

Linear relationship between carbon and nitrogen isotope ratios along simple food chains in marine environments

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To examine the relationship between carbon and nitrogen stable isotope (SI) ratios ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of zooplankton, we analyzed samples collected bimonthly from March to October 2009, from the euphotic layers of the Oyashio current along the A-line in the western North Pacific. Isotopic ratios of higher trophic levels such as predatory zooplankton and/or long-lived zooplankton varied little with season, while those of short-lived zooplankton were variable on the $\delta^{15}\text{N}$ – $\delta^{13}\text{C}$ map. We also analyzed preserved samples taken from the warm-core ring 86-B derived from the Kuroshio extension region. Although the zooplankton groups in the two regions exhibited different values in $\delta^{15}\text{N}$, the $\delta^{15}\text{N}$ versus $\delta^{13}\text{C}$ slopes for each ecosystem do not show significant differences. Statistical analysis conducted together with previously published data from the Antarctic Ocean and the Gulf of Alaska suggested a similar $\delta^{15}\text{N}$ versus $\delta^{13}\text{C}$ slope throughout the four regions. We attributed this common slope to physiological aspects of feeding processes (e.g. the kinetic isotope effects inherent in the processes of amino acid synthesis). The common pattern for all four oceanic regions suggests that SIs may be used to elucidate general patterns in ecosystems and biogeochemical cycles.

KEYWORDS: stable isotopes; nitrogen; carbon; food chain; isotopic fractionation

INTRODUCTION

Over the past 20 years, rapid progress in satellite remote sensing, automatic field observation systems, computer simulation, and the development of ecosystem analytical methods such as stable isotope (SI) studies and genomic sequencing have allowed ecologists to observe natural ecosystems from entirely new perspectives. For example, using satellite-derived data, we can

clarify the distributions of phytoplankton community structures and primary production at a global scale (Behrenfeld and Falkowski, 1997; Behrenfeld *et al.*, 2005; Alvain *et al.*, 2008; Hirata and Brewin, 2009; Okamoto *et al.*, 2010). Considerable progress has also been made in marine ecosystem modeling, although there remain large discrepancies in ecosystem structure

between models and observations (Aita *et al.*, 2007; Sumata *et al.*, 2010). For ecosystem structure, complex processes within food chains are elucidated by energy flux, but this does not provide information at species, metabolic or molecular levels. Genetic analysis is mainly applied to clarify positions in relation to phylogeny and ecosystem evolution. On the other hand, SI analysis is a useful tool for the study of biogeochemical cycles as well as ecosystem structures. We can determine the structure of food webs and the interactions between organisms using distributions and variation in C/N isotope ratios together with their fractionations (Wada, 2009).

Variation in the SI ratios of nitrogen ($\delta^{15}\text{N}$) and carbon ($\delta^{13}\text{C}$) are well studied for single-feeding processes: $\delta^{15}\text{N}$ is enriched by $\sim 3\text{‰}$ – 4‰ per trophic level (TL) (DeNiro and Epstein, 1981; Minagawa and Wada, 1984; Fry, 1988). In subtropical and tropical seas, where phytoplankton grow under nitrogen-deficient conditions in the euphotic layer, blue–green algae fix molecular nitrogen and exhibit $\delta^{15}\text{N}$ values of approximately -2‰ (Minagawa and Wada, 1986; Carpenter *et al.*, 1997). At high latitudes, phytoplankton $\delta^{15}\text{N}$ exhibit rather low values of -1‰ to 3‰ by nitrogen isotope fractionation under high nitrate concentrations with $\delta^{15}\text{N}$ of ca. 6‰ , whereas nitrate $\delta^{15}\text{N}$ increases with decreasing utilization of nitrate by phytoplankton (e.g. Sigman and Casciotti, 2001). The $\delta^{15}\text{N}$ values of algae differ widely from -1‰ to 10‰ depending on the forms of inorganic nitrogen utilized. Wada and Hattori (Wada and Hattori, 1991) broadly divided phytoplankton in the North Pacific into three types based on $\delta^{15}\text{N}$ and the three major forms of nitrogenous compounds utilized: NO_3^- , NH_4^+ and N_2 . In marine environments, the primary producer (phytoplankton) provides the base of the food chain for zooplankton and fishes, in turn affecting the $\delta^{15}\text{N}$ values of animals at higher TLs. Chiba *et al.* (Chiba *et al.*, 2008) examined regional differences in $\delta^{15}\text{N}$ of major copepod species in the subarctic North Pacific. Areal $\delta^{15}\text{N}$ of *Neocalanus* spp. was high in the western and eastern North Pacific (7‰ – 10‰) and low in the central North Pacific (2‰ – 4‰) reflecting the gradient of nitrate concentration.

Phytoplankton have lower $\delta^{13}\text{C}$ values than coastal C4 plants, such as eelgrass (average $\delta^{13}\text{C}$ is about -10‰ , generally ranging from -15‰ to -3‰ ; Fry and Sherr, 1984). This difference is used to determine the carbon source of the primary producer and consumer and whether the carbon source is terrestrial, coastal or open-ocean (Fry, 2006). For $\delta^{13}\text{C}$ in the ocean, McConnaughey and McRoy (McConnaughey and McRoy, 1979) investigated food-web structure from the viewpoint of fractionation of carbon isotopes in the

Bering Sea. They reported that stepwise $\delta^{13}\text{C}$ enrichment is 1.5‰ per TL. Rau *et al.* (Rau *et al.*, 1983) showed that the average increase in $\delta^{13}\text{C}$ per TL ranged from 0.7‰ to 1.4‰ for samples from eastern equatorial Pacific and California coastal waters, whereas about 1‰ $\delta^{13}\text{C}$ per TL was reported for rearing systems and sea grass meadows (DeNiro and Epstein, 1978; Rau *et al.*, 1983; Fry and Sherr, 1984).

