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Determination of aquatic food-web structure based on compound-specific nitrogen isotopic composition of amino acids

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Abstract

The nitrogen isotopic composition ($\delta^{15}N$) of amino acids is potentially useful as an alternative method for estimating the trophic levels of organisms in food webs. However, because this "amino acid method" has been constructed from the observations of only a few case studies of food-consumer combinations in previous studies, the universality of the approach remains unclear. In this study, we investigated the $\delta^{15}N$ signatures of amino acids in 17 photoautotrophs and the trophic relationships during four controlled feeding experiments using green algae, zooplankton, and fish. The results are consistent with those reported in previous studies, implying that the amino acid method can be applied to a variety of organisms. From these and previously published data, we estimate the two factors (β, isotope differences among amino acids in primary producers; Δ, the ^{15}N-enrichment factor for each trophic level) required to calculate the trophic level. Based on the lowest error ($1\sigma = 0.12$) in the estimated trophic level, we conclude that a comparison of the $\delta^{15}N$ values for glutamic acid and phenylalanine is most useful in calculating precise estimates of the trophic level, using the following equation: trophic level ($TL_{Glu/Phe}$) = $(\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - 3.4)/7.6 + 1$.

Introduction

The stable isotope analysis of bulk organic materials has been used to investigate food-web structures in a number of ecological studies (Fry 2006). In particular, the nitrogen isotopic composition ($\delta^{15}N$) of bulk organisms and their tissues has been widely used to identify the trophic levels of organisms and the nitrogen flow in food webs (e.g., Hobson and Welch 1992; Yoshii et al. 1999; Ogawa et al. 2001). The trophic level (TL) is generally estimated by Eq. 1, based on the empirical observation that the $\delta^{15}N$ values for bulk organisms and their tissues tend to increase by ~3.4‰ with each trophic level (e.g., DeNiro and Epstein 1981; Minagawa and Wada 1984; Post 2002).

$$TL_{bulk} = (\delta^{15}N_{consumer} - \delta^{15}N_{producer})/3.4 + 1 \quad (1)$$

However, this "bulk method" involves several pitfalls, which often lead to large errors in estimating the trophic level. First, the ^{15}N-enrichment factor, an increase of ~3.4‰ with each trophic level, varies with different species, physiology, and trophic ecology (e.g., Vander Zanden and Rasmussen 2001; McCutchan et al. 2003; Martinez del Rio et al. 2009). For example, DeNiro and Epstein (1981) reported a large variation in the ^{15}N-enrichment factor, from –0.5‰ to +9.2‰, for different animals, including insects and mammals. McCutchan et al. (2003) also reported that the ^{15}N-enrichment factor ranged from –2.1‰ to +5.4‰ for insects and fish. Second, it is necessary to characterize the $\delta^{15}N$ values for primary producers to estimate the trophic level, but this task is difficult in

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many cases. For example, primary producers in aquatic environments (e.g., cyanobacteria and algae) show spatial and temporal variability in their $\delta^{15}N$ values (more than 10‰ in some cases, which is equivalent to approximately three times the ^{15}N-enrichment factor; e.g., Hannides et al. 2009) because of their assimilation of various nitrogen sources (i.e., N_2, NO_3^-, NH_4^+) and the short life span of the organisms (e.g., Bronk and Glibert 1993; Rolff 2000; Dore et al. 2002). In a study conducted in the Baltic Sea, Rolff (2000) reported an annual range in $\delta^{15}N$ values for phytoplankton of 0‰ to 7‰ and a range for zooplankton of 3‰ to 10‰. Therefore, because the collected phytoplankton usually represent only a snapshot of the natural environment, spatial and temporal integration must be considered to obtain realistic $\delta^{15}N$ estimates for primary producers in food webs (e.g., O'Reilly et al. 2002).

Therefore, this method was recently improved with the analysis of the nitrogen isotope compositions of amino acids (McClelland and Montoya 2002; McClelland et al. 2003; Chikaraishi et al. 2007). McClelland and Montoya (2002) determined the $\delta^{15}N$ values for individual amino acids hydrolyzed from a green alga and its consumer zooplankton in experiments in culture. The authors observed large ^{15}N enrichments (~7‰) in some amino acids (e.g., glutamic acid) and little changes in others (e.g., phenylalanine) with each trophic level, and initially suggested that a comparison of the $\delta^{15}N$ values of these two types of amino acids in organisms might indicate their trophic level in the food web. Chikaraishi et al. (2007) observed the same relationship between macroalgae and gastropods in a natural marine coastal environment, and proposed a mechanism that might affect the trophic relationships deduced from the $\delta^{15}N$ values (Fig. 1): certain amino acids (e.g., alanine, valine, isoleucine, and glutamic acid) are enriched in ^{15}N (by up to 10‰) as a result of isotopic fractionation during metabolic transamination (because it cleaves carbon-nitrogen bond), whereas other amino acids (e.g., methionine and phenylalanine) show little change in their $\delta^{15}N$ values because their dominant metabolic processes neither forms nor cleaves bonds related to the nitrogen atom. The trophic level is estimated based on the $\delta^{15}N$ values of amino acids (Fig. 2), according to Eq. 2:

$$TL_{x/y} = (\delta^{15}N_x - \delta^{15}N_y - \beta_{x/y})/(\Delta_x - \Delta_y) + 1 \quad (2)$$

where $\beta_{x/y}$ represents the isotope difference between amino acids x and y in the primary producers (trophic level = 1.0), and Δ_x and Δ_y represent the ^{15}N-enrichment factors with each trophic level for amino acids x and y, respectively. The subscripts x and y indicate "trophic" and "source" amino acids that show a large ^{15}N enrichment and little change in $\delta^{15}N$ values with each trophic level, respectively. Important advantages of this "amino acid method" are as follows: (1) the trophic level is estimated based on two amino acids from a single organism; (2) it inherently reflects an integrated value for the $\delta^{15}N$ of the primary producers that are actually eaten by consumers in the studied food webs; and (3) the large

