Factors for controlling stable isotopic composition of amino acids of marine organisms: Implication to aquatic ecosystem studies

海洋生物に含まれるアミノ酸の安定同位体比を変化させる要因の解明

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ABSTRACT

Introduction

Marine ecosystems have been damaged by human activities, including the extinction of organisms by overfishing, the concentration of pollutants by biomagnification, and the acidification of ocean by CO_2 emission. These problems affect marine ecosystems in many ways, including alteration of the trophic position (TP) and energy consumption of organisms. TP of organisms is useful for determining biomagnification. However, in previous studies, there is an issue on the determination, because calculating the accurate TP of organisms is challenging. Also, energy consumption of organisms is useful for estimating the effect of ocean acidification on organisms. However, there is an issue on the estimation, because evaluating the accurate energy consumption is challenging.

Scientific objectives

We have two scientific objectives in this study. First, to evaluate the effect of multiple nitrogen sources on the isotopic composition of organisms in food webs, we chose Sagami Bay, where is affected by Kuroshio Current, agriculture fertilization, and industrial nitrogen, as a site for the three following studies: charactering food web structure for fish communities (Chapter II, 2-1), comparing nitrogen isotope ratios ($\delta^{15}N$) between tissue types (Chapter II, 2-2), and illustrating isoscape map of coastal areas (Chapter II, 2-3). Secondly, to evaluate the effect of pH on the isotopic composition of organisms, we reared fish, gastropods, and corals in the artificial

seawater with pH7.3-8.1, as samples for evaluating the change in the energy consumption of organisms between pHs (Chapter III).

Effect of multiple nitrogen sources on the isotopic composition of organisms in food webs (Chapter II)

1. The small standard deviation of TP for 14 species collected from Sagami Bay suggests that compound-specific isotope analysis of amino acids (CSIA-AA) is useful for calculating TP of organisms and illustrating food web structure even for complex ecosystems.
2. The $\delta^{15}N$ values of amino acids in scale, fin, shell, and yolk are almost identical to those corresponding amino acids in muscle. This result suggests that these four tissues can be used as alternative samples to muscle in CSIA-AA, but other tissues (e.g., blood and bone) cannot.
3. From the isoscape map illustrated, we found a large variation in the $\delta^{15}N_{Baseline,phe}$ value across Sagami Bay, which reflects the incorporation of isotopically-distinct nitrogen sources to Sagami Bay.

Effect of low pH on the isotopic composition of aquatic organisms (Chapter III)

Changes in the $\delta^{15}N$ value of amino acids between pHs reveal that, in the end of energy consumption, there are different effects among species to the seawater pH: (1) pH7.3 is positively affected to the fish and gastropods; (2) pH7.6-8.1 is negligibly affected to the corals; and (3) pH lower than 7.6 is negatively affected to the corals.

CHAPTER I.

General Introduction

Marine ecosystems have been damaged by human activities, including the extinction of organisms by overfishing, the concentration of pollutants by biomagnification, and the acidification of ocean by CO_2 emission (Wren and Stephenson, 1991; Jackson et al. 2001; Conley et al. 2009). In generally, extinction is the termination of a species of organisms. One of major reasons for the extinction is the destruction of natural ecosystems by human activities. For example, Ostrom et al. (2017) reported that the trophic position (TP) of Hawaiian petrel *Pterodroma sandwichensis* is declined ~0.4 units from last 100 years to modern. These results suggest that size of food webs in North Pacific Ocean has shrank because of overfishing. In the Ph.D. thesis, I mainly focus on the study related to biomagnification and ocean acidification. Biomagnification is an increase in the concentration of pollutants (e.g., Hg, N, and P) among organisms along food chain. Scientists have a lot of efforts during the last three decades, to identify the degree of biomagnification for each pollutant in food webs (Giesy et al. 2001; Tomy et al. 2004). TP of organisms is a factor for determining biomagnification. However, there is an issue on the determination in the previous studies, as the calculation of TP of organisms is challenging. On the other hand, ocean acidification is a decrease in the pH of seawater because the uptake of CO_2 from atmosphere. Scientists have a lot of efforts during the last three decades, to evaluate the effect of ocean acidification on the organism and food webs.

Energy consumption of organisms is a factor for estimating the effect of ocean acidification. However, there is an issue on the estimation, because evaluation of the

energy consumption is challenging. Compound-specific isotope analysis of amino acids (CSIA-AA) is one of potential powerful tools for solving these two issues on the determination and estimation, because this analysis allows us accurately to calculate TP and evaluate energy consumption of organisms in environments.

Nitrogen atoms have two stable isotopes that are ^{15}N and ^{14}N, and the relative atomic abundance are 0.365% and 99.635% for former and latter, respectively (Fry, 2006). The nitrogen isotope ratios (i.e., $^{15}N/^{14}N$ = ~0.0036) have a large variation among samples in natural environments. The main reason for this variation is the isotope fractionation during chemical and biological reactions in natural environments, as organisms preferentially use ^{14}N comparing to ^{15}N in metabolisms (Fry, 2006). Nitrogen isotope ratios are conventionally expressed in parts per thousand (‰) as the difference between the ratios in sample and those in the international standard, according to the following equation (1-1):

$$\delta^{15}N = \left(\frac{\left(\frac{^{15}N}{^{14}N}\right)_{Sample}}{\left(\frac{^{15}N}{^{14}N}\right)_{Standard}} - 1 \right) \times 1000\ (‰) \qquad (1\text{-}1)$$

The nitrogen isotope ratios ($\delta^{15}N$) are particularly useful in ecological studies, for example, changes in the ratio from diet to consumer species is correlated with the trophic position and the energy consumption of organisms in food webs.

At present, there are several methods to calculate TP of organisms, including observation, stomach content analysis, and stable isotope analysis (e.g., DeNiro and Epstein, 1981; Williams and Martinez, 2004). For the former two methods, they can directly evaluate the proportion of diet species for consumers, but such diet information is snapshot comparing to the whole diets of consumers. Stable isotope analysis including bulk (e.g., muscle or whole samples) nitrogen isotope analysis and CSIA-AA allows us to know the integrated $\delta^{15}N$ values of diets (e.g., hour-day scale for phytoplankton, day-week scale for zooplankton, month-year scale for fish) (e.g., Sweeting et al. 2005; Tiselius and Fransson, 2016). Since 1980s, bulk nitrogen isotope analysis has been used to calculate TP of the organisms in food webs. However, the bulk analysis has two major drawbacks that potentially cause large errors in the TP calculation. First, the $\delta^{15}N$ values of primary producers have a large variation in many cases. Second, the trophic discrimination factor (TDF) varies among different samples (e.g., Cabana and Rasmussen, 1996; Fourqurean et al. 1997; Vander Zanden et al. 1997). To reduce the errors on TP calculation, CSIA-AA has been used since 2007 (Chikaraishi et al. 2007; McCarthy et al. 2007; Popp et al. 2007). There are two types of amino acids, 'trophic' and 'source' for changing the $\delta^{15}N$ values in trophic transfer along food webs. 'Trophic' AAs (e.g., glutamic acid: Glu) show a large ^{15}N enrichment (3-8‰) during trophic transfer, whereas 'source' AAs (e.g., phenylalanine, Phe) show a small ^{15}N enrichment (0-1‰) (Chikaraishi et al. 2007). Difference in the ^{15}N enrichment between "trophic" and 'source' AAs has been used to calculate TP of

organisms in food webs. However, little is known the applicability of CSIA-AA for calculating TP of organisms in complex ecosystems with isotopically-distinct nitrogen sources.

At present, there are several methods to access the energy consumption of organisms, including observation, oxygen consumption analysis, and stable isotope analysis. Munday et al. (2012) reported that juvenile coral trout *Plectropomus leopardus* spent 75% and 10% of their life time in shelters when CO_2 concentration in seawater are 490 μatm and 960 μatm, respectively. The Authors suggested that high activity levels potentially cause a high energy consumption for this species in seawater with CO_2 concentration is 960 μatm. However, in general, results of observation may frequently contain human error on the identification of organism behaviors such as turning, moving, jumping, swimming, hiding, etc. and evaluation of the results are not directly correlated the energy consumption. Moreover, in the master thesis of mine, I measured the oxygen consumption rate (OCR) of ♀ *Epinephelus fuscoguttatus* × ♂ *E. lanceolatus* hybrid grouper juveniles under difference in the temperature and salinity. We found that the OCR is correlated positively with temperature and negatively with salinity. These results suggest that the energy consumption of organisms potentially changes with environmental factors (Xing et al. 2019). However, because the oxygen consumption analysis is a snapshot analysis, we need the integration of in turn snapshots to understand the oxygen consumption at week to year scale for the

organisms in experiments. Also, in many case, pure culture (without any bacterium and alga) is difficult for the oxygen consumption experiments.

To evaluate the energy consumption of organisms in ocean acidification, we hypothesize that CSIA-AA will be available, because change in the $\delta^{15}N$ value of amino acid in organisms is caused by metabolic breakdown of amino acids to produce life energy and therefore mirrors the energy consumption in organisms. The organisms may receive stresses in specific environments where physical, chemical, and biological factors are different from natural environments, therefore the energy consumption of organisms potentially change, probably to adapt such environments.

The Ph.D. thesis is composed of two studies, 1) calculating the accurate TP of organisms in food webs, and 2) evaluating the energy consumption of organisms in ocean acidification. For former, we demonstrate the applicably of CSIA-AA in a complex environment, evaluate the variation in the $\delta^{15}N$ value of amino acids among tissue types, and illustrate the spatial variation in the $\delta^{15}N$ value of primary producers in the complex environment. This study will become the basis of my future science, as well as contribute advance in stable isotope ecology. For latter, we have first attempt to access the energy consumption of organisms, which includes designing a constant pH aquarium system, and evaluating energy consumption of organisms. This study will also become the basis of my future science, as well as contribute better understanding for ocean acidification.

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CHAPTER II.

Illustration of Food Webs in a Complex Ecosystem, via Stable Isotope Analysis of Amino Acids

2-1. Trophic hierarchy of coastal marine fish communities viewed via compound-specific isotope analysis of amino acids

Abstract

Coastal marine ecosystems are very complex and composed of myriad organisms, including offshore, coastal, and migratory fish occupying diverse trophic positions (TPs) in food webs. The illustration of trophic hierarchy based on the TP and resource utilization of individual organisms remains challenging. In this study, we applied compound-specific isotope analysis of amino acids to estimate the TP and isotopic baseline (i.e., $\delta^{15}N$ values of primary resources at the base of food webs) for 13 fish and 1 squid species in a coastal area of Sagami Bay, Japan, where a large diversity in the isotopic baseline is caused by an admixture of ocean currents and artificial nitrogen inputs. Our results indicate that the TP of fish and squid varies between 2.9 and 3.9 (i.e., omnivorous, carnivorous, and tertiary consumers), with low variation within individual species. Moreover, the $\delta^{15}N$ values of phenylalanine revealed the diversity of isotopic baselines between and within species. Low values (7.8−10.3‰) and high values (18.6−19.2‰), with a small variation ($1\sigma < 1.0$‰), were found in 2 offshore species and 3 coastal species, respectively. In contrast, highly variable values (9.8−19.7‰), with large variation within species ($1\sigma > 1.0$‰), were found for the remaining 9 migratory species. These results represent evidence of differential trophic exploitation

of habitats between offshore and coastal species, particularly among individuals of migratory species, that were all collected in a single area of Sagami Bay.

Key words:

Trophic position, habitat, food web, nutrient input, compound-specific isotope analysis, amino acids, nitrogen isotope

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1. Introduction

For the last several decades, marine ecosystems have been increasingly impacted by human activities, including eutrophication of coastal areas by nutrient inputs, ocean acidification by CO_2 emissions, increased concentrations of pollutants by biomagnification, and the extinction of organisms by overfishing (Wren and Stephenson, 1991; Jackson et al. 2001; Conley et al. 2009). These problems affect marine ecosystems in many ways, including alterations of the food webs (e.g., Ostrom et al. 2017; Morra et al. 2019).

The characterization of the trophic positions (TPs) of organisms and their use of resources within food webs is useful in understanding changes in marine ecosystems (e.g., Wada et al. 1987; Vander Zanden and Rasmussen, 1996; Post, 2002; Fry, 2006). The TPs of organisms have long been estimated by stomach content analysis, based on the direct identification of diets that the organisms feed on. However, this method has several drawbacks, as it provides only a snapshot of feeding, is biased toward easily detectable prey species, and does not always allow clear identification of digested items. To solve these issues, stable isotope analysis of bulk nitrogen in protein-rich tissues of organisms has been used since the 1980s, based on the increase in nitrogen stable isotope ratios ($\delta^{15}N$) between food and consumers (e.g., DeNiro and Epstein, 1981; Minagawa and Wada, 1986). The use of stable isotopes allows us to see the integrated prey information at the turnover time of the tissue: days to weeks for zooplankton (Tiselius and Fransson, 2016) and months to years for fish (Sweeting et al. 2005).

However, this bulk isotope method is not always useful for the study of food webs, particularly for coastal marine ecosystems. In coastal marine ecosystems, the primary producers (mostly phytoplankton) can utilize multiple isotopically distinct inorganic nitrogen sources (e.g., NH_4^+ and NO_3^-) to synthesize the amino acids required to produce biomass protein, which is transferred to upper trophic levels by consumers such as zooplankton and fish. The large difference in the life span and integration time of the isotopes between phytoplankton and fish makes it difficult to compare the $\delta^{15}N$ values between primary producers and consumers, which is further complicated when the latter exploit different habitats (e.g., Cabana and Rasmussen, 1996; Fourqurean et al. 1997; Vander Zanden et al. 1997; O'Reilly et al. 2002; Post, 2002).

To avoid asynchronies in the life spans between primary producers and consumers, compound-specific isotope analysis of amino acids (CSIA-AA) has been proposed as an alternative tool for calculating the TPs of organisms (e.g., Chikaraishi et al. 2007; McCarthy et al. 2007; Popp et al. 2007). This method is based on predictable trophic increases in the $\delta^{15}N$ values between amino acids along food chains (Gaebler et al. 1966; McClelland and Montoya, 2002; Steffan et al. 2015; McMahon and McCarthy, 2016). Such increases (trophic discrimination factor, TDF) allow for estimating the TP from the $\delta^{15}N$ values of amino acids within a single organism, thus avoiding the problem with bulk isotope analysis caused by differences in the life span between primary producers and consumers, and therefore illustrating the food web structure from TPs in marine ecosystems (e.g., Chikaraishi et al. 2014). However, CSIA-AA has not yet been

applied to coastal areas where isotopically distinct multiple inorganic nitrogen forms are used as nitrogen sources for primary producers and incorporated into food webs. In such environments, the estimations of TP by the bulk method are less precise in assessing the dynamics of coastal ecosystems (e.g., Rolff 2000; Carscallen et al. 2012). Sagami Bay (Japan) is a complex environment in which nutrient dynamics are largely affected by ocean currents, agricultural fertilization, and industrial nitrogen fixation, which translates into a large diversity in the $\delta^{15}N$ values of primary producers (Fujiki et al. 2004; Baek et al. 2009). This diversity has resulted in some serious underestimations of the TPs of starfish, bivalves, and gastropods, which were estimated to be autotrophs according to bulk isotope analysis (Won et al. 2007). Setting the wrong baseline also led to serious overestimation of the TP for the planktivorous Japanese anchovy *Engraulis japonicus* (e.g., James 1988), which was estimated to be predatory according to the bulk isotope analysis (Miyachi et al. 2015).

In this study, we applied CSIA-AA to a collection of fish and squid species from Sagami Bay to estimate their TPs and evaluate their dependence on isotopically distinct inorganic nitrogen inputs. Moreover, based on the TPs and the $\delta^{15}N$ values of amino acids of these organisms, the diversity of isotopic baselines (i.e., $\delta^{15}N$ value of primary resources at the base of food webs) for these species appears to represent differences in their preferential habitat at the level of species and individuals for this coastal area.

2. Materials and methods

2.1. Sample collection

In May 2010, we used a fixed shore net to collect 13 fish and 1 squid species (Table 2-1-1) from a coastal area in Sagami Bay, Japan (35° 37’ N, 139° 25’ E) (Fig. 2-1-1). Sagami Bay is located in the southwest of Tokyo Bay, and is connected to the Pacific Ocean. Two major rivers (Sakawa and Hayakawa) near the study site deliver a large amount of agricultural nutrients from farms (including rice, vegetable, and fruit farms) to Sagami Bay. The squid *Todarodes pacificus*, the bluefin searobin *Chelidonichthys spinosus*, and the marbled flounder *Pseudopleuronectes yokohamae* were collected as representatives of offshore-pelagic, offshore-benthic, and migrating-benthic species, respectively. The other fish were classified as coastal- or migrating-pelagic species based on body shapes (i.e., fusiform for 8 species and compressiform for 3 species, Table 2-1-1); fusiform fishes are characterized by a streamlined body shape for swimming long distances across offshore and coastal areas, whereas compressiform species are characterized by a laterally compressed body shape for producing quick bursts of speed for living in coastal areas (e.g., Roy et al. 2007). All studied fish and squid are ammonotelic, and we analyzed only adult fish and squid in the present study. The collected samples were cleaned with filtered seawater to remove surface contaminants and stored at −20°C before analysis.

Table 2-1-1. The $\delta^{15}N$ values of amino acids, the $\delta^{15}N_{Baseline,phe}$ values, and the TP for fish and squid investigated in this study. Dash line (--): the form of fish does not belong to compressiform and fusiform; n.d.: not detectable. CA: *Cypselurus agoo*, CS: *Chelidonichthys spinosus*, EJ: *Evynnis japonica*, ET: *Etrumeus teres*, LJ: *Lateolabrax japonicus*, PA: *Psenopsis anomala*, PJ: *Pneumatophorus japonicus*, PT: *Parapristipoma trilineatum*, PY: *Pseudopleuronectes yokohamae*, SC: *Stephanolepis cirrhifer*, TJ: *Trachurus japonicus*, TM: *Thamnaconus modestus*, TP: *Todarodes pacificus*, UH: *Uraspis helvola.* C: compressiform, F: fusiform, BLP: $\delta^{15}N_{Baseline,phe}$.