Evidence suggests that SIs have the potential to reveal complex interactions, including trophic interactions and energy or mass flow through ecological communities (Peterson and Fry, 1987; Kling *et al.*, 1992; Cabana and Rasmussen, 1996). However, precise examinations of trophic fractionation have been mostly limited to freshwater ecosystems (Vander Zanden and Rasmussen, 1999; Post *et al.*, 2000; Post, 2002). At present, the magnitude of trophic fractionation of carbon isotopes in natural ecosystems remains unclear and requires further study with emphasis on kinetic isotope fractionation during feeding processes in marine ecosystems. For example, $\Delta\delta^{13}\text{C}$ per TL seems to be low in lakes and coastal areas and higher in pelagic areas of the ocean (Wada *et al.*, 1987; Post, 2002).

The $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ of animals are thus greatly affected by their feeding processes within the food chain. Based on this fact, we focus on the ratio of C and N trophic fractionation per TL ($\Delta\delta^{15}\text{N}/\Delta\delta^{13}\text{C}$), which could be obtained as the slope between different TLs in plots of $\delta^{15}\text{N}$ versus $\delta^{13}\text{C}$. In this study, we analyzed isotopic ratios of plankton samples taken from the Oyashio waters and from warm-core ring (WCR) 86-B. The results of isotopic ratios and comparison with $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ ratios for both regions are described in the ‘Results’ section. In the ‘Discussion’ section, we discuss the $\Delta\delta^{15}\text{N}/\Delta\delta^{13}\text{C}$ slope using our data combined with data from the Antarctic Ocean (Wada *et al.*, 1987) and the Gulf of Alaska (Kaeriyama, 2004). If we could better understand both carbon and nitrogen trophic fractionation within ecosystems, SIs may help to elucidate general patterns in ecosystems and biogeochemical cycles.

METHOD

Study area (Oyashio)

Plankton samples were collected in the Oyashio waters along the A-line monitoring transect ($38^\circ 00'\text{N}$ – $42^\circ 50'\text{N}$, $144^\circ 50'\text{E}$ – $147^\circ 50'\text{E}$; Fig. 1a) of the Fisheries Research Agency (FRA) of Japan. The transect extends from the cold Oyashio current to the warm Kuroshio current in the western North Pacific. The Oyashio flows southward along the Kuril Islands, and its first and

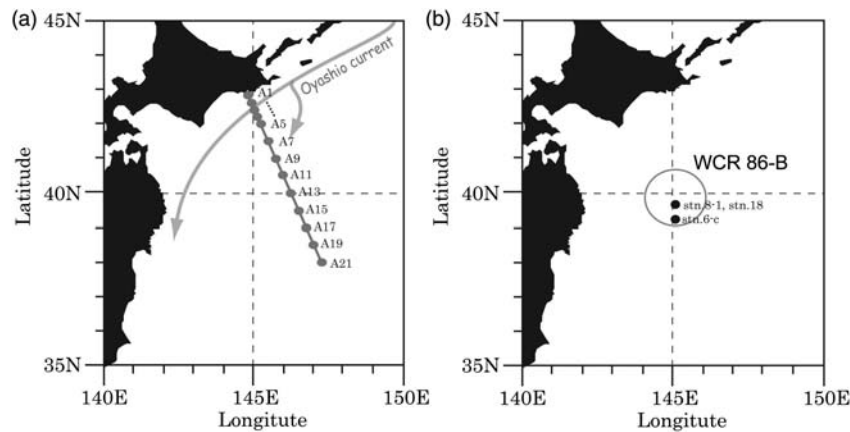


Fig. 1. Sampling area and location of the sampling stations of the A-line (a) and WCR 86-B (b).

second branches fork near Sanriku, Japan. In this region, ~ 100 km diameter warm eddies, called WCRs, extend from the Kuroshio current (Okuda, 1991).

Samples were collected along the A-line bimonthly from March to October 2009, during cruises of the R.V. *Wakataka-maru* of the Tohoku National Fisheries Research Institute (FRA, WK-09-03 and WK-09-07 cruises), the R.V. *Kaiyo-maru* of the Fisheries Agency (KY-09-07 cruise) and the R.V. *Hokko-maru* of the Hokkaido National Fisheries Research Institute, FRA (HK-09-10 cruise).

Study area (WCR 86-B)

Samples from the WCR, which formed from the meanders of the Kuroshio extension in mid-March 1986 (Fig. 1b; Saino, 1992; Tsuda and Nemoto, 1992), were collected during the WCR 86-B cruise KH-87-4 of the *Hakuho-Maru* of the University of Tokyo, from 1 to 25 September 1987. In the WCR, thermostad waters of $\sim 10^\circ\text{C}$ were observed from 75 to 350 m at the center of the water mass (Saino, 1992), and water-column nitrate concentrations were $< 0.2 \mu\text{M}$. Samples were collected from the surface and 600 m depth using 10 horizontal Motoda (MTD) nets (Motoda, 1971) of 56 cm mouth diameter and 0.33 mm mesh size (Tsuda and Nemoto, 1992; Hasumoto, 2006). After collection, all samples were desiccated and preserved in sealed glass containers. We used zooplankton samples from the upper 150 m for analysis of isotopic ratios and comparison with $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ ratios of the Oyashio.

Temperature and nitrate and chlorophyll *a* concentrations (Oyashio)

Vertical temperature profiles were measured at all stations with a CTD (conductivity–temperature–depth;

Sea Bird Electronics, WA, USA), and water samples for analyses of nitrate and chlorophyll *a* concentrations were collected using Niskin bottles. Water samples for nitrate were immediately frozen at -20°C until analysis using a nutrient analyzer AACS-III (BRAN + LUEBBE, Norderstedt, Germany). Water samples were filtered with Whatman GF/F filters for chlorophyll *a* analysis; samples underwent extraction with *N,N*-dimethylformamide (DMF). Fluorescence was then measured using a fluorometer (Turner Designs, Sunnyvale, CA, USA; Holm-Hansen *et al.*, 1965; Kasai *et al.*, 2001; Saito *et al.*, 2002). Along the A-line, we identified the Oyashio waters based on temperatures at 100 m depth, following Kawai (Kawai, 1972) and Odate (Odate, 1994); water masses with temperatures below 5°C at 100 m depth were classified as the Oyashio waters.