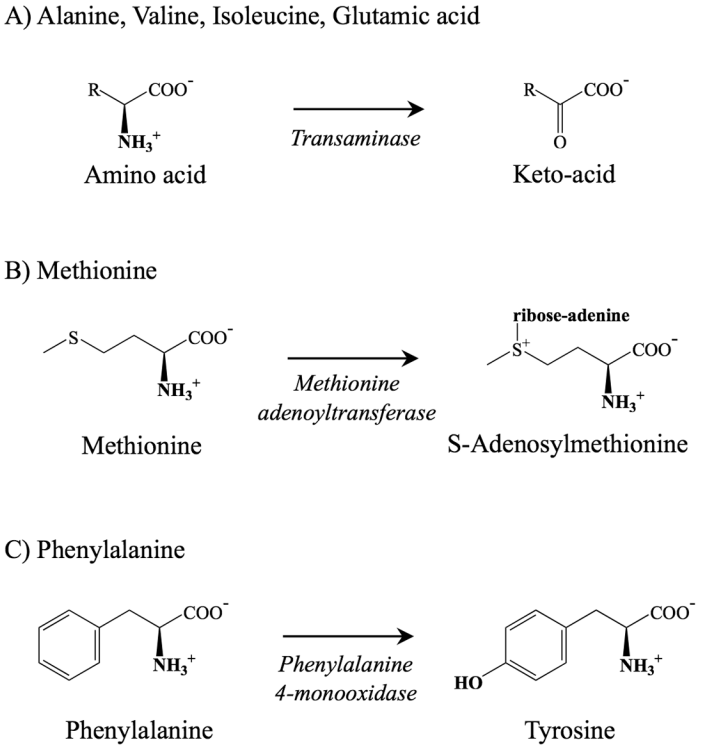

Fig. 1. Nitrogen isotopic fractionation during amino acid metabolism (after Chikaraishi et al. 2007)

denominator (e.g., 7‰ to 8‰ in some cases) in Eq. 2 potentially has the effect of reducing uncertainty associated with variations in the ^{15}N-enrichment effect with different samples. Of these, the first advantage is particularly significant because, unlike the bulk method, it means that the amino acid method does not require the characterization of the $\delta^{15}N$ values of the primary producers to estimate the trophic level. Moreover, a relatively small sample size (nanomolar amounts of nitrogen) is generally required for the nitrogen isotope analysis of amino acids, by using gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS). Thus, the amino acid method facilitates the estimation of trophic levels even from small samples, thereby extending our understanding of the actual structures of food webs and nitrogen flow in natural environments. For this reason, the amino acid method has recently been applied to clarify the trophic levels of organisms in various ecological studies, such as those of shrimp in the sub-Antarctic archipelago (Pakhomov et al. 2004), krill in the Antarctic (Schmidt et al. 2004, 2006), plankton in the central Pacific (McCarthy et al. 2007), yellowfin tuna in the eastern tropical Pacific (Popp et al. 2007), and zooplankton near Hawaii (Hannides et al. 2009). These previous studies have demonstrated that the amino acid method give a good estimate of the trophic level of marine organisms even from spatially and temporally variable environments. In particular, Hannides et al. (2009) demonstrated that the bulk $\delta^{15}N$ values of zooplankton near Hawaii temporally vary by up to 10‰

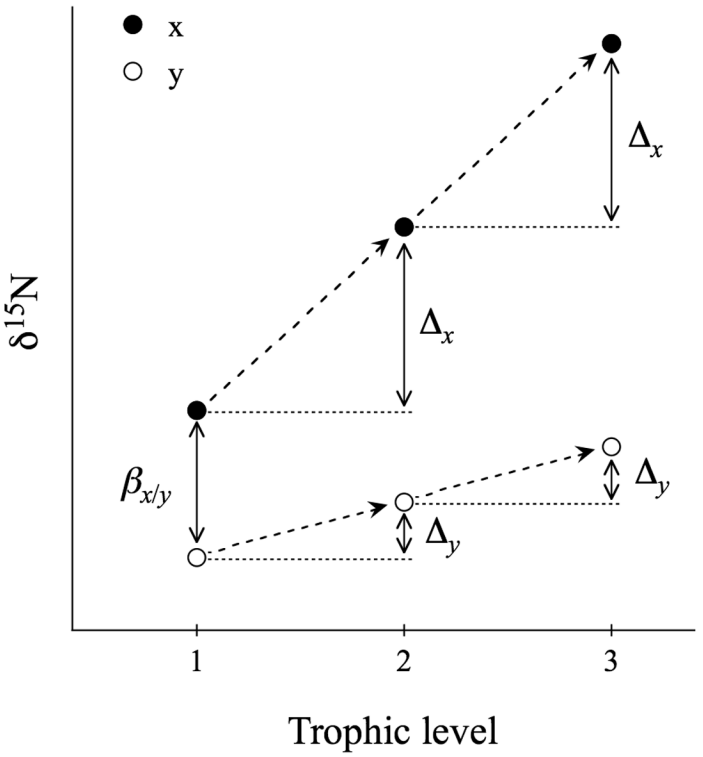

Fig. 2. Schematic illustration of the relationship between the nitrogen isotopic composition of amino acids and trophic levels, when samples are collected in one food web with a uniform isotope baseline. $\beta_{x/y}$ represents the isotope difference between the amino acids *x* (filled symbols) and *y* (open symbols) in primary producers. Δ_x and Δ_y are the ^{15}N-enrichment factors through the trophic levels for each amino acid.

but the trophic level estimated by the amino acid method stays consistent over a period of 10 years.

Despite the advantages discussed above, the universality of this approach remains uncertain. In fact, the method was constructed based on observations from only a few case studies of food-consumer combinations (i.e., controlled feeding experiments using cultured green algae and zooplankton by McClelland and Montoya 2002, and a natural macroalgae-gastropod combination by Chikaraishi et al. 2007). In particular, limited information is available regarding the β and Δ values and their variability among organisms, despite the fact that they are essential factors in calculating trophic levels and understanding their precision. If the β and Δ values vary across different samples, the amino acid method could lead to large errors in estimating trophic levels. It is also unclear which amino acid pair is the most accurate in making precise estimates of trophic levels. In fact, although the majority of studies (Pakhomov et al. 2004; Schmidt et al. 2006; Hannides et al. 2009) used glutamic acid (Glu) and phenylalanine (Phe), with a $\beta_{Glu/Phe}$ value of 4‰, a Δ_{Glu} value of 7‰, and a Δ_{Phe} value of 0‰, Popp et al. (2007) used glutamic acid and glycine (Gly), with a $\beta_{Glu/Gly}$ value of 0‰, a Δ_{Glu} value of 7‰, and a Δ_{Gly} value of 0‰. It also remains unclear whether the trophic relationship in terms of $\delta^{15}N$ values between primary producers and primary consumers (McClelland and Montoya 2002; Chikaraishi et al. 2007) can simply be extrapolated to other sets of organisms, particularly between primary consumers and secondary consumers (e.g., between zooplankton and fish). This must be directly confirmed with controlled feeding experiments before the amino acid method is extensively used as a routine method of estimating the trophic levels of organisms in natural food webs.

In this study, to confirm the universality of the amino acid method, we directly determined the isotope differences among amino acids (i.e., $\beta_{x/y}$) in 17 (12 cultured and 5 natural) photoautotrophs (cyanobacteria and algae) and the ^{15}N-enrichment factor for each trophic level (i.e., Δ_x and Δ_y) in four controlled feeding experiments using green algae, zooplankton, and fish. From these and previously published data (e.g., McClelland and Montoya 2002; Chikaraishi et al. 2007), we estimated the values for the factors in Eq. 2 (i.e., $\beta_{x/y}$, Δ_x, and Δ_y) and determined the most appropriate amino acid pair for precise estimates of trophic levels.