Scientific name	Form	Length (cm)	$\delta^{15}N$									BLP	TP
			Ala	Gly	Val	Leu	Ile	Pro	Ser	Glu	Phe		
EJ	C												
1		15.8	41.4	20.3	33.9	40.4	37.6	41.8	15.4	41.2	19.0	18.1	3.5
2		15.8	40.9	20.8	35.6	40.2	37.9	42.0	18.3	41.2	18.6	17.6	3.5
3		15.8	41.5	19.2	34.0	37.3	36.4	45.9	19.5	42.0	19.2	18.2	3.6
CS	--	--											
1		25.3	n.d.	14.5	29.1	25.7	30.1	n.d.	n.d.	31.2	8.5	7.4	3.5
2		25.3	n.d.	15.1	27.1	25.0	30.8	n.d.	n.d.	29.1	7.1	6.2	3.4
3		25.3	n.d.	15.1	30.9	25.6	29.6	n.d.	n.d.	29.2	7.8	6.8	3.4
UH	F												
1		16.7	38.5	14.1	42.4	30.7	34.3	34.6	13.1	37.2	17.5	16.7	3.1
2		16.7	36.0	15.6	47.4	30.2	30.0	32.4	14.4	38.9	19.7	18.8	3.1
3		16.7	34.8	14.6	46.8	37.0	36.7	38.6	12.4	41.8	22.0	21.1	3.2
SC	C												
1		16.2	38.9	16.3	36.0	27.8	26.9	34.6	10.9	37.6	18.5	17.7	3.1
2		16.2	38.0	20.8	38.4	27.2	29.0	36.1	14.6	37.1	19.3	18.6	2.9
3		16.2	36.0	18.7	39.0	32.3	30.2	43.7	11.5	37.1	18.1	17.2	3.1
PA	C												
1		17.1	39.9	13.2	39.9	39.5	38.2	n.d.	n.d.	40.7	20.2	19.3	3.3
2		17.1	38.1	10.5	38.2	38.3	37.9	n.d.	n.d.	40.9	19.4	18.5	3.4
3		17.1	38.0	7.5	40.3	37.7	39.0	n.d.	n.d.	40.3	18.1	17.1	3.5

PY	F												
1		16.3	34.3	18.1	33.0	28.4	27.9	30.8	n.d.	30.3	12.5	11.7	2.9
2		16.3	27.7	19.1	33.8	24.8	31.3	30.1	n.d.	26.2	8.1	7.4	2.9
3		16.3	30.7	17.7	28.5	32.0	24.8	26.8	n.d.	30.1	10.4	9.6	3.1
TJ	F												
1		18.6	34.9	6.7	29.6	32.6	29.7	n.d.	n.d.	35.0	14.8	13.9	3.2
2		18.6	31.9	6.3	27.0	27.5	23.8	n.d.	n.d.	29.5	9.9	9.1	3.1
3		18.6	36.6	8.2	32.2	39.6	28.3	n.d.	n.d.	36.7	17.1	16.3	3.1
PJ	F												
1		27.6	38.0	4.9	38.4	34.3	32.0	n.d.	n.d.	34.1	14.9	14.1	3.1
2		27.6	36.0	3.3	32.1	37.1	30.3	n.d.	n.d.	31.9	12.2	11.4	3.1
3		27.6	38.5	2.5	42.4	35.2	29.0	n.d.	n.d.	37.4	17.0	16.1	3.2
PT	F												
1		14.9	32.0	8.3	30.1	31.6	26.3	n.d.	n.d.	33.5	13.5	12.6	3.2
2		14.9	27.4	11.4	25.3	27.1	25.7	n.d.	n.d.	29.7	10.5	9.7	3.1
3		14.9	24.4	12.0	21.9	26.5	23.0	n.d.	n.d.	25.0	5.5	4.6	3.1
TM	F												
1		26.2	23.5	13.7	28.1	29.8	27.0	n.d.	n.d.	32.2	12.6	11.8	3.1
2		26.2	26.6	18.4	24.1	33.8	25.9	n.d.	n.d.	28.5	9.9	9.1	3.0
3		26.2	30.7	21.0	23.4	31.4	26.6	n.d.	n.d.	30.9	10.4	9.5	3.3
CA	F												
1		26.2	26.8	3.6	28.8	28.5	29.8	n.d.	8.5	29.4	11.7	11.0	2.9
2		26.2	23.3	6.1	33.7	27.6	30.9	4.0	11.1	27.2	9.0	8.3	2.9
3		26.2	24.4	4.9	34.0	23.7	30.5	3.8	13.5	28.3	10.4	9.6	2.9
TP	--	--											
1		19.9	38.1	4.6	32.9	34.6	28.5	37.4	11.1	33.5	10.1	9.0	3.6
2		19.9	41.1	7.4	32.9	37.8	30.5	37.6	11.8	32.9	10.6	9.6	3.5
LJ	F												
1		34.7	45.5	12.3	40.8	40.2	35.6	34.3	n.d.	41.0	15.1	13.9	4.0
2		34.7	44.3	13.8	40.3	39.6	35.3	35.8	n.d.	43.2	17.9	16.8	3.9
3		34.7	46.2	13.3	41.0	37.1	33.3	39.3	n.d.	40.0	14.7	13.5	3.9
ET	F												
1		19.4	30.1	3.9	36.6	30.4	32.0	n.d.	4.3	32.1	12.7	11.8	3.1
2		19.4	32.2	3.8	30.4	30.9	30.5	n.d.	-2.0	32.0	12.9	12.0	3.1
3		19.4	35.0	1.8	39.4	34.0	30.4	n.d.	4.3	35.2	15.5	14.6	3.1

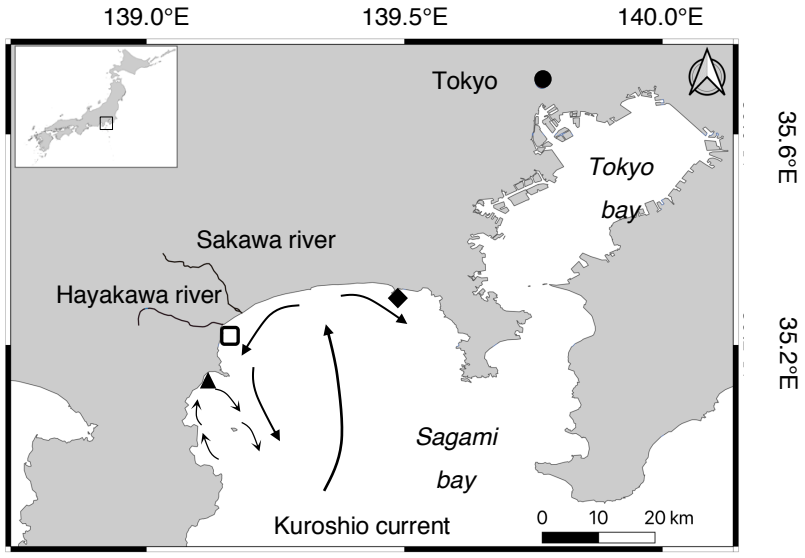

Fig. 2-1-1.

Sagami Bay, Japan. Open square marks the sampling site for this study. Sites of previous studies marked as follows: filled triangle, Chikaraishi et al. (2014); filled diamond, Miyachi et al. (2015). Arrows represent the Kuroshio and its branched currents.

2.2. Isotope analysis

Muscle tissues were taken from these samples and prepared for the isotopic analysis. The nitrogen isotopic composition of amino acids was determined by the procedure in Chikaraishi et al. (2009). In brief, the muscle tissues were hydrolyzed with 12 N HCl at 110°C for overnight (>12 hours). The hydrolysate was washed with *n*-

hexane/dichloromethane (3/2, v/v) to remove hydrophobic constituents. Then, derivatizations were performed sequentially with thionyl chloride/2-propanol (1/4) and pivaloyl chloride/dichloromethane (1/4). The *N*-pivaloyl/isopropyl (Pv/iPr) esters of amino acids were extracted with *n*-hexane/dichloromethane (3/2, v/v). The nitrogen isotopic composition of amino acids was determined by gas chromatography/isotope ratio mass spectrometry (GC/IRMS) (Chikaraishi et al. 2014). To assess the reproducibility of the isotope measurement and obtain the amino acid isotopic composition, reference mixtures of nine amino acids (alanine, glycine, leucine, norleucine, aspartic acid, methionine, glutamic acid, phenylalanine, and hydroxyproline) with known $\delta^{15}N$ values (–26.6‰ to 45.7‰, Indiana University, Shoko science co.) were derivatized to Pv/iPr esters and analyzed after every four to six samples runs, and three pulses of reference N_2 gas were discharged into the IRMS instrument at the beginning and end of each chromatography run for both reference mixtures and samples. The isotopic composition of amino acids in samples was expressed relative to atmospheric nitrogen (AIR) on scales normalized to known $\delta^{15}N$ values of the reference amino acids. The accuracy and precision for the reference mixtures were always 0.0‰ (mean of Δ) and 0.4‰-0.7‰ (mean of 1σ), respectively, for sample sizes of $\geqslant$1.0 nmol N. We note that glutamine was converted into glutamic acid during the acid hydrolysis, resulting in that the $\delta^{15}N$ value of glutamic acid was combined glutamic acid itself and α- amino group of glutamine (Chikaraishi et al. 2009). In this study, we obtained the $\delta^{15}N$ value of six amino acids (glycine, valine, leucine,

isoleucine, glutamic acid, and phenylalanine) for all species and three amino acids (alanine, proline, and serine) for several species based on the peak intensity and separation on the GC/IRMS chromatogram.

2.3. Calculation of TP and isotopic baseline

The TP of samples was calculated based on the comparison of large (3‰-8‰) and small (0‰-1‰) trophic discrimination factors (TDF) between trophic (e.g., glutamic acid and alanine) and source amino acids (e.g., phenylalanine and methionine), respectively (e.g., Chikaraishi et al. 2007; McCarthy et al. 2007; Popp et al. 2007). Indeed, the distinct TDF between glutamic acid and phenylalanine has been frequently used to calculate the TP of diverse organisms including fish (Chikaraishi et al. 2014; Steffan et al. 2015), based on the following equation:

$$TP = (\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - \beta) / TDF_{Glu-Phe} + 1 \qquad (2\text{-}1\text{-}1)$$

where $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ represent the $\delta^{15}N$ values of glutamic acid and phenylalanine, respectively, in a studied organism, β represents a constant offset between $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values in primary producers (e.g., 3.4‰, Chikaraishi et al. 2009), $TDF_{Glu-Phe}$ represents the difference in the TDF between glutamic acid and phenylalanine in consumers (e.g., 7.6‰, Chikaraishi et al. 2009).

Moreover, the $\delta^{15}N$ values of source AAs in high TP consumers represent the mean $\delta^{15}N$ value of primary producers (i.e., $\delta^{15}N_{Baseline}$, which is approximately equal to the mean $\delta^{15}N$ value of inorganic nitrogen in tropical and temperate areas, e.g., Altabet et

al. 1994; Minagawa and Wada 1986) (Fig. 2-1-2). Because there is a small TDF in phenylalanine (e.g., 0.4‰ for each trophic level, Chikaraishi et al. 2009), the equation (2-1-2) were used to minimize this effect, and the $\delta^{15}N_{Baseline,phe}$ values were obtained to compare the resource utilization among samples:

$$\delta^{15}N_{Baseline,Phe} = \delta^{15}N_{Phe} - 0.4\ (TP - 1) \qquad (2\text{-}1\text{-}2)$$

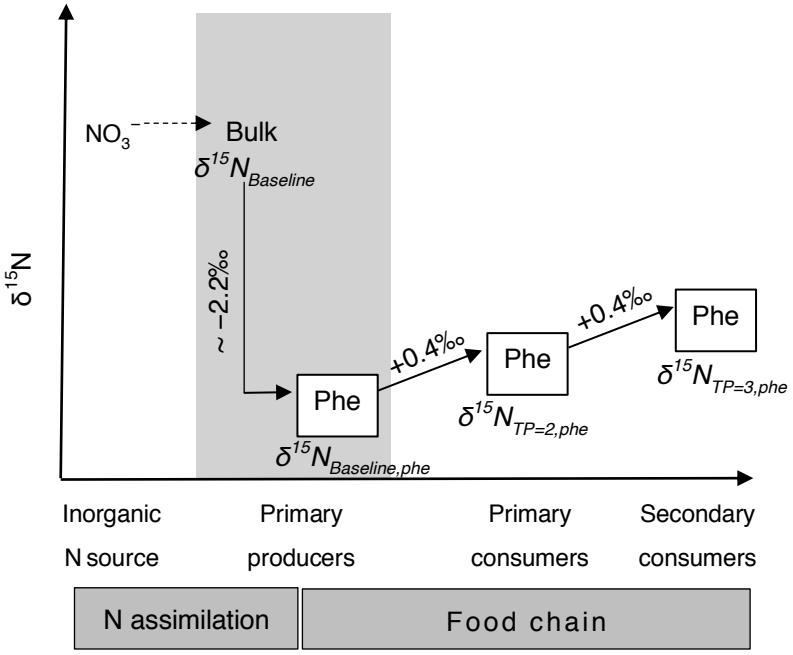

Fig. 2-1-2.

Relationship of $\delta^{15}N_{Phe}$, $\delta^{15}N_{Baseline}$, and $\delta^{15}N_{Baseline,phe}$. Phe: phenylalanine, TP: trophic position. Shaded bar: isotopic fractionation associated with phenylalanine synthesis in primary producers.

3. Results

The $\delta^{15}N$ values vary from 5.5‰ to 22.0‰ for phenylalanine (14.0 ± 4.3‰, as mean ± 1σ standard deviation) and from 25.0‰ to 43.2‰ for glutamic acid (34.4 ± 5.1‰) in the studied species (Table 2-1-1). Among these species, the chicken grunt *Parapristipoma trilineatum* and the seabass *Lateolabrax japonicus* have the lowest and highest $\delta^{15}N$ values of glutamic acid ($\delta^{15}N_{Glu}$), respectively, whereas *P. trilineatum* and the whitetongue jack *Uraspis helvola* have the lowest and highest $\delta^{15}N$ values of phenylalanine ($\delta^{15}N_{Phe}$), respectively.

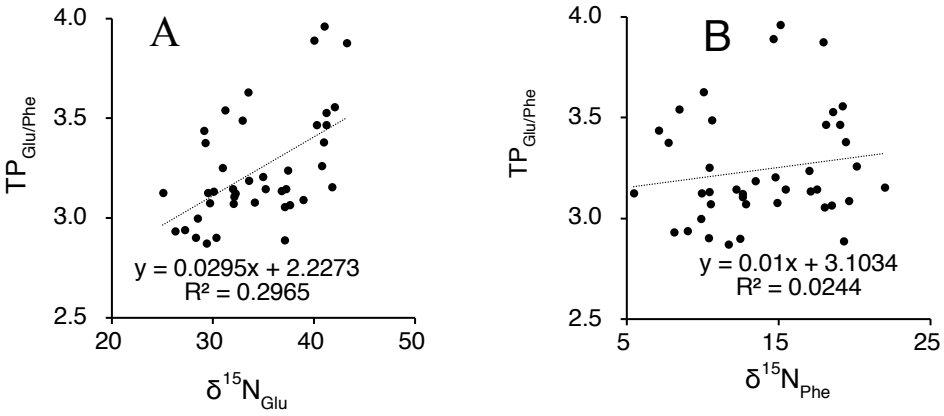

Fig. 2-1-3.

The relationship of (A) $TP_{Glu/Phe}$ and $\delta^{15}N_{Glu}$ and (B) $TP_{Glu/Phe}$ and $\delta^{15}N_{Phe}$ for the investigated fish and squid.

The mean $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values for each species of thirteen fish and one squid (Fig. 2-1-4) are plotted along the trophoclines that defined from equation (1) with a slope of 1.0 for each TP and an interval of 7.6‰ for each trophic transfer in food webs

(e.g., Chikaraishi et al. 2014; Bowes and Thorp 2015; Nielsen et al. 2015; Steffan et al. 2013). Notwithstanding a large variation in the $\delta^{15}N_{Phe}$ value, the TP values vary only between 2.9 (e.g., flyingfish *Cypselurus agoo*) and 3.9 (*L. japonicus*) (Table 2-1-1, Fig. 2-1-4), and do not correlate with the $\delta^{15}N_{Baseline,phe}$ values ($R^2 = 0.02$, $p = 0.4$). The TP values significantly but weakly correlate with the $\delta^{15}N$ values of glutamic acid ($R^2 = 0.30$, $p = 0.0002$, Fig. 2-1-3A) and do not correlate with those of phenylalanine ($R^2 = 0.02$, $p = 0.318$, Fig. 2-1-3B). For example, the bluefin searobin *Chelidonichthys spinosus* and the crimson seabream *Evynnis japonica* have the same TP value (3.5 ± 0.1 and 3.5 ± 0.1, respectively), although there are large differences in their $\delta^{15}N_{Glu}$ (29.8 ± 1.2 and 41.5 ± 0.5, respectively) and $\delta^{15}N_{Phe}$ values (7.8 ± 0.7 and 18.9 ± 0.3, respectively) between these two species (Table 2-1-1).

The $\delta^{15}N_{Baseline,phe}$ values (13.1 ± 4.3‰) follow the $\delta^{15}N_{Phe}$ values, ranging from 4.6‰ (*P. trilineatum* 3) to 21.1‰ (*U. helvola* 3) among studied samples (Table 2-1-1). The variability in the $\delta^{15}N_{Baseline,phe}$ value is species-dependent, as exemplified by the smallest ($1\sigma = 0.6$‰) and largest ($1\sigma = 8.0$‰) variation for *E. japonica* and *P. trilineatum*, respectively. The TP does not correlate with the $\delta^{15}N_{Baseline,phe}$ values ($R^2 = 0.02$, $p = 0.4$).

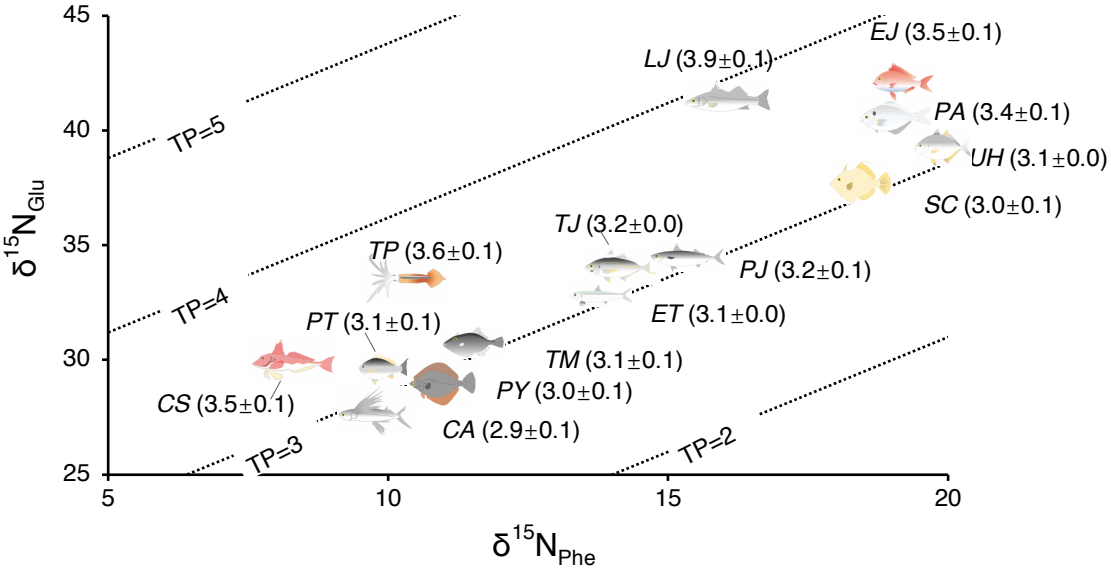

Fig. 2-1-4.