Dominant zooplankton taxa (Oyashio)

In the Oyashio region, copepods are the most important species, constituting over 80% of total mesozooplankton biomass, followed by chaetognaths, euphausiids and amphipods (Ikeda *et al.*, 2008). Four species of chaetognaths (*Sagitta elegans*, *Eukrohnia hamata*, *Eukrohnia bathypelagica* and *Eukrohnia fowleri*) occur in the Oyashio waters, and only *S. elegans* occurs shallower than 250 m (e.g. Ikeda *et al.*, 2008). Kim *et al.* (Kim *et al.*, 2009) found that 80% of the abundance of three species of euphausiids in the Oyashio region was composed of *Euphausia pacifica*. Among copepods, the four dominant species, *Neocalanus cristatus*, *Neocalanus flemingeri*, *Neocalanus plumchrus* and *Eucalanus bungii*, exhibit different life cycles and vary seasonally in abundance (Mackas and Tsuda, 1999; Tsuda *et al.*, 1999; Kobari *et al.*, 2003). In particular, the life histories of *E. bungii* and *Neocalanus* spp. differ substantially. For *Neocalanus* spp., the peak

abundance of *N. cristatus* C5 occurred from May to July, when lipid storage was primarily observed in the C5 stage (Tsuda *et al.*, 2004). Mature populations descend to below 500 m depth and spawn from October to December in the Oyashio region. Hatched populations in the deep layer ascend to the surface layers and continue to graze and grow into adults in the mixed layer. Larger *N. cristatus* remain for ~6 months in the mixed layer, but smaller *N. flemingeri* stay for ~1.5 months (Miller *et al.*, 1984; Kobari and Ikeda, 1999; Tsuda *et al.*, 1999, 2004). *Eucalanus bungii*, on the other hand, spawns near the surface layers during the spring bloom. Larvae of *E. bungii* prey on phytoplankton during the season of high primary production and algal blooms (Miller *et al.*, 1984; Tsuda *et al.*, 2004; Kobari *et al.*, 2007).

Collection of plankton samples (Oyashio)

We collected specimens of copepods, amphipods, euphausiids and chaetognaths for SI analyses, with particular emphasis on dominant species in the western North Pacific. Samples were collected from 150 m depth to the surface using vertical tows of a NORPAC net (45 cm mouth diameter, 0.33 mm mesh size) at a speed of 0.5 m s⁻¹. After collection, samples were filtered through a 0.33 mm mesh net; plankton on the net were immediately frozen at -80°C onboard until analysis. In the laboratory, all samples were thawed and rinsed quickly with a NaCl solution as isotonic seawater to remove bicarbonate (HCO₃⁻) or inorganic carbon salts and were then sorted. Copepods were classified under a stereomicroscope into four species: *N. cristatus*, *N. flemingeri*, *N. plumchrus* and *E. bungii*. We used copepodite stage C5 for isotopic analysis. A considerable amount of algae was also collected in May by vertically towing a NORPAC net from 150 m to the surface. Algae were sorted using tweezers under a stereomicroscope and were also analyzed for isotopic ratios.

Sample preparation (Oyashio and WCR 86-B)

Zooplankton and algal samples were dried at 60°C for more than 12 h. All samples were ground into a fine powder using an agate mortar and pestle. Lipid fractions were removed because of their generally low δ¹³C values relative to whole organisms (DeNiro and Epstein, 1977; Monson and Hayes, 1982). Lipids were extracted and removed from samples with 1 mL methanol, 1 mL dichloromethane/methanol (7:1) and 1 mL dichloromethane/methanol (10:1) using an ultrasonicator (20 min) prior to isotopic analyses (Ohkouchi *et al.*, 1997).

Isotopic analysis (Oyashio and WCR 86-B)

Carbon and nitrogen isotopic ratios (δ¹³C and δ¹⁵N) of zooplankton and algae were determined using an elemental analyzer/isotope-ratio mass spectrometer (EA/IRMS, ThermoFinnigan FlashEA1112, ConFloIII, DeltaPlusXP; Ohkouchi *et al.*, 2005; Ogawa *et al.*, 2010) at the Institute of Biogeosciences, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), the Japan Chemical Analysis Center (Thermo Fisher Scientific, Flash2000, GC-IsoLink, Delta V advantage) or at SI Science Co., Ltd. (Thermo Fisher Scientific, Flash2000, ConFloIV, Delta V). The lipid-free samples were transferred to pre-cleaned tin capsules and then introduced into the EA/IRMS system. Isotope values are reported in standard δ-notation relative to the international standards:

$$\delta X(\text{‰}) = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 10^3,$$

where X is ¹³C or ¹⁵N, and R the isotopic ratio of ¹³C/¹²C or ¹⁵N/¹⁴N of the sample and standard, respectively. Samples were referenced to the following standards: Vienna-PeeDee Belemnite limestone for carbon and atmospheric nitrogen (AIR) for nitrogen. International and/or in-house standard materials (tyrosine, proline, alanine and glycine) were measured alongside samples to calibrate the isotope data (Ogawa *et al.*, 2010; Sato and Suzuki, 2010). The analytical errors associated with the standard materials were less than ± 0.2‰ for both carbon and nitrogen.