Materials and procedures

In this study, we examined the nitrogen isotopic composition of the amino acids hydrolyzed from 26 organisms, including 17 photoautotrophs (cyanobacteria and algae) and 4 consumers (zooplankton and fish) in controlled feeding experiments, and 5 consumers (crab and fish) collected from a natural marine coastal environment (Tables 1–3). The cyanobacteria *Anabaena cylindrica*, *Nostoc* sp., *Oscillatoria sancta*, and *Synechococcus* sp. were grown in batch culture in BG-11 medium with or without $NaNO_3$ at room temperature, and *Acaryochloris marina* was grown in batch culture in filtered seawater with IMK medium containing NH_4Cl at 25°C. The green algae *Donaliella* sp. and *Boergesenia forbesii* were grown in batch culture in sterilized prefiltered natural seawater supplemented with f/2 medium and artificial seawater (Rohtomarine) at 20°C, respectively. Further detailed information about the culture of these organisms was described by Rippka et al. (1979), Miyashita et al. (1997), and Nomaki et al. (2005). The National Center for Stock Enhancement of the Fisheries Research Agency of Japan (NCSE, FRA) provided the other cultured samples, including the green alga *Chlorella* sp., the zooplankton *Brachionus plicatilis* (rotifer), and the juvenile fish *Sebastes schlegli* (scorpion fish, 1.2 cm in length, 18 days old after yolk exhaustion) and *Paralichthys olivaceus* (bastard halibut, 1.0 cm in length, 10 days old after yolk exhaustion). In these cultures, *B. plicatilis* was grown grazing on *Chlorella* as a single food source and was harvested as a single food source for *S. schlegli* and *P. olivaceus* (Table 2). In general, the rapid growth rate of fish larvae dictates a high amino acid requirement from diet. Although the protein turnover rate has not been carefully estimated, contribution of yolk-derived amino acids could be considerably small to our cultured fish (e.g., Rønnestad et al. 2003). As natural samples, the cyanobacterium *Nostoc*

Table 1. Nitrogen isotopic composition of amino acids in primary producers.

Sample	Type	$\delta^{15}N$ (‰, relative to Air)											$TL_{Glu/Phe}$*
		Bulk	Ala	Gly	Val	Leu	Ile	Pro	Ser	Met	Glu	Phe	
This study													
Cyanobacteria													
Acaryochloris marina	Culture	3.4	4.4	–0.8	6.2	4.1	2.5	4.4	–1.2	n.d.†	5.1	0.8	1.1
Anabaena cylindrica	Culture (N_2 fixation)	–1.8	0.2	–5.8	3.0	0.3	0.6	n.d.	–6.4	n.d.	1.1	–3.0	1.1
Nostoc sp. (#1)	Culture (N_2 fixation)	–0.9	–1.4	–3.6	0.8	–0.8	n.d.	–0.6	–6.2	n.d.	–0.2	–3.8	1.0
Nostoc sp. (#2)	Culture	–6.0	–4.7	–5.6	–4.0	–7.0	–4.6	–5.4	–13.4	n.d.	–6.1	–8.8	0.9
Nostoc commune	Natural	–0.9	–0.2	–11.4	–1.1	–1.3	–1.0	–1.5	–10.8	n.d.	–1.6	–4.6	1.0
Oscillatoria sancta	Culture	–1.7	–1.3	–4.5	1.9	–0.6	–0.2	–3.4	–9.3	–3.4	1.3	–1.7	0.9
Synechococcus sp. (#1)	Culture	5.5	5.2	–0.2	6.1	4.0	4.8	6.3	–2.1	n.d.	5.6	2.2	1.0
Synechococcus sp. (#2)	Culture	2.6	–0.9	–5.4	0.9	0.2	1.2	1.1	–7.3	–5.4	1.2	–2.6	1.0
Green algae													
Boergesenia forbesii	Culture	5.9	5.7	0.2	6.7	4.9	5.4	5.4	–4.2	0.7	5.8	2.4	1.0
Chlorella sp. (#1)	Culture	–2.9	–3.9	–5.5	–2.8	–6.5	–3.9	–5.6	–9.1	n.d.	–5.4	–7.3	0.8
Chlorella sp. (#2)	Culture	–2.8	–4.2	–4.8	–2.8	–6.6	–3.8	–4.5	–9.8	n.d.	–4.9	–7.4	0.9
Donaeliella sp. (#1)	Culture	2.1	2.9	–8.6	5.8	5.9	3.8	5.5	–4.4	n.d.	4.6	0.1	1.1
Donaeliella sp. (#2)	Culture	2.6	6.0	5.4	7.3	5.5	4.2	7.5	–4.8	n.d.	5.1	1.5	1.0
Prasiola japonica	Natural	3.4	2.6	0.1	4.2	3.9	2.7	3.2	–0.5	–1.1	4.2	0.3	1.1
Ice algae‡	Natural	14.3	13.0	4.3	14.4	n.d.	13.2	12.2	0.7	n.d.	13.0	10.1	0.9
Red algae													
Gelidium japonicum	Natural	7.3	10.4	0.4	10.6	8.5	9.4	10.5	0.6	3.8	9.2	6.6	0.9
Brown algae													
Sargassum filicinum	Natural	5.6	7.9	–0.8	9.3	4.6	7.2	9.0	1.6	2.5	8.2	4.4	1.0
References													
Cyanobacteria§													
Trichodesmium sp.	Culture (N_2 fixation)	–1.7	–3.2	–2.1	–0.5	–0.3	–0.7	–1.6	–8.9	–4.4	–1.4	–3.6	0.8
Green algae‖													
Tetraselmis suecica (#1)	Culture	–1.9	0.5	–4.7	1.8	–0.4	–0.8	0.1	–7.8	n.d.	–0.4	–5.3	1.2
Tetraselmis suecica (#2)	Culture	–1.7	–1.6	–4.4	1.0	–1.5	–1.3	–0.4	–7.8	n.d.	–0.1	–4.3	1.1
Red algae¶													
Binghamia californica	Natural	6.5	6.9	–0.8	8.3	4.9	6.4	7.3	–1.4	0.9	8.6	3.6	1.2
Gelidium japonicum	Natural	6.5	8.2	2.9	7.7	5.7	7.0	7.3	–3.3	3.2	9.3	4.7	1.2
Brown algae¶													
Sargassum filicinum	Natural	5.6	7.3	–1.5	8.4	2.8	5.8	7.2	–1.6	2.0	6.3	4.1	0.8
Undaria pinnatifida	Natural	5.6	8.0	–0.7	6.8	5.1	5.7	5.4	–2.1	2.0	6.9	4.1	0.9
Diatom#													
Rhizosolenia sp.	Natural	n.d.	6.3	–3.5	10.6	4.3	5.6	2.6	0.6	n.d.	5.7	2.6	1.0

