previously
904 recognized. In addition, knowledge of muscle turnover time in bluefin tuna (Madigan et al., 2012b)
905 sets limits on how quickly a migrant bluefin tuna would reach a nitrogen isotope steady state with
906 the new environment (and thus be classified as a resident based on CSIA-AA), and allows the date of
907 migrant arrival to be estimated. Madigan et al. (2014) found that the proportion of recent migrants to
908 residents decreased with increasing age, which is critical information for effectively managing this
909 heavily fished species. Unlike a radiogenic isotopic tracer that has finite utility for studying animal
910 migration in the ocean, CSIA-AA can be used *ad infinitum* and in other species. For example, the
911 same isotopic differences in the North Pacific Ocean were used to distinguish apparent eastern and
912 western Pacific migratory groups of endangered leatherback sea turtles (*Dermochelys coriacea*),
913 which provided unique evidence for foraging area philopatry among turtles nesting in Indonesia
914 (Seminoff et al., 2012). These CSIA-AA results clarify the interpretation of bulk tissue isotopic
915 variability in populations, and can be used to recognize and trace movements of many highly
916 migratory pelagic species.
917
918 4.4.3. Case study 3: Deep-sea coral
919 As Earth's climate changes, there is a growing need to put these changes and their
920 subsequent effects on ecosystem structure and function into a greater historical context (Corno et al.,
921 2007; Hoegh-Guldberg and Bruno, 2010). One of the most exciting new applications for CSIA-AA
922 is in paleoceanography, where parameters originally developed for ecology are being adapted as new
923 paleo-proxies in novel protein-rich archives. Biogenic skeletons of proteinaceous deep-sea corals
924 provide a remarkable geochemical archive of information about the structure and function of past
925 ocean ecosystems (Druffel, 1997; Robinson et al., 2014). These globally distributed corals represent
926 "living sediment traps", recording geochemical information about recently exported organic
927 materials in their exquisitely preserved accretionary protein skeletons (Roark et al., 2009; Guilderson
928 et al., 2013). Much of the recent work with proteinaceous deep-sea corals has focused on isotope
929 analysis of total skeletal material as a proxy for changes in surface ocean conditions (*e.g.*, Sherwood
930 et al., 2005; Williams et al., 2007; Hill et al., 2014). However, CSIA-AA results can provide
931 unprecedented reconstruction of past ocean conditions (Sherwood et al., 2011, 2014; Schiff et al.,
932 2014; Strzepek et al., 2014; McMahon et al., 2015c; Williams et al., 2017). The $\delta^{15}N_{SAA}$ values of
933 these consumers provide particularly faithful records of baseline nitrogen sources and cycling that
934 are otherwise seldom preserved in paleorecords.
935 Sherwood et al. (2011) first applied CSIA-AA to deep-sea corals to distinguish between
936 temporal (decadal to centennial) changes in nitrogen sources, while constraining changes in the
937 trophic structure of proteinaceous deep-sea corals in the Scotia-Maine region of the Northwest

Atlantic Ocean. They used the $\delta^{15}N_{SAA}$ values of *Primnoa resedaeformis* coral as a proxy for increasing nitrate levels in the region, associated with externally driven shifts in slope water source partitioning over the last 100 years. Given that slope water circulation in the Scotia-Maine region is linked with broader scale climate variability associated with the North Atlantic Oscillation (Loder et al., 2001; Pershing et al., 2001), these authors concluded that changes in nitrate source partitioning may be tied to recent, human-caused changes in global climate.

More recently, Sherwood et al. (2015) determined $\delta^{15}N_{bulk}$ and $\delta^{15}N_{AA}$ values recorded in the skeletons of the very long-lived (>1,000 years) deep-sea proteinaceous corals *Kulamanamana haumeaae* collected from the Hawaiian Archipelago. After nearly a millennium of minor oscillations, coral $\delta^{15}N_{bulk}$ values decreased dramatically in the last 150 years. Using $\delta^{15}N_{Phe}$ as a proxy for baseline isotopic composition, Sherwood et al. (2015) calculated the relative contribution of N_2-fixation to export production in the North Pacific Subtropical Gyre. They found that increasing N_2-fixation in the subtropical gyre recently observed in the modern instrumental record (Karl et al., 1997, 2001) is a continuation of a much longer centennial-scale trend, resulting in a 17% to 27% increase in N_2-fixation since the end of the Little Ice Age and the onset of the Industrial Era. These authors suggested that this increase in N_2-fixation might be attributed to Northern Hemisphere climate change since the end of the Little Ice Age (Wilson et al., 2006; Mann et al., 2008).

In a complementary study of *K. haumeaae* in the North Pacific Subtropical Gyre, McMahon et al. (2015c) reconstructed the first high-resolution records of changing plankton community composition over the past millennium, using the AA carbon isotope fingerprinting approach of Larsen et al. (2009, 2013). This study revealed three major plankton regimes corresponding to Northern Hemisphere climatic periods over the past 1,000 years. The most recent regime, which began during the warming and stratification period following the end of the Little Ice Age (1850 CE; Corno et al., 2007; Dore et al., 2008), was characterized by an increase of approximately 47% in the contribution of exported POM from N_2-fixing cyanobacteria. These data support the growing body of evidence that the last 150 years in the North Pacific Subtropical Gyre have seen a major, and likely unique shift in plankton community dynamics and nitrogen cycling associated with the end of the Little Ice Age. These studies illustrate the power of CSIA-AA approaches to reconstructing past ocean ecosystem dynamics and biogeochemical cycling.

4.5. CSIA-AA as an indicator of organic matter source and degradation state

4.5.1. Patterns in microbial $\delta^{15}N_{AA}$ variability

The majority of OM in natural environments is not in living organisms, but exists as detrital OM (*e.g.*, Hedges, 1992; Eglinton and Repeta, 2004). Thus, production, alteration, and degradation of detrital OM are key components in biogeochemical cycles, especially for carbon

and nitrogen, and they also play important roles in ecosystems. AAs represent a major fraction of nitrogenous detritus, and are vital in biogeochemical cycles of OM in various environments such as ocean water columns (Cowie and Hedges, 1994; McCarthy et al., 1996), marine sediments (Keil et al., 2000), and soils (Schulten and Schnitzer, 1997). Therefore, $\delta^{15}N_{AA}$ values and patterns also represent novel indicators for the sources and degradation state of detrital OM, especially for organic nitrogen. In contrast to CSIA-AA in animal ecology (Section 4), however, CSIA-AA studies of detrital OM must consider not only food chain processes, but also the subsequent effects of metabolism of chemotrophic microbes (both heterotrophs and chemoautotrophs) on $\delta^{15}N_{AA}$ values and patterns. This remains a frontier area of CSIA-AA applications, and exactly how $\delta^{15}N_{AA}$ patterns are altered by microbial processes remains an area of active research. Importantly, in contrast to metazoans, the metabolic plasticity of microbes allows for multiple means of AA acquisition, including *de novo* synthesis, salvage incorporation (*i.e.*, uptake and incorporation of existing AAs into bacterial biomass), as well as selected resynthesis (*i.e.*, heterotrophic synthesis). This metabolic diversity is likely the reason that observed microbial $\delta^{15}N_{AA}$ fractionation patterns are substantially more complex than metazoans. Based on the literature results, we propose that $\delta^{15}N_{AA}$ patterns resulting from microbial heterotrophy can be classified into four main categories, and that these patterns can be used as a conceptual framework for interpreting $\delta^{15}N_{AA}$ values in detrital OM. Patterns indicating different microbial metabolisms may include changes in *TP*, $\delta^{15}N_{SAA}$ values, and an additional parameter, ΣV. Here, ΣV is a proxy for total heterotrophic resynthesis, and is defined as $\Sigma V = 1/n \sum \mathrm{Abs}(\chi_{AA})$, where deviation of each TAA is $\chi = \delta^{15}N_{AA} - \delta^{15}N$ of average Ala, Asp, Glu, Ile, Leu, and Pro, and n is the total number of TAAs used in the calculation (McCarthy et al., 2007, Fig. 14).

Pattern 1: Algae-like $\delta^{15}N_{AA}$ patterns from *de novo* AA synthesis: Pure culture experiments with microbes have shown that when chemotrophic microbes (*i.e.*, both heterotrophs and chemoautotrophs, including Eukarya, Bacteria, and Archaea) synthesize AAs *de novo* from inorganic nitrogen, the relative $\delta^{15}N_{AA}$ pattern normalized to $\delta^{15}N_{Glu}$ is very similar to that of algae (Fig. 14a, Yamaguchi, 2013). Applying standard formulas discussed above on such material indicates low *TP* values and low ΣV values, just as in fresh algal biosynthetic (Yamaguchi, 2013). Just as for algal production, the absolute $\delta^{15}N$ values depend on that of the nitrogen source and isotopic fractionation during uptake and synthesis of Glu (*e.g.*, Hoch et al., 1992; Fogel and Cifuentes, 1993; Chikaraishi et al., 2007; Ohkouchi and Takano, 2014), which is the sole source of most nitrogen in the other AAs (Bender, 2012).

De novo synthesis of AAs from inorganic nitrogen by chemotrophic microbes might contribute greatly to detrital OM in some environments. For example, in environments with carbon-rich OM and abundant inorganic nitrogen, such as forest litter, some heterotrophic microbes

use inorganic nitrogen as the main nitrogen source for AA synthesis. Another example is environments where chemoautotrophic microbes are the dominant primary producers, such as submarine hydrothermal vents. The algae-like *de novo* $\delta^{15}N_{AA}$ pattern of chemotrophic microbes could be useful for explaining $\delta^{15}N_{AA}$ values and patterns of detrital OM in such environments, although the effect of microbial heterotrophy must also be considered (see pattern 2-4).

These results show that most algae and chemotrophic microbes covering the three domains of life generally show similar $\delta^{15}N_{AA}$ patterns for *de novo* AA synthesis. However, some differences in specific AAs may exist between domains or between microbial species (McCarthy et al., 2013; Yamaguchi, 2013; Maki et al., 2014). To use $\delta^{15}N_{AA}$ patterns as indicators for specific microbial groups, further microbial culture experiments are needed to verify interspecies differences and to understand the variation of microbial $\delta^{15}N_{AA}$ values mechanistically in terms of AA metabolic pathways.

Pattern 2: Animal-like changes in $\delta^{15}N_{AA}$ values (increases in *TP* value): Heterotrophic microbes can use existing AAs in environments (sometimes specific AAs) by metabolizing AAs as carbon and nitrogen sources for resynthesis, or by salvage incorporation. The enzymatic degradation processes of AAs, such as deamination or transamination, cause nitrogen isotopic fractionation (*e.g.*, Macko and Estep, 1984; Macko et al. 1986). Experiments using axenic cultures of heterotrophic microbes across the three domains (Eukarya, Bacteria, and Archaea) have shown that the pattern of TDF_{AA} between microbial biomass and substrates (free AAs or complex media containing proteins) can be similar to that of animals, as evidenced by large positive TDF_{TAA} (*e.g.*, +6 to +8‰ in Glu) and the small TDF_{Phe} (~0‰) (Steffan et al., 2015a; Yamaguchi, 2013, Fig. 14b). These results suggest that when microbes incorporate AAs from the environment, the AAs in the microbial biomass and the microbially produced OM show higher *TP* values, which would be distinct from the algae-like *de novo* $\delta^{15}N_{AA}$ pattern (pattern 1).

However, the mechanisms behind the apparently similar *TDF* patterns may differ between animals and heterotrophic microbes, because these organisms often use different metabolic AA pathways. For example, the proposed mechanism for the small, stable TDF_{Phe} in animals *via* the phenylalanine hydroxylase pathway (Chikaraishi et al., 2007) would not apply to many microbes, which can synthesize Phe, and do not have this pathway (Yamaguchi, 2013). Alternatively, the small TDF_{Phe} in heterotrophic microbes may arise from the high energetic cost of Phe biosynthesis, which would strongly suppress Phe synthesis and degradation and result in the salvage incorporation of Phe from the culture media (Yamaguchi, 2013; Akashi and Gojobori, 2002). To better understand the mechanisms of the heterotrophic changes in microbial $\delta^{15}N_{AA}$ values, we propose that it is important to examine the AAs that were not analyzed in these first culture experiments (*e.g.*, Met, Thr, Tyr, etc.), and to directly compare $\delta^{15}N_{AA}$ patterns in microbes that have different metabolic AA

pathways, as has been done for $\delta^{13}C_{AA}$ (Scott et al., 2006).