Data analysis (Oyashio and WCR 86-B)

In the Oyashio waters, seasonal differences in isotopic ratios (δ¹⁵N and δ¹³C) of zooplankton were analyzed using one-way analysis of variance (ANOVA) and analysis of covariance (ANCOVA). ANCOVA was used to test for differences among the slopes (δ¹⁵N/δ¹³C) of the regression lines among seasons or oceanic regions. Regression analysis was used to examine the overall trend in the δ¹⁵N and δ¹³C of animals in food chains. δ¹³C was treated as a covariate, and sampling region was the independent variable. The interaction between δ¹³C and sampling region had no significant effects on the δ¹⁵N of the samples and was therefore discarded from the analyses. In cases where ANCOVA tests were significant, the overall significance of differences among the sampled seasons or regions was tested using Tukey's honestly significant difference (HSD) *post hoc* tests at α = 0.05. For each sample, linear regression analysis was also applied to examine the relationships of δ¹⁵N with δ¹³C. All statistical analyses were conducted using JMP

software (version 8.0.2 for Windows, SAS Institute, Inc., Cary, NC, USA).

RESULTS

Seasonal changes and environmental conditions on the A-line

We collected samples during four cruises in March, May, July and October. Seasonal changes in average temperature as well as nitrate and chlorophyll *a* concentrations of the water column are shown with corresponding mixed layer depths (MLDs; Fig. 2). In March, the mean water temperature of the Oyashio region at stations A2–A5 was $1.1^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ (mean \pm SD), and nitrate concentrations were $23.4 \pm 3.1 \mu\text{M}$. The mean water temperature increased from $2.4^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ (A2–A6) to $12.3^{\circ}\text{C} \pm 1.4^{\circ}\text{C}$ (A2–A4.5) in May (spring) to July (summer), whereas nitrate concentrations decreased

from 18.0 ± 4.9 to $6.9 \pm 4.4 \mu\text{M}$. During this time, chlorophyll *a* decreased from $4.9 \pm 3.1 \text{ mg m}^{-3}$ in May to $0.64 \pm 0.33 \text{ mg m}^{-3}$ in July. The spring bloom appeared to occur in May. In October (autumn), the mean water temperature and nitrate concentration were $12.2^{\circ}\text{C} \pm 2.4^{\circ}\text{C}$ (A3–A5) and $5.8 \pm 2.2 \mu\text{M}$ (A3–A7), respectively. Saito *et al.* (Saito *et al.*, 2002) sampled the A-line from 1990 to 1998 and found that nitrate and silicate decreased after April and was lowest in August or October.

In March and May, a WCR formed south of 42.5°N , and between A5 and A7, where the temperature of the water column increased to $\sim 7^{\circ}\text{C}$, whereas the nitrate concentration was $10 \mu\text{M}$. Chlorophyll *a* at the central part of the WCR, A7, was $2.1 \pm 0.3 \text{ mg m}^{-3}$, higher than that measured in the Oyashio region ($0.35 \pm 0.02 \text{ mg m}^{-3}$) at the same time. These results show that photosynthetic activity was high in the WCR. In the WCR formed around A7–A11 in May, the mean water temperature was $\sim 8^{\circ}\text{C}$ higher than that of the Oyashio