Cross-plot of the $\delta^{15}N$ values of glutamic acid (Glu) and phenylalanine (Phe). Lines represent integer trophic positions (TPs, i.e., 2, 3, 4, and 5) estimated with equation (2-1-1). Numbers in brackets show the mean ± SD of TP for the species. See Table 2-1-1 for fish abbreviations.

4. DISCUSSION

4.1. Food web structure

The trophoclines defined by the $\delta^{15}N$ values of trophic and source amino acids have been used to understand the food web structure, not only in terms of trophic position but also in the simultaneous interpretation of isotopic baselines (e.g., Chikaraishi et al. 2014; Bowes and Thorp, 2015; Steffan et al. 2015; Nielsen et al. 2015). In two dimensional trophocline space, all samples are expected to fall within a relatively vertical line when there is a single food chain starting from isotopically uniform primary producers in the studied area. Alternatively, the samples are expected to distribute along the trophoclines, according to their proximities to the mean of their diet resources, when there are multiple food chains that have multiple, isotopically distinct primary producers (Chikaraishi et al. 2014). For instance, the $\delta^{15}N$ values of phenylalanine can be directly used as isotopic baseline for illustrating the trophocline, because of its small TDF (0.4‰ for each trophic level) (e.g., Chikaraishi et al. 2014; Ohkouchi et al. 2017).

In this study, the trophocline space defined by the $\delta^{15}N$ of glutamic acid and phenylalanine illustrates a food web structure with TP varying from 2.9 to 3.9 among species. Nine species (e.g., *P. yokohamae*) are secondary consumers (TP = 3.1 ± 0.1), one species (i.e., *L. japonicus*) is a tertiary consumer (TP = 3.9 ± 0.0), and the other four species occupies intermediate positions (TP = 3.5 ± 0.1). These results are consistent with the expected TP of the studied fish and squid species, as reported in the literatures: for instance, *L. japonicus* and the sardine *Etrumeus teres* were characterized

as zooplanktivorous and piscivorous fish, respectively (Nip et al. 2003; Falautano et al. 2006); also, the estimated TP of *P. trilineatum* (TP = 3.1 ± 0.1) in the studied area is equivalent to the value reported by Chikaraishi et al. (2014) for this species collected from a different area in Sagami Bay (TP = 2.9 ± 0.1).

Based on the $\delta^{15}N$ values of phenylalanine (i.e., isotopic baseline), we infer the existence of multiple distinct food chains for the fish and squid species investigated. The offshore-pelagic and offshore-benthic species (e.g., *P. yokohamae*) belong to food chains characterized by low baseline values (6.8‰ to 9.3‰), the compressiform species (e.g., *E. japonica*) belong to food chains with high baseline values (17.8‰ to 18.3‰), and fusiform species (e.g., *L. japonicus*) belong to food chains with intermediate baseline values (9.6‰ to 18.9‰). These results are consistent with our expectation of a large variation in the $\delta^{15}N$ baseline value in Sagami Bay (Won et al. 2007; Miyachi et al. 2015).

4.2 Effect of $\delta^{15}N_{Baseline,phe}$ values on the TP

A large variation of the $\delta^{15}N$ values at the base of food webs theoretically represents diversity in the $\delta^{15}N$ value of nutrient sources. Alterations in these sources pose potential risks for the preservation of ecological conditions, primary production, and ultimately food web structures (e.g., Cabana and Rasmussen, 1996; Hicks et al. 2016; Baeta et al. 2017). In this study, variability in the $\delta^{15}N_{Baseline,phe}$ value between individuals of a single species is species dependent (Table 2-1-1). Indeed, a small

variation in the $\delta^{15}N_{Baseline,phe}$ value is found for all the offshore-pelagic and offshore-benthic species, as well as for the three compressiform species (Fig. 2-1-5 a and b, respectively), whereas the eight fusiform species (Fig. 2-1-5 c) have a much larger variability. In contrast, for all species investigated in this study, the variation in the TP within species is small ($1\sigma = 0.1$) (Fig. 2-1-4). For instance, no substantial difference in the TP is found among three individuals of *P. trilineatum* (3.1 ± 0.1‰), despite their variable $\delta^{15}N_{Baseline,phe}$ values (9.0 ± 4.1‰). However, *E. japonica* has a low variability in both TP (3.5 ± 0.1‰) and $\delta^{15}N_{Baseline,phe}$ value (17.9 ± 0.3‰) among the three specimens investigated. In addition to the no correlation between TP and $\delta^{15}N_{Baseline,phe}$ values when all species were pooled, these results suggest that the $\delta^{15}N_{Baseline,phe}$ values do not affect the TP of organisms in Sagami Bay. Although the investigation of the underlying cause is out of the scope of this study, our findings imply that the participation of different sources of inorganic nitrogen has not affected the size of food chains in the study area.

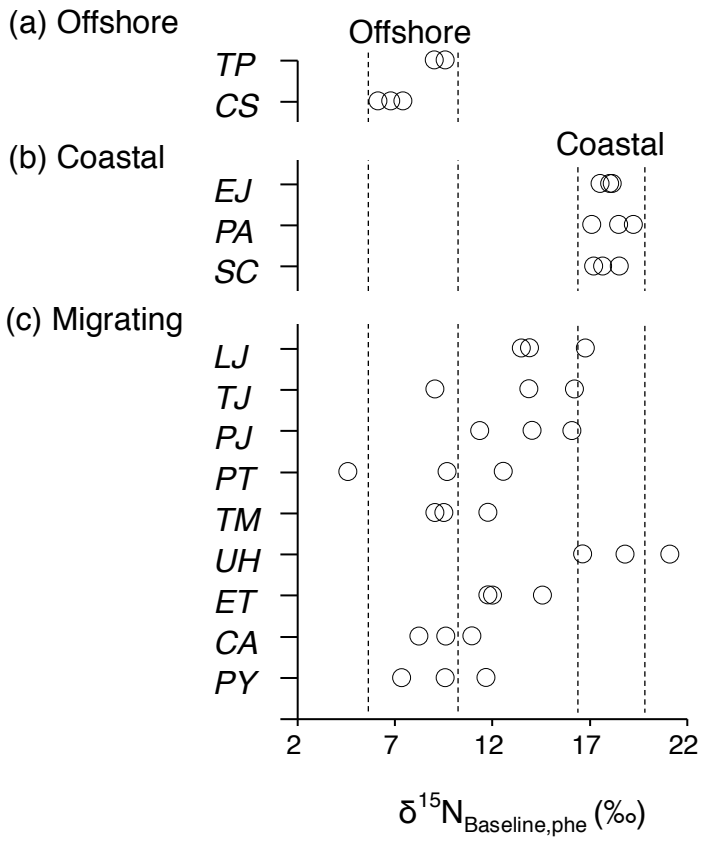

Fig. 2-1-5.

Diversity in the $\delta^{15}N$ with respect to their habitats (i.e., offshore vs. coastal, and pelagic vs. benthic). Vertical dashed lines demarcate the coastal and offshore regions. See Table 2-2-1 for fish abbreviations

4.3 Habitats

Agricultural runoff (e.g., rice, vegetables, and fruits), as well as artificial dams, and wastewater treatment plants frequently cause anoxic conditions. In these conditions, denitrification produces N_2 gas, enriched in ^{14}N and leaving behind a pool of ^{15}N-enriched nitrate (e.g., Kellman and Marcel, 2003; Sebilo et al. 2006). The abundance of nutrients with high $\delta^{15}N$ is temporally dependent on agricultural cycles and precipitation, and they are incorporated into the Bay and mixed with the coastal water by ocean currents. The spatial-dependence of such inputs is likely explanation of the difference between the results of this study, with high and variable $\delta^{15}N_{Baseline,phe}$ (13.0 ± 4.1‰), and the low and homogeneous values (4.6 ±1.1‰) found in the previous study site (Chikaraishi et al. 2014) within in Sagami Bay. This is simply consistent with our expectation that isotopically-distinct multiple inorganic nitrogen forms are introduced in the food web of our study site but not in the previous site. We conclude that our study site has been considerably affected by agricultural- and artificially-derived nutrient inputs, while the latter has been affected by natural sources of nutrients, with negligible inputs of agricultural- and artificially-derived nutrients (Chikaraishi et al. 2014).

Low $\delta^{15}N_{Baseline,phe}$ values for *T. pacificus* and *C. spinosus* (Fig.2-1-5) can be attributed to their preferential use of offshore-pelagic and offshore-benthic habitats, respectively (Watanabe et al. 1996 and Byun et al. 2018), where a lower contribution from agricultural and artificial nutrient inputs relative to the coastal area can be expected. In contrast, high $\delta^{15}N_{Baseline,phe}$ values of three compressiform fish species (i.e.,

E. japonica, the Pacific rudderfish *Psenopsi anomala*, and the threadsail filefish *Stephanolepis cirrhifer*) inhabiting the coastal area (Yamaoka et al. 2003; Wang et al. 2004), can be attributed to a large contribution from agricultural and artificial nutrient inputs. The other nine fish are migrating fusiform and benthic species, as they are found in a variety of habitats extending from offshore to coastal areas (Wada 2007; Fuji et al. 2011). The use of a wide range of habitats is consistent with highly variable $\delta^{15}N_{Baseline,phe}$ values overlapping those of specialized offshore and coastal species. Interestingly, there is a large individual variability in the $\delta^{15}N_{Baseline,phe}$ value within the migrating fusiform species. In the case of *T. japonicus*, the $\delta^{15}N_{Baseline,phe}$ values of three individuals were 9.1‰, 13.9‰, and 16.3‰. Applying a two-end member mixing model, with reference baseline values of 7.8‰ and 18.0‰ for offshore and coastal habitats, respectively, we can estimate the offshore: coastal habitat ratio of these specimens as 9:1, 2:3, and 1:4, respectively. These results imply that each of individuals uses both habitats differently, maybe because they pertained to independent groups or subpopulations of this species. Based on these results, the large difference in the $\delta^{15}N_{Baseline,phe}$ value among the species in this study reveals diversity in the use of habitats (i.e., offshore vs. costal) at the level of species and of individuals, respectively, even though they are collected from a single study site.

5. Conclusion

The CSIA-AA method provides greater resolution to trophic position estimation (vs. traditional bulk methods, per Chikaraishi et al. 2009), and has been used to elucidate the trophic structure of food webs characterized by two isotopically-distinct primary producers (algae vs. seagrass: Choi et al. 2017, algae vs. terrestrial plants: Ishikawa et al. 2018). In the present study, we illustrated the trophic tendencies of fish and squid species with a wide range of isotopically-distinct primary producers (i.e., $\delta^{15}N_{Baseline,phe}$ ranging from 4.6‰ to 21.2‰), and show a large diversity in the habitat use among and within species. These findings further proof the applicability of the CSIA-AA method to food web studies in which there is a large variation among $\delta^{15}N_{Baseline}$ values, which can cause large errors in trophic position estimation when using the traditional bulk isotope method.

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2-2. **Tissue-specific $\delta^{15}N$ values of amino acids in aquatic organisms: Implication to the ecological food web studies**

Abstract

Compound-specific isotope analysis of amino acids (CSIA-AA), particularly for nitrogen in muscle tissues of organisms, has been used for identifying trophic position (TP) of them and baseline (e.g., plants, phytoplankton, etc.) of food webs to illustrate trophic transfer and linkage in ecosystems. However, little is known whether or not other tissues such as scale and blood can be employed as samples for the CSIA-AA. In this study, we compared the tissue-specific $\delta^{15}N$ values of amino acids in several aquatic organisms including fish (e.g., for muscle, scale, blood, bone, etc.), gastropods (i.e., for muscle vs shell protein), and a turtle (i.e., for yolk vs embryo) to evaluate applicability of these tissues in the CSIA-AA. The results reveal that the $\delta^{15}N$ values of amino acids in muscle are substantially identical to those in scale, fin, and yolk of fish as well as in shell of gastropods, but not to those in internal tissues (e.g., blood, embryo, etc.). Based on these results, we concluded that the former tissues (e.g., scale and shell) are applicable as alternatives to muscle whereas the latter tissues will be required further evaluation, if we can use them in the CSIA-AA.

This section was already submitted to *Res. Org. Geochem.*

1. Introduction

Compound-specific isotope analysis of amino acids (CSIA-AA) of nitrogen isotopes (i.e., $\delta^{15}N$ values) has been employed as a powerful tool in ecological food web studies, in particular through identifying the trophic position (TP) of organisms and baseline (e.g., plants and phytoplankton as diet resources) of food webs, based on the distinct elevation in the $\delta^{15}N$ value (i.e., trophic discrimination factor, TDF) among amino acids in consumers during each trophic transfer (e.g., Chikaraishi et al. 2007; McCarthy et al. 2007; Popp et al. 2007). The illustration of TP and diet resources among organisms in food webs simply provides a valuable framework for evaluating diet-consumer relationships and tracking nutrient flow in food webs (e.g., Lindeman 1942).

Whole or muscle tissues of organisms have been employed commonly as samples for the CSIA-AA, although the collection of these tissues from target organisms inflicts serious physical damage in the life of these organisms. This is a problem in the application of CSIA-AA if we have limitations to access whole or muscle tissues of organisms, or if we investigate isotopic characteristics changing with time in a single organism. For example, several scientists have reported the $\delta^{15}N$ values of amino acids from other tissues such as scale, fin, and blood, which are generally non-lethally compared to muscle, in their studies (e.g., Germain et al. 2013; Chikaraishi et al. 2014; Sugaya et al. 2019). Moreover, we simply expect that some other specific tissues in the pre-hatching stage may have different $\delta^{15}N$ values of amino acids compared to the

post-hatching stage, because of difference in the nutrient source between these stages (i.e., yolk and diets, respectively). However, little is known whether or not these specific tissues are applicable as alternatives to muscle (i.e., the substantially same values to muscle, as plotted on 1:1 line) if we apply these tissues to the CSIA-AA in diverse fields of science.

In this study, we evaluated the tissue-specific $\delta^{15}N$ values of amino acids in several aquatic organisms including fish (e.g., for muscle vs scale, blood, bone, etc.), gastropods (i.e., for muscle vs shell protein), and a turtle (i.e., for yolk vs embryo). Based on the results, we discussed which tissues are applicable as alternatives to muscle for the CSIA-AA.

2. Materials and Methods

2.1. Materials

For comparing the $\delta^{15}N$ values of amino acids between muscle and other tissues within a single fish, the Japanese horse mackerel *Trachurus japonicus* (approximately 20 cm length) was collected by fishing from a beach in Yugawara (35°08′N, 139°07′ E), Japan, and dissected to nine tissues: muscle and scale (dorsal-muscle and-scale); fin (caudal fin); eye; heart; gill; liver; blood; and bone (dorsal spine).

For comparing the values between muscle and shell protein, the published data on the three gastropods (i.e., Japanese abalone *Haliotis discus*, horned turban *Turbo sazae*, and commercial trochus *Omphalius pfeifferi*) are available in the previous study

(Chikaraishi et al. 2014). The data for the latest growth increment of shell (approximately 5 mm width) were used in that study to minimize possible effect of variation in the $\delta^{15}N$ value for whole life of the gastropods.

For comparing the values between yolk and embryo, the false kelpfish *Sebastiscus marmoratus* and the hawksbill sea turtle *Eretmochelys imbricate* were used in this study. The *S. marmoratus* (approximately 20 cm length), an ovoviviparous fish, was collected by fishing from a stony shore in Yugawara, Japan, and dissected to three parts: muscle (dorsal-muscle); yolk and embryo (in developed egg). The *E. imbricata* was reared in Okinawa Churaumi Aquarium. Its developed egg was collected and dissected to two parts: embryo's leg and yolk.

2.2. CSIA-AA

The samples were dried with a freeze-drier and kept in –20°C before analysis. The nitrogen isotopic composition of amino acids was determined by the procedure in Chikaraishi et al. (2009). In brief, the sample was hydrolyzed with 12 N HCl at 110°C for overnight (>12 hours). The hydrolysate was washed with *n*-hexane/dichloromethane (3/2, v/v) to remove hydrophobic constituents. Then, derivatizations were performed sequentially with thionyl chloride/2-propanol (1/4) and pivaloyl chloride/dichloromethane (1/4). The $\delta^{15}N$ values of amino acids was determined by gas chromatography/isotope ratio mass spectrometry (GC/IRMS). The isotopic composition of amino acids in samples was expressed relative to atmospheric nitrogen

($\delta^{15}N_{AIR}$ = 0 ‰) on scales normalized to known $\delta^{15}N$ values of the reference amino acids (Chikaraishi et al. 2009). The accuracy and precision for the reference mixtures were always 0.0‰ (mean of Δ) and 0.4‰ to 0.7‰ (mean of 1σ), respectively, for sample sizes of ≥1.0 nmol N. In this study, we obtained the $\delta^{15}N$ values of 7 amino acids (alanine, glycine, valine, leucine, isoleucine, glutamic acid, and phenylalanine) for all tissues and 3 amino acids (proline, serine, and methionine) for several tissues based on the peak intensity and separation on the GC/IRMS chromatogram.

3. Results and discussions

3.1 Seven tissues within a single fish

The $\delta^{15}N$ values of amino acids in muscle vary from −1.9‰ (glycine) to +25.8‰ (isoleucine) in the *T. japonicus* examined (Table 2-2-1). Amino acids in scale and fin have substantially the same $\delta^{15}N$ values to muscle (paired T – Test: $t = -1.3$, $df = 6$, $p = 0.24$ for scale; $t = 1.7$, $df = 7$, $p = 0.13$ for fin) (Table 2-2-2), which illustrates the 1:1 relationship in the $\delta^{15}N$ value between muscle and scale (Fig. 2-2-1A) as well as between muscle and fin (Fig. 2-2-1B). In contrast, amino acids in bone and eye have higher (by from 2.2‰ to 4.1‰) and lower (by from 2.4‰ to 4.1‰) $\delta^{15}N$ values, respectively, than corresponding amino acids in muscle (Table 2-2-1), illustrating a poor 1:1 relationship in the $\delta^{15}N$ value between tissue types ($t = -12.7$, $df = 6$, $p = 0.00002$ for bone; $t = 7.2$, $df = 6$, $p = 0.0004$ for eye) (Table 2-2-2). Amino acids in the other tissues have lower $\delta^{15}N$ values (by from 1.0‰ to 12.6‰) than corresponding

amino acids in muscle, except for phenylalanine in gill (Table 2-2-1), illustrating a poor 1:1 relationship in the $\delta^{15}N$ value between tissue types (e.g., $t = 6.3$, $df = 6$, $p = 0.0008$ for heart). For comparing in the other tissues, a strong 1:1 relationship is found between eye and liver and between eye and heart (Table 2-2-2).