Pattern 3: Scattered changes in $\delta^{15}N_{AA}$ values (large increase in ΣV value): Although pure culture experiments have demonstrated that the heterotrophic microbes can show $\delta^{15}N_{AA}$ changes similar to those of animals, the microbial $\delta^{15}N_{AA}$ changes in natural environments may also show patterns that are more scattered (Fig. 14c). For example, incubation experiments of natural marine microbes with algal DOM showed that microbial DOM reworking caused $\delta^{15}N_{AA}$ changes that were more scattered than those observed in pure culture experiments and in animals (Calleja et al., 2013). Large ^{15}N enrichment was observed for some AAs, such as Gly (>10‰), and small ^{15}N enrichment was observed for some TAAs such as Ile (~0‰). Similarly, incubation of plant materials in salt marsh sediments also showed highly scattered $\delta^{15}N_{AA}$ changes caused by microbial OM reworking and replacement, but little change in Phe (Fogel and Tuross, 1999). Microcosm experiments of an alga and a phagotrophic protist showed a scattered *TDF* pattern in the protist (*e.g.*, +8‰ for Ala and ~0‰ for Glu, Gutierrez-Rodriguez et al., 2014).

These "scattered" $\delta^{15}N_{AA}$ changes caused by heterotrophic microbial resynthesis of only selected AAs can be quantified by relative ΣV values, (as defined above by the average deviation in the $\delta^{15}N$ values of the trophic AAs, Ala, Asp, Glu, Ile, Leu, and Pro; McCarthy et al., 2007). Changes in ΣV values caused by microbial OM reworking may also be decoupled from changes in $TP_{Glu/Phe}$ values, because the microbially-mediated changes in $\delta^{15}N_{Glu}$ values may be small in some settings, relative to changes in other source AA (*e.g.*, Gutierrez-Rodriguez et al., 2014). Thus, large increase of ΣV values decoupled with $TP_{Glu/Phe}$ values has been hypothesized as a characteristic marker of microbial reworking (McCarthy et al., 2007). In contrast, while ΣV values also increase in animal trophic steps to some extent, the increase of ΣV values in animals are relatively small and usually coupled with increase of $TP_{Glu/Phe}$ values (McCarthy et al., 2007). The AAs used to calculate ΣV values may also vary, because some AAs often cannot be measured depending on analytical protocols and the status of samples. Therefore, relative inter-sample trends in ΣV values would be typically interpreted as diagnostic for relative degradation, whereas exact values are only generally comparable among studies.

Mechanisms for selected AA $\delta^{15}N$ changes, leading to "scattered" $\delta^{15}N_{AA}$ changes linked to microbial reworking of OM in natural settings are still poorly understood, but we suggest several hypotheses. First, the quality of OM substrates, particularly AA content and AA imbalances between substrates and microbial biomass, may be an important factor controlling the $\delta^{15}N_{AA}$ changes by heterotrophic microbes, as has been suggested for animals (Chikaraishi et al., 2015; McMahon et al., 2015a, see Section 3.2.1). For example, substantial effects of the C:N ratio (*i.e.*, AA content) of substrates on the microbial $\delta^{15}N_{AA}$ patterns were reported in microbial culture experiments using a single AA as the nitrogen source (Maki et al., 2014). Second, a mixture of *de novo* AA synthesis

from inorganic nitrogen, coupled with direct AA incorporation from the environment (*i.e.*, combination of patterns 1 and 2) could also cause scattered $\delta^{15}N_{AA}$ values, due to selective microbial resynthesis of specific AAs. This mixed metabolism may be particularly important in settings with abundant available inorganic nitrogen. Third, the diversity of microbial AA metabolic pathways itself could also be a cause of the variation in $\delta^{15}N_{AA}$ patterns. Finally, while only internal processes within microbial cells are considered in the above three hypothesis, mixing between microbially-produced OM and residue of original substrate also needs to be considered for reworking of detrital OM. Because the patterns of $\delta^{15}N_{AA}$ fractionation may be different between intercellular and extracellular processes (see pattern 4), mixing of the two different OM pools could complicate $\delta^{15}N_{AA}$ patterns. To use the ΣV value properly as an indicator of heterotrophic microbial OM reworking, it is important to reproduce the scattered $\delta^{15}N_{AA}$ changes in highly controlled culture experiments with heterotrophic microbes whose AA metabolic pathways are well characterized. Such future controlled experiments should particular address if ΣV changes can be linked to specific AA, whose $\delta^{15}N$ values change under specific conditions. In addition, for assessing the factors controlling ΣV changes, it is important to culture microbes with substrates containing varying AA contents and compositions, or with substrates containing both inorganic nitrogen and AAs.

Pattern 4: Similar $\delta^{15}N_{TAA}$ and $\delta^{15}N_{SAA}$ increases, possibly by extracellular protein hydrolysis: The last $\delta^{15}N_{AA}$ pattern that has been observed is very different from any others discussed: linked increases in $\delta^{15}N$ values for both TAAs and SAAs (including Phe), with similar amplitudes for all AA, possibly due to isotopic fractionation associated with extracellular protein hydrolysis to oligomers (Fig. 14d) (Hannides et al., 2013). To assimilate AAs in natural environments, heterotrophic microbes usually need to conduct extracellular hydrolysis to degrade proteins into small molecules such as free AAs or small peptides (Hoppe et al., 2002). If preferential cleavage of ^{14}N-C peptide bonds in proteins occurs during microbial extracellular hydrolysis, the residual AAs in the proteins should show ^{15}N enrichment (Silfer et al., 1992). Furthermore, if nitrogen isotopic fractionation during peptide bond hydrolysis is similar among peptide bonds between various AAs, there should be similar increases in $\delta^{15}N$ values for TAAs and SAAs. Hannides et al. (2013) proposed this mechanism to explain the $\delta^{15}N_{AA}$ values of suspended POM observed in the mesopelagic ocean (Section 4.5.2), noting that $\delta^{15}N_{AA}$ changes across all AAs were consistent with a simple Raleigh distillation mechanism, suggesting an external (as opposed to metabolic) fractionation process. It has been suggested that extracellular protein hydrolysis by heterotrophic microbes plays an important role in the biogeochemical cycles in many environments (*e.g.*, Arnosti, 2011); thus, the effect of this mechanism on the $\delta^{15}N_{AA}$ values of detrital OM might be critical in various environments.

However, nitrogen isotope fractionation of AAs during peptide bond hydrolysis has been

experimentally investigated only for the abiotic hydrolysis of glycylglycine (Silfer et al., 1992), and there has been no experimental study of changes in $\delta^{15}N_{AA}$ during peptide bond hydrolysis by microbes. Future experimental studies using various microbes or enzymes are needed to verify this hypothesized $\delta^{15}N_{AA}$ pattern resulting from extracellular protein hydrolysis. Such studies must carefully separate measurement of microbial biomass from partially-hydrolyzed substrate in order to isolate the origins of the patterns described above.

4.5.2. Case studies: Suspended particles in the ocean

As discussed above Section 4.5.1, $\delta^{15}N_{AA}$ analysis of detrital OM can provide a direct molecular-level view of $\delta^{15}N_{bulk}$ values of OM. In the ocean, early studies documented large increases in $\delta^{15}N_{bulk}$ values of POM from the mesopelagic surface ocean (*e.g.*, Saino and Hattori, 1980; Altabet et al., 1991). Hannides et al. (2013) evaluated the mechanisms of a $\delta^{15}N_{bulk}$ increase by applying CSIA-AA to POM in the North Pacific Subtropical Gyre. Their key observation was one of large similar increases in $\delta^{15}N$ values of SAAs and TAAs between the surface and mesopelagic POM. This resulted in constant *TP* values of POM with depth. The ΣV values also remained low and stable with depth. Thus, they concluded that the inclusion of high-*TP* material or heterotrophic microbial biomass in the POM pool (*i.e.*, patterns 2 and 3) is unlikely to be the mechanism of ^{15}N enrichment for mesopelagic POM in the North Pacific Subtropical Gyre. They also suggested that microbial utilization of ^{15}N-enriched nitrate in the midwater as a nitrogen source for *de novo* AA synthesis (*i.e.*, contribution of pattern 1) is not likely to be a major contributor to the $\delta^{15}N$ depth trends of POM.

Hannides et al. (2013) also proposed that isotopic fractionation associated with heterotrophic degradation, probably driven by extracellular hydrolysis of protein (pattern 4), controls the $\delta^{15}N_{AA}$ values of midwater POM. The smaller magnitude of ^{15}N enrichment in Lys, which is around half that of most AAs, is consistent with the proposed hydrolytic mechanism, because Lys was the only measured AA with both an amide and an amino nitrogen (Hannides et al., 2013). However, the values for Thr do not appear consistent with the extracellular protein hydrolysis hypothesis. The depth changes in $\delta^{15}N_{Thr}$ values were very small in the POM measured by Hannides et al. (2013). In contrast to Lys, there is no obvious explanation for the $\delta^{15}N_{Thr}$ values. There is no experimental data on the nitrogen isotopic effect on Thr during microbial heterotrophic processes; thus, future studies on $\delta^{15}N_{Thr}$ during microbial degradation, including extracellular protein hydrolysis and heterotrophic resynthesis, will be important to explain the anomalous $\delta^{15}N_{Thr}$ signature in POM and to clarify POM transformation processes in the ocean.

Comparing $\delta^{15}N_{AA}$ and $\delta^{15}N_{bulk}$ would also provide useful new information about the biogeochemical cycling of organic nitrogen, including nitrogen fractions other than AAs. Specifically, $\delta^{15}N$ values of total hydrolysable AAs ($\delta^{15}N_{THAA}$) can be used as a proxy for total

proteinaceous $\delta^{15}N$ values, estimated as the molar-weighted average of individual $\delta^{15}N_{AA}$ values (McCarthy et al., 2013; Calleja et al., 2013; Batista et al., 2014). When concentrations of AAs and bulk nitrogen are known, $\delta^{15}N$ values of the nitrogen fraction other than THAA (non-THAA) can be calculated by $\delta^{15}N$ mass balance. Accurate quantification of AAs and bulk nitrogen is, however, essential for these mass-balance calculations, but has been absent from many past CSIA-AA studies. We suggest that the concentration of AAs and bulk nitrogen should be routinely reported in future CSIA-AA studies, to better understand the relationship between $\delta^{15}N$ values of THAA and non-THAA in organisms and detrital OM (*e.g.*, Cowie and Hedges, 1992; Amelung and Zhang, 2001).

5. Future work and challenges

We have reviewed the current "state of the art" of using nitrogen isotopic composition of AAs for estimating the *TP* of organisms, as well as broader applications to terrestrial and marine ecology and biogeochemical cycling. The CSIA-AA method provides information on diet sources that is more precise than classical bulk isotope methods and is now rapidly expanding into a number of fields, such as biomagnification of toxic chemicals (*e.g.*, polychlorinated biphenyls) through the food web (Ohkouchi et al., 2016), and nitrogen exchange between symbionts and host organisms (Maeda et al., 2012). Although the advantages of CSIA-AA for studying a wide range of ecosystems are clear, at the same time the methods remain relatively new, and will benefit greatly from further improvement and development. We suggest the following as being among the main problems which need to be addressed in future studies.

1) A prerequisite for the wider application of this tool for accurately estimating *TP* is a robust knowledge of the magnitude of TDF_{AA}, especially, but not only, for well documented source and trophic amino acid pairings such as Phe and Glu. As discussed in Section 3.2, the most appropriate $\Delta_{Glu/Phe}$ values, for instance, for calculating *TP* in specific situations is still open to debate. In some cases, the CSIA-AA approach based on current understanding of TDF_{AA} has not produced ecologically realistic *TP* values (*e.g.*, penguins in Lorrain et al. (2009), elasmobranchs in Dale et al. (2011), dragonfish in Choy et al. (2012), killer whales in Matthews and Ferguson (2014), sperm whales in Ruiz-Cooley et al. (2014)). The following questions thus need to be addressed regarding the trophic discrimination of amino acids. A) Is the TDF_{AA} of a given AA value constant or variable across a wide variety of food webs? B) Do TDF_{AA} values decrease with increasing *TP* (Hetherington et al., 2016)? C) Are TDF_{AA} value more constant in the terrestrial environment than in the aquatic environment, as suggested by the work of Steffan et al. (2013, 2015a)? And most broadly, it will be critical to determine to what extent TDF_{AA} variations depend on the specific biochemistry and physiology of organisms and their diet, as suggested may be the case by the feeding experiments of McMahon et al. (2015a) and Chikaraishi et al. (2015). To answer these

questions, further work focused on understanding the biochemical, physiological, and ecological mechanisms underlying TDF_{AA} variability is required.

2) In natural environments, microorganisms play critical roles in the food web. Although several studies have examined explicitly aspects of these roles (*e.g.*, Steffan et al. 2017), the effects of microbial activity on the isotopic compositions of AAs require further evaluation. Knowledge of these effects is extremely important, particularly in terms of understanding complex microbially-driven nitrogen cycling in ocean and soil environments using CSIA-AA.