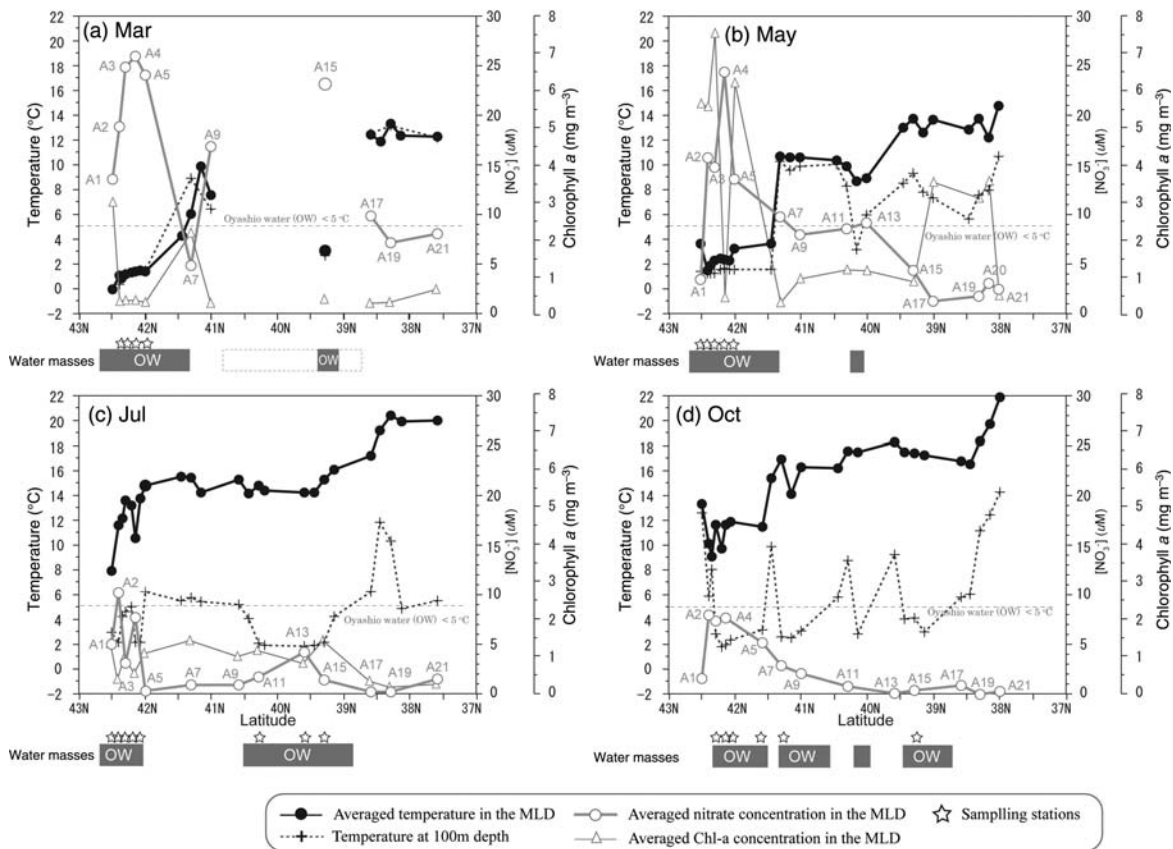


Fig. 2. Seasonal and latitudinal variation in the physical, chemical and biological characteristics of the A-line of the western subarctic North Pacific. Averaged temperature (filled circles), nitrate (open circles) and chlorophyll *a* concentration (open triangles) in the MLD. The MLD is defined as the depth at which the potential density (σ_t) becomes 0.125 kg m^{-3} higher than the surface value (Aita *et al.*, 2007). Along the A-line, we distinguished between the Oyashio and Kuroshio waters based on the temperature at 100 m depth, where the isotherm is relatively stable, with little seasonal variation. Following Odate (Odate, 1994), we classified water masses with temperatures (at 100 m; plus symbol) below 5°C as the Oyashio. Gray bar shows water masses of the Oyashio waters (OW). The symbol star represents plankton sampling stations.

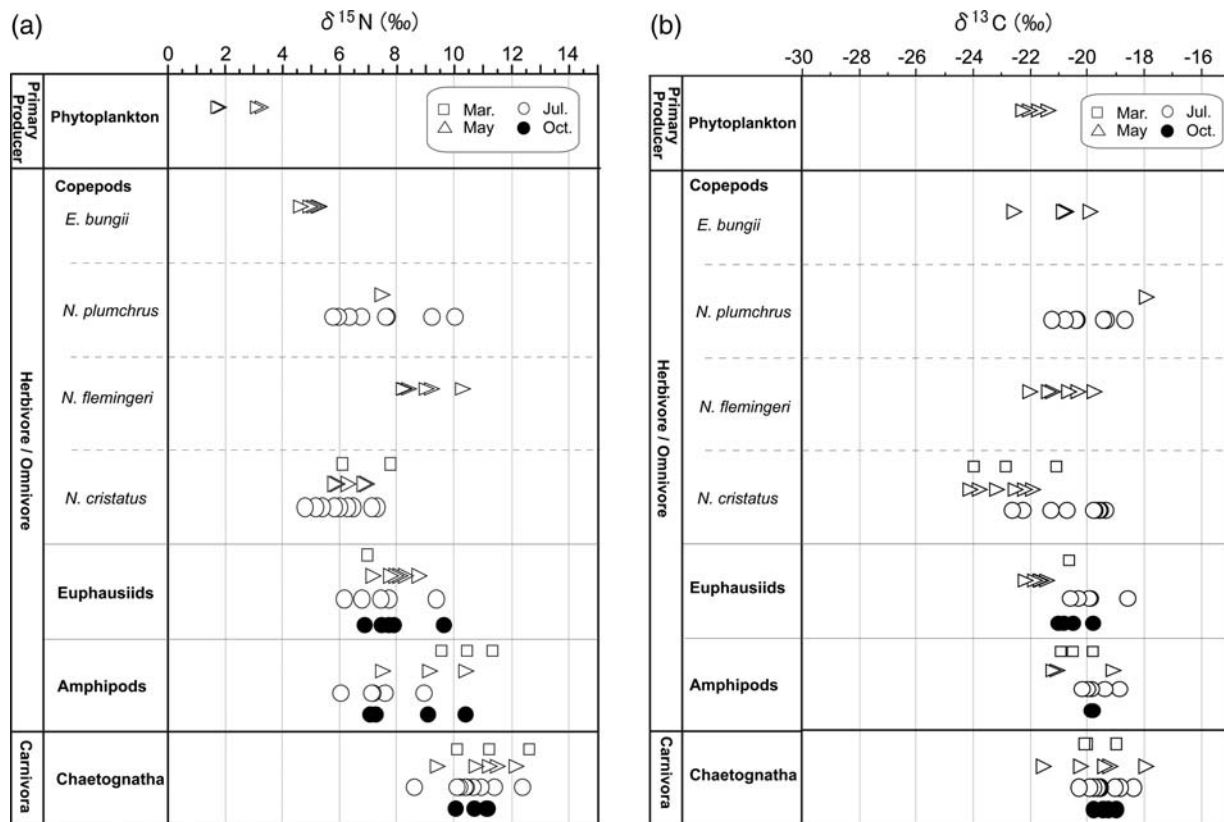


Fig. 3. Distribution of (a) nitrogen and (b) carbon isotope ratios of algae and dominant species of zooplankton collected from the A-line in the western subarctic North Pacific.

hand, the $\delta^{13}\text{C}$ of *N. cristatus*, euphausiids and amphipods differed significantly among seasons (ANOVA, $F = 8.83$, $df = 2/15$, $P < 0.0029$ for *N. cristatus*; $F = 10.52$, $df = 3/13$, $P < 0.0009$ for euphausiids; $F = 8.30$, $df = 3/11$, $P < 0.0037$ for amphipods), but that of other zooplankton did not ($P > 0.05$).

In May, we collected *E. bungii* and three species of *Neocalanus* spp. (indicated by open triangle in Fig. 3a). The $\delta^{15}\text{N}$ value of *E. bungii* was lowest, at $5.1 \pm 0.3\text{‰}$ (mean \pm SD). The $\delta^{15}\text{N}$ increased in the following order: *N. cristatus*, $6.5 \pm 0.5\text{‰}$; *N. plumchrus*, 7.4‰ ; and *N. flemingeri*, $8.9 \pm 0.8\text{‰}$. For algae and zooplankton, the observed yearly range of $\delta^{13}\text{C}$ was -23‰ to -19‰ , which was narrower than that of $\delta^{15}\text{N}$ (2‰ to 12‰). Annual mean $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ increased from copepods to euphausiids to chaetognaths (Table I). Figure 4a–d presents the relationship between $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ and the linear regression line for zooplankton in the Oyashio waters. ANCOVA tests for seasonal differences of $\delta^{15}\text{N}/\delta^{13}\text{C}$ ratios among seasons (using the data in Table I) were inconclusive.