*Trophic level calculated using the amino acid method with the following equation: $TL_{Glu/Phe} = (\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - 3.4)/7.6 + 1$; *see* text.
†n.d., not determined.
‡Mixture of *Chlorella* sp., *Raphidonema nivale*, and *Chloromonas* sp.
§Data from McClelland et al. (2003).
‖Data from McClelland and Montoya (2002).
¶Data from Chikaraishi et al. (2007).
#Data from McCarthy et al. (2007).

commune and the green alga *Prasiola japonica* were collected from the grounds of Kyoto University, Japan (34°54′N, 135°48′E) in March 2008 and from the Naka River in Shizuoka, Japan (34°45′N, 138°47′E) in January 2008, respectively. The red macroalga *Gelidium japonica* and the brown macroalga *Sargassum filicinum* were collected from water at a depth of 2-4 m along the sea coast near Yokohama, Japan (35°08′N, 139°07′E) in May 2006. Ice algae (a mixture of *Chlorella* sp., *Raphidonema nivale*, and *Chloromonas* sp.) were collected from Langhovde, Antarctica (69°53′S, 39°48′E) during the activities of the summer party of the 47th Japan Antarctica Research Expedition from December 2005 to February 2006. The microbial community within the ice algae was preliminarily identified by gene analysis by Fujii et al. (2006). We also collected three

Table 2. Nitrogen isotopic composition of amino acids in heterotrophs.

Sample	Type	Food	$\delta^{15}N$ (‰, relative to Air) Bulk	Ala	Gly	Val	Leu	Ile	Pro	Ser	Met	Glu	Phe	TL_{Bulk}*	$TL_{Glu/Phe}$†
This study															
Zooplankton															
Brachionus plicatilis (#3)	Culture	*Chlorella* sp. (#1)‡	–2.0	0.8	–4.7	3.5	–2.9	0.5	2.3	–5.9	n.d.§	2.2	–7.0	1.3	1.8
Brachionus plicatilis (#4)	Culture	*Chlorella* sp. (#2)‡	–1.5	2.5	–4.7	4.1	–1.9	0.7	3.2	–5.4	n.d.	3.4	–7.0	1.4	1.9
Fish															
Paralichthys olivaceus	Culture	*B. plicatilis* (#4)	2.0	5.4	7.8	9.4	3.7	7.2	9.1	–4.1	n.d.	12.8	–6.3	2.4	3.1
Sebastes schlegeli	Culture	*B. plicatilis* (#3)	2.5	10.0	–1.0	6.5	3.7	4.7	8.2	1.6	n.d.	11.2	–6.8	2.6	2.9
References															
Zooplankton‖															
Brachionus plicatilis (#1)	Culture	*T. suecica* (#1)‡	0.1	4.8	–3.2	4.5	2.8	3.3	4.3	–7.1	n.d.	6.5	–4.2	1.6	2.0
Brachionus plicatilis (#2)	Culture	*T. suecica* (#2)‡	–0.2	4.0	–4.1	5.6	2.0	2.4	3.3	–6.9	n.d.	6.4	–4.8	1.4	2.0
Gastropod¶															
Batillus cornutus	Natural	Brown algae‡	7.8	16.6	4.8	15.1	12.7	14.3	14.1	7.0	3.1	16.4	5.0	1.6	2.0
Haliotis discus	Natural	Brown algae‡	6.0	12.6	2.8	12.7	6.1	9.5	12.9	–0.6	1.9	13.2	4.3	1.1	1.7
Omphalius pfeifferi	Natural	Brown algae‡	7.4	15.1	3.6	11.4	8.9	9.0	11.7	2.4	2.5	14.7	4.4	1.5	1.9

*Trophic level calculated using the bulk method: $TL_{Bulk} = (\delta^{15}N_{Bulk\ in\ sample} - \delta^{15}N_{Bulk\ in\ primary\ producer})/3.4 + 1$ (e.g., Minagawa and Wada 1984).
†Trophic level calculated using the amino acid method: $TL_{Glu/Phe} = (\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - 3.4)/7.6 + 1$; *see* text.
‡$\delta^{15}N$ values of amino acids in these algae are reported in Table 1.
§n.d., not determined.
‖Data from McClelland and Montoya (2002).
¶Data from Chikaraishi et al. (2007).

crabs, *Pachygrapsus crassipes* (6 cm in length), *Plagusia dentipes* (18 cm in length), and *Percnon planissimum* (10 cm in length), and two fish, *Girella punctata* (rudder fish, 16 cm in length), and *Acanthopagrus schlegeli* (black porgy, 22 cm in length), from the seacoast near Yokohama, Japan (35°08′N, 139°07′E) (Table 3). These samples were cleaned with distilled water to remove contaminants and stored at –20°C. The whole sample (for all photoautotrophs, zooplankton, and cultured fish), small pieces of muscular tissue from walking leg (for natural crabs), and scale (for natural fish) were used for the isotope analysis.

The samples described above were prepared for the compound-specific nitrogen isotope analysis of their amino acids with HCl hydrolysis followed by *N*-pivaloyl/isopropyl (Pv/iPr) derivatization, according to the procedures described by Chikaraishi et al. (2007). In brief, each sample was hydrolyzed with 12 N HCl at 100°C, and the hydrolysate was washed with *n*-hexane/dichloromethane (6:5, v/v) to remove any hydrophobic constituents, such as lipids. After derivatization with thionyl chloride/2-propanol (1:4, v/v) and subsequently with pivaloyl chloride/dichloromethane (1:4, v/v), the Pv/iPr derivatives of the amino acids were extracted with *n*-hexane/dichloromethane (6:5, v/v). The nitrogen isotopic composition of the individual amino acids was determined by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) using a ThermoFinnigan Delta plus XP isotope ratio mass spectrometer coupled to an Agilent Technologies 6890N gas chromatograph *via* combustion and reduction furnaces (e.g., Hayes et al. 1990; Brand et al. 1994; Merritt and Hayes 1994). The analytical conditions for GC/C/IRMS were described by Chikaraishi et al. (2007, 2008).

Nitrogen isotopic compositions are expressed in δ notation against atmospheric N_2 (Air). Standard mixtures of eight $\delta^{15}N$-known amino acids (alanine, glycine, valine, leucine, aspartic acid, serine, glutamic acid, and phenylalanine) were analyzed every 4-5 GC/C/IRMS runs to confirm the reproducibility of the isotope measurements. The analytical errors (1σ) for the standards were always better than 0.5‰ when a minimum sample of 30 ng N was used. We determined the $\delta^{15}N$ values for 10 amino acids (alanine, glycine, valine, leucine, isoleucine, proline, serine, methionine, glutamic acid, and phenylalanine) because they always eluted separately with baseline resolution on the chromatograms (Fig. 3). The $\delta^{15}N$ value for glutamic acid includes a contribution from the α-amino group of glutamine because glutamine is converted to glutamic acid during acid hydrolysis. The isotopic compositions of other amino acids were not determined in the procedures because aspartic acid and threonine partly coeluted on the chromatogram, and arginine, cysteine, histidine, lysine, tyrosine, and tryptophan were not detected on the chromatogram, probably because of their degradation or low recovery during the procedures.