3) It is still difficult to estimate precisely the *TP* of multivorous feeders that integrate aquatic and terrestrial food webs feeders such as humans. In some cases, such as Naito et al. (2010), the $\delta^{15}N_{Phe}$ value can be used to distinguish between aquatic and terrestrial food sources, whereas in other cases it cannot. Development of techniques which will help expand the application of CSIA-AA tools across food webs could open broad new applications in both ecological and archaeological contexts.

4) Although to date most CSIA-AA studies have relied heavily on the isotopic compositions of just two AAs, Glu and Phe, to determine *TP*, we need a more holistic application of the technique, such as by embracing the diversity in TDF_{AA} in 1) above, to fully exploit the utility of AA data for interpreting the diet and physiology of organisms (*e.g.*, Bradley et al., 2015; Nielsen et al. 2015).

5) Currently, we know very little about how D-AAs affect $\delta^{15}N_{AA}$ values. Because D-AAs are subject to different metabolic pathways, they should have distinct isotopic compositions from L-AAs (Engel and Macko, 1986; Takano et al., 2010; Chan, 2016), which may affect the overall $\delta^{15}N_{AA}$ value, even if they are minor components.

Finally, we note that in addition to nitrogen isotopic composition, carbon isotopic composition of AAs can provide an independent measure of sources and metabolic processes, and has immense potential to help resolve some of the challenges outlined above. Furthermore, recent advances in measuring the radiocarbon of AAs may also provide detailed information on carbon transfer from the environment to consumers. This latter technique may be especially useful for soil ecosystems, where old carbon potentially makes significant contributions to microbial substrates, and should also be helpful for adding chronological information to the food web, as well as for identifying the source of AAs from various pools. While such applications are beyond the scope of the current review, development of appropriate methods is ongoing (*e.g.*, Marom et al., 2014; Takano et al., 2015; Bour et al., 2016). Ultimately, combining CSIA-AA with such new tools offers the promise of extraordinarily high-resolution delineation of food webs in space and time, as well as the potential to quantify food web linkages between and within aquatic and terrestrial systems at a new level of precision.

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Table legends

Table 1. A summary of three types of derivatized amino acids used for the nitrogen isotopic analysis.

Figure captions

Fig. 1. Derivatization of amino acids for the nitrogen isotope analysis by GC/IRMS: Schemes of (a) basic chemical reaction, (b) TFA/AA/iPr ester, (c) Pv/AA/iPr ester, and (d) MOC AA ester.

Fig. 2. Ester exchange between amino acid derivatives and ethyl acetate.

Fig. 3. A representative chromatogram of GC/IRMS analysis of the nitrogen isotope analysis of amino acids as *N*-pivaloyl *O*-isopropyl esters.

Fig. 4. Schematic illustrations of the trophic position (*TP*) estimates by (a) bulk and (b) CSIA-AA methods. In the bulk method, the δ^{15}N values of consumers at the same *TP* frequently vary, due to temporal or spatial change in the δ^{15}N value at the basis of food web. In contrast, CSIA-AA method can estimate *TP* independent of change in the δ^{15}N value at the basis of food web (after Naito et al., 2016).

Fig. 5. (a) Initial steps of the dominant metabolism for glutamic acid and phenylalanine in animals, and (b) schematic illustrations of the relationship between δ^{15}N values of amino acids (Glu and Phe) and trophic position in aquatic and terrestrial food webs (after Chikaraishi et al., 2009, 2010).

Fig. 6. Schematic of two aquatic food webs differentiated based on the δ^{15}N values of Phe and Glu. Changes in baseline nitrogen sources cause each food web to be separated along the source amino acid axis; here an oceanic food web based on phytoplankton production (P) supported by *e.g.* N_2-fixation (low $\delta^{15}N_{Phe}$) is separated from a benthic food web based on macroalgae production (M) supported by *e.g.* upwelling or terrestrial run-off (high $\delta^{15}N_{Phe}$). The potential for 'trophic omnivory' can be evident as non-integer *TP*s; here the oceanic food web depicts potential ontogenetic changes in CSIA-AA-derived *TP* for fish across two *TP*s (anchovy and tuna). An example is also provided of a mobile apex predator (the tiger shark, *Galeocerdo cuvier*; *Ga*) potentially integrating across oceanic and benthic food webs at a given *TP*, leading to intermediate $\delta^{15}N_{SAA}$ value.

Fig. 7. Two examples of food web analysis by $\delta^{15}N_{AA}$: a) the coastal marine (a stony shore) ecosystem in Japan (Chikaraishi et al., 2014), and b) Lake Baikal (Ohkouchi et al., 2015).

Fig. 8. (a) Concentrations of nitrate (black) and phosphate (red) observed in the hypolimetic water in the north basin of Lake Biwa, Japan. (b) Trophic position of gobiid fish Isaza (*Gymnogobius isaza*) estimated by the $\delta^{15}N_{AA}$. (c) $\delta^{15}N$ values of bulk muscular tissue, Glu, and Phe of formalin-fixed Isaza specimens. $\delta^{15}N$ values of bulk sediments were also shown (data from Ogawa et al. 2001). A grey band indicates the major eutrophication period in Lake Biwa (1960-1980, Ogawa et al., 2013)

Fig. 9. Fungi can be carnivorous. Here, the fungus *Beauveria bassiana* has subdued and killed a caterpillar (larval *Spodoptera frugiperda*). The trophic position of the fungus is 3.0 (Steffan et al., 2015a), because functionally, this fungus is a strict carnivore.

Fig. 10. Isotopic approaches have been used to decode carnivore impacts on key ecosystem metrics, such as primary productivity (after Steffan et al., 2015b). Heterotrophic feeding induces trophic cascades, which directly and indirectly influence other trophic groups. The trophic tendency of any given species, coupled with its resource capture efficiency (% consumption of resource base), permits estimation of the consumers' impacts on plant protection.

Fig. 11. Nitrogen isotopic compositions of Phe and Glu of Holocene hunter-gatherers in Japanese archipelago. a) Kitakogane shell midden located near the coastal line of Hokkaido (Early Jomon period, *ca.* 6000-5300 cal BP) and b) Tochibara rockshelter site located at inland Nagano (Initial Jomon period, *ca.* 9100-9700 cal BP). Note that Kitakogane humans exhibit $\delta^{15}N_{Phe}$ closer to marine fauna than terrestrial fauna suggesting their strong reliance on marine foods while Tochibara humans exhibit $\delta^{15}N_{Phe}$ comparable to those of terrestrial fauna suggesting their reliance exclusively on terrestrial foods (Naito et al., 2010b, 2013b).

Fig. 12. Nitrogen isotopic compositions of Phe and Glu for Neanderthal and animal remains from Spy and Scladina caves in Pleistocene Belgium (Naito et al., 2016b).

Fig. 13. $\delta^{15}N_{bulk}$ trends (A) and $\delta^{15}N_{Phe}$-calibrated baseline $\delta^{15}N$ isoscape (B) along the California coast, based on selected CSIA-AA within high-density bulk sampling of littoral mussels.

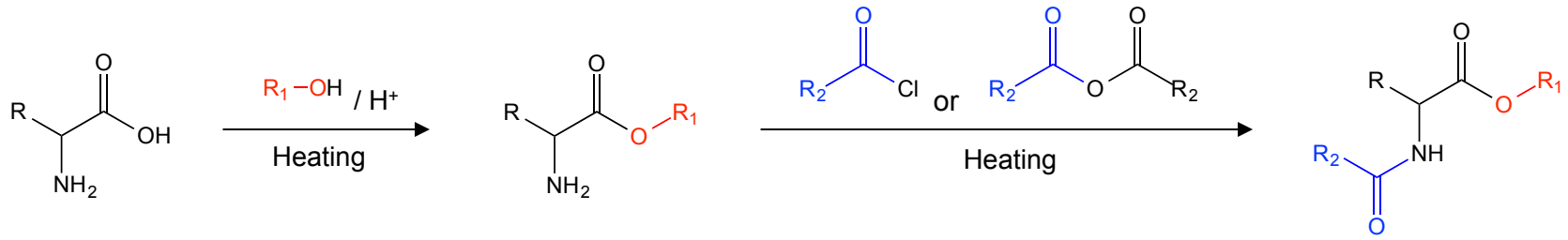
Fig. 14. Conceptual diagrams describing the proposed four patterns of $\delta^{15}N_{AA}$ fractionation of chemotrophic microbes (for details, see Section 4.5.1 in the main text). Eight AAs which have been commonly analyzed are selected for the diagrams. (a) Pattern 1. The $\delta^{15}N_{AA}$ pattern of *de novo* AA synthesis from inorganic nitrogen by chemotrophic microbes (closed circles: microbial biomass), which was observed in the pure culture experiments (Yamaguchi, 2013). The $\delta^{15}N_{AA}$ values are normalized to the $\delta^{15}N_{Glu}$ value. (b) Pattern 2. The $\delta^{15}N_{AA}$ fractionation pattern of

heterotrophic microbes relative to preformed AA in substrates, which was observed in the pure culture experiments (red squares: microbial biomass) (Stefan et al., 2015; Yamaguchi, 2013). The $\delta^{15}N_{AA}$ values of the substrates in b, c, and d (open circles) are set as the average pattern of algae (Chikaraishi et al., 2009; McCarthy et al., 2013), and are normalized to the $\delta^{15}N_{Glu}$ value. (c) Pattern 3. A possible example of the scattered $\delta^{15}N_{AA}$ fractionation by heterotrophic microbes relative to substrates in some settings (blue triangles: degraded materials), hypothesized from the results of incubation or microcosm experiments (Fogel and Tuross, 1999; Calleja et al., 2013; Gutierrez-Rodriguez et al., 2014). Note that the $\delta^{15}N$ fractionation value of each AA in this pattern is not well constrained and is likely variable. (d) Pattern 4. A hypothesized $\delta^{15}N_{AA}$ fractionation pattern during extracellular protein hydrolysis by heterotrophic microbes (green squares: residue of hydrolysis) (Hannides et al., 2013). Note that the magnitude of $\delta^{15}N$ fractionation would be variable, depending on the character of substrates and the degree of degradation.

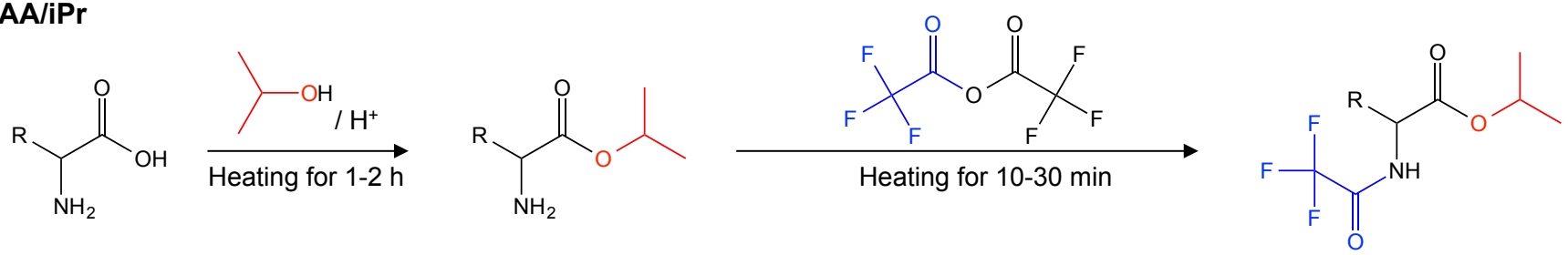
	TFA/AA/iPr	Pv/AA/iPr	MOC/AA ester
Available solvent	DCM	DCM	DCM or MeOH
Toxicity	High	Very high	High
Stability at -20℃	1-2 years	1-2 years	1-2 weeks
Volatility	High	Low	Very high