Table 2-2-1. The $\delta^{15}N$ values of amino acids for *T. japonicus* investigated in this study. n.d.: not detectable.

	$\delta^{15}N$								
	Ala	Gly	Val	Leu	Ile	Pro	Met	Glu	Phe
Muscle	23.4	-1.9	19.4	20.9	25.8	24.6	n.d.	25.5	5.0
Scale	23.7	-2.2	20.5	20.7	26.3	n.d.	4.9	25.2	5.8
Fin	22.1	-3.1	17.5	19.1	24.2	26.9	n.d.	24.1	5.1
Eye	17.8	-5.4	14.2	14.3	15.4	n.d.	n.d.	19.8	0.6
Heart	15.5	-6.9	14.3	14.3	16.5	n.d.	n.d.	17.5	3.5
Gill	20.1	-3.0	15.5	16.6	17.7	n.d.	n.d.	21.8	5.9
Liver	14.9	-9.7	14.6	13.3	16.1	n.d.	n.d.	16.3	2.4
Blood	14.7	-11.8	11.5	11.3	13.1	n.d.	n.d.	15.1	1.7
Bone	25.8	1.0	22.7	24.0	28.0	n.d.	n.d.	29.0	9.1

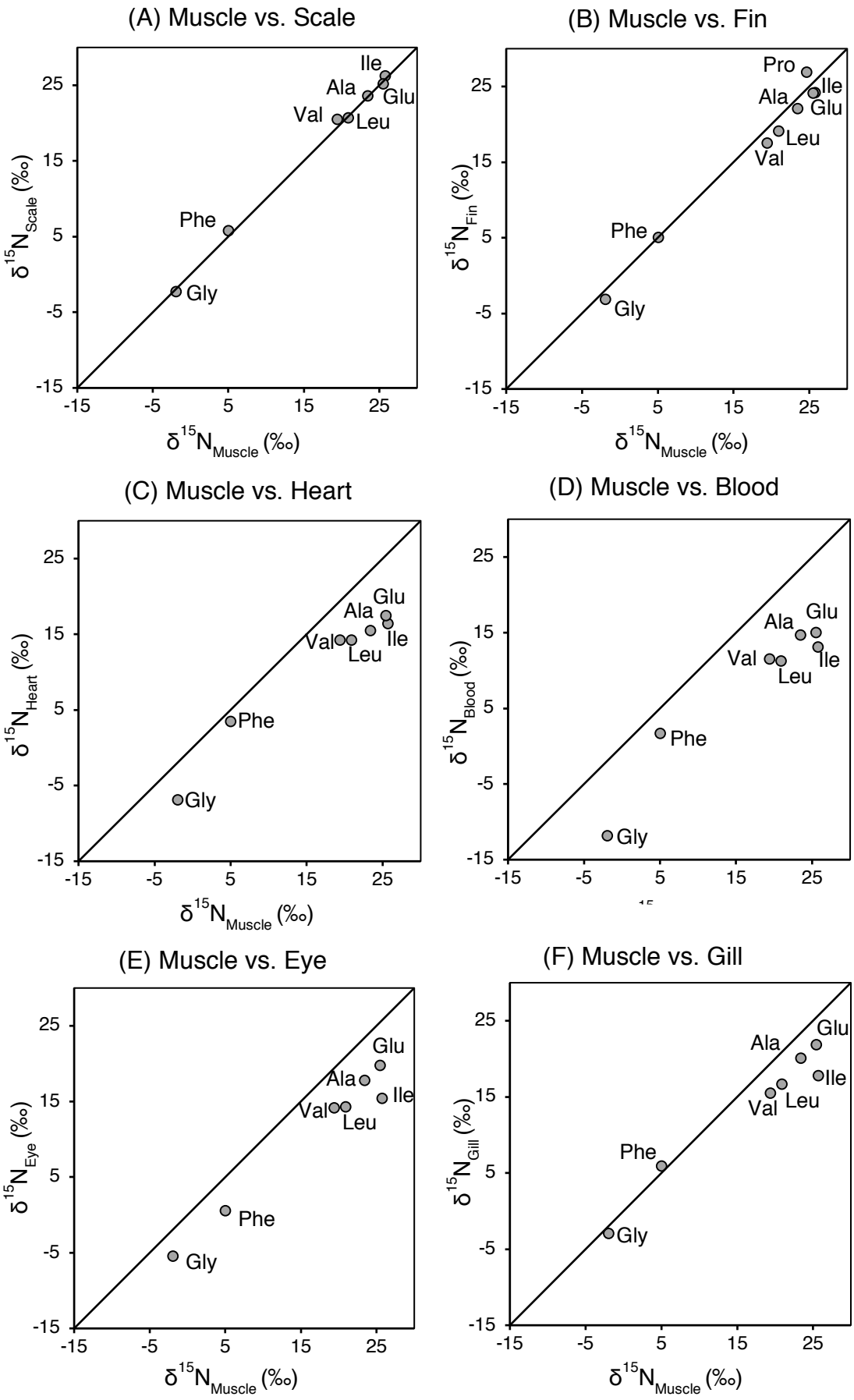
Table 2-2-2. *P* values of paired T-Test between tissues for *T. japonicus* investigated in this study.

	Muscle	Scale	Fin	Eye	Heart	Gill	Liver	Blood	Bone
Muscle									
Scale	0.24								
Fin	0.13	0.002							
Eye	0.0004	0.0004	0.0009						
Heart	0.0008	0.0004	0.001	0.69					
Gill	0.02	0.015	0.0605	0.002	0.0018				
Liver	0.0003	0.0001	0.0004	0.2	0.04	0.003			
Blood	0.0002	0.0001	0.0002	0.014	0.001	0.0001	0.005		
Bone	0.00002	0.0001	0.000001	0.00001	0.00003	0.0004	0.00001	0.00001	

$P > 0.05$: no significant different; $0.01 < P < 0.05$: significant different; $P < 0.01$: highly significant different.

In the previous study, Chikaraishi et al. (2009) reported little difference in the $\delta^{15}N$ value of amino acids between muscle and scale in a fish (Half-lined cardinal *Apogon semilineatus*). Little difference was also reported in the $\delta^{15}N$ value of bulk (i.e., total nitrogen) between muscle and fin of the seahorses *Hippocampus guttulatus* (Valladares and Planas, 2012). This little difference in our study thus is consistent with that in these previous studies. In contrast, although no literature is reported the $\delta^{15}N$ values of amino acids in fish blood, Germain et al. (2013) reported that the values of glutamic acid in blood serum for harbor seal *Phoca vitulina* is significantly lower than the expected values in muscle or whole body. This could be consistent with our results that the 1:1 relationship in the $\delta^{15}N$ value between muscle and the blood is poor for the examined fish. Moreover, the concentration and sources of free amino acids (including small-size peptides) in fish blood can change daily or weekly depending on recent diets (German et al. 2010). Even though the turnover of heart, liver, and gill themselves is monthly-year scale (e.g., Hilderbrand et al. 1996; Buchheister et al. 2010), these tissues have large volume of blood compared to muscle tissues. Thus, variation in the concentration and sources may lead to difference in the $\delta^{15}N$ value of amino acids between blood and muscle. Also, the bone (Hahn et al. 2012) and eye (Lynnerup et al. 2008) are a unique tissue that the $\delta^{15}N$ values of amino acids may not much change from larva to adult stages, because of little or no turnover. In addition, we still poorly understand why the strong positive 1:1 relationship in the $\delta^{15}N$ value is found between heart and eye as well as between liver and eye. Based on these results, we concluded that the scale and fin of

fish are applicable as alternatives to muscle, whereas the other tissues will be required further evaluation if we apply the CSIA-AA in food web studies.


(A) Muscle vs. Scale
Ile
Ala
Glu
Val
Leu
Phe
Gly
δ15NScale (‰)
δ15NMuscle (‰)
(B) Muscle vs. Fin
Pro
Ile
Ala
Glu
Leu
Val
Phe
Gly
δ15NFin (‰)
δ15NMuscle (‰)
(C) Muscle vs. Heart
Glu
Ala
Val
Ile
Leu
Phe
Gly
δ15NHeart (‰)
δ15NMuscle (‰)
(D) Muscle vs. Blood
Ala
Glu
Val
Ile
Leu
Phe
Gly
δ15NBlood (‰)
(E) Muscle vs. Eye
Glu
Ala
Ile
Val
Leu
Phe
Gly
δ15NEye (‰)
δ15NMuscle (‰)
(F) Muscle vs. Gill
Ala
Glu
Ile
Val
Leu
Phe
Gly
δ15NGill (‰)
δ15NMuscle (‰)

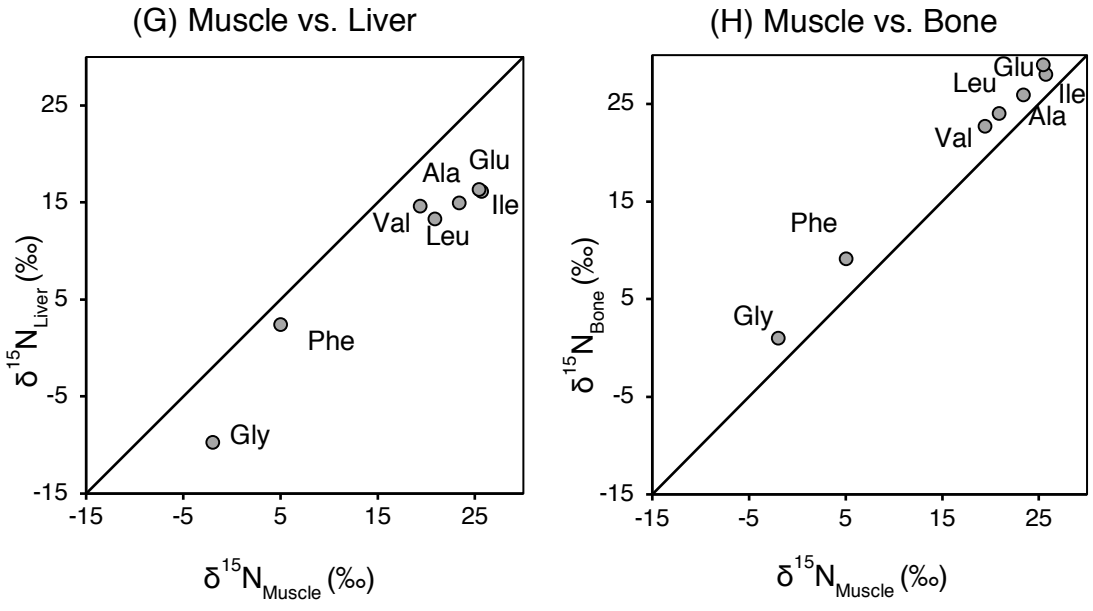


Fig. 2-2-1.

The relationship in the $\delta^{15}N$ value between tissue types in a *T. japonicus*: (A) scale; (B) fin; (C) heart; (D) Blood; (E) eye; (F) gill; (G) liver; and (H) bone. The line represents 1:1 relationship.

3.2 Shell protein of gastropods

The $\delta^{15}N$ values of amino acids in muscle vary from −0.6‰ (serine) to +13.2‰ (glutamic acid) for the *H. discus*, from +3.1‰ (methionine) to +16.6‰ (alanine) for the *T. sazae*, and from +2.4‰ (serine) to +15.1‰ (alanine) for the *O. pfeifferi* (Table 3). Amino acids in shell protein have substantially the same $\delta^{15}N$ values to those in muscle for these gastropods (Table 2-2-3), which illustrates the 1:1 relationship in the $\delta^{15}N$ value between tissue types ($t = 2.06$, d$f = 25$, $p = 0.48$) (Fig. 2-2-2).

Table 2-2-3. The $\delta^{15}N$ values of amino acids for gastropods investigated in this study (data from Chikaraishi et al., 2014). n.d.: not detectable.

		$\delta^{15}N$									
		Ala	Gly	Val	Leu	Ile	Pro	Ser	Met	Glu	Phe
H. discus	Muscle	12.6	2.8	12.7	6.1	9.5	12.9	-0.6	1.9	13.2	4.3
	Shell	12.7	2.6	12.4	5.5	9.3	12.7	0.1	n.d.	13.1	3.8
T. sazae	Muscle	16.6	4.8	15.1	12.7	14.3	14.1	7.0	3.1	16.4	5.0
	Shell	16.2	3.9	14.9	12.9	14.7	n.d.	7.7	n.d.	15.9	4.6
O. pfeifferi	Muscle	15.1	3.6	11.4	8.9	9.0	11.7	2.4	2.5	14.7	4.4
	Shell	15.4	3.0	11.7	8.5	9.8	11.1	3.3	n.d.	15	4.0

It is hypothesized that amino acids and their $\delta^{15}N$ values have long been preserved in hard tissues (e.g., shell and bone) but not in soft tissues (e.g., muscle) after the organisms die. Several previous studies indeed applied the CSIA-AA to the hard tissues such as bone and feather for evaluating trophic ecology on historical animal samples (mammal bone including human bone, Naito et al. 2010; bird bone, Ostrom et al. 2017; bird feather, Morra et al. 2019). However, there are two major issues in the application of hard tissues to ecological studies: whether or not the $\delta^{15}N$ values of amino acids in hard tissues 1) can be used instead of those in muscle; and 2) can be preserved for long time. Our results indicate that the $\delta^{15}N$ values of amino acids in shell are substantially identical to those of the corresponding amino acids in muscle within a single gastropod (Fig. 2-2-2). Thus, the $\delta^{15}N$ values of amino acids in hard tissues, particularly in gastropod shells, can be used instead of those in muscle for application of CSIA-AA, which can clarify the former issue. Based on our results, we can expect that a piece of gastropod shell on beach is useful as alternative to muscle if muscle is not accessible

with specific reasons (e.g., protection). Moreover, historical changes in the $\delta^{15}N$ value of amino acids may also be found in hard tissues that preserved for long time. As mentioned above, because the breakdown of amino acids and associated alternation of their $\delta^{15}N$ values are poorly understood, further studies are required to clarify the latter issue. If this issue will be clarified, we can apply hard tissues as convenient historical samples in ecological studies.

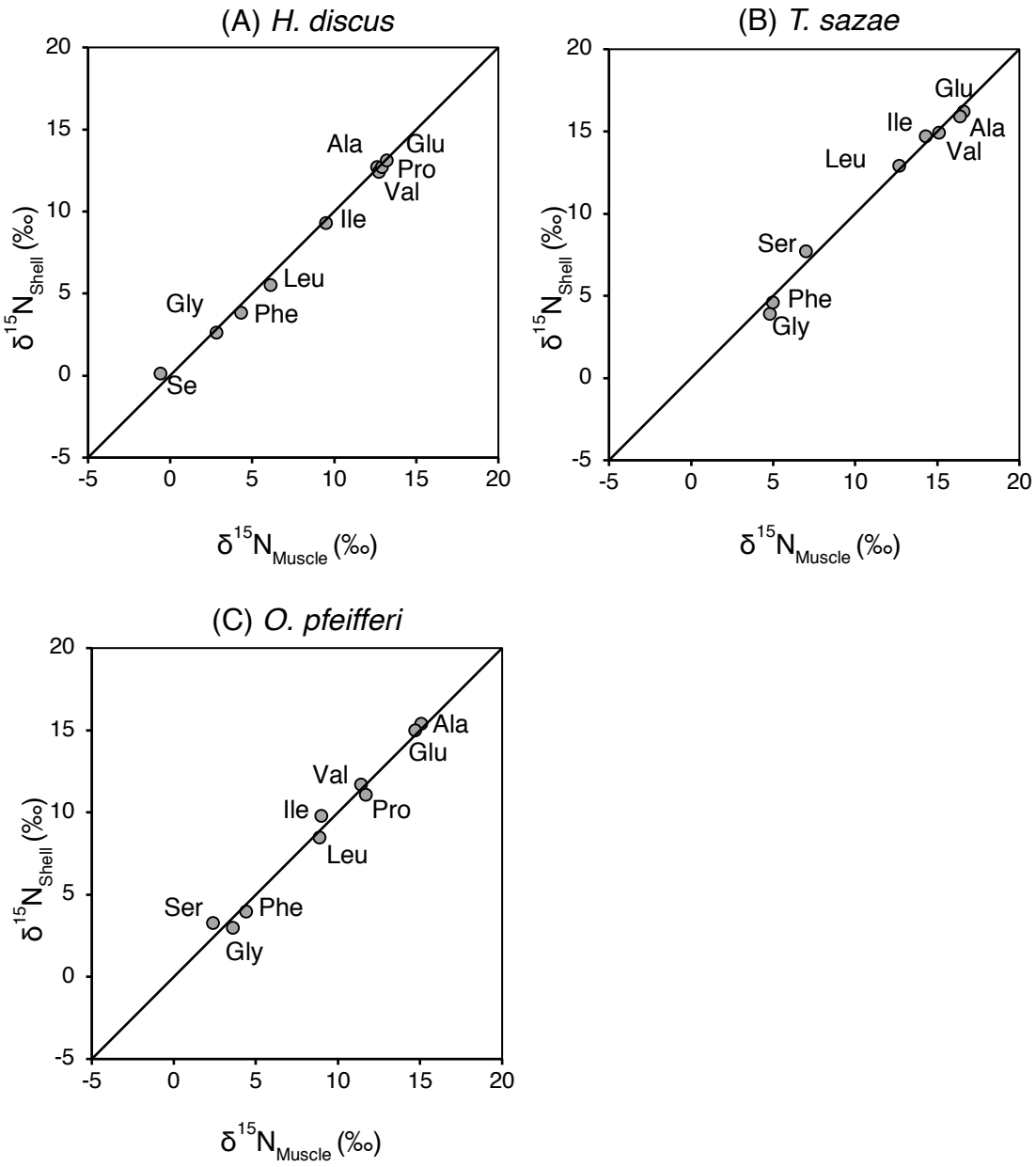


Fig. 2-2-2.

The relationship in the $\delta^{15}N$ value between muscle and shell protein in a gastropod for three species: (A) *Haliotis discus*; (B) *Turbo sazae*; and (C) *Omphalius pfeifferi*. The line represents 1:1 relationship.