Assessment and discussion

Distribution of $\delta^{15}N$ among the amino acids of primary producers (β)—The $\delta^{15}N$ values of the amino acids from the photoautotrophs are summarized in Table 1, together with previously published data for cyanobacteria (McClelland et al. 2003),

Table 3. Nitrogen isotopic composition of crab and fish from the seacoast near Yokohama, Japan.

		$\delta^{15}N$ (‰, relative to Air)												
Sample	**Type**	**Bulk**	**Ala**	**Gly**	**Val**	**Leu**	**Ile**	**Pro**	**Ser**	**Met**	**Glu**	**Phe**	TL_{Bulk}*	$TL_{Glu/Phe}$†
Crab														
Pachygrapsus crassipes	Natural	7.8	15.9	3.7	15.4	10.7	9.4	15.4	3.3	1.9	19.3	3.8	1.5	2.6
Plagusia dentipes	Natural	10.1	14.9	6.0	16.3	13.0	9.9	16.9	6.4	2.6	20.4	5.1	2.2	2.6
Percnon planissimum	Natural	8.4	14.1	7.5	12.8	12.9	12.3	16.4	6.6	1.8	17.9	4.9	1.7	2.3
Fish														
Acanthopagrus schlegeli	Natural	11.1	20.0	7.4	19.9	19.4	21.5	21.9	11.2	2.4	25.6	4.9	2.4	3.3
Girella punctata	Natural	11.1	20.7	6.3	20.2	18.1	19.8	19.4	11.5	1.6	22.0	4.4	2.4	2.9

*Trophic level calculated using the bulk method: $TL_{Bulk} = (\delta)^{15}N_{Bulk\ in\ sample} - (\delta)^{15}N_{Bulk\ in\ primary\ producer})/3.4 + 1$ (e.g., Minagawa and Wada 1984)
†Trophic level calculated using the amino acid method: $TL_{Glu/Phe} = (\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - 3.4)/7.6 + 1$; *see* text.

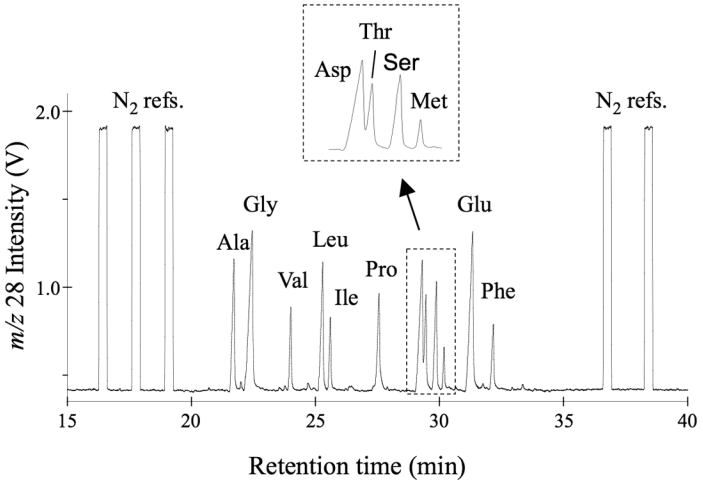

Fig. 3. Representative *m/z* 28 chromatogram of a GC/C/IRMS analysis of amino acids as Pv/iPr derivatives (brown alga, *S. filicinum*). Abbreviations: alanine (Ala), glycine (Gly), valine (Val), leucine (Leu), isoleucine (Ile), proline (Pro), aspartic acid (Asp), threonine (Thr), serine (Ser), methionine (Met), glutamic acid (Glu), and phenylalanine (Phe).

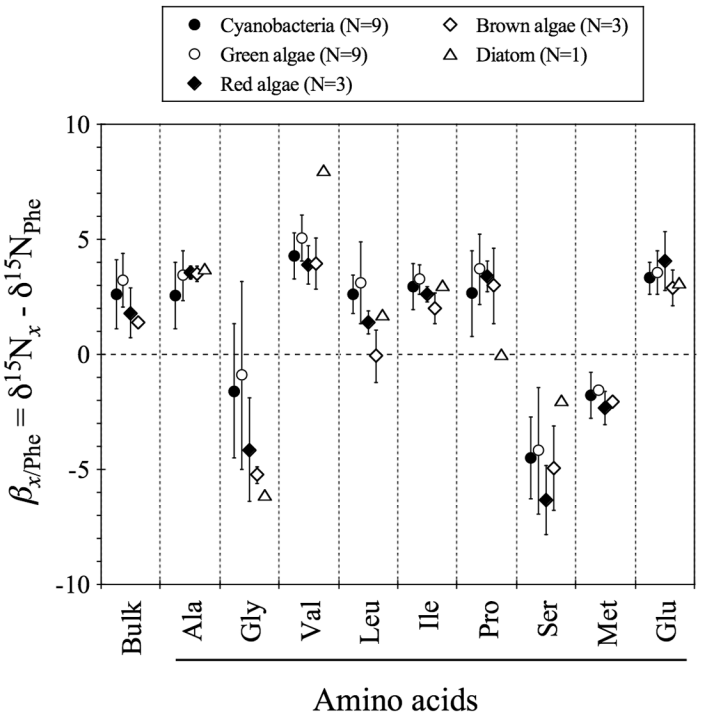

Fig. 4. β Patterns for photoautotrophs. Symbols and bars represent the means and variations (1σ) of the β values for a single taxon of primary producers.

green algae (McClelland and Montoya 2002), red and brown macroalgae (Chikaraishi et al. 2007), and a diatom (McCarthy et al. 2007). A wide range in $\delta^{15}N$ values (from –13.4‰ to 14.4‰) was observed for the photoautotrophs (Table 1), reflecting both the assimilation of isotopically variable nitrogen species and the unique isotopic fractionation associated with amino acid synthesis and metabolism (e.g., McClelland and Montoya 2002; Chikaraishi et al. 2007). To determine the β values for individual amino acids, we normalized the $\delta^{15}N$ values of the amino acids according to their difference from that of phenylalanine, as shown in the $\beta_{x/Phe}$ ($\beta_{x/Phe} = \delta^{15}N_x - \delta^{15}N_{Phe}$) values in Fig. 4. We used phenylalanine as the source amino acid (*see* below).