Table 1

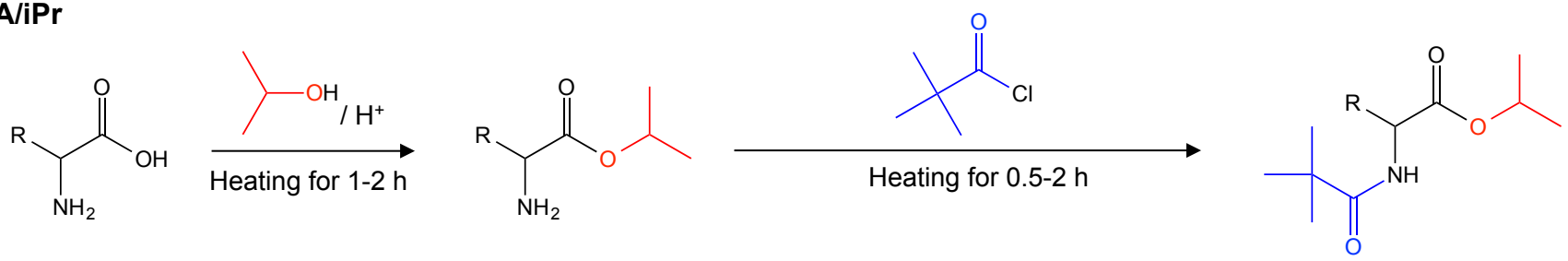
(a) Basic chemical reaction



(b) TFA/AA/iPr



(c) Pv/AA/iPr



(d) MOC AA ester

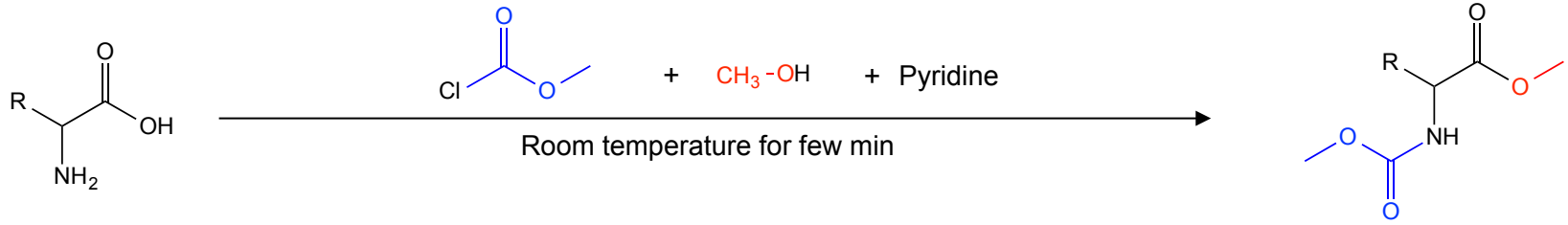
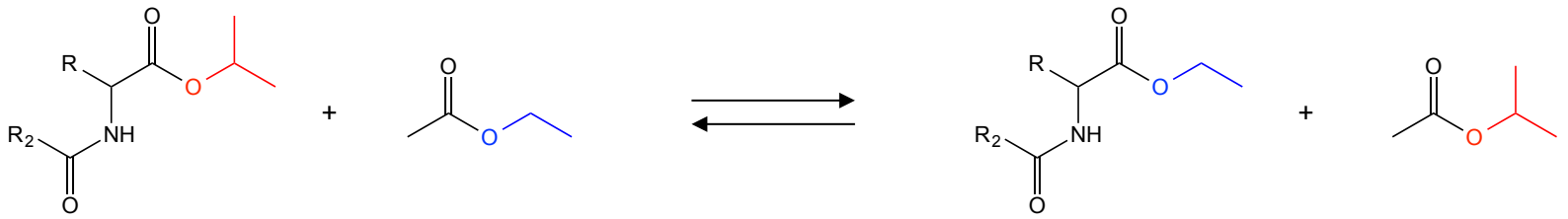
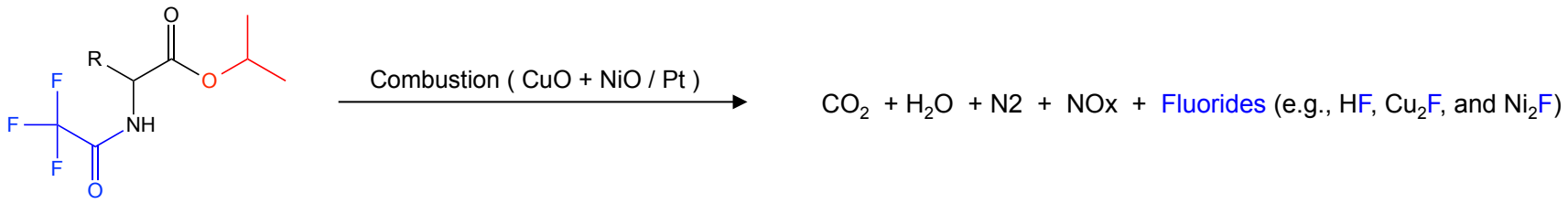


Fig. 1

(a) Exchange of ester group



(b) Combustion of TFA/AA/iPr



(c) MOC derivatives of glutamic acid

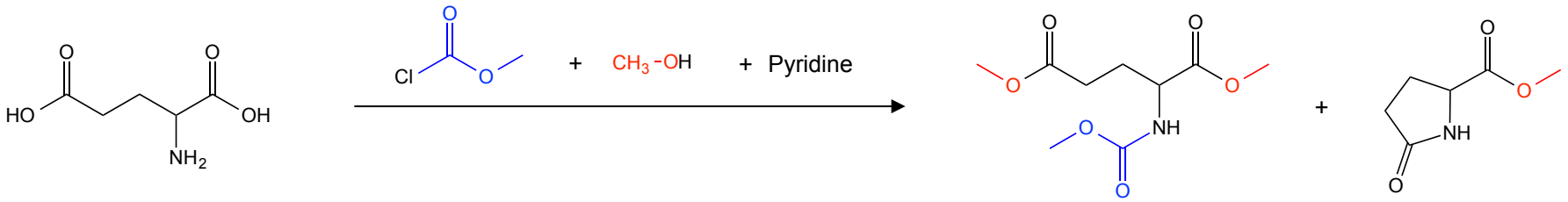


Fig. 2

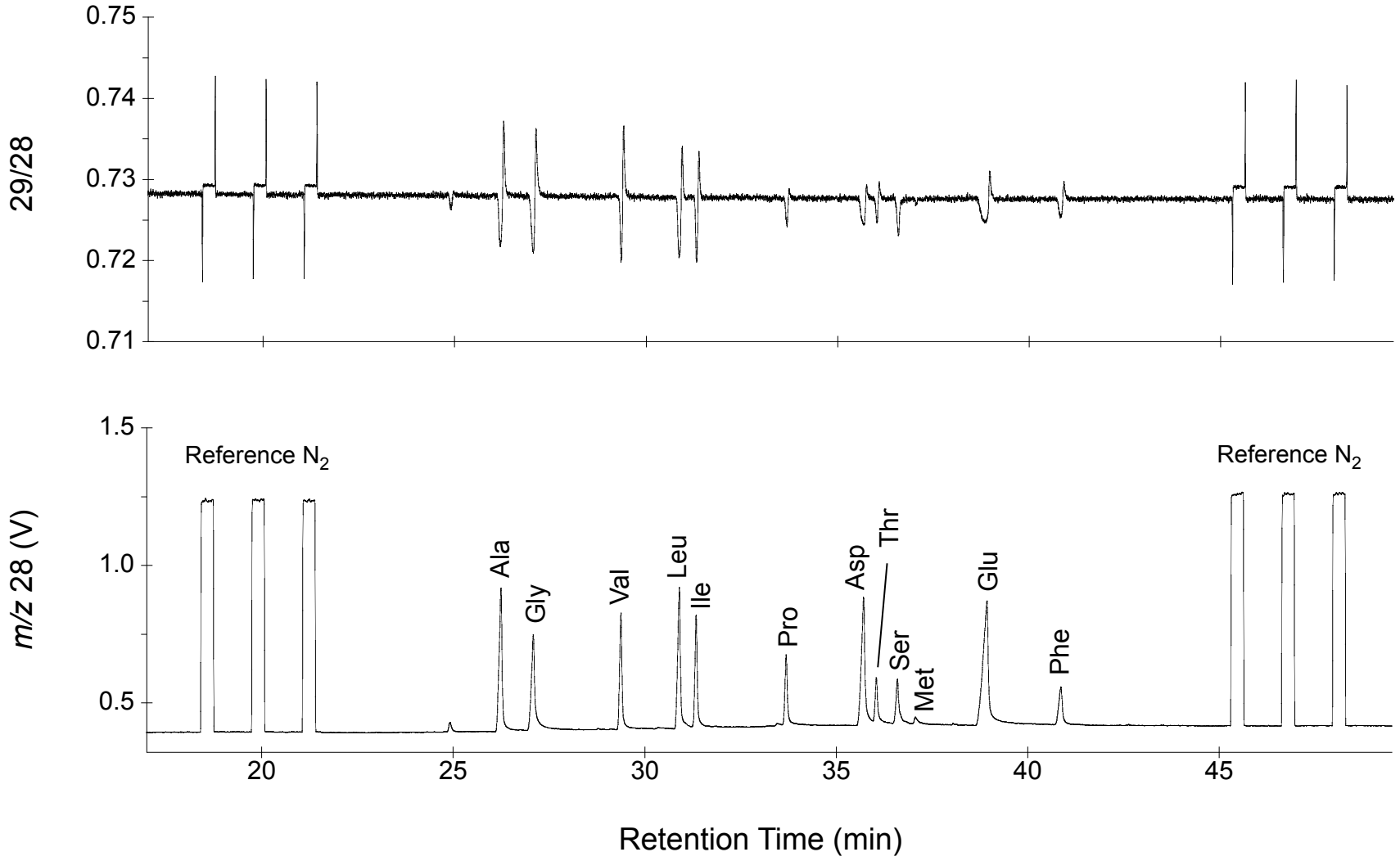
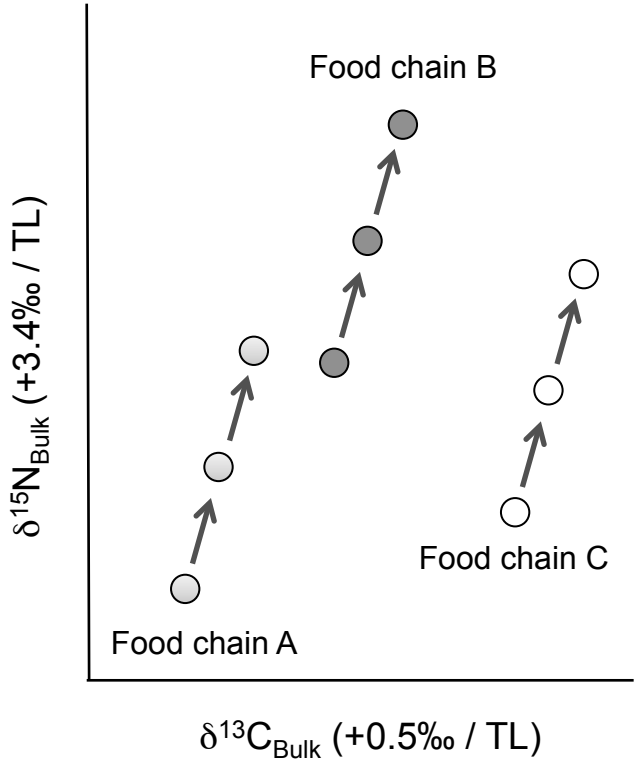

29/28
0.75
0.74
0.73
0.72
0.71
m/z 28 (V)
1.5
1.0
0.5
Reference N2
Reference N2
Ala
Gly
Val
Leu
Ile
Pro
Asp
Thr
Ser
Met
Glu
Phe
20
25
30
35
40
45
Retention Time (min)

Fig. 3

(a) Bulk method



(b) CSIA-AA method

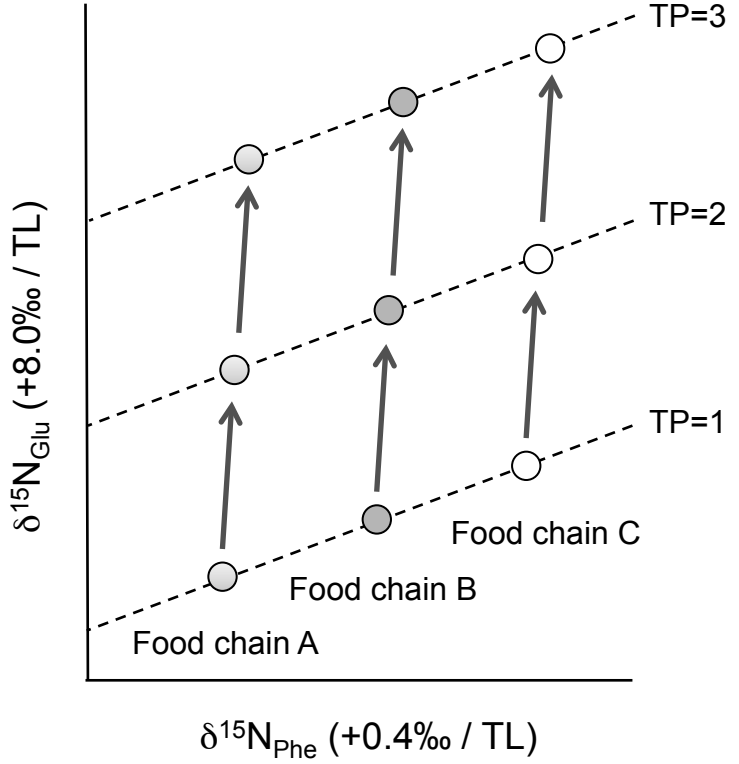
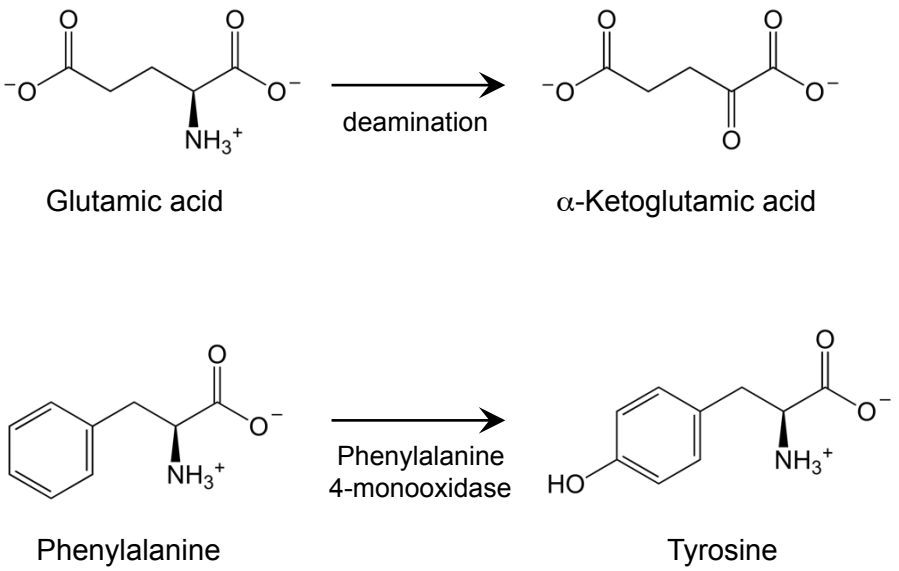