Differences in $\delta^{15}\text{N}/\delta^{13}\text{C}$ ratios of zooplankton between Oyashio and WCR 86-B

The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for each species and sampling location in the WCR 86-B are presented in Table II (mean value of plankton samples) and Supplementary Table SII (list of each species and location). The preserved samples included salps, copepods, amphipods and chaetognaths. Euphausiids were not observed in the upper 150 m of the WCR 86-B. For $\delta^{15}\text{N}$, amphipods had the highest value at 9.8‰ , whereas copepods and chaetognaths had nearly the same value (8.4 – 8.6‰). A comparison of $\delta^{15}\text{N}$ – $\delta^{13}\text{C}$ on the WCR 86-B and Oyashio maps (Fig. 5) reveals that values for zooplankton in the Oyashio varied more widely, especially for copepods. The slope of the fitted linear regression was steeper for zooplankton from the Oyashio. However, the ANCOVA did not reveal significant differences ($P > 0.05$) between the Oyashio and WCR 86-B or conclusive evidence that their respective slopes were the same ($P > 0.05$).

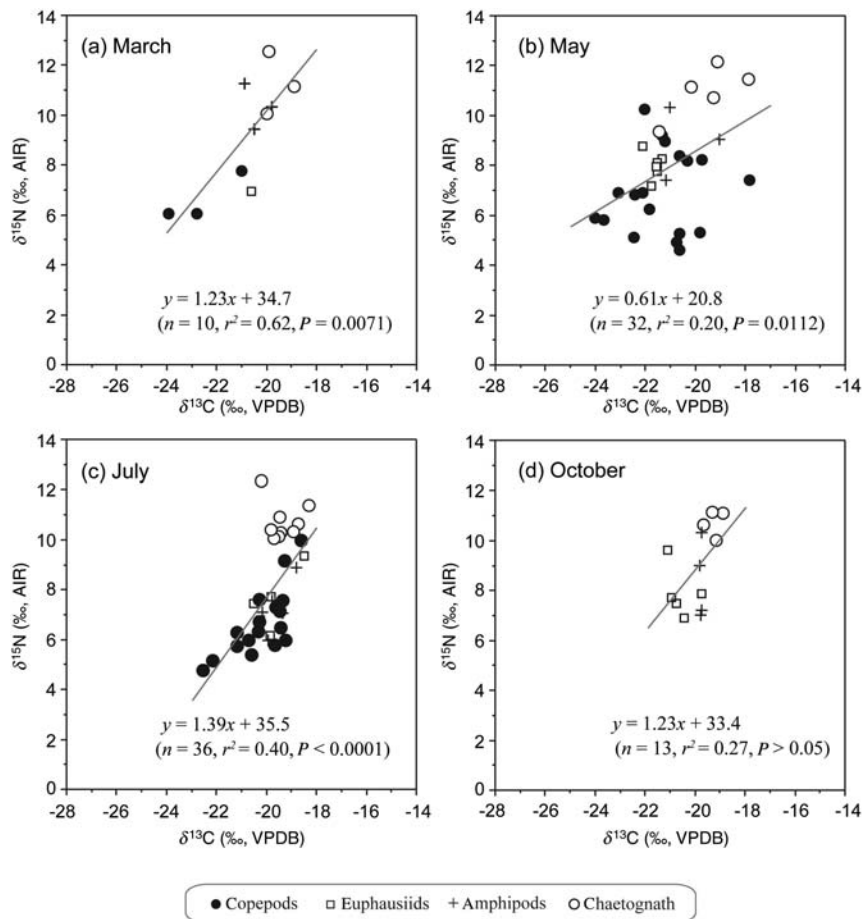


Fig. 4. Relationship between $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for zooplankton at the A-line in the Oyashio waters from March to October. Solid lines are derived from linear regressions of each month. Numbers in parentheses indicate the number of samples used for the analyses. The slopes of the lines in March, May, July and October were 1.23, 0.61, 1.39 and 1.23, respectively. The correlation coefficient, r^2 , for mean $\delta^{13}\text{C}$ versus $\delta^{15}\text{N}$ was 0.62 for March, 0.20 for May, 0.40 for July and 0.27 for October. The symbol filled circle represents copepods; square, euphausiids; plus, amphipods; open circle, chaetognaths.

DISCUSSION

Distributions of nitrogen and carbon isotope ratios of algae and dominant species of zooplankton

Discrepancies exist in the observed $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values of each group of zooplankton within the Oyashio region (Fig. 3a and b). Two factors may contribute to this result: differences in life cycle according to body size and differences in the depths of occurrence. Euphausiids, which have a long life cycle of 17–28 months, and chaetognaths, a top predator among zooplankton, exhibited nearly the same isotopic ratios across seasons (Figs 3 and 4). In contrast, the isotopic ratios of amphipods were similar to those of chaetognaths in March but were nearly the same as those of copepods from May to July. Zooplankton with short life

Table II: Nitrogen and carbon isotope ratios of zooplankton collected at WCR 86-B in the western North Pacific from 1 to 25 September 1987

Species	$\delta^{15}\text{N}$ (‰)	\pm SD	$\delta^{13}\text{C}$ (‰)	\pm SD	<i>n</i>
Phytoplankton	n.d.		n.d.		
Salps	6.7	0.6	-22.3	0.4	7
Copepods	8.6	0.3	-20.0	0.4	2
Amphipods	9.8	0.8	-19.5	1.2	2
Chaetognath	8.4		-19.3		1

All species were collected upper 150 m and values are mean \pm SD. 'n' indicates the number of samples analyzed (not population size). 'n.d.' indicates no data.