Although the amino acids showed large variations in β values, ranging from –9.3‰ to +8.0‰, the β patterns were similar among the five taxa of photoautotrophs (Fig. 4). No substantial differences were observed between the cultured and natural samples or between the samples grown on various nutrients (i.e., N_2, NO_3^-, NH_4^+). Alanine, valine, leucine, isoleucine, proline, and glutamic acid were commonly enriched in ^{15}N relative to phenylalanine, whereas serine and methionine were depleted in ^{15}N (even though the values differed among the amino acids). In contrast, glycine generally showed a large variation in β values across the zero line (–8.8‰ to +3.9‰), even within a single taxon of photoautotrophs. This phenomenon has also been reported in previous studies (Chikaraishi et al. 2007; McCarthy et al. 2007). Based on the expanded dataset for the 25 photoautotrophs listed in Table 1, including natural and cultured algae and cyanobacteria, which are phylogenically distant from the algae and show large diversity even within a taxon, we suggest

Table 4. Summary of the mean original isotope difference between amino acid and phenylalanine (β) and ^{15}N-enrichment factor along trophic level (Δ) and their variability (1σ).

	$\beta_{x\text{-Phe}}$		Δ	
	Average	1σ	Average	1(σ)
Bulk	2.6	1.3	2.1	1.3
Alanine	3.2	1.2	6.1	2.1
Glycine	–2.3	3.4	3.7	3.9
Valine	4.6	1.2	5.0	1.7
Leucine	2.3	1.6	4.8	2.0
Isoleuicine	2.9	0.8	4.8	1.7
Proline	3.1	1.7	6.1	1.6
Serine	–4.6	2.2	3.6	3.0
Methionine	–2.0	0.6	0.5	0.6
Glutamic acid	3.4	0.9	8.0	1.2
Phenylalanine	—	—	0.4	0.5

that the β pattern is a general signature for aquatic primary producers. This implies that the β value reflects the biosynthetic and metabolic processes affecting amino acids and that difference in taxa and nutrient sources of photoautotrophs plays only a minor role. The mean β values and variations (1σ) for the photoautotrophs are summarized in Table 4.

^{15}N-Enrichment factor for amino acids at each trophic level (Δ)—The nitrogen isotopic compositions of the amino acids from consumer samples in the feeding experiments are summarized in Table 2, together with data from previous consumer-food combination studies of zooplankton (McClelland and Montoya 2002) and gastropods (Chikaraishi et al. 2007). The amino acid $\delta^{15}N$ values vary widely from –7.1‰ to +16.6‰ for the consumer samples (Table 2), reflecting both the isotopic composition of the food sources and the isotopic fractionation during the metabolism of the amino acids by the consumers (McClelland and Montoya 2002; Chikaraishi et al. 2007). The ^{15}N-enrichment factors for each amino acid for single shifts in trophic levels (Δ, $\Delta = \delta^{15}N_{consumer} - \delta^{15}N_{food}$) are shown in Fig. 5.

Although the amino acids showed large variations in Δ values, ranging from –0.5‰ to +12.4‰, the Δ patterns were similar or consistent for all food-consumer combinations, even including zooplankton-fish combinations. Alanine, valine, leucine, isoleucine, proline, and glutamic acid were commonly enriched in ^{15}N (up to 9.8‰) relative to the corresponding amino acids in the food sources, whereas methionine and phenylalanine showed little difference (Δ values of –0.5‰ to 1.1‰). In contrast, glycine and serine showed relatively large variations in Δ values, ranging from 0.2‰ to 12.4‰ depending on the sample (e.g., for glycine, slight differences from 0.2‰ to 1.5‰ for zooplankton, but much greater ^{15}N-enrichment from 3.8‰ to 12.4‰ for gastropods and fish). Thus, the trophic relationship between primary producers and primary consumers in terms of the $\delta^{15}N$ values

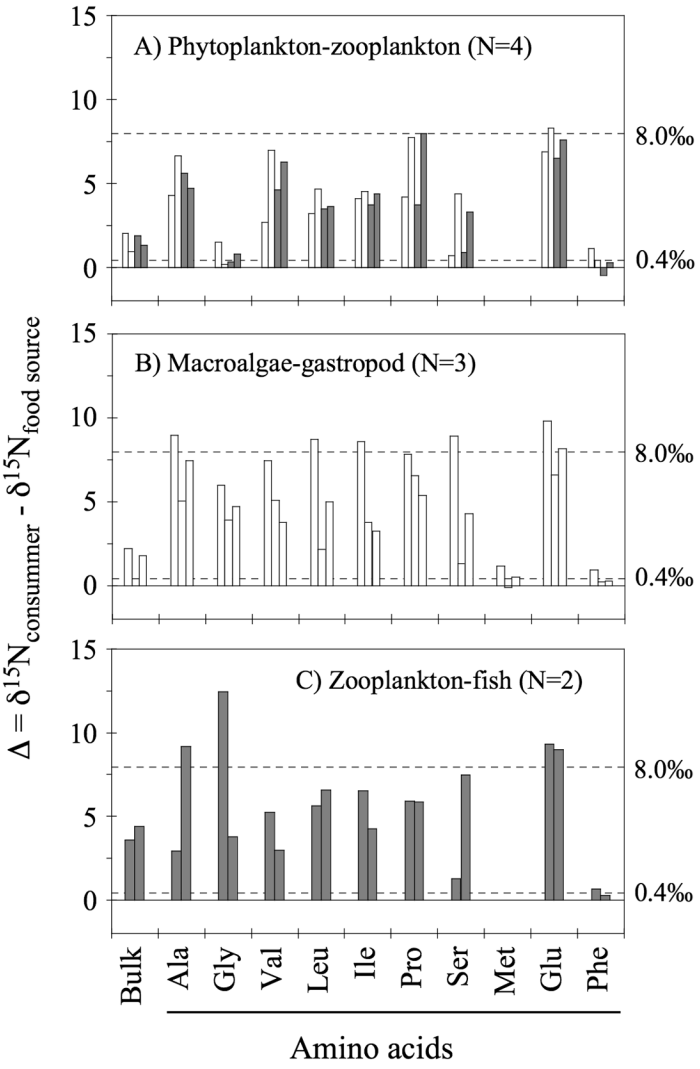

Fig. 5. ^{15}N-enrichment factors (Δ) between consumer and food for (A) phytoplankton-zooplankton, (B) macroalgae-gastropod, and (C) zooplankton-fish combinations. Different pillars represent the different samples: filled and open pillars represent data in the present study and previous studies (McClelland and Montoya 2002; Chikaraishi et al. 2007), respectively.

(McClelland and Montoya 2002; Chikaraishi et al. 2007) was observed for the green algae–zooplankton combination and even for the zooplankton-fish combination in this study, implying that the trend in the ^{15}N-enrichment of amino acids is very similar or consistent for a wide range of consumers, even at higher trophic levels in food webs. The mean Δ values and their variations (1σ) for the consumer samples are listed in Table 4.