3.3 Embryo of fish and turtle

The $\delta^{15}N$ values of amino acids in mother's muscle of the *S. marmoratus* vary from +4.6‰ (phenylalanine) to +30.5‰ (glutamic acid). They are almost identical to those of corresponding amino acids in the remaining yolk attached with the embryo (Table 2-2-4), which illustrates the 1:1 relationship in the $\delta^{15}N$ value between tissue types (t = – 1.0, df = 7, p = 0.35) (Fig. 2-2-3A), but not to those of corresponding amino acids in the developing embryo (t = – 4.9, df = 7, p = 0.002) (Fig. 2-2-3B). Except for phenylalanine and glycine, seven amino acids (alanine, valine, leucine, isoleucine, glutamic acid, proline, and serine) in the embryo's leg muscle of the *E. imbricata* have higher $\delta^{15}N$ values (by from 3.5‰ to 8.0‰) than corresponding amino acids in the remaining yolk (Table 2-2-4). These seven amino acids plot far from the 1:1 line in the $\delta^{15}N$ value between tissue types (t = 4.4, df = 8, p = 0.002) (Fig. 2-2-3C).

Table 2-2-4. The $\delta^{15}N$ values of amino acids for *S. marmoratus and E. imbricata* investigated in this study. n.d.: not detectable.

		$\delta^{15}N$									
		Ala	Gly	Val	Leu	Ile	Pro	Ser	Met	Glu	Phe
S. mamoratus	Muscle	28.1	10.2	26.2	29.9	26.2	28.1	n.d.	n.d.	30.5	4.6
	Yolk	28.3	10.5	26.1	30.9	26.0	28.4	n.d.	n.d.	30.2	4.6
	Embryo	35.5	12.4	33.5	32.8	32.3	34.8	n.d.	1.4	39.4	5.0
E. imbricata	Embryo	18.2	14.4	18.9	20.2	20.7	22.3	13.6	n.d.	18.9	3.4
	Yolk	10.6	14.7	13.2	15.5	12.9	14.3	10.1	n.d.	12.2	3.7

The typical life cycle of fish and turtle is comprised of serval stages including embryo, larva, juvenile, and adult. Embryo uses yolk as a solo nutrient source in eggs, whereas the others feed on diets. It is known that the $\delta^{15}N$ values of amino acids in the later stages are determined by those in diets and isotopic fractionation associated with metabolism in consumer themselves. Similar to this, the $\delta^{15}N$ values of amino acids in embryo are potentially far different from those in yolk, because of active metabolism during the development in eggs. Based on a large difference in the $\delta^{15}N$ value between remaining yolk and developing embryo, we predict that this difference is attributable to metabolism of yolk amino acids during the development for these two investigated species. Takizawa and Chikaraishi (2017) reported that deciduous plant *Cerasus lannesiana* used storage amino acids for the leaf flush under no/less photosynthetic activities. Moreover, because of little isotopic fractionation on phenylalanine during metabolism, no substantial difference in the $\delta^{15}N$ value of phenylalanine between remaining yolk and developing embryo is consistent with that the nutrient of embryo is derived only from yolk. On the other hand, the $\delta^{15}N$ values of amino acids in the remaining yolk of *S. marmoratus* is almost identical to those of mother's muscle. This is likely consistent with previous study that Hirahara et al. (2015) reported no substantial difference in the $\delta^{15}N$ value of amino acids between undeveloped egg and mother's body of the calanoid copepod *Acartia steueri*. These results of the previous and this studies suggest that the remaining yolk can, but developing embryo cannot, be

useful alternatives to muscle for the CSIA-AA, independent of any developmental stages of embryo.

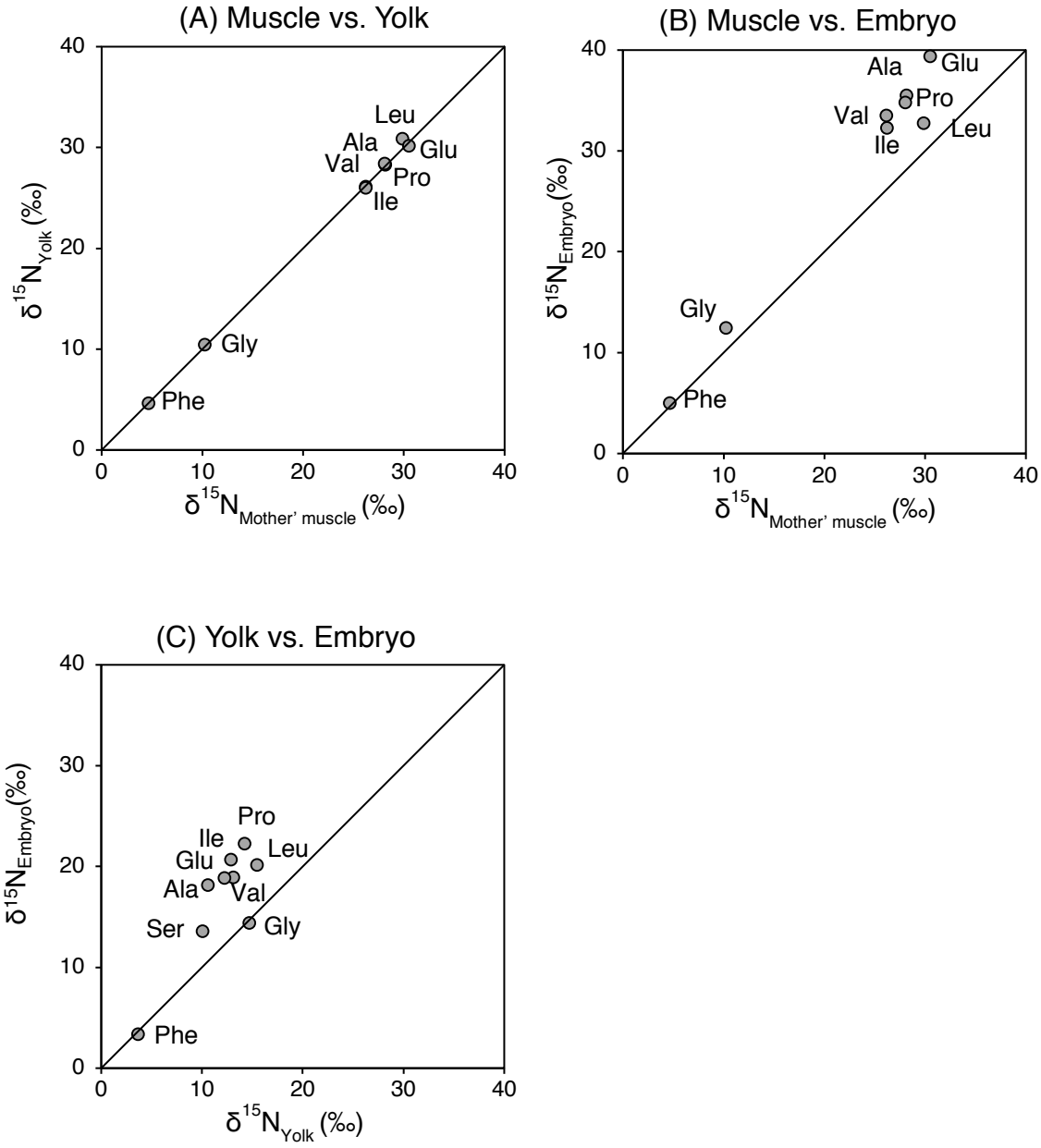


Fig.2-2-3.

The relationship in the $\delta^{15}N$ value between tissue types in *S. marmoratus*: (A) muscle vs. yolk; (B) muscle vs. embryo; and in *E. imbricate* (C) yolk vs. embryo. The line represents 1:1 relationship.

4. Conclusion

Muscle of organisms is generally used as samples in the food web studies. However, not only muscle but also other tissues such as scale and fin are potentially used as alternative samples. In this study, based on the tissue-specific $\delta^{15}N$ values of amino acids in several aquatic organisms including fish, gastropods and a turtle, we evaluated which tissues are useful as samples in CSIA-AA. The results suggest that scale, fin, shell, and yolk are applicable as alternatives to muscle, whereas many internal tissues (e.g., heart, liver, blood, and embryo) will be required further evaluation if we need to use them in the CSIA-AA.

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2-3. A baseline $\delta^{15}N$ isoscape for Sagami Bay based on compound specific isotope analysis of amino acids in local fish

Abstract

Compound-specific isotope analysis of nitrogen within amino acids in long-life consumer samples provides an isoscape map for the temporally-integrated isotope ratios of primary producers ($\delta^{15}N_{Primary\ producer}$) at the basis of food webs, which can be employed as essential information in ecological studies particularly for elucidating the trophic relationship among species in ecosystems. In this study, based on nitrogen isotope ratios of amino acids in two fish species ($\delta^{15}N_{Fish\ amino\ acids}$), we illustrated the isoscape map along Sagami Bay where has complex inputs of isotopically-distinct nitrogen sources derived from ocean current and human activities. The isoscape map illustrated show a spatial variation in the $\delta^{15}N_{Primary\ producer}$ value across Sagami Bay, as low (~2‰), middle (~12‰), and high (~16‰) values are attributable to nutrients found in ocean current, released from industrial, and experienced by denitrification, respectively. Moreover, the trophic position calculated by the $\delta^{15}N_{Fish\ amino\ acids}$ values shows that these two fish (generalist species for both) have kept a constant TP at the species level in Sagami Bay where is expected to have a large heterogeneity in the biomass size and species diversity of primary producers caused by multiple nitrogen sources inputs.

Keywords: isoscape map; nitrogen sources; isotopic composition; food webs; ecosystem; generalist

1. Introduction

The isotope ratios (δ values) of light elements such as hydrogen, nitrogen, and oxygen vary in systematically across natural environments. Geospatial δ map (termed isoscape map) for nitrogen of primary producers (i.e., baseline of food chains) can identify the source of isotopically-distinct nitrogen and quantify the contribution of them among organisms in food webs, which has been employed as basic information in a number of ecological studies, particularly for describing the trophic relationship of species, assessing biomagnification risk of pollutant, understanding the impact of nutrient inputs, and tracing the migration of organisms in ecosystems (Hansson et al. 1997; Aurioles et al. 2006; Newsome et al. 2007; Matsubayashi et al. 2020).

It is known that isotope ratios for the bulk nitrogen of organisms are explained by two major factors: the isotope ratios of baseline of food webs ($\delta^{15}N_{Baseline}$) and the elevation of isotope ratios of each trophic transfer (which is generally called by trophic discrimination factor, TDF), and therefore are given by the following equation (2-3-1):

$$\delta^{15}N_{Organism} = \delta^{15}N_{Baseline} + (TP_{Organism} - 1)\ TDF \qquad (2\text{-}3\text{-}1)$$

where $TP_{Organism}$ represents the trophic position of organisms in food webs. According to this equation, the $\delta^{15}N_{Baseline}$ values can be simply found by the isotope analysis of organisms with the TP of 1.0. The $\delta^{15}N$ values of phytoplankton ($\delta^{15}N_{Phytoplankton}$) and plants ($\delta^{15}N_{Plant}$) therefore have been used for illustrating isoscape

map in ecological studies (Costanzo et al. 2001; Brault et al. 2018). However, the $\delta^{15}N_{Phytoplankton}$ values frequently have a significantly large temporal variation in coastal areas, which is attributable to three major reasons as follows: 1) isotopically-distinct multiple nitrogen sources (e.g., nitrate vs. ammonia, natural vs. artificial) are inputted with temporally different proportion; 2) preferential assimilation of ^{14}N by phytoplankton accelerates temporal variation in the $\delta^{15}N$ value of the remaining pool of the nitrogen sources; 3) short life (i.e., hour-day scale) of phytoplankton records only snapshot of the temporal variation on the nitrogen sources into their $\delta^{15}N$ values. The isoscape map illustrated with the $\delta^{15}N_{Phytoplankton}$ values thus is not always useful in a number of studies, because the $\delta^{15}N_{Baseline}$ values mirror the temporal variation in the $\delta^{15}N_{Phytoplankton}$ value. In contrast, because consumers particularly for long life (i.e., month-year scale) ones do not sensitive to such temporal variation in the $\delta^{15}N_{Phytoplankton}$ value (Cabana et al. 1996), the $\delta^{15}N$ values of consumers ($\delta^{15}N_{Consumer}$), instead of phytoplankton, are potentially useful for illustrating isoscape map, if TP of the consumers is known. However, knowing the TP of consumers is also challenging in many cases, because the equation (1) requires the $\delta^{15}N_{Phytoplankton}$ values to calculate the TP of consumers. Thus, illustrating isoscape map itself is still challenging, and a common issue prior to the use of isoscape map in application studies.

During the last two decades, compound-specific isotope analysis of amino acids (CSIA-AA) in consumer samples has been used to estimate the $\delta^{15}N_{Baseline}$ values of

food webs (equation 2-3-2) (Xing et al. 2020), and to calculate the TP of organisms (equation 2-3-3) (Chikaraishi et al. 2009), in which the estimation and calculation are independent of the $\delta^{15}N_{Phytoplankton}$ values:

$$\delta^{15}N_{Baseline,phe} = \delta^{15}N_{Phe} - 0.4\ (TP - 1) \qquad (2\text{-}3\text{-}2)$$

$$TP = (\delta^{15}N_{Glu} - \delta^{15}N_{Phe}) / (8.0 - 0.4) + 1 \qquad (2\text{-}3\text{-}3)$$

where $\delta^{15}N_{Baseline,phe}$ represents the $\delta^{15}N$ values of phenylalanine in primary producers, and $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ represent the measured $\delta^{15}N$ values for glutamic acid and phenylalanine in a single consumer, respectively. It is known that the $\delta^{15}N_{Baseline,phe}$ values are lower by approximately 2.2‰ than the $\delta^{15}N_{Baseline}$ values, because of isotopic fractionation associated with phenylalanine synthesis in primary producers (Chikaraishi et al. 2009). Thus, the $\delta^{15}N_{Baseline,phe}$ values (equation 2-3-2) are estimated as long-time integration of the $\delta^{15}N_{Baseline}$ values in environments. Moreover, it is known that the $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values are increased by 8.0‰ and 0.4‰ at each step along the trophic transfer in food webs, respectively, because of isotope fractionation associated with amino acid metabolism (i.e., deamination and hydroxylation, respectively) in consumers (Chikaraishi et al. 2007). Thus, the TP (equation 2-3-3) of consumers are calculated without any consideration of temporal variation in the $\delta^{15}N_{Phytoplankton}$ value (Xing et al. 2020). It is mentioned that there are two types of consumers (i.e., migrating and local) in the coastal environments. The $\delta^{15}N_{Baseline,phe}$ values from the former species may apparently reduce spatial variation in the $\delta^{15}N_{Phytoplankton}$ value on illustrating isoscape map (Germain et al. 2013), because they can across several areas where have

isotopically-distinct nitrogen sources. Thus, isoscape map illustrated with the $\delta^{15}N_{Baseline,phe}$ values from latter species will be useful directly to provide temporally-integrated $\delta^{15}N_{baseline}$ values and isotopically-distinct nitrogen sources among areas within a coastal environment.

The isoscape map illustrated will be further useful as basic information to compare the contribution of nitrogen sources into the TP of organisms among different areas in coastal environments. Variation in the TP of organisms within a single species may be explained by the utilization of isotopically-distinct nitrogen sources in food webs where they grow. For instance, Choi et al. (2020) reported that there is a small and large variation in the TP among rivers for a specialist pike gudgeon *Pseudogobio esocinus* ($1\sigma = 0.2$) and for a generalist largemouth bass *Micropterus salmoides* ($1\sigma = 0.6$), respectively. This variation in the TP of freshwater fish suggests that adaptabilities to the environment changes (e.g., diverse of diets) is low for specialist (e.g., *P. esocinus*) and high for generalist species (e.g., *M. salmoides*). The high adaptability of *M. salmoides* implies that the TP of generalist species in freshwater environments can sensitively record the changes in nitrogen sources for growth areas. Because the TP of organisms is calculated accurately and precisely based on CSIA-AA (i.e., equations 2-3-2 and 2-3-3) for isoscape illustration, variation in the TP of local fish investigated will be simultaneously evaluated whether or not the TP of marine organisms is dependent on the nitrogen source and its related change in the food web community along coastal environments.

In this study, to illustrate isoscape map, we collected the fish striped beakfish *Oplegnathus fasciatus* and kusafugu *Takifugu niphobles* (representative local generalist species in stony and sandy areas, respectively) along Sagami Bay where has isotopically-distinct multiple inorganic nitrogen sources, and measured the $\delta^{15}N$ values of glutamic acid and phenylalanine in these two fish species. Moreover, to understand how impact nitrogen sources on the TP of these two marine generalist species, we compared the TP of individual specimens among different areas.

2. Method and materials

2.1. Collection of fish

On February 2015, we collected the local fish *O. fasciatus* from 7 sites in rocky-areas (site 1, 2, 3, 4, 6, 9, and 10 in Fig.2-3-2) and *T. niphobles* from 3 sites in sandy-areas (site 5, 7, and 8 in Fig.2-3-2) by fishing along Sagami Bay (three individuals for each site). These two fish species are generalists that can feed on multiple species as diets (wang et al. 2006; Kono et al. 2008). Several pieces of dorsal scales for *O. fasciatus* and a small part of tail fin for *T. niphobles* were sampled for the isotope analysis, and were stored at –20°C before the analysis.

Sagami Bay is situating on the south-west of Tokyo, and is surrounded by the land for west (Izu Peninsula), north (Shonan District), and east (Miura and Boso Peninsula), by the mouth of Tokyo Bay for north-east between Miura Peninsula and Boso Peninsula, and by the Pacific Ocean for south. There are two big rivers (Sakawa River and Hayakawa River) between the Izu Peninsula and the Shonan District. There are many

agricultural fields (including rice, vegetable, and fruit farms) located around these two rivers, and a dam constructed on the upstream and a wastewater treatment plant connected to the mouth of Sakawa River.