spans (1 month to <1 year), such as copepods and amphipods, were widely spaced on the $\delta^{15}\text{N}$ – $\delta^{13}\text{C}$ map (Fig. 4). Populations with large body sizes and that live in surface layers for long periods of time show less

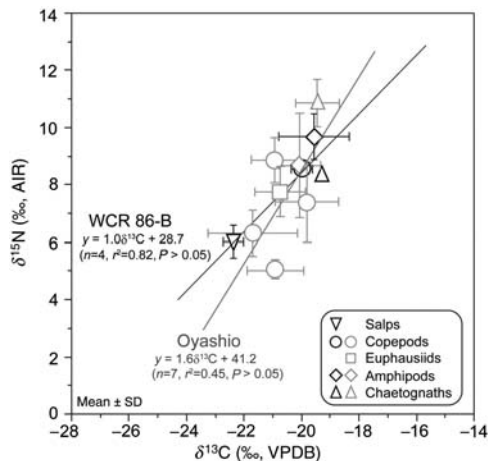


Fig. 5. Relationship between $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for zooplankton aggregated by group from the Oyashio waters (annual means, Table I, shown in gray) and WCR 86-B (from Table II, shown in black). Solid lines are derived from linear regressions.

variability in their isotope ratios than those with small body sizes that prey over shorter time spans. One reason for this pattern may be that for large-bodied populations with longer life spans, isotopic ratios reflect the mean integrated value of feeding over a longer period (Chikaraishi *et al.*, 2007); thus, the $\delta^{15}\text{N}$ of euphausiids and chaetognaths varied little with season.

The nitrate concentration in seawater changes remarkably in the mixed layer depending on changes in physical conditions and uptake of nitrate by phytoplankton. Hence, in the present study, $\delta^{15}\text{NO}_3^-$ may increase with decreases in nitrate concentration, particularly in the euphotic layer (Saino and Hattori, 1980; Saino, 1992; Sigman and Casciotti, 2001). Isotope fractionation is correlated with the growth rate (μ ; Takahashi *et al.*, 1991; Wada and Hattori, 1991). The growth rate of oceanic phytoplankton and the nitrogen isotope fractionation factor in nitrate assimilation (α) are negatively related: α decreases from 1.015 to 1.001 as μ increases, until isotope fractionation becomes negligibly small at the fastest growth rates (Wada and Hattori, 1991). Thus, phytoplankton $\delta^{15}\text{N}$ values differ depending on the depth at which phytoplankton grow. If zooplankton inhabit different depth ranges seasonally as shown by Takahashi *et al.* (Takahashi *et al.*, 2008), the $\delta^{15}\text{N}$ of zooplankton will also differ depending on the depth of sampling. Because we sampled using 150 m vertical tows in this study, we might have collected a mixed population from the surface layers to below the mixed layer within the same sampling net. We suspect that this dependence on sampling depth contributed to the observed variability of zooplankton $\delta^{15}\text{N}$.

Carbon and nitrogen trophic discrimination factor in the Oyashio waters

Wada *et al.* (Wada *et al.*, 1987) showed a clear linear relationship between the chemical parameter of isotopic ratios and the ecological parameter of TL. Mearns (Mearns, 1982) defined TL by the origin or food of small prey items identified from stomachs of predators from three Pacific Ocean food webs. He assumed that $\text{TL} = 2.0$ for calanoid copepods as phytoplankton feeders and that $\text{TL} = 3.5$ for chaetognaths preying on larval fish rather than zooplankton (Mearns, 1982). We estimated inherent ratios of trophic fractionation of $\delta^{15}\text{N}$ ratios in the Oyashio based on linear regressions of observed $\delta^{15}\text{N}$ for zooplankton versus Mearns' assumed TLs.

In the Oyashio, we collected algae, *E. bungii*, *Neocalanus* spp., chaetognaths and other zooplankton in May. Therefore, the TL of algae was 1.0, and the TL of zooplankton was estimated by assuming a TL of 2.0 for *E. bungii*, which had the lowest value among the copepods, and a TL of 3.5 for chaetognaths (Mearns, 1982). The TL for other zooplankton was estimated based on their observed mean $\delta^{15}\text{N}$ values in May, assuming a linear relationship between $\delta^{15}\text{N}$ and TL. The enrichment of $\delta^{15}\text{N}$ per TL in the Oyashio (in May) was $3.5 \pm 0.2\text{‰}$ (mean \pm SE; $r^2 = 0.91$, $P < 0.01$). Using the TL estimated from $\delta^{15}\text{N}$, the mean amplitude of $\delta^{13}\text{C}$ enrichment per TL was $0.9 \pm 0.3\text{‰}$ ($r^2 = 0.16$, $P < 0.05$). These values agree with the $3 \pm 1\text{‰}$ per TL for $\delta^{15}\text{N}$ reported by DeNiro and Epstein (DeNiro and Epstein, 1981), Minagawa and Wada (Minagawa and Wada, 1984) and Fry (Fry, 1988), whereas $\delta^{13}\text{C}$ enrichment per TL was consistent with values of 0.7–1.5‰ per TL (average of 1‰) according to McConnaughey and McRoy (McConnaughey and McRoy, 1979) and Rau *et al.* (Rau *et al.*, 1983).