Trophic level estimation—As summarized in Table 4, the mean Δ values for methionine and phenylalanine were close to zero, with little variation among the consumers (0.5 ± 0.6‰ for methionine, 0.4 ± 0.5‰ for phenylalanine). This finding probably reflects the small magnitude of isotopic fractionation during the specific metabolic processes of each

amino acid, meaning that bonds to the nitrogen atoms in the amino acids are neither formed nor broken (Fig. 1). This is in contrast to the significant isotopic fractionation that occurs during deamination, which is a dominant metabolic process for other amino acids (*see* Chikaraishi et al. 2007). Thus, methionine and phenylalanine are candidates for the source amino acids used to estimate trophic levels. However, because methionine is commonly less abundant than phenylalanine, the $\delta^{15}N$ value of methionine was not determined for approximately half the samples analyzed in this study (Tables 1–3). Accordingly, we used phenylalanine as the source amino acid to estimate trophic levels.

The mean Δ values for the other amino acids (i.e., other than methionine and phenylalanine) are significantly positive (3.6‰ to 8.0‰; Table 4), which directly reflects the isotopic fractionation during metabolic deamination (Macko et al. 1986; Chikaraishi et al. 2007). Therefore, these amino acids are ideally suited as trophic amino acids for estimating trophic levels. $TL_{x/y}$ is calculated with Eq. 2 using the β and Δ values listed in Table 4. For example, the $TL_{x/y}$ values based on alanine (Ala), valine (Val), isoleucine (Ile), proline (Pro), and glutamic acid (Glu) are calculated with Eqs. 3-7, respectively:

$$TL_{Ala/Phe} = (\delta^{15}N_{Ala} - \delta^{15}N_{Phe} - 3.2)/5.7 + 1 \quad (3)$$

$$TL_{Val/Phe} = (\delta^{15}N_{Val} - \delta^{15}N_{Phe} - 4.6)/4.6 + 1 \quad (4)$$

$$TL_{Ile/Phe} = (\delta^{15}N_{Ile} - \delta^{15}N_{Phe} - 2.9)/4.4 + 1 \quad (5)$$

$$TL_{Pro/Phe} = (\delta^{15}N_{Pro} - \delta^{15}N_{Phe} - 3.1)/5.7 + 1 \quad (6)$$

$$TL_{Glu/Phe} = (\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - 3.4)/7.6 + 1 \quad (7)$$

However, as summarized in Table 4, glycine and serine show relatively small Δ values (<3.7‰) and large standard deviations for both the β (1σ > 2.2‰) and Δ values (1σ > 3.0‰), leading to large errors in the estimated trophic levels. In contrast, glutamic acid has the largest Δ value (8.0‰) and only small variations in both the β (1σ = 0.9‰) and Δ values (1σ = 1.2‰), thereby minimizing the error involved in estimating the trophic level. It is also expected that intermediate Δ values (4.8 to 6.1‰) and relatively small variations in both the β (1σ < 1.7‰) and Δ values (1σ < 2.1‰) for the other amino acids will result in errors intermediate between those for glycine (or serine) and glutamic acid in estimating the trophic level. Thus, the error involved in estimating the trophic level could be dependent on the magnitude of the Δ value and the degree of variation in the β and Δ values. For example, as shown in Fig. 6, when this equation is applied to the samples listed in Tables 1 and 2, for which the actual trophic levels are known (i.e., photoautotrophs = 1.0, zooplankton and gastropods = 2.0, and fish = 3.0), the trophic level is estimated with the greatest precision when glutamic acid is used (1σ = 0.12), but is estimated with poor precision when the other amino acids are used (particularly, 1σ = 1.05 for glycine and 0.77 for serine). The estimation error (1σ = 0.12) for glutamic acid is approximately half that for alanine

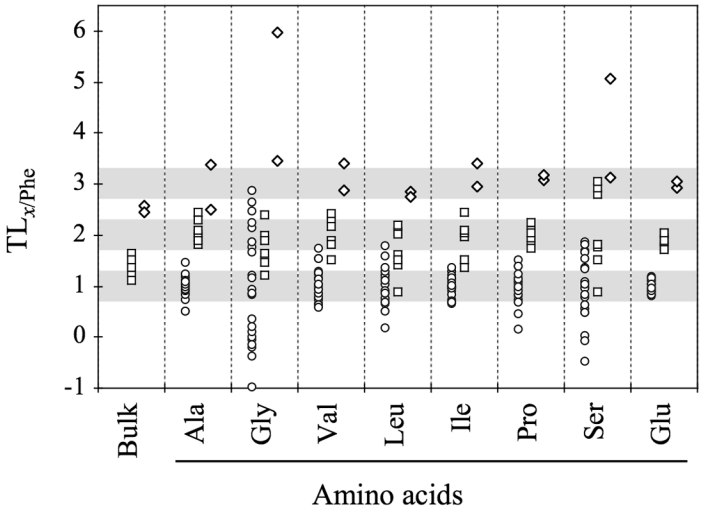

Fig. 6. Estimated trophic levels for those samples listed in Tables 1 and 2, calculated using the bulk and amino acid methods. The circle, square, and diamond symbols indicate primary producers (actual trophic level = 1.0), zooplankton and gastropods (actual trophic level = 2.0), and fish (actual trophic level = 3.0), respectively. The marked area indicates the actual trophic level with an error of 0.3.

(1σ = 0.23), valine (1σ = 0.28), isoleucine (1σ = 0.24), or proline (1σ = 0.26). Therefore, we conclude that the use of the glutamic acid–phenylalanine pair with Eq. 7 is the most promising combination for the precise estimation of the trophic level.

Eq. 7 is very similar to the equation generally applied in previous studies using glutamic acid and phenylalanine with a $\beta_{Glu/Phe}$ value of 4‰, a Δ_{Glu} value of 7‰, and a Δ_{Phe} value of 0‰ (Pakhomov et al. 2004; Schmidt et al. 2006; Hannides et al. 2009). Both equations provide similar results in estimating the trophic level. In the other words, this similarity proves the universality of the Eq. 7. Glycine and serine were also previously used as source amino acids for trophic level assessments by McCarthy et al. (2007) and Popp et al. (2007). The trophic levels of zooplankton estimated using these amino acids were similar to or consistent with that estimated using the glutamic acid–phenylalanine pair (Hannides et al. 2009). However, glycine and serine show a larger variation in the Δ value across different samples (Fig. 5) and are not useful as source amino acids for estimating trophic levels.

Application of the amino acid method to a natural food web—As an example of applying the amino acid method to a natural food web, we investigated 14 organisms from a natural coastal marine environment (sea coast near Yokohama, Japan). Macroalgae and gastropods were examined as a representative primary producer–consumer (i.e., herbivore) couple in this environment and the $\delta^{15}N$ values of their individual amino acids are listed in Tables 1 and 2. Crabs and fish were examined as representatives of higher trophic-level consumers (i.e., omnivores and carnivores, respectively), and the $\delta^{15}N$ values of their individual amino acids are summarized in Table 3.