Fig. 4

(a) First step in metabolism



(b) Trophic enrichment in ^{15}N

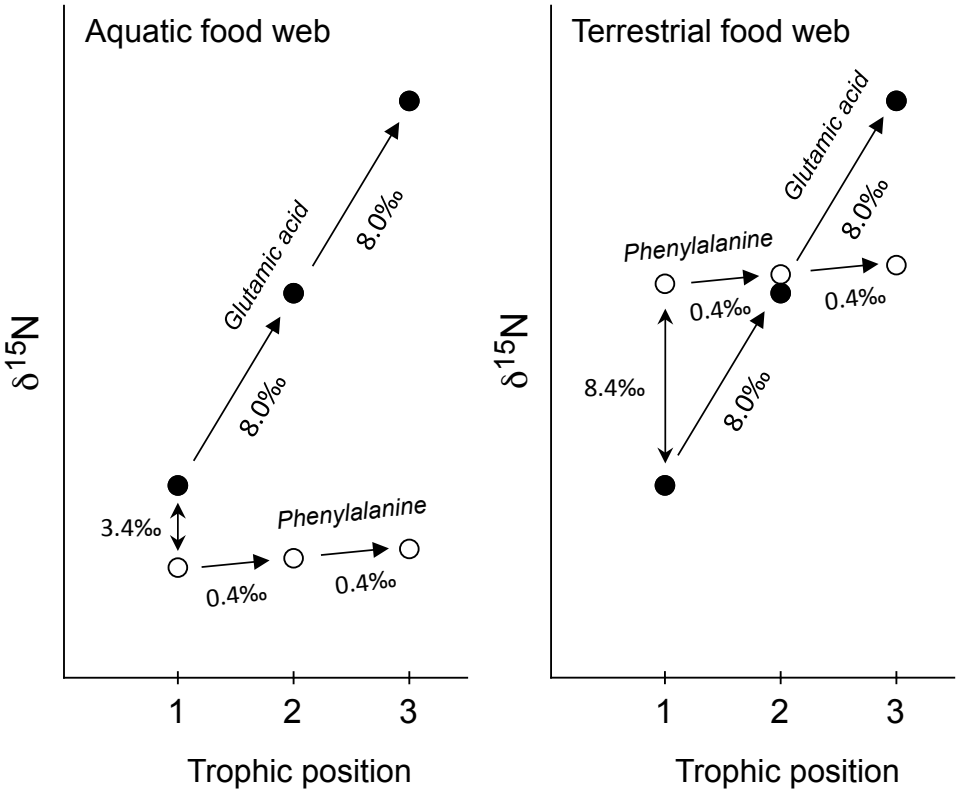


Fig. 5

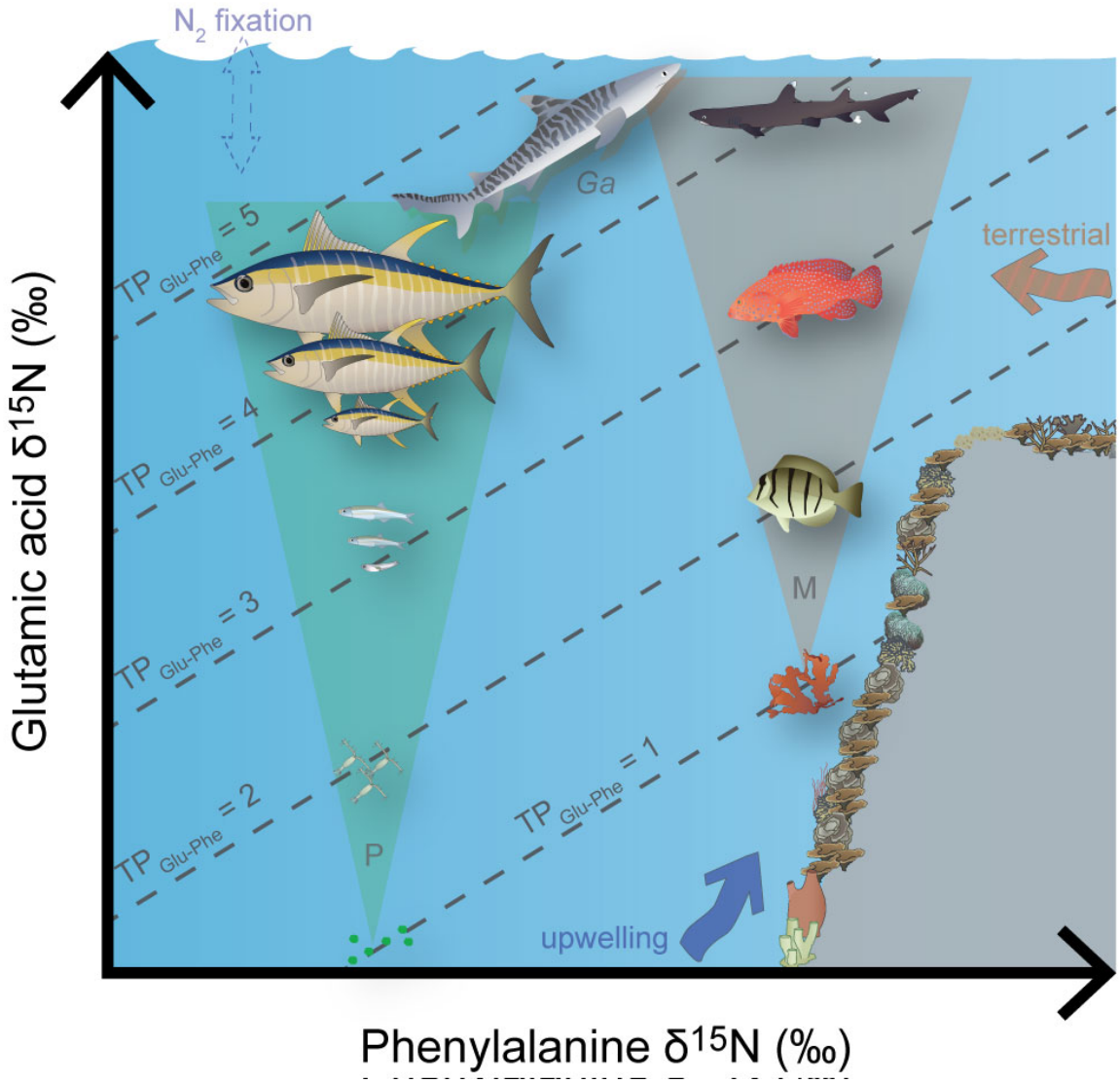

N2 fixation
Ga
terrestrial
TP Glu-Phe = 5
TP Glu-Phe = 4
TP Glu-Phe = 3
TP Glu-Phe = 2
TP Glu-Phe = 1
M
P
upwelling
Glutamic acid δ15N (‰)
Phenylalanine δ15N (‰)

Fig. 6

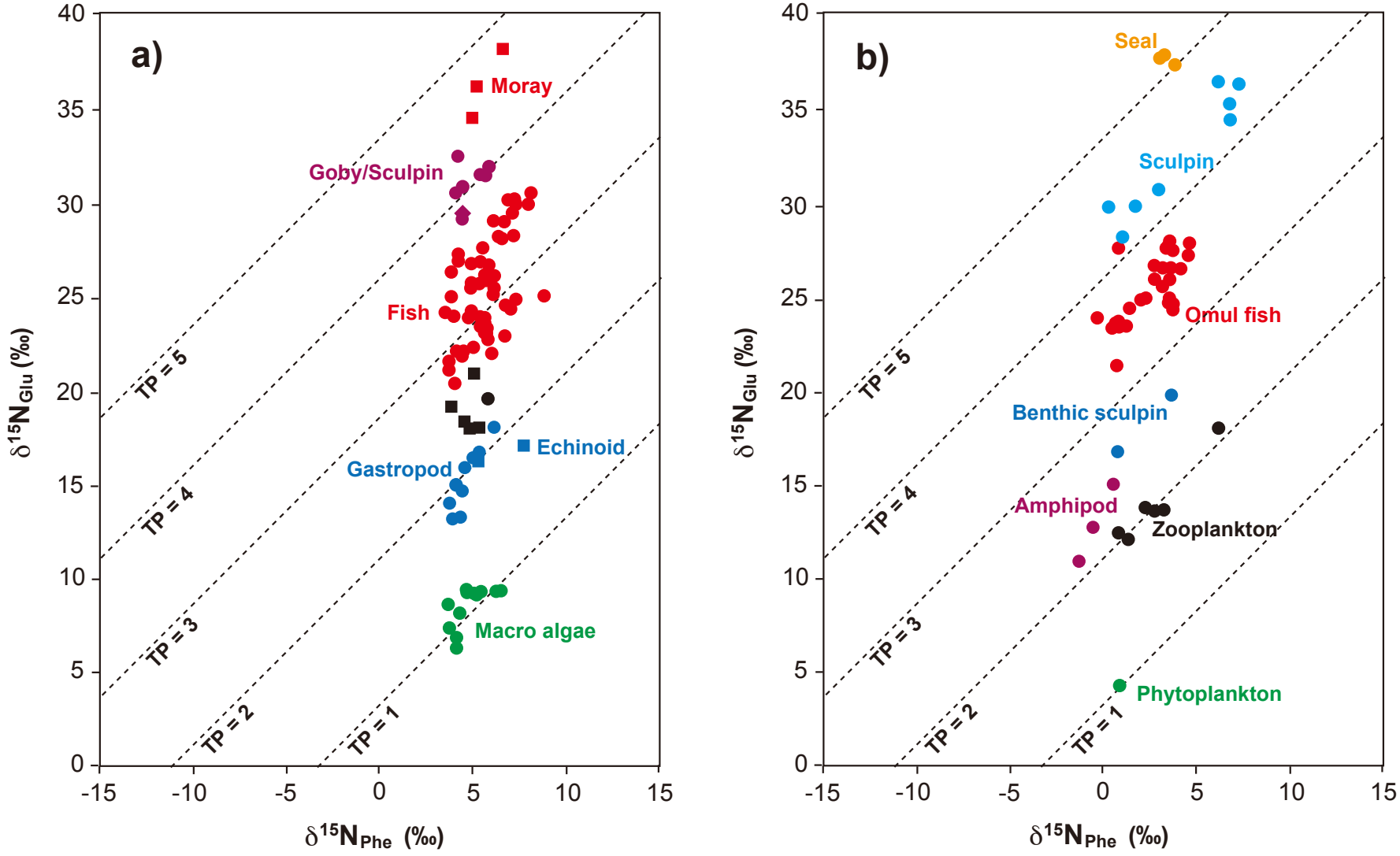

a)
Moray
Goby/Sculpin
Fish
Echinoid
Gastropod
Macro algae
TP = 5
TP = 4
TP = 3
TP = 2
TP = 1
δ15NGlu (‰)
δ15NPhe (‰)
b)
Seal
Sculpin
Omul fish
Benthic sculpin
Amphipod
Zooplankton
Phytoplankton
TP = 5
TP = 4
TP = 3
TP = 2
TP = 1
δ15NGlu (‰)
δ15NPhe (‰)