2.2. Analysis of the $\delta^{15}N$ values of amino acids

The nitrogen isotopic composition of amino acids was determined by the procedure in previous study (Chikaraishi et al. 2009). In brief, the sample was dried with a freeze-drier for overnight (>12 hours), and the dried sample was hydrolyzed with 12 N HCl at 110°C for overnight (>12 hours). The hydrolysate was washed with *n*-hexane/dichloromethane (3/2, v/v) to remove hydrophobic constituents. Derivatization of amino acids were performed sequentially with thionyl chloride/2-propanol (1/4) and pivaloyl chloride/dichloromethane (1/4) to produce *N*-pivaloyl isopropyl esters. The $\delta^{15}N$ values of amino acid derivatives were determined by gas chromatography/isotope ratio mass spectrometry (GC/IRMS). The isotopic composition of amino acids in samples was expressed relative to atmospheric nitrogen ($\delta^{15}N_{Air}$ = 0‰) on scales normalized to known $\delta^{15}N$ values of the reference amino acids (Chikaraishi et al. 2014). The accuracy and precision for the reference mixtures were always 0.0‰ (mean of Δ) and 0.4‰ to 0.7‰ (mean of 1σ), respectively, for sample sizes of ≥1.0 nmol N. In this study, we obtained the $\delta^{15}N$ values of two amino acids (glutamic acid, and phenylalanine) for these two fish species to estimate the $\delta^{15}N_{Baseline,phe}$ values with the equation (2-3-2) and to calculate the TP with the equation (2-3-3), and a mean value for three individuals was used.

2.3 Illustration of baseline $\delta^{15}N$ isoscape map

Ocean Data View (ODV) (Schlitzer, R., https://odv.awi.de, 2019) was used to illustrate baseline $\delta^{15}N$ isoscape map. The $\delta^{15}N_{Baseline,phe}$ values of fish, and the latitude and longitude of sampling sites were inputted into ODV, with a mode DIVA gridding for the gridded field, 66 for X scale lengths, 83 for Y scale lengths, 50 for signal-to-noise ratio, and 3.0 for quality limit.

3. Results

The $\delta^{15}N$ values in *O. fasciatus* vary from 1.8‰ to 18.2‰ for phenylalanine (7.3 ± 6.1‰, as mean ± 1σ standard deviation) and from 23.5‰ to 40.1‰ for glutamic acid (29.3 ± 6.0‰). Based on these values, the $\delta^{15}N_{Baseline,phe}$ values estimated are from 0.84‰ to 17.23‰ (6.3 ± 6.2‰) (Table 2-3-1). The $\delta^{15}N$ values in *T. niphobles* vary from 9.3‰ to 17.0‰ for phenylalanine (12.4 ± 4.1‰) and from 29.8‰ to 37.4‰ for glutamic acid (32.8 ± 4.0‰). Based on these values, the $\delta^{15}N_{Baseline,phe}$ values estimated are from 8.4‰ to 16.1‰ (11.5 ± 4.1‰) (Table 2-3-1). The TP is almost the same for *O. fasciatus* among these 7 sites (3.4 ± 0.1) as well as for *T. niphobles* among these 3 sites (3.2 ± 0.1) (Table 2-3-1), even though the $\delta^{15}N_{Baseline,phe}$ values have a large variation within species, showing no correlation between TP and the $\delta^{15}N_{Baseline,phe}$ values ($R^2 = 0.16$ for *O. fasciatus* and $R^2 = 0.04$ for *T. niphobles*, Fig 2-3-1).

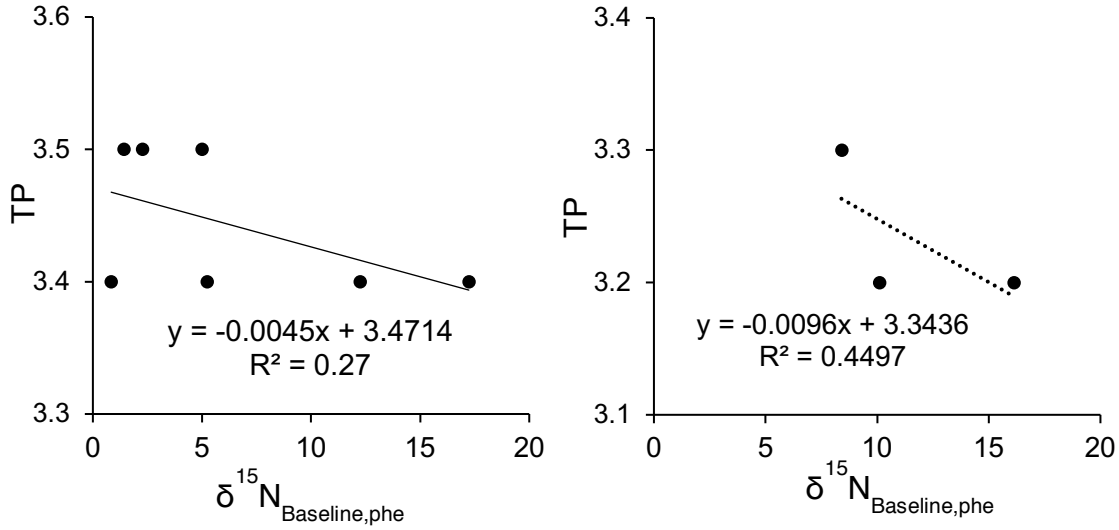

Fig 2-3-1.

The relationship between the TP of organisms and the $\delta^{15}N_{Baseline,phe}$ of Sagami Bay.

Table 2-3-1. The $\delta^{15}N$ values of amino acids, the $\delta^{15}N_{Baseline}$ values, and the TP for *Oplegnathus fasciatus* investigated in this study.

Species	Lat (N)	Lon (E)	$\delta^{15}N$			TP
			Glu	Phe	Baseline	
O. fasciatus	34.75	138.77	28.4	6.0	5.00	3.5
	34.68	138.98	23.5	1.8	0.84	3.4
	34.78	139.05	24.6	2.4	1.41	3.5
	35.15	139.13	27.9	6.2	5.24	3.4
	35.23	139.14	40.1	18.2	17.23	3.4
	35.14	139.63	34.7	13.2	12.25	3.4
T. niphobles	34.98	139.76	25.8	3.3	2.29	3.5
	35.16	139.14	31.2	10.9	10.01	3.2
	35.26	139.19	37.4	17.0	16.11	3.2
	35.31	139.32	29.8	9.3	8.40	3.3

There is a large spatial variation in $\delta^{15}N_{Baseline,phe}$ value on the isoscape map illustrated, which are classified into the three groups:

(1) around rivers mouth, the $\delta^{15}N_{Baseline,phe}$ values are high (16.11‰ for site 7 and 17.23‰ for site 6);

(2) around the east coast of Izu Peninsula, the $\delta^{15}N_{Baseline,phe}$ values are low (0.84‰ for site 2 and 1.44‰ for site 3);

(3) around Tokyo Bay mouth, the $\delta^{15}N_{Baseline,phe}$ values are intermediate (12.25‰ for station 9) between (1) and (2).

4. Discussion

4.1. Nitrogen sources of ecosystems

To discuss the nitrogen sources in ecosystems on the isoscape map illustrated with the $\delta^{15}N_{Baseline,phe}$ values, it should be care for original difference in the $\delta^{15}N$ value among nitrate ($\delta^{15}N_{Nitrate}$) in the ocean, primary producers ($\delta^{15}N_{Primary\ producer}$) in food webs, and phenylalanine ($\delta^{15}N_{Baseline,phe}$) in primary producers. It is known that the $\delta^{15}N_{Primary\ producer}$ values mirror the $\delta^{15}N_{Nitrate}$ values in the ocean surface, because of little isotopic fractionation associated with the assimilation of nitrogen in primary producers (Sigman et al. 2019). The $\delta^{15}N_{Baseline,phe}$ values are, however, changed to negative values by 2.2‰ from the $\delta^{15}N_{Primary\ producer}$ values, because of isotopic fractionation associated with the biosynthesis of phenylalanine in primary producers (Chikaraishi et al. 2009). For example, a previous study reported that the $\delta^{15}N_{Primary\ producer}$ values range from 6.5‰ to 12.3‰ in Sagami Bay (Won et al. 2007), which

accounts for 4.3‰ to 10.1‰ as the $\delta^{15}N_{Baseline,phe}$ values. In this study, isoscape map illustrated show us a large variation in $\delta^{15}N_{Baseline,phe}$ value ranging from 0.8‰ to 17.2‰ (Table 2-3-1), which are explained by inputs of isotopically-distinct multiple nitrogen sources into Sagami Bay.

The high $\delta^{15}N_{Baseline,phe}$ values (~16‰) were found in the areas around the river mouths (Fig. 2-3-2). These high values are probably caused by the nutrient inputs from rivers, in which farms (particularly rice fields), dam, and wastewater treatment plant frequently produce anoxic water. Such anoxic water induce denitrification that preferentially releases ^{14}N to atmosphere and leaves ^{15}N in the remaining pool of the nutrients (Kellman et al. 2003; Sebilo et al. 2006).

The low $\delta^{15}N_{Baseline,phe}$ values (~2‰) were found around the east coast of Izu Peninsula (Fig. 2-3-2). These low values indicate that the major nitrogen sources in this area are attributable to natural nutrients in the ocean, but not to agricultural and artificial nutrients derived from terrestrial environments. Indeed, the $\delta^{15}N_{Nitrate}$ values reported were 2‰-3‰ for subsurface water for the branch (that enter Sagami Bay) of Kuroshio Current (Yamazaki et al. 2016), which accounts for –0.2‰ to 0.8‰ as the $\delta^{15}N_{Baseline,phe}$ values. The $\delta^{15}N_{Baseline,phe}$ values observed in this study are slightly higher than that expected for Kuroshio Current, but the values observed are lower than mean $\delta^{15}N_{Baseline,phe}$ values (i.e., ~3‰) estimated for global ocean. These results thus indicate that the low $\delta^{15}N_{Baseline,phe}$ values (~2‰) found around the east coast of Izu Peninsula is explained by the contribution from nutrient derived from Kuroshio Current.

The intermediate $\delta^{15}N_{Baseline,phe}$ values (~12‰) were found around Tokyo Bay mouth (Fig. 2-3-2). Tokyo Bay is one of the most eutrophicated bay in Japan (Sukigara et al. 2005), because human activities around Tokyo have resulted in that a large amount of industrial nutrients is released to the environments including Tokyo Bay. There is no reported for the mean and variation in the $\delta^{15}N$ value for these industrial nutrients, because of a huge diversity in the nutrient source in them. However, a previous study reported that the $\delta^{15}N$ values are 9.5‰ for microphytobenthos in Tokyo Bay (Kanaya et al. 2013), implying that the $\delta^{15}N$ values of industrial nutrients are potentially higher than the $\delta^{15}N_{Nitrite}$ values in ocean current and lower than the $\delta^{15}N$ values of nutrients supplied from Sakawa River and Hayakawa River to Sagami Bay.

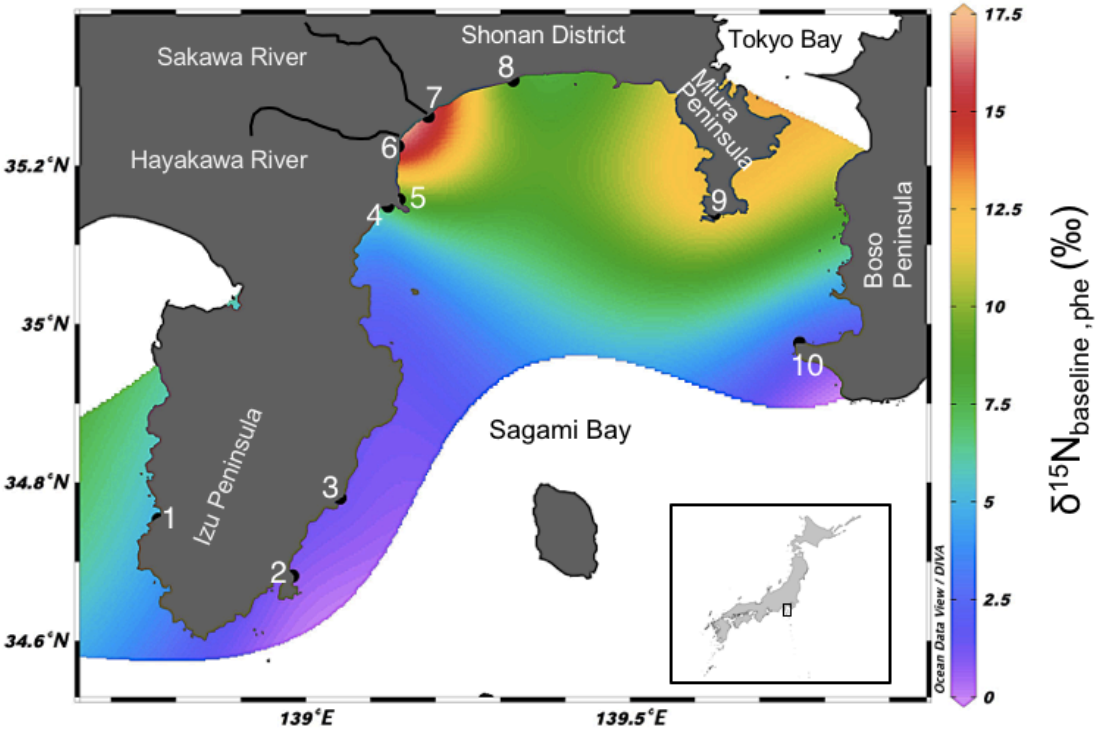

Fig. 2-3-2. $\delta^{15}N_{Baseline,phe}$ isoscape map of Sagami Bay. Color gradient bar indicates the $\delta^{15}N_{Baseline,phe}$ values. Number 1-10 represents the sampling sites.

4.2. The Impact of nitrogen sources on TP of fish

It is known that nutrients are key factors controlling for ecosystems, as the biomass size and species diversity for both primary producers and consumers and therefore the size of food webs may be affected by even a little change in the nutrient. For example, the release of artificial nutrients to coastal water frequently results in the blooming of specific phytoplankton (e.g., which is called red tide) (Anderson et al. 1997). The TP of consumers also may be affected by change in the nutrient, particularly for that of generalist species because these species potentially feed on multiple diets with diverse TP and potentially place on different energetical hierarchy in food webs.

The isoscape map in this study shows a large variation in the $\delta^{15}N_{Baseline,phe}$ value ranging from 0.8‰ to 17.2‰, and illustrates at least three different nutrient sources (i.e., with high, middle, and low values of $\delta^{15}N$) in ecosystems. Such a large difference in the $\delta^{15}N_{Baseline,phe}$ value simply supports that there is heterogeneity in the biomass size and species diversity of primary producers among areas within Sagami Bay, and make a general assumption that TP of generalist species potentially changes with the heterogeneity among areas. Indeed, in the case of river, the TP is variable from 2.7 to 3.9 for the generalist fish largemouth bass *Micropterus salmoides* among diverse environments (Choi et al. 2020). However, the $\delta^{15}N$ values of glutamic acid and phenylalanine measured in this study clearly indicate little variation in the TP within species (3.4 ± 0.1 for *O. fasciatus* and 3.2 ± 0.1 for *T. niphobles*), implying that TP of these species is independent of nutrient sources in the seawater and of their inducing

heterogeneity in the primary producers among areas. These results do not support the general assumption and is inconsistent with the previous study for freshwater fish, and suggest a specific mechanism to keep the constant TP for marine fish against the heterogeneity in the primary producers. Although specific reason is unknown at this moment, we predict that there is a preferential place on the energetical hierarchy in food webs even for generalist species and that these species have kept identical place even if the biomass size and species diversity for primary producers are changed by nutrient sources.

5. Conclusion

Based on the stable nitrogen isotope analysis of glutamic acid and phenylalanine in two generalist fish collected from Sagami Bay, an isoscape map for temporally-integrated $\delta^{15}N_{Primary\ producer}$ values was illustrated. The isoscape map illustrated show a large variation in the $\delta^{15}N_{Baseline,phe}$ value, ranging from 0.8‰ to 17.2‰, which are classified into the three groups: high values for river mouth; low values for east coast of Izu peninsula; and intermediate values for Tokyo Bay mouth, and are explained by inputs of isotopically-distinct multiple nitrogen sources. The high $\delta^{15}N_{Baseline,phe}$ values (~16‰) are probably caused by the nutrient inputs from rivers, in which the nutrients experienced denitrification in anoxic water. The low $\delta^{15}N_{Baseline,phe}$ values (~2‰) are attributable to natural nutrients mainly derived from Kuroshio Current. The intermediate $\delta^{15}N_{Baseline,phe}$ values (~12‰) are explained by the industrial nutrients released from human activities around Tokyo.

The TP of two fish species were also compared among different areas in Sagami Bay where has a large variation in $\delta^{15}N_{Baseline,phe}$ value. For both fish species, no substantially difference in the TP is found at the species level, with no correlation between TP and the $\delta^{15}N_{Baseline,phe}$ values. Thus the fish investigated can keep the constant TP against the heterogeneity in the biomass size and species diversity for primary producers in ecosystems, resulting in that they can stay preferential place on the energetical hierarchy in food webs.

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CHAPTER III.

Effect of pH on the Trophic Discrimination of $^{15}N/^{14}N$ for Amino Acids in Marine Organisms

Abstract

Releasing CO_2 from the industrial decreases ocean pH already by 0.1 during the last two century and will further by 0.3 at the end of 21 century. However, little is known about the effect of pH decrease on marine organisms and food webs. In this study, we reared fish, gastropods, and corals in the artificial seawater with pH7.3-8.1 for 2-5 months, and evaluated the change in the energy consumption between pHs based on the $\delta^{15}N$ values of amino acids in these organisms. Changes in the $\delta^{15}N$ value of amino acids between pHs suggest that energy consumption of fish and gastropods in the seawater with pH7.3 is lower than that with pH8.1. In contrast, the energy consumption of corals is negligibly changed in the seawater with pH7.6-8.1 but increased in the seawater pH lower than 7.6. These results reveal that, in the end of energy consumption, there are different effects among species to the seawater pH: (1) pH7.3 is positively affected to the fish and gastropods; (2) pH7.6-8.1 is negligibly affected to the corals; and (3) pH lower than 7.6 is negatively affected to the corals.

1. Introduction

Ocean acidification is one of the environment problems that caused by human activities. As a consequence of the increase of CO_2 emission into the atmosphere, the concentration of CO_2 in ocean is increasing, resulting in that of H^+ in seawater. Ciais et al. (2013) reported that 30 ± 7% of the CO_2 that released to atmosphere had been taken

up by ocean from industrial revolution to 2011. This CO_2 released from the industrial decreases ocean pH already by 0.1 during the last two century and will further by 0.3 at the end of 21 century if the emission rate of CO_2 does not change (IPCC, 2020).

In general, we predict that ocean acidification has negative effects on marine organisms. For example, Hoegh-Guldberg et al. (2007) reported that the coral reef ecosystem will disappear if the CO_2 level increases from 375 ppm to >500 ppm in Great Barrier Reef. At this moment, few studies reported that the ocean acidification has positive effects on marine organisms, and that these organisms are mainly primary producers. For example, Chen and Durbin (2007) reported that the photosynthesis rate of marine centric diatom *Thalassiosira pseudonana and oceanic diatom Thalassioira oceanica* significantly increase when the pH decreases from 9.4 to 7.0.