The trophic levels were calculated with Eq. 7 based on the $\delta^{15}N$ values for glutamic acid and phenylalanine.

As mentioned above, the $\delta^{15}N$ values for primary producers generally vary in natural aquatic environments (e.g., Rolff 2000; O'Reilly et al. 2002). This variability was seen in the $\delta^{15}N$ values for the bulk materials and the individual amino acids of macroalgae in the present study (Fig. 7). The bulk $\delta^{15}N$ values for the macroalgae varied by 1.7‰ (from 5.6‰ to 7.3‰), which was equivalent to half the ^{15}N-enrichment factor in the bulk method (i.e., 3.4‰; Minagawa and Wada 1984). Moreover, the ^{15}N-enrichment factor for the bulk materials is also generally very variable, depending on the organisms (e.g., DeNiro and Epstein 1981; Vander Zanden and Rasmussen 2001; McCutchan et al. 2003). For these reasons, the trophic level estimated by the bulk method (TL_{Bulk}) represents a significant underestimation of the value expected for gastropods (the trophic level of herbivores should be 2.0) and crabs (the trophic level of omnivores should be greater than 2.0) in this environment.

However, the $TL_{Glu/Phe}$ values, ranging from 0.8 to 1.2 for macroalgae and from 1.7 to 2.0 for gastropods, are almost consistent with the actual trophic levels of primary producers and herbivores, respectively. Moreover, the $TL_{Glu/Phe}$ values, ranging from 2.3 to 2.6 for crabs and from 2.9 to 3.3 for fish, are considered to be adequate values for the trophic levels of omnivores and carnivores, respectively. Thus, the trophic levels estimated with the amino acid method using Eq. 7 reflect the actual food-web structure in a natural aquatic environment.

Comments and recommendations

The application of the amino acid method allows the estimation of the trophic level based on the $\delta^{15}N$ values of two amino acids from a single organism, potentially yielding an error smaller than that associated with the bulk method. In this study, we have confirmed the universality of this approach based on various samples of photoautotrophs (e.g., cyanobacteria, green, red, and brown algae) and controlled feeding experiments using algae-zooplankton and zooplankton-fish combinations, and have estimated the values for the essential factors (i.e., $\beta_{x/y}$, Δ_x, and Δ_y in Eq. 2) in calculating the trophic level. Based on the lowest error ($1\sigma = 0.12$) obtained in the estimated trophic levels for the investigated samples, we recommend that a comparison of the $\delta^{15}N$ values of glutamic acid and phenylalanine is most useful in obtaining precise estimates of the trophic levels of organisms, using the general equation: $TL_{Glu/Phe} = (\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - 3.4)/7.6 + 1$.

An important advantage of the amino acid method is that it does not require the characterization of the $\delta^{15}N$ values of the primary producers, which facilitates the estimation of the trophic levels of organisms and thus extends our understanding of the actual structures of food webs and the nitrogen flow in natural environments. Moreover, only a nanomolar amount of nitrogen is required for the precise determination of the isotopic composition of a single amino acid by GC/C/IRMS. For example, 0.1–1.0 mg samples were used in the present study. The method allows the estimation of trophic levels from small samples, such as microorganisms, the growth layers of fish scales, and the remnants of protein in fossil bones.

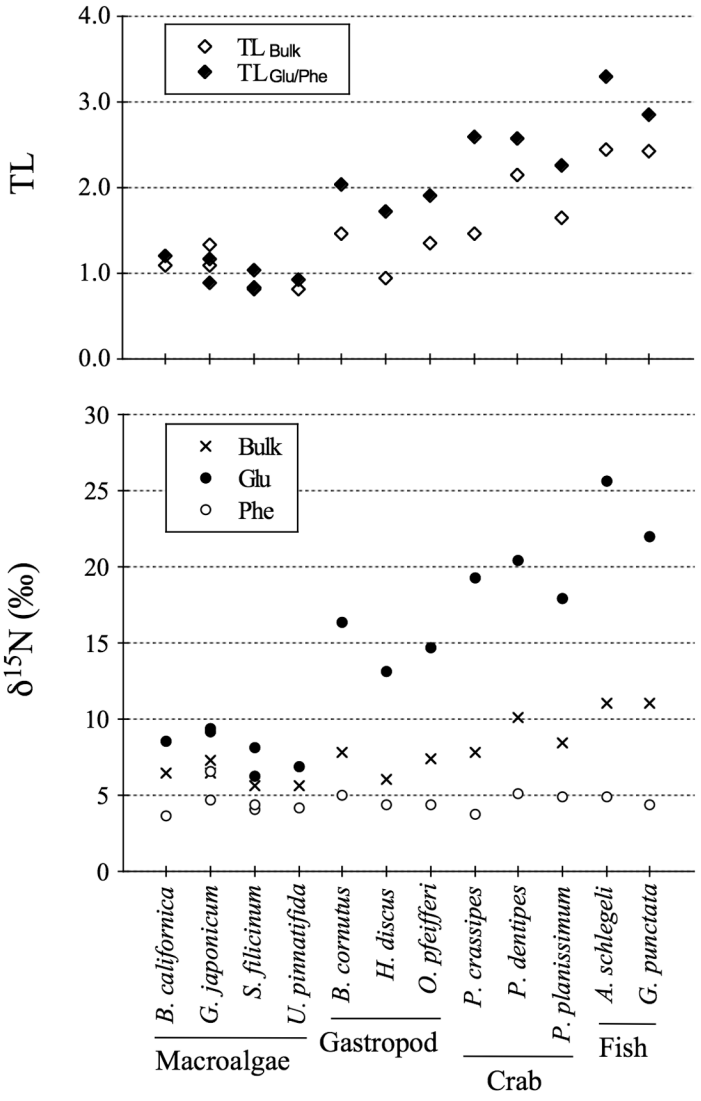

Fig. 7. $\delta^{15}N$ values for bulk material (cross), glutamic acid (filled circles), and phenylalanine (open circles) in 14 organisms from a natural marine coastal environment along the sea coast near Yokohama, Japan. Also shown are the estimated trophic levels calculated using the bulk (Eq. 1 in text) and amino acid methods (Eq. 7 in text). The mean bulk $\delta^{15}N$ value for six macroalgae (i.e., 6.2‰) supplies the $\delta^{15}N$ value for bulk primary producers in the bulk method.

However, sample preparation and isotope analysis of amino acids by GC/C/IRMS is time-consuming and relatively costly compared with bulk isotope analysis by traditional EA/IRMS methods, which may be a disadvantage of the amino acid method (i.e., we cannot easily apply it to a large number of samples at this stage). Combined with further improvements and optimization of sample preparation and

instrument condition, the amino acid method will maximize its advantage and should be widely used as a powerful alternative tool for identifying the trophic levels of organisms in a number of ecological food-web studies.

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