Fig. 7

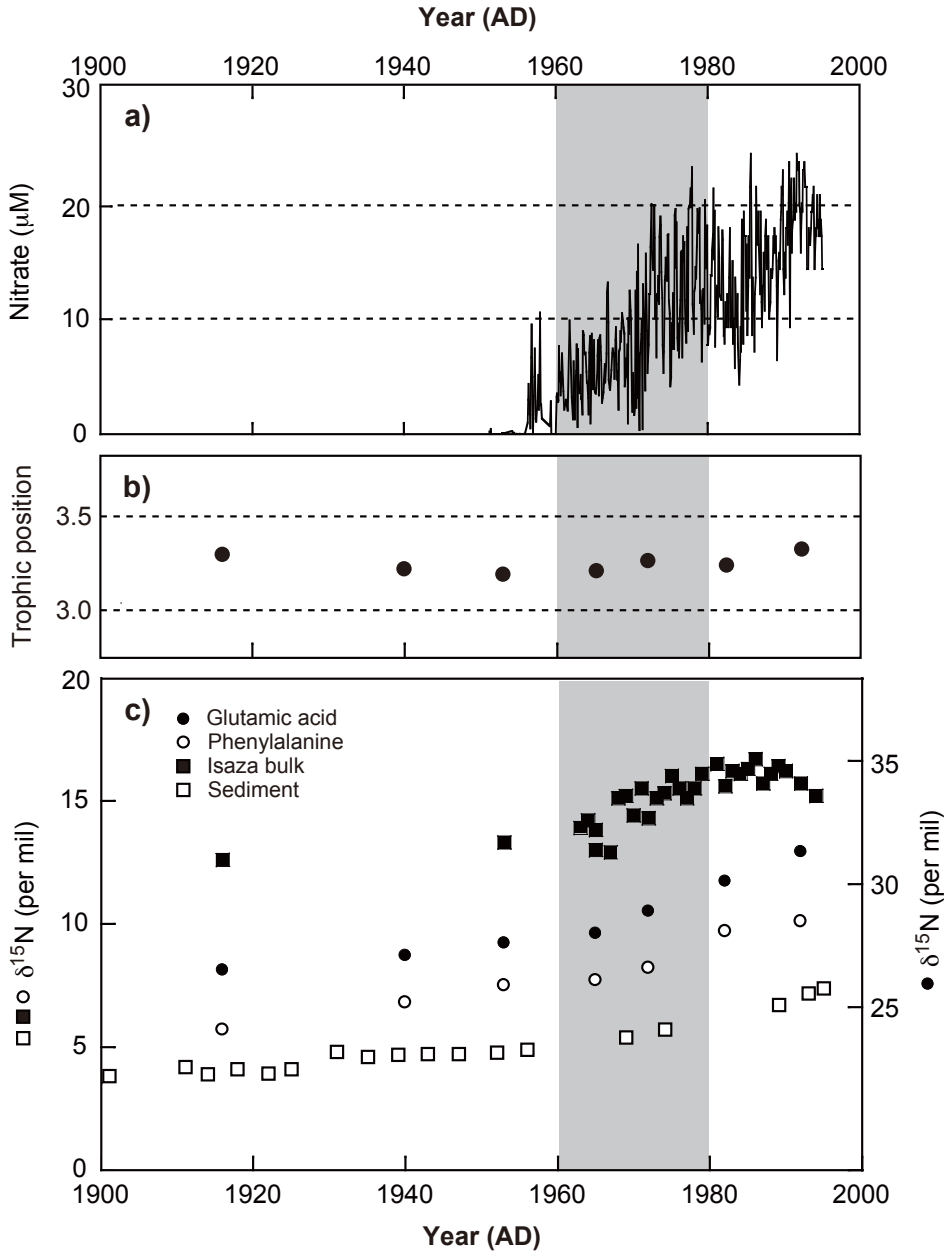

Year (AD)
1900
1920
1940
1960
1980
2000
a)
Nitrate (μM)
30
20
10
0
b)
Trophic position
3.5
3.0
c)
Glutamic acid
Phenylalanine
Isaza bulk
Sediment
□ ■ ○ δ15N (per mil)
● δ15N (per mil)
20
15
10
5
0
35
30
25
Year (AD)

Fig. 8

Fig. 9

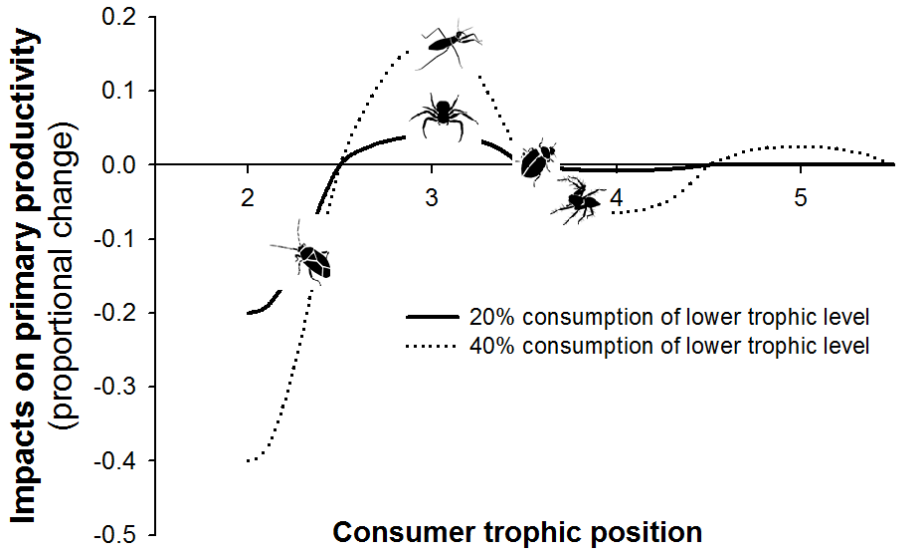
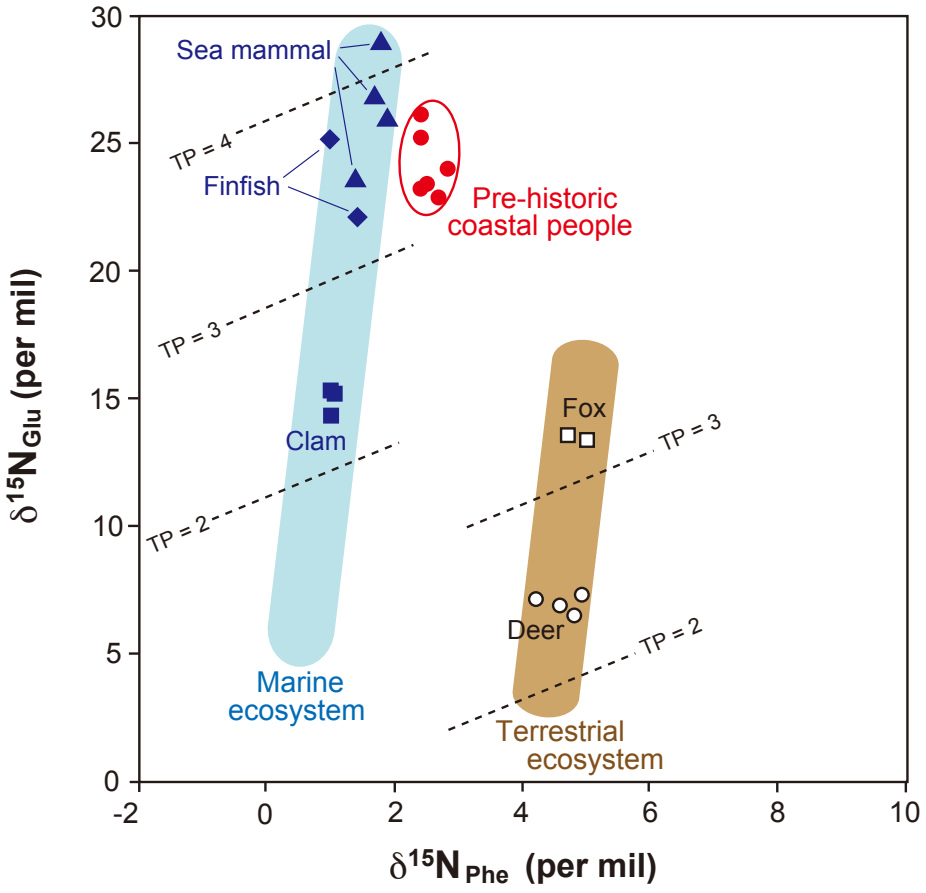

Impacts on primary productivity
(proportional change)
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
-0.5
2
3
4
5
20% consumption of lower trophic level
40% consumption of lower trophic level
Consumer trophic position

Fig. 10


Sea mammal
Finfish
Clam
Pre-historic
coastal people
Fox
Deer
Marine
ecosystem
Terrestrial
ecosystem
TP = 4
TP = 3
TP = 2
TP = 3
TP = 2
δ15NGlu (per mil)
δ15NPhe (per mil)

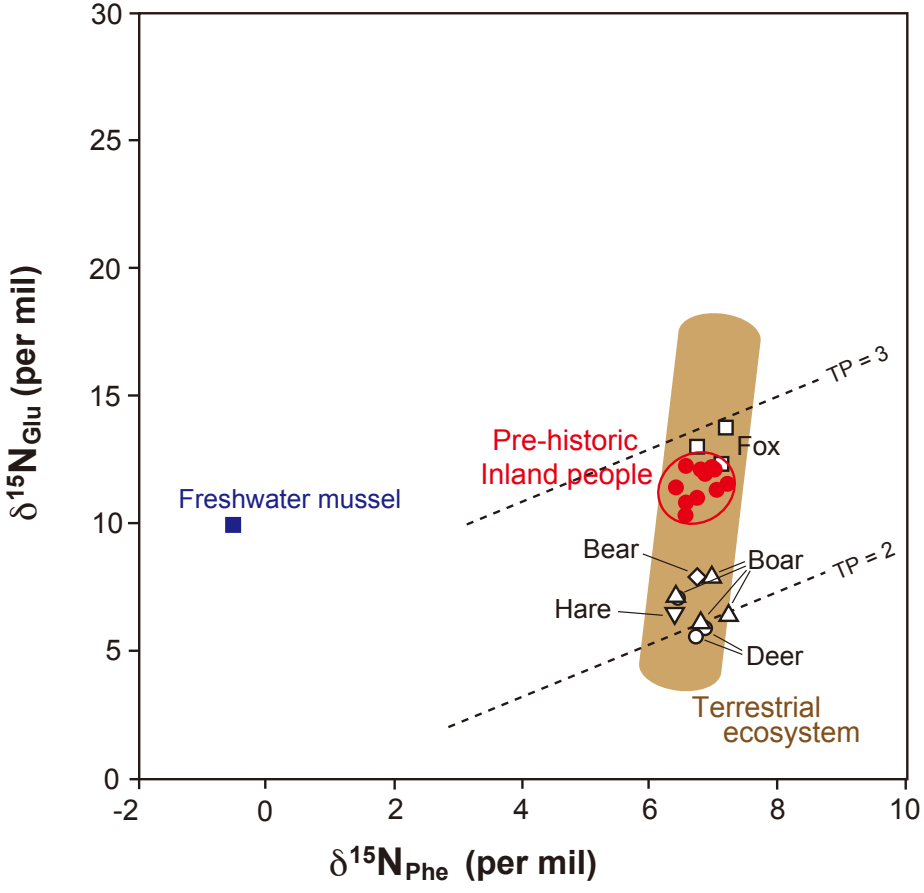

Freshwater mussel
Pre-historic
Inland people
Fox
Bear
Boar
Hare
Deer
Terrestrial
ecosystem
TP = 3
TP = 2
δ15NGlu (per mil)
δ15NPhe (per mil)