Moreover, consider the CO_2 concentration in the history of earth, we found that the CO_2 level is basically higher than present in the last 600 million years, which means that ocean pH is lower than present for almost always in that period. For example, CO_2 level in Devonian and Cretaceous were approximately 9 and 5 times, respectively, higher than present (Mackenzie, 1997), resulting in the ocean pH are 0.6 and 0.4 units, respectively, lower than present (Kump et al. 2009). Thus, the effect of ocean acidification on the marine organisms and food webs is a common question in the biogeochemical studies.

However, the nitrogen isotopic composition of amino acids ($\delta^{15}N$) will be available as an effective tool to evaluate the effect of ocean acidification, because change in the

nitrogen isotope ratio of amino acids ($\Delta\delta^{15}N$) from diet to consumers positively correlates with energy consumption of consumers. It is known that the $\Delta\delta^{15}N$ is caused by the isotopic fractionation in deamination of amino acids to produce life energy in organisms. Indeed, the nitrogen isotopic composition of consumer ($\delta^{15}N_{Consumer}$) is explained as the following equation (3-1):

$$\delta^{15}N_{Consumer} = \delta^{15}N_{Diet} + \Delta\delta^{15}N \qquad (3\text{-}1)$$

where $\Delta\delta^{15}N$ ($\Delta\delta^{15}N = \delta^{15}N_{t=x} - \delta^{15}N_{t=0}$) is the enrichment in ^{15}N on amino acids in organism from those in their diet. In general, the $\Delta\delta^{15}N$ is constant (e.g., 8.0‰ for glutamic acid) during each trophic transfer along food chain, but potentially change in specific environments where physical, chemical, and biological factors are different sizes compared to present environments. In the low pH environments, organisms may receive stresses and therefore change energy consumption in the life. Moreover, it is known that the concentration of NH_3 and NH_4^+ will decrease and increase, respectively, when the ocean pH decreases. Thurston et al. (1981) reported that the toxic level of NH_3 is higher than NH_4^+ for rainbow trout *Salmo gairdneri* and fathead minnows *Pimephales promelas*. Thus, the concentration of NH_3 potentially also is one reason for controlling the energy consumption of organisms.

In this study, to evaluate the effect of ocean acidification on energy consumption of organisms, we reared 3 fish and 1 gastropod species for 5 months under pH8.1 (control) and pH7.3, and 1 coral species for 2 months under pH8.1 (control), pH7.6, pH7.5, and pH7.4, and measured the $\Delta\delta^{15}N$ in these organisms.

2. Materials and Method

2.1 Raising samples

In this study, we reared 5 marine species. These species can be separated into 3 groups, including free-roaming species (i.e., the fish *Chromis viridis* and *Abudefduf sordidus*), benthic species (i.e., the goby *Stonogobiops nematodes* and the gastropod *Strombus luhuanus*), and coral (i.e., the coral *Briareidae* sp.). The larva of *A. sordidus* was collected from Sagami Bay (Japan) on October 2019, and of other species were bought from a company. These 3 fish are reared for 5 months with only isotopically uniformed commercial pellets that are homogenized before use. The gastropods are also reared for 5 months in the same aquarium of fish. The diets of gastropods are mainly the remaining pellets and faeces of fish, but rarely algae attached on the glasses of aquarium. The corals are reared for 2 months without diet, because this species has symbiotic algae that supply photosynthetic products to the host corals. The dorsal scale of *C. viridis* and *A. sordidus*, the dorsal muscle of *S. nematodes*, the foot muscle of *S. luhuanus*, and the newly-growing part of *Briareidae* sp. were used for analysis in this study.

2.2 Designing of aquarium system

We designed an aquarium system that has a constant pH of seawater for experimental periods (i.e., 5 months for fish and gastropod and 2 months for coral) (Fig. 3-1). The system is composed of 4 main units: plastic bags, a drip chamber, an aquarium, and a tank on floor 4, 3, 2, and 1, respectively, which are connected with silicone tube

(i.d. = 5 mm) from top to down (Fig. 3-1). Plastic bags are filled with pH5.6 artificial seawater that are saturated with CO_2, are tightly sealed without air, and are used for supplying the pH5.6 seawater to the drip chamber through silicone tube. The plastic bags are shrunk after 2-4 days, and then replaced with new plastic bags. A drip chamber is used to control the water flow dropping onto the surface of seawater in aquarium. For example, the rate of water flow is 50 and 90 drops per minute to make seawater with pH7.7 and pH7.3, respectively. The aquarium is used for rearing fish, gastropods, and corals in a constant pH. An air-stone is used for providing oxygen, a water pump is used for circulating the water, plastic wools and bolls are used for filtering the water, a LED lamp (with 42 white, 10 blue, and 10 red LEDs) is used for providing a light-dark cycle in 12-12 hours, and a heater is used for keeping 23 °C in the aquarium. A silicone tube for waste line is used for removing the seawater into tank with the same water flow from the drip chamber to the aquarium, to keep the constant volume of seawater in the aquarium.

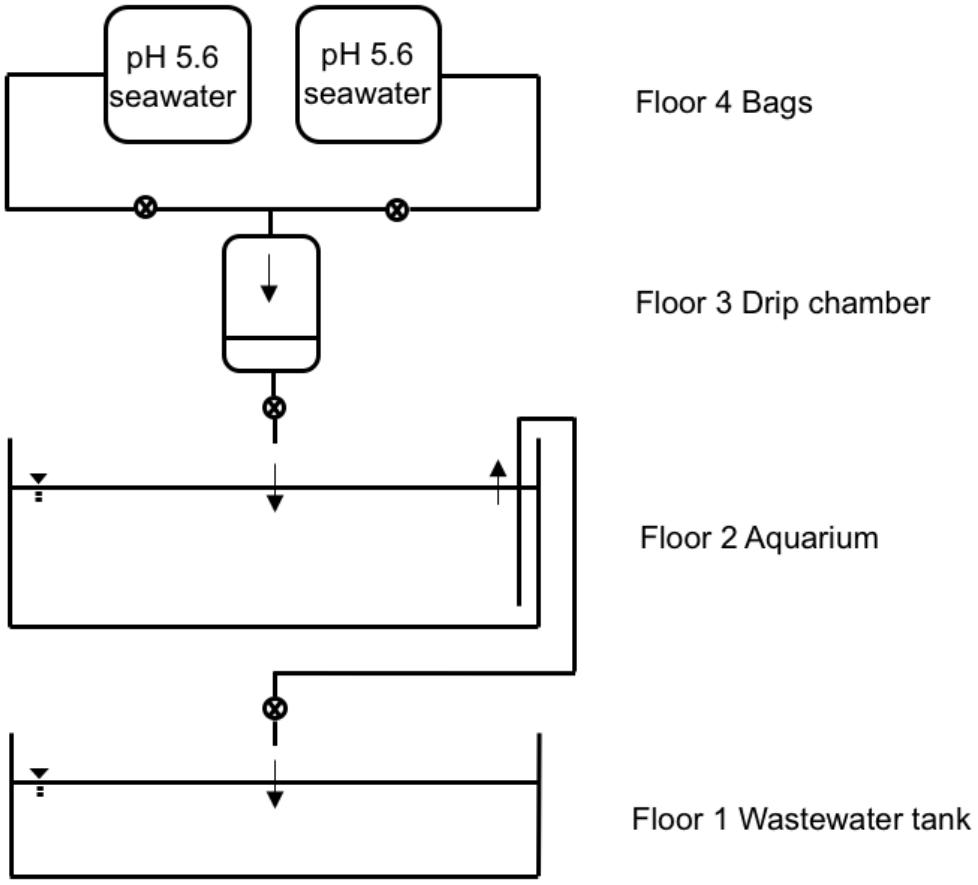


Fig 3-1.

The schematic of aquarium system.

2.3 Analyzing of the $\delta^{15}N$ values of amino acids

The nitrogen isotopic composition of amino acids was determined by the procedure in previous study (Chikaraishi et al. 2009). In brief, the sample was dried with a freeze-drier for overnight (>12 hours), and the dried sample was hydrolyzed with 12 N HCl at 110°C for overnight (>12 hours). The hydrolysate was washed with *n*-hexane/dichloromethane (3/2, v/v) to remove hydrophobic constituents. Derivatization of amino acids were performed sequentially with thionyl chloride/2-propanol (1/4) and pivaloyl chloride/dichloromethane (1/4) to produce *N*-pivaloyl isopropyl esters. The

$\delta^{15}N$ values of amino acid derivatives were determined by gas chromatography/isotope ratio mass spectrometry (GC/IRMS). The isotopic composition of amino acids in samples was expressed relative to atmospheric nitrogen ($\delta^{15}N_{Air}$ = 0‰) on scales normalized to known $\delta^{15}N$ values of the reference amino acids (Chikaraishi et al., 2014). The accuracy and precision for the reference mixtures were always 0.0‰ (mean of Δ) and 0.4‰ to 0.7‰ (mean of 1σ), respectively, for sample sizes of ≥1.0 nmol N. In this study, we obtained the $\delta^{15}N$ values of five amino acids (i.e., alanine, glycine, valine, glutamic acid, and phenylalanine) for the all five species.

2.4 Evaluating energy consumption of organisms

2.4.1 Evaluating energy consumption of fish and gastropods

We measured the $\delta^{15}N$ values of 5 amino acids in fish and gastropods, and calculated the $\Delta\delta^{15}N_{Consumer}$ values for each amino acid in these species between pH7.3 and pH8.1, as the following equation (3-2):

$$\Delta\delta^{15}N_{Consumer} = (\delta^{15}N_{7.3} - \delta^{15}N_{Diet}) - (\delta^{15}N_{8.1} - \delta^{15}N_{Diet}) \qquad (3\text{-}2)$$

where $\delta^{15}N_{7.3}$ and $\delta^{15}N_{8.1}$ represent the $\delta^{15}N$ values of an amino acid in consumers that are reared in pH7.3 and pH8.1 seawater, respectively, $\delta^{15}N_{Diet}$ represents the $\delta^{15}N$ values of an amino acid in diets. Because the $\delta^{15}N_{Diet}$ values are identical between pH7.3 and pH8.1 experiments, the equation (3-2) is given by the following equation (3-3),

$$\Delta\delta^{15}N_{Consumer} = (\delta^{15}N_{7.3} - \delta^{15}N_{8.1}) \qquad (3\text{-}3)$$

According to the Rayleigh model, change in the $\delta^{15}N$ values of amino acids from diets to consumers (i.e., $\delta^{15}N_{7.3} - \delta^{15}N_{Diet}$ and $\delta^{15}N_{8.1} - \delta^{15}N_{Diet}$) is dependent of the flux of

breakdown of amino acids to produce life energy for consumers, as the following equations (3-4) and (3-5):

$$\delta^{15}N_{7.3} - \delta^{15}N_{Diet} = 1000 \times [\, F_{7.3}{}^{(\alpha - 1)} - 1] \qquad (3\text{-}4)$$

$$\delta^{15}N_{8.1} - \delta^{15}N_{Diet} = 1000 \times [\, F_{8.1}{}^{(\alpha - 1)} - 1] \qquad (3\text{-}5)$$

where α represents the isotopic fractionation factor associate with the breakdown of amino acids in consumers, and F represents the flux of amino acids resisted to the breakdown (in which 1−F approximately mirrors energy consumption) in the consumers. The α has a single value ranging between 0 and 1for each amino acid in any pH condition, but the F varies between 0 and 1 for amino acids among conditions. The equations (3-3) to (3-5) are combined to the following equation (3-6):

$$\Delta\delta^{15}N_{Consumer} = 1000 \times [F_{7.3}{}^{(\alpha - 1)} - F_{8.1}{}^{(\alpha - 1)}] \qquad (3\text{-}6)$$

Based on the equation (3-6), positive and negative for the $\Delta\delta^{15}N_{Consumer}$ values can be explained by that fish and gastropods much and less, respectively, consumed amino acids as energy sources in the experiments.

2.4.2 Evaluating energy consumption of coral

For coral experiments, $\delta^{15}N_{Diet}$ in the equation (3-2) should be replaced with the $\delta^{15}N$ values of symbioses algae ($\delta^{15}N_{Algae}$) in corals, as given by the following equation (3-7):

$$\Delta\delta^{15}N_{Coral} = (\delta^{15}N_{low\text{-}pH} - \delta^{15}N_{Algae,low\text{-}pH}) - (\delta^{15}N_{8.1} - \delta^{15}N_{Algae,8.1}) \qquad (3\text{-}7)$$

where $\Delta\delta^{15}N_{Coral}$ represents the $\Delta\delta^{15}N$ values of amino acids in corals, $\delta^{15}N_{Algae,low\text{-}pH}$ and $\delta^{15}N_{Algae,8.1}$ represent the $\delta^{15}N$ values of an amino acid in corals that are reared in low pH (i.e., pH7.4, 7.5, or 7.6) and pH8.1 seawater, respectively.

Unlike the change of equation from (3-2) to (3-3) for consumer samples, the $\delta^{15}N_{Algae}$ values are not able to delete in equation (3-7), because the $\delta^{15}N_{Algae,low\text{-}pH}$ values are potentially different from the $\delta^{15}N_{Algae,8.1}$ values and vary during the experiment for 2 months. It is simply thought that the variation in the $\delta^{15}N_{Algae}$ values is caused by photosynthesis activities of symbiotic algae in corals, input rate of pH5.6 seawater into aquarium, and initial and final biomass size of symbiotic algae in the experiments. Because phenylalanine has a little change in the $\delta^{15}N$ value from diet to consumer species (Chikaraishi et al. 2007), we can use the normalization of the $\delta^{15}N$ values of amino acids (i.e., alanine, glycine, valine, and glutamic acid) with the value of phenylalanine ($\delta^{15}N_{Phe}$) to evaluate the energy consumption of coral samples.

Instead of the equation (3-3) for consumer samples, the following equations (3-8) to (3-10) are used for coral samples:

$$\Delta\delta^{15}N_{Coral} = [\delta^{15}N_{Coral,AA,low\text{-}pH} - (\delta^{15}N_{Alage,phe,low\text{-}pH} + \beta)] - [\delta^{15}N_{Coral,AA,8.1} - (\delta^{15}N_{Alage,phe,8.1} + \beta)] \quad (3\text{-}8)$$

$$\delta^{15}N_{Alage,AA,low\text{-}pH} = \delta^{15}N_{Alage,phe,low\text{-}pH} + \beta \quad (3\text{-}9)$$

$$\delta^{15}N_{Alage,AA,8.1} = \delta^{15}N_{Alage,phe,8.1} + \beta \quad (3\text{-}10)$$

where $\delta^{15}N_{Coral,AA,low\text{-}pH}$ and $\delta^{15}N_{Coral,AA,8.1}$ represent the $\delta^{15}N$ values of an amino acid (i.e., alanine, glycine, valine, or glutamic acid) of coral in low pH and pH8.1

experiments, respectively, and $\delta^{15}N_{Alage,phe,low\text{-}pH}$ and $\delta^{15}N_{Alage,phe,8.1}$ represent the $\delta^{15}N$ values of phenylalanine of coral in low pH and pH8.1 experiments, respectively, β represents the difference between the $\delta^{15}N_{AA}$ values and the $\delta^{15}N_{Phe}$ values in algae. β is a constant value for each amino acid, which is reported in previously (Chikaraishi et al., 2009).

Similar to the consumers experiment, according to the Rayleigh model, change in the $\delta^{15}N$ values of amino acids from algae to corals is calculated as the following equations (3-11) and (3-12):

$$\delta^{15}N_{Coral,AA,low\ pH} - \delta^{15}N_{Alage,AA,low\text{-}pH} = 1000 \times [F_{Low\ pH}^{(\alpha-1)} - 1] \quad (3\text{-}11)$$

$$\delta^{15}N_{8.1AA} - \delta^{15}N_{Alage,AA,8.1} = 1000 \times [F_{8.1}^{(\alpha-1)} - 1] \quad (3\text{-}12)$$

The equations (3-8) to (3-12) are combined to the following equation (3-13):

$$\Delta\delta^{15}N_{Coral} = 1000 \times [F_{Low\ pH}^{(\alpha-1)} - F_{8.1}^{(\alpha-1)}] \quad (3\text{-}13)$$

Based on the equation (3-13), positive and negative for the $\Delta\delta^{15}N_{Coral}$ values can be also explained by that corals much and less, respectively, consumed amino acids as energy sources in the experiments.

3. Results

The $\Delta\delta^{15}N_{Consumer}$ values calculated are ranging from −3.4‰ (glutamic acid) to +0.3‰ (phenylalanine) for the *C. viridis*, from −4.0‰ (valine) to +0.4‰ (alanine) for the *A. sordidus*, from −4.2‰ (valine) to +0.4‰ (glycine) for the *S. nematodes*, and from −2.25‰ (glycine) to −0.1‰ (phenylalanine) for the *S. luhuanus* (Table 3-1). The analytic error (1σ) of $\delta^{15}N$ values of amino acids in this study is better than 0.8‰,

resulting in that the error of $\Delta\delta^{15}N_{Consumer}$ is better than 1.1‰ based on the propagation of 1σ on the equation (3-3). The $\Delta\delta^{15}N_{Consumer}$ values of valine (-1.3‰) and glutamic acid (-3.4‰) for *C. viridis*, of valine (-4.0‰) for *A. sordidus*, of alanine (-1.7‰), valine (-4.2‰), and glutamic acid (-1.5‰) for *S. nematodes,* of glycine (-2.3‰) for *S. luhuanus* are significantly negative values from 0‰ with the propagation error (1σ = 1.1‰). The $\Delta\delta^{15}N_{Consumer}$ values of the other amino acids in these samples are no substantial difference from 0‰ with the propagation error (Fig 3-2).

Table 3-1. The $\delta^{15}N$ values of amino acids, and the $\Delta\delta^{15}N_{Consumer}$ values between pH8.1 and pH7.3 for 3 fish and 1 gastropod.

		Ala	Gly	Val	Glu	Phe
C. viridis	pH8.1	20.0	-0.8	22.1	22.5	2.8
	pH7.3	18.9	-0.6	20.8	19.1	3.1
	$\Delta\delta^{15}N_{Consumer}$	-1.1	0.2	-1.3	-3.4	0.3
A. sordidus	pH8.1	24.5	2.6	22.0	23.6	3.1
	pH7.3	24.9	2.0	18.0	22.6	3.2
	$\Delta\delta^{15}N_{Consumer}$	0.4	-0.6	-4.0	-1.0	-0.2
S. nematodes	pH8.1	26.3	8.0	27.7	25.5	8.5
	pH7.3	24.6	8.4	23.5	24.0	8.6
	$\Delta\delta^{15}N_{Consumer}$	-1.7	0.4	-4.2	-1.5	0.1
S. luhuanus	pH8.1	19.0	12.6	18.3	19.2	11.8
	pH7.3	18.3	10.3	18.1	18.8	11.7
	$\Delta\delta^{15}N_{Consumer}$	-0.7	-2.3	-0.2	-0.4	-0.1

The $\Delta\delta^{15}N_{Consumer}$ values are calculated with equation 3-3.