Fig. 11

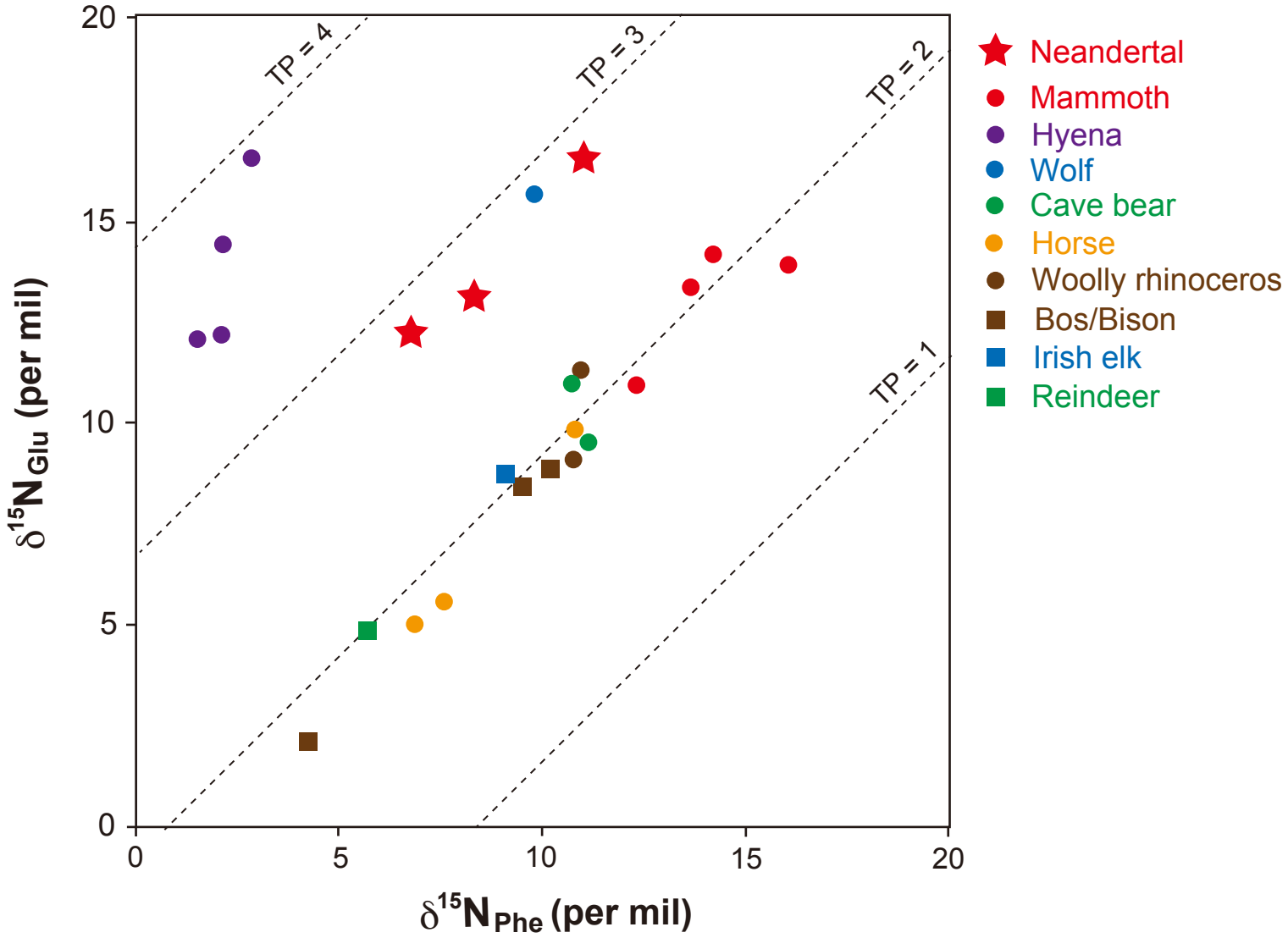
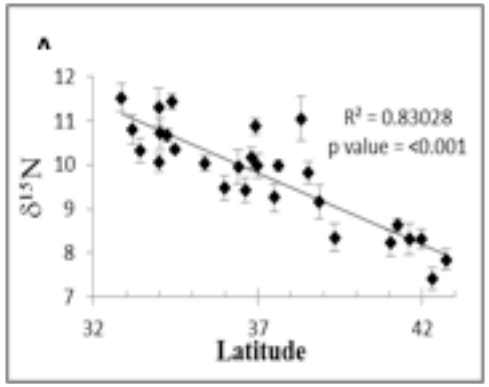

20
15
10
5
0
0
5
10
15
20
TP = 4
TP = 3
TP = 2
TP = 1
δ15NGlu (per mil)
δ15NPhe (per mil)
Neandertal
Mammoth
Hyena
Wolf
Cave bear
Horse
Woolly rhinoceros
Bos/Bison
Irish elk
Reindeer

Fig. 12



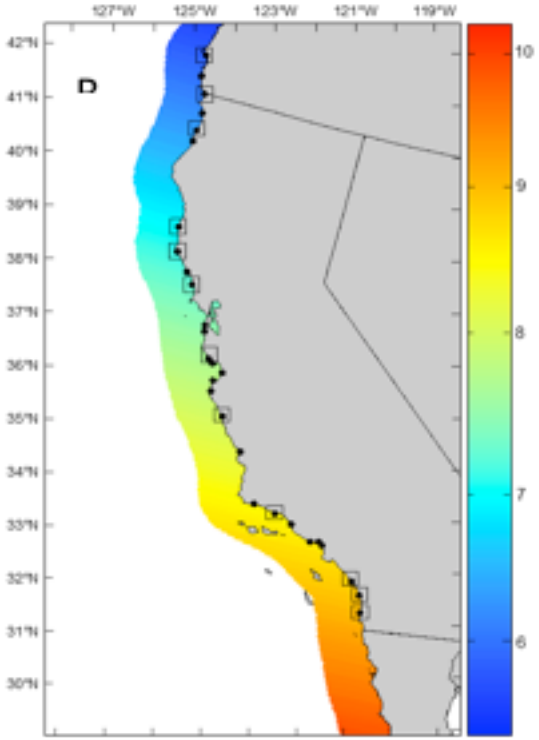


Fig. 1: Bulk $\delta^{15}N$ trends (A) and a $\delta^{15}N_{Phe}$ calibrated baseline $\delta^{15}N$ isoscape (B) along the CA coast,

Fig. 13

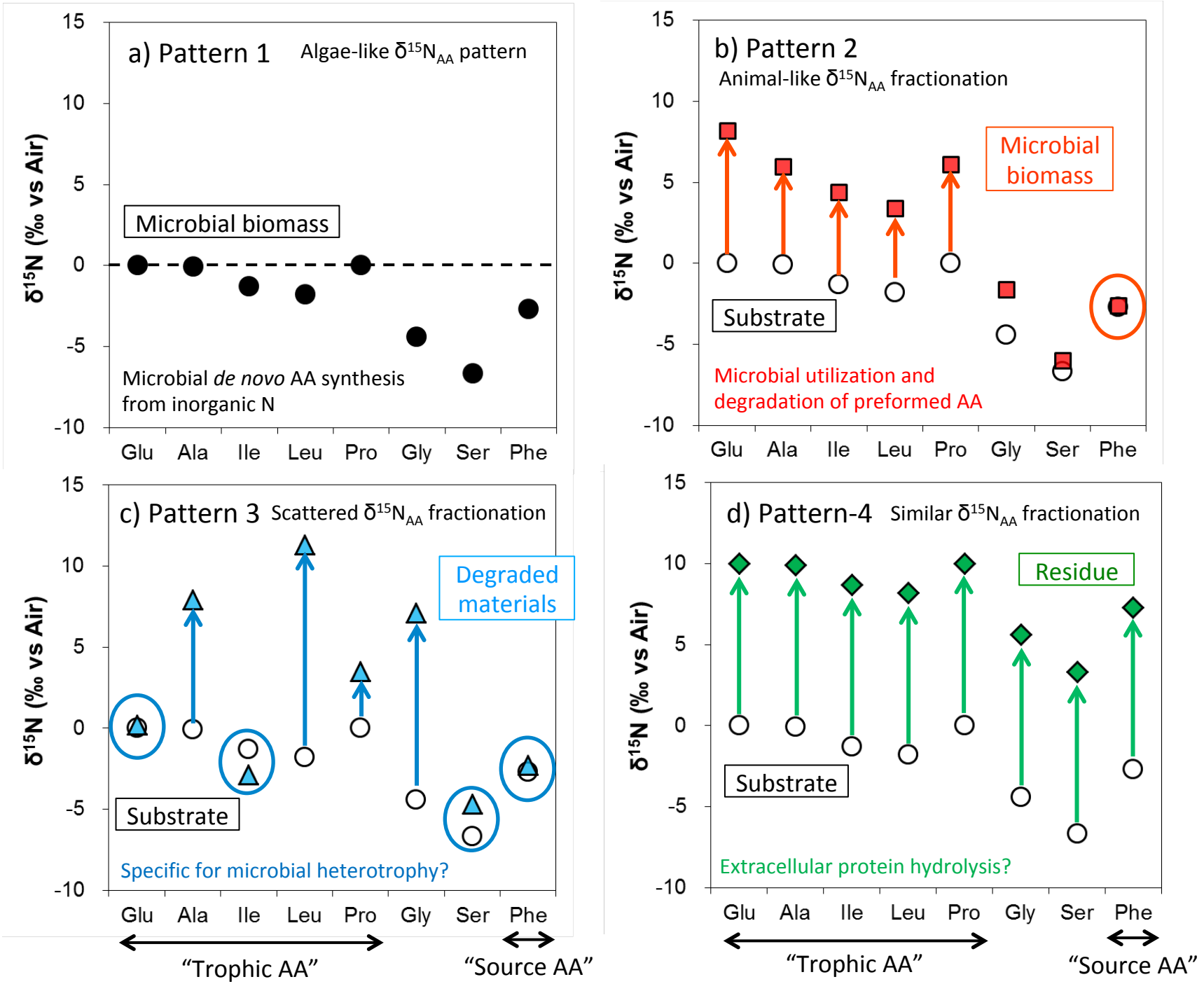

a) Pattern 1
Algae-like δ15NAA pattern
Microbial biomass
Microbial de novo AA synthesis from inorganic N
b) Pattern 2
Animal-like δ15NAA fractionation
Microbial biomass
Substrate
Microbial utilization and degradation of preformed AA
c) Pattern 3
Scattered δ15NAA fractionation
Degraded materials
Substrate
Specific for microbial heterotrophy?
d) Pattern-4
Similar δ15NAA fractionation
Residue
Substrate
Extracellular protein hydrolysis?
δ15N (‰ vs Air)
15
10
5
0
-5
-10
Glu
Ala
Ile
Leu
Pro
Gly
Ser
Phe
“Trophic AA”
“Source AA”

Fig. 14

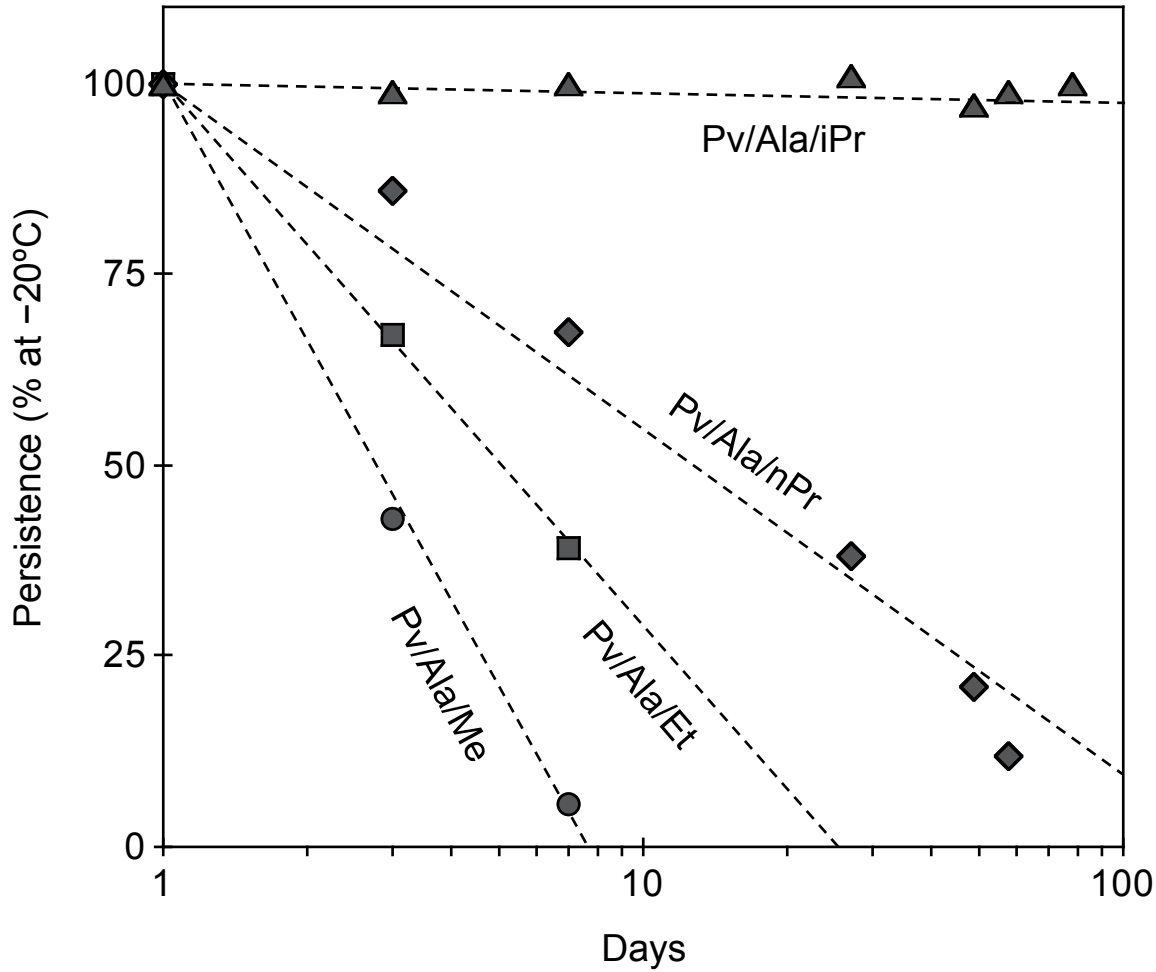


Fig. S1 Comparison of stabilities of various pivaloyl esters of alanine. Triangle: methyl ester (Pv/Ala/Me), diamond: ethyl ester (Pv/Ala/Et), squire: *n*-propyl ester (Pv/Ala/nPr), circle: isopropyl ester (Pv/Ala/iPr).

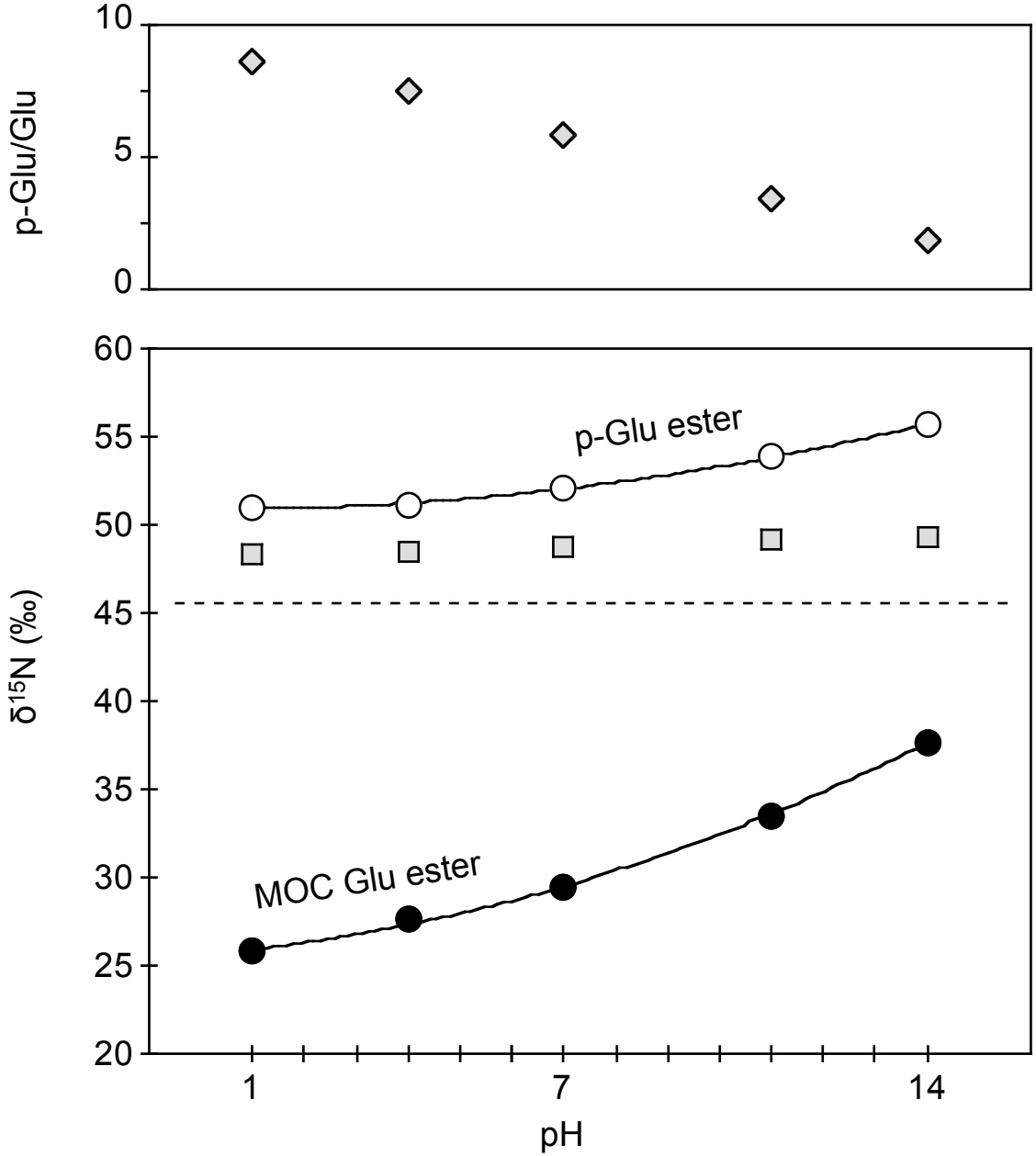


Fig. S2. Isotopic fractionation of MOC ester derivatization of Glu. The MOC ester (MOC Glu ester, filled circle) is depleted in ^{15}N whereas the cyclic ester (p-Glu ester, open circle) is enriched in ^{15}N. Molar ratio of these two derivatives (p-Glu/Glu) has a negative correlation with *p*H during the derivatization, however, the mass balanced values (gray square) do not equal to the reference isotopic composition of glutamic acid (+45.7‰, broken line).

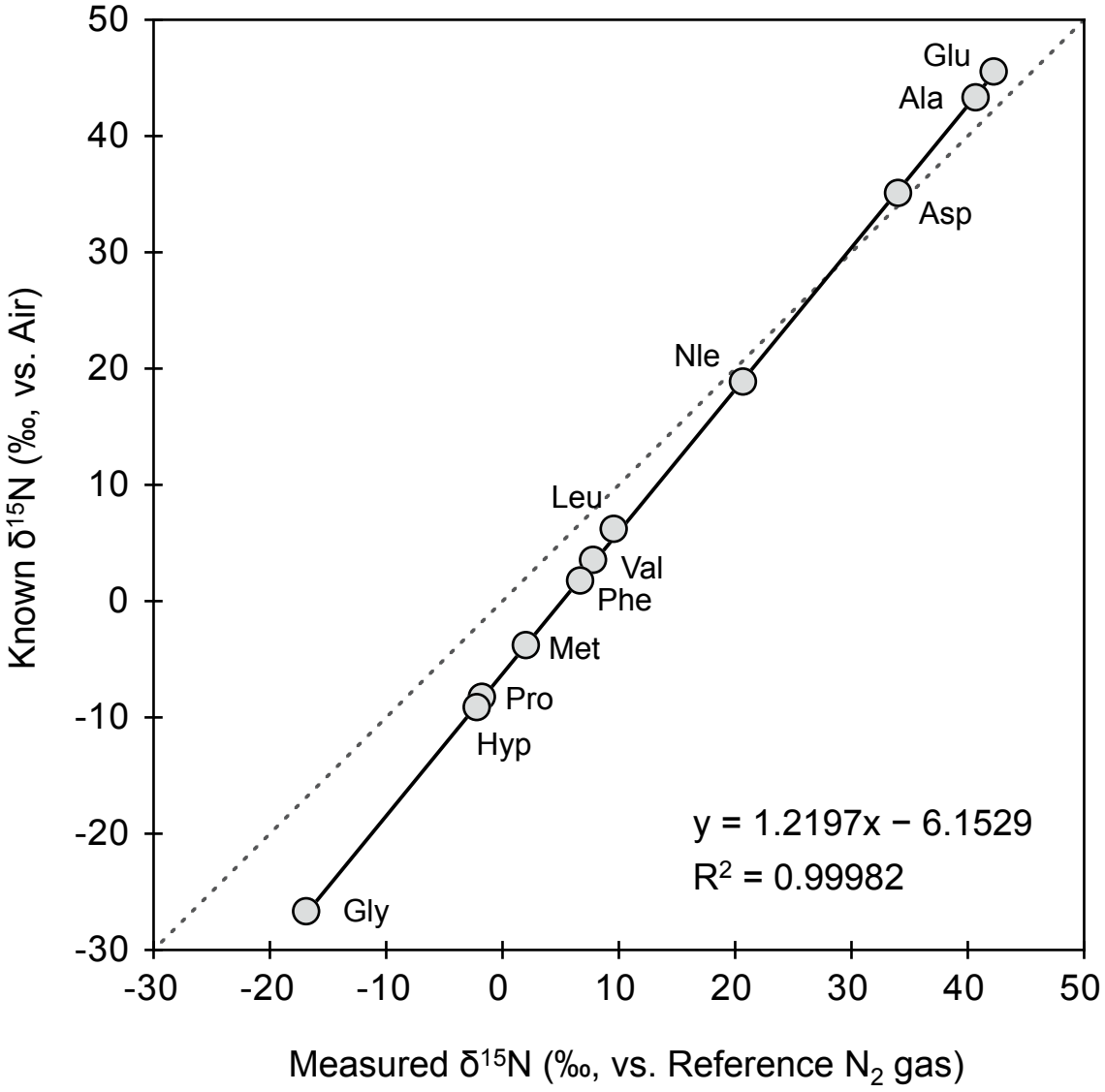


Fig. S3. A regression line between known (‰, *vs.* AIR) and measured values (‰, *vs.* reference N_2 gas).

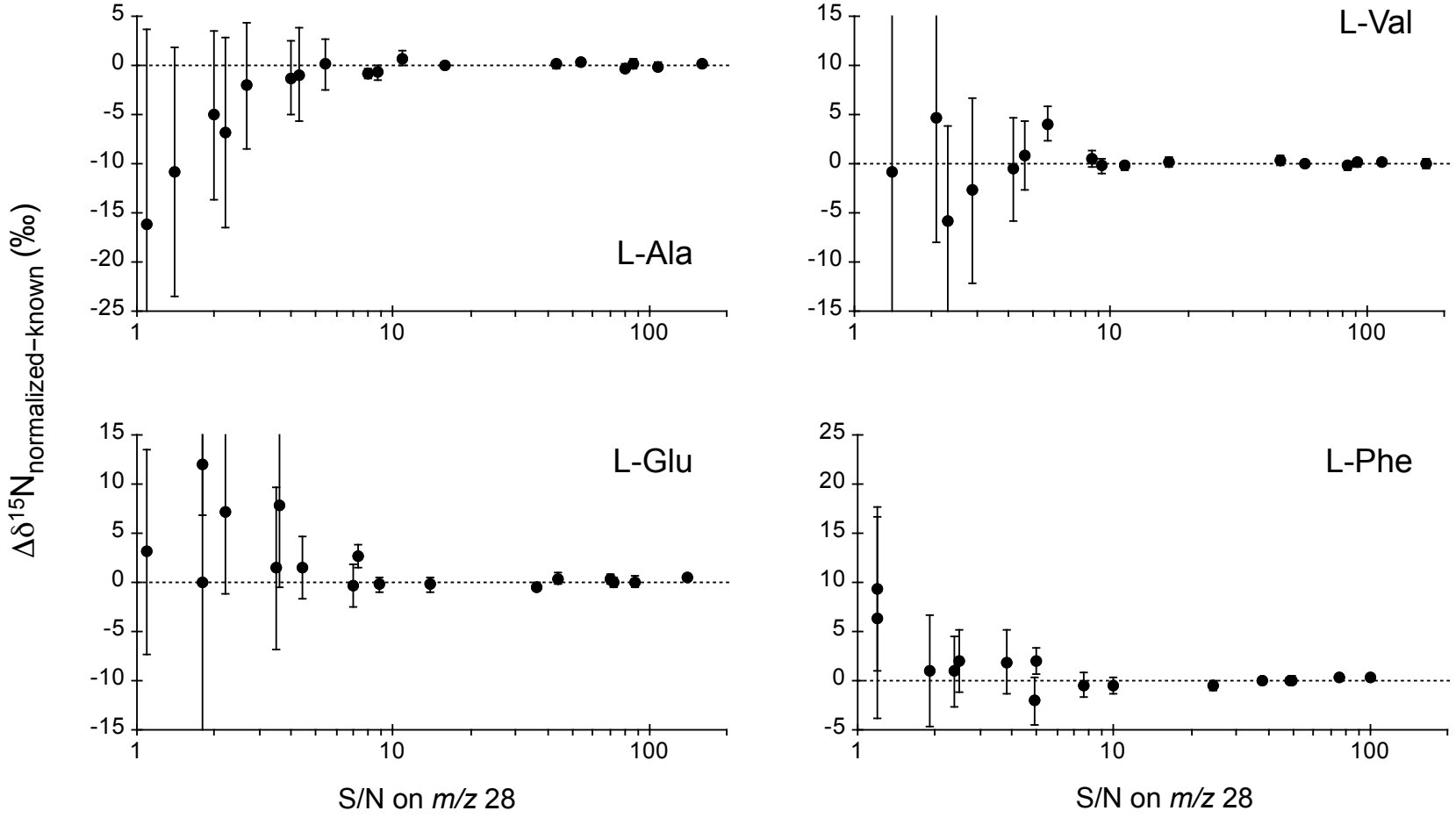


Fig. S4. The accuracy and precision of the nitrogen isotope analysis of amino acids by GC/IRMS with respect to the signal/noise (S/N) ratio (Chan et al., 2016).