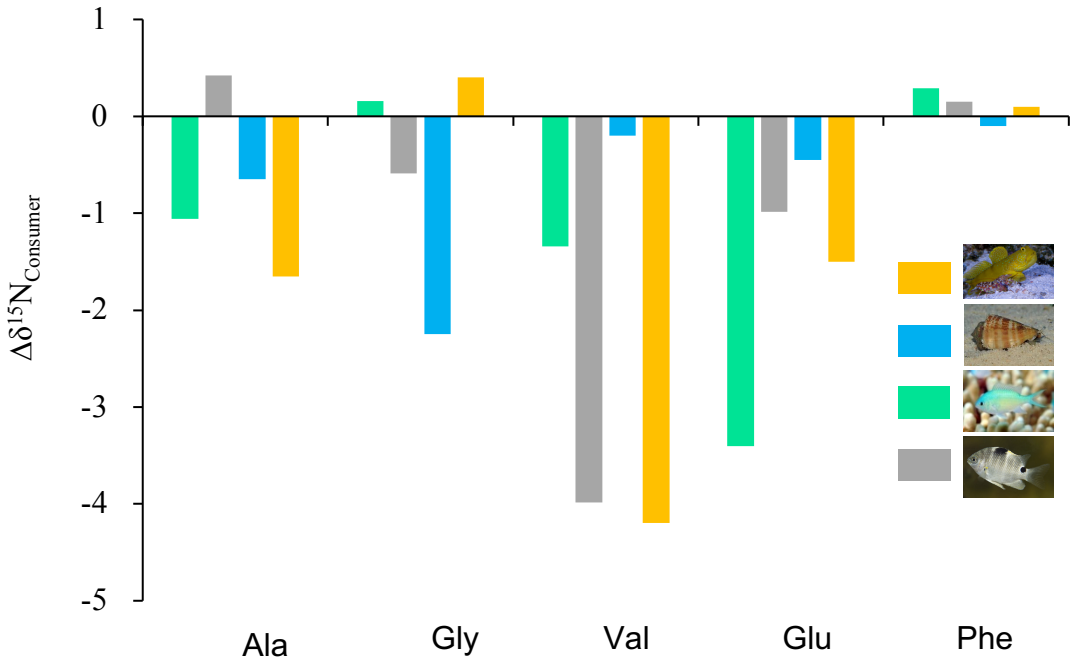


Fig 3-2.

The $\Delta\delta^{15}N_{Consumer}$ values between pH8.1 and pH7.3 for 3 fish and 1 gastropod. The yellow, blue, green, and grey bar represent *S. nematodes*, *S. luhuanus*, *C. viridis*, and *A. sordidus*, respectively.

The $\Delta\delta^{15}N_{Coral}$ values calculated are ranging from −1.3‰ (glutamic) to +0.9‰ (glycine) for pH7.6 experiment, from −1.1‰ (glycine) to +2.5‰ (glycine) for pH7.5 experiment, from −0.3‰ (glutamic acid) to +5.5‰ (glycine) for pH7.4 experiment (Table 3-2). The error of $\Delta\delta^{15}N_{Coral}$ is better than 1.6‰ based on the propagation of 1σ on the equation (3-7). The $\Delta\delta^{15}N_{Coral}$ values of 4 amino acids in pH7.6 experiment are no substantial difference from 0‰ with the propagation error (1σ = 1.6‰). The $\Delta\delta^{15}N_{Coral}$ values of glycine for pH7.5 experiment, and of alanine, glycine, and valine

for pH7.4 experiment are significantly positive values from 0‰ with the propagation error (Fig 3-3).

Table 3-2. The $\delta^{15}N$ values of amino acids, and the $\Delta\delta^{15}N_{coral}$ values for coral under different pH.

	Ala	Gly	Val	Glu	Phe
pH8.1	8.2	2.3	9.2	9.6	4.9
pH7.6	8.7	5.9	10.7	10.2	7.1
$\Delta\delta^{15}N_{Coral}$	-1.2	0.9	0.6	-1.3	--
pH7.5	8.9	6.1	11.8	11.6	6.6
$\Delta\delta^{15}N_{Coral}$	-1.1	2.5	1.3	0.7	--
pH7.4	13.3	9.7	13.0	11.2	6.8
$\Delta\delta^{15}N_{Coral}$	3.2	5.5	1.9	-0.3	--

The $\Delta\delta^{15}N_{Coral}$ values are calculated with equation 3-7.

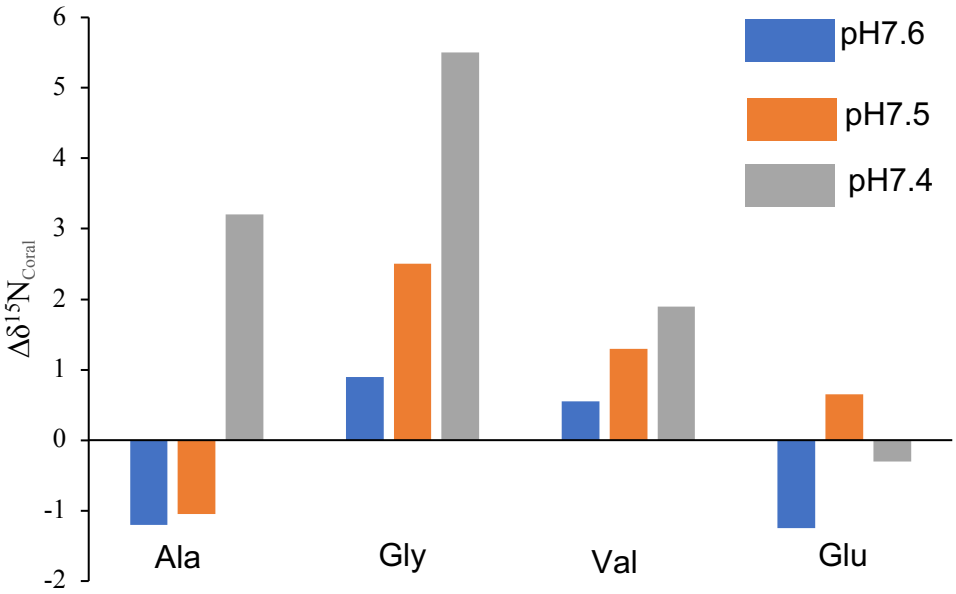


Fig 3-3.

The $\Delta\delta^{15}N_{Coral}$ values for coral under different pH. The blue, orange, and grey bar represent pH7.6, 7.5, and 7.4, represently.

4. Discussion

4.1 Evaluating the energy consumption of fish and gastropod

Negative $\Delta\delta^{15}N_{Consumer}$ values can be explained by that consumers less consumed amino acids as energy sources in the experiments. The negative $\Delta\delta^{15}N_{Consumer}$ values for 7 amino acids in these samples suggest that the energy consumption of these species is smaller in pH7.3 experiment than that in normal condition pH8.1. On other words, these results induce that the requirement of life energy for these species probably lower in pH7.3 experiment than that in normal condition pH8.1. It is simply thought that the low requirement of life energy is a positive effect on consumers, probably for several factors including growth rate and activities.

This is not consistent with previous studies, because many recent studies reported that ocean acidification have negative effects on marine organisms. For example, Munday et al. 2008) reported the larva clownfish *Amphiprion percula* will lose olfactory capacity if they are reared seawater with pH7.6. Even though we have many unclear points about the reason for these positive effect, we suggest the following two hypotheses potentially to explain these results:

Hypothesis 1: Fish and gastropods need more energy to keep the balance of pH between inside and outside of cells in pH8.1 than that in pH7.3. Hirata et al. (2003) reported that the blood pH is ~7.45 for a freshwater fish, osorezan dace *Tribolodon hakonensis*. Although we do not know the blood pH for the investigated fish and gastropods, we assumed that the blood pH for these species are closer to pH7.3 than pH8.1, implying that the interval of pH between inside and outside of cells is reduced

in the pH7.3 seawater. This small interval can be explained why the energy consumption is lower in pH7.3 experiment than that in pH8.1 experiment. Moreover, the $\Delta\delta^{15}N_{Consumer}$ of individuals amino acids is different among species, which may suggest that process to adapt the low pH is somewhat different among species.

Hypothesis 2: Fish and gastropod need more energy to against the toxic condition in pH8.1. It is known that the toxicity of NH_3 is higher than NH_4^+ for aquatic organisms (Silva et al., 2013). Based on $K_b = 1.8 \times 10^{-5}$, the rate of NH_4^+/ NH_3 increases from 14.3 to 90.2 when pH decreases from pH8.1 to pH7.3. Thurston et al. (1981) reported that the toxic level of pH8.5 seawater is 10 times higher than pH7.5 seawater. Thus, in this study, the toxic level in pH8.1 experiment is significantly higher than pH7.3 experiment, which means that fish and gastropods need less energy to against the toxic in pH7.3 than normal condition pH8.1.

4.2 Evaluating the energy consumption of coral

We would like to mention that, because it is generally required to have skill for keeping and growing corals in laboratory for several months, we did not have enough samples to evaluate the effect of ocean acidification on corals in this study. Indeed, we tried to prepare several species of corals in laboratory during the last two years. However, we cannot keep corals in the experiments, as almost 80% of corals died particularly for the control experiment (i.e., pH8.1). Therefore, at this moment, we have thought that the $\Delta\delta^{15}N_{Coral}$ values found in this study are used as preliminary data, and

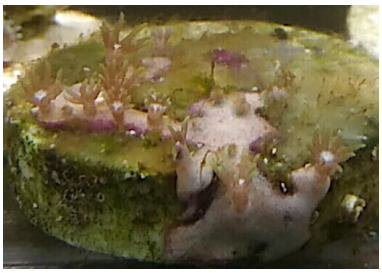
that further experiments are required to conform the finding and explanation in this study.

There is a large difference in the trend of the $\Delta\delta^{15}N$ values between corals (Fig. 3-2) and other consumers (Fig. 3-3). The $\Delta\delta^{15}N_{Coral}$ values sift toward positively by up to 5.5‰ and does not have negative values over the range of errors, whereas the $\Delta\delta^{15}N_{Consumer}$ values sift toward negatively by up to 4.2‰ and does not have positive values over the range of errors. This difference suggests that the response to ocean acidification in corals is different from that in fish and gastropods.

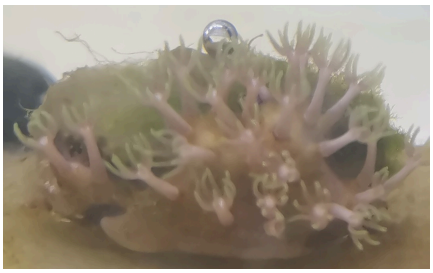
In the experiment with pH7.6, change in the $\Delta\delta^{15}N_{Coral}$ value is negligible, which is simply explained by that the energy consumption level of corals in pH7.6 is almost the same to that in pH8.1 (Fig. 3-3). These results clearly indicate that change in the pH from 8.1 to7.6 is no effect for corals. These results are not consistent with the negative effects to pH decrease in previous studies. For example, Anthony et al. (2008) reported a negative effect that the bleaching rates for staghorn coral *Acropora intermedia* in pH7.85-7.95 and pH7.6-7.7 conditions are ~20% and ~40%, respectively. Because supplying photosynthetic products is significantly reduced under such high bleaching rates, we think that consumption rate of organic pools (e.g., protein) in corals is increased and that the $\Delta\delta^{15}N_{Coral}$ values are also increased. However, we did not find any positive number for the $\Delta\delta^{15}N_{Coral}$ values in this study. Unlike fish and gastropods, it is thought that the effects regarding to pH balance between inside and outside of cells and the toxicity of NH_3 probably are not effective to corals. For corals, because the

symbiotic algae can use CO_2 and NH_3 for photosynthesis, pH and NH_3 concentration of seawater around corals frequently change a lot. If it is case, corals potentially have a function for adapting to the seawater with the pH7.6-8.1, which may be a simply explanation to find negligible change in the $\Delta\delta^{15}N_{Coral}$ values in this study.

In the experiments under pH7.6, the $\Delta\delta^{15}N_{Coral}$ values are significantly increased for glycine in pH7.5, and for alanine, glycine, and valine in pH7.4 (Fig. 3-3). These results suggest that reducing pH under 7.6 start negative effect to corals. This negative effect is probably similar to that found in previous studies (e.g., Hoegh-Guldberg et al. 2007; Anthony et al., 2008). Moreover, during the experiment in this study, polyps of corals is decreased in color (from green to white) less in pH7.5 and much in pH7.4 for seawater (Fig. 3-4). This decrease in color suggests that the abundance of symbiotic algae in corals is reduced for the seawater pH lower than pH7.6, and can simply explain that energy consumption of corals in excess to its input from symbiotic algae induces the significantly positive $\Delta\delta^{15}N_{Coral}$ values found in this study.

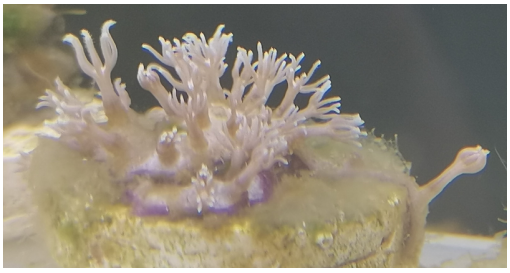


pH8. 1



pH7. 6

pH7. 5



pH7. 4

pH7. 3

Fig 3-4.

The condition for coral under different pH.

5. Conclusion

The $\Delta\delta^{15}N_{Consumer}$ values for 7 amino acids in fish and gastropods are significantly negative values, which suggests that energy consumption of these species in pH7.3 experiment is lower than that in pH8.1 experiment. Even though we have many unclear points for the reasons, we hypotheses: 1) difference in the pH between inside and outside of cells in pH7.3 is smaller than that in pH8.1, resulting in smaller energy consumption in pH7.3 than pH8.1 to keep the pH of cells for fish and gastropods; and 2)concentration of NH_3 in seawater with pH7.3 is lower than that with pH8.1, resulting in smaller energy consumption in pH7.3 than pH8.1 to against the toxicity of NH_3 in seawater for fish and gastropods

The $\Delta\delta^{15}N_{Coral}$ values are close to 0‰ for pH7.6 experiment, which suggests that energy consumption of corals does not change in pH7.6-8.1. This is probably caused by that corals potentially have a function for adapting to the seawater with the pH7.6-8.1 and low concentration of NH_3 due to photosynthetic activities of symbiotic algae. However, the $\Delta\delta^{15}N_{Coral}$ values for 4 amino acids in pH 7.5 and 7.4 are significantly positively values, which suggests that reducing pH under 7.6 start negative effect to corals, probably because energy consumption of corals is larger than energy supply as photosynthetic products from symbiotic algae in seawater pH lower than 7.6.

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CHAPTER IV.

General Conclusions

In the Ph.D. thesis, a main purpose is learning the scientific tools and skills to study the effect of pollutants (e.g., toxic chemicals, nitrogen, phosphorus, CO_2, etc.) on marine ecosystems. For this purpose, we did serval studies during the last three years. For the first studies, we evaluated the effect of multiple nitrogen sources on the isotopic composition of organisms in food webs. This studies includes three parts, i.e., charactering the food web structure for fish communities, comparing nitrogen isotope ratios between tissue types, and illustrating isoscape map of coastal areas. Based on the $\delta^{15}N$ values of amino acids of organisms collected from Sagami Bay, we illuminated that:

1. The small variation of TP within species collected from Sagami Bay suggests that CSIA-AA is useful for calculating TP of organisms and illustrating food web structure even for complex ecosystems with isotopically-distinct nitrogen sources inputs.
2. The $\delta^{15}N$ values of amino acids in scale, fin, shell, and yolk are almost identical to those corresponding amino acids in muscle. These results suggest that these four tissues can be used as alternative samples to muscle in CSIA-AA, but other tissues (e.g., blood and bone) cannot.
3. From the isoscape map illustrated, we found a large variation in the $\delta^{15}N_{Baseline,phe}$ value across Sagami Bay, which reflects the incorporation of isotopically-distinct nitrogen sources.

Based on the described above, the calculation of TP of organisms by CSIA-AA is useful for studying the effect of pollutants along food webs. After I going back to China, I will continue the research related to Ph.D. thesis because the marine environments along China are increasing at risk and many environments are more complex than Sagami Bay. At this moment, I have two ideas for further studies: (1) collecting a same species in coastal areas from north to south of China. Based on the $\delta^{15}N$ values of this species, we can illustrate the isoscape map of China coastal areas and understand the contribute of nitrogen sources for these areas; (2) collecting a same species in several isolated lakes. Based on the $\delta^{15}N$ values of this species, we can understand the effect of different environments on the trophic position of single species.

Moreover, to evaluate the effect of ocean acidification on the isotopic composition of organisms, we did the second studies during the last three years. For these studies, we reared 3 fish and 1 gastropod species in pH 8.1 and pH 7.3 for five months, and 1 coral species in pH 8.1-7.4 for 2 months, and measured the $\Delta\delta^{15}N$ values in these species. Based on the measured $\Delta\delta^{15}N$, we illuminated that:

(1) The $\Delta\delta^{15}N_{Consumer}$ values for 7 amino acids are significantly negative values for fish and gastropods, which suggests that energy consumption of these species in pH7.3 experiment is lower than that in pH8.1 experiment;

(2) The $\Delta\delta^{15}N_{Coral}$ values are close to 0‰ for pH7.6 experiment, which suggests that energy consumption of corals does not change in pH7.6-8.1. However, the $\Delta\delta^{15}N_{Coral}$ values for 4 amino acids in pH 7.5 and 7.4 are significantly positively

Values which suggests that reducing pH under 7.6 start negative effect to corals.

Based on the described above, we can make sure that ocean acidification does not only have negative effect on organisms, even though we still have many unclear points. For coral experiment, I would like to note that this is preliminary results, because keeping coral in aquarium system is quite difficult even for 2 months, especially for control experiments. Thus, after I going back to China, I will continue the research related to Ph.D. thesis, as the following two studies: (1) evaluating the effect of temperature on organisms in marine environments, and (2) evaluating the effect of pH on calcified corals. The increase of concentration of CO_2 in atmosphere results in the increase of seawater temperature and the decrease of seawater pH. Thus, it should understand the effect of temperature and pH on organisms if we accurate evaluate ocean acidification. In this Ph.D. thesis, we used non-calcified coral species. Thus, I expect that, for different coral types, the effect of pH will be potentially different.

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