$\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ map for food chains in the Oyashio, WCR 86-B, Antarctic Ocean and Gulf of Alaska

To test whether each ecosystem exhibits a distinct linear relationship between $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ ratios along the food chain, we combined our data with data from the Antarctic Ocean (AO; Wada *et al.*, 1987, Supplementary Table SIII) and the Gulf of Alaska (ALS; digitized and redrawn from Fig. 1 of Kaeriyama, 2004). We examined the combined data for differences among the four oceanic regions. Figure 6 combines data from Fig. 5 [the Oyashio (OY) and WCR 86-B] with those from the AO and ALS. Because different species were collected at each station,

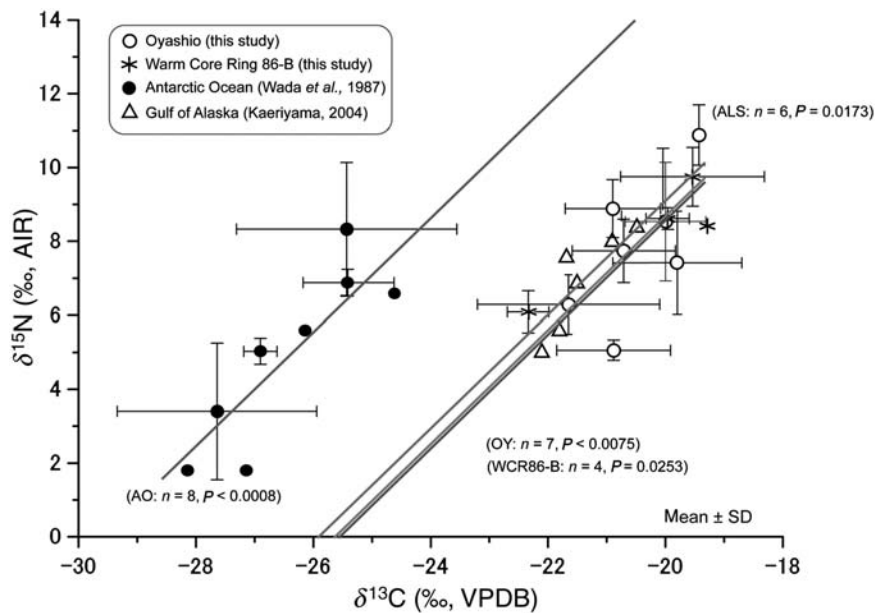


Fig. 6. Relationship between $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for zooplankton and fish from four oceanic regions: Oyashio: OY (open circle), WCR 86-B (asterisk), Antarctic Ocean: AO (filled circle; taken from Wada *et al.*, 1987) and the Gulf of Alaska: GA (open triangle; mean data redrawn from Kaeriyama, 2004). Symbols represent means \pm SD, and solid lines are fits for each region from ANCOVAs.

Table III: Results of ANCOVA on $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ of zooplankton in four oceanic regions: Oyashio, WCR 86-B, Antarctic Ocean and Gulf of Alaska

Parameter	df	F	P-value
(a) Response variable: $\delta^{15}\text{N}$ (‰)			
$\delta^{13}\text{C}$ (‰)	1	24.7808	<0.001
Regions	3	5.3422	0.0089
$\delta^{13}\text{C} \times$ regions	3	0.5183	0.6753
(b) Response variable: $\delta^{13}\text{C}$ (‰)			
$\delta^{15}\text{N}$ (‰)	1	32.5640	<0.001
Regions	3	66.1239	<0.001
$\delta^{15}\text{N} \times$ regions	3	1.4537	0.2624
(c) Response variable: $\delta^{15}\text{N}$ (‰)			
$\delta^{13}\text{C}$ (‰)	1	37.4618	<0.001
Regions	3	5.5583	0.0061
(d) Response variable: $\delta^{13}\text{C}$ (‰)			
$\delta^{15}\text{N}$ (‰)	1	37.4618	<0.001
Regions	3	65.8374	<0.001

Significant effects at $P = 0.05$ are indicated in bold.

we aggregated the data by zooplankton group (salps, copepods, euphausiids, amphipods and chaetognaths) and used the average values for each group in the analysis.

The $\delta^{15}\text{N}-\delta^{13}\text{C}$ map of each region indicates that the range of $\delta^{15}\text{N}$ was 1‰–15‰, and the range of $\delta^{13}\text{C}$ was generally -24‰ to -18‰ , except for low $\delta^{13}\text{C}$ values in the AO ($\delta^{15}\text{N} = 0\text{‰}-11\text{‰}$; $\delta^{13}\text{C} = -33\text{‰}$ to -23‰). ANCOVA tests using the respective isotopic ratios as the response variable revealed no significant interactions

between oceanic region and either $\delta^{15}\text{N}$ or $\delta^{13}\text{C}$ (Table IIIa and b; $P > 0.05$). A common $\delta^{15}\text{N}/\delta^{13}\text{C}$ slope was found, with only the intercept differing among the four oceanic regions (Table IIIc and d and linear regression line in Fig. 6; $P < 0.001$). For $\delta^{15}\text{N}$ as a response variable, the regression yielded:

$$\delta^{15}\text{N} = 1.53[\pm 0.25]\delta^{13}\text{C} + 40.9[\pm 5.6] + (\text{respective constant for each region}),$$

where the constant values for each region were OY: -1.71 ($P = 0.0075$), WCR 86-B: -1.61 ($P = 0.0253$), AO: 4.49 ($P = 0.0008$) and ALS: -1.17 ($P = 0.0173$).

Tukey's HSD *post hoc* tests for differences among the slopes of the fitted lines revealed that AO differed significantly from OY, WCR 86B and ALS (Fig. 7). Both the subarctic North Pacific and Antarctic Oceans are typical high-nutrient, low-chlorophyll regions where iron limits biological productivity (Martin and Fitzwater, 1988; Sohrin *et al.*, 2000; Bowie *et al.*, 2001; Tsuda *et al.*, 2003). The seasonal amplitudes of temperature and nitrate concentrations in the Gulf of Alaska are smaller than those in the Oyashio, but both regions are located in the same subarctic North Pacific gyre. In contrast, surface water temperature of the Antarctic Ocean is -0.5°C to 2°C (Wada *et al.*, 1987), and productivity is rather low, despite high nitrate and silicate concentrations (e.g. Garcia *et al.*, 2010). In the AO, phytoplankton are limited by low light intensity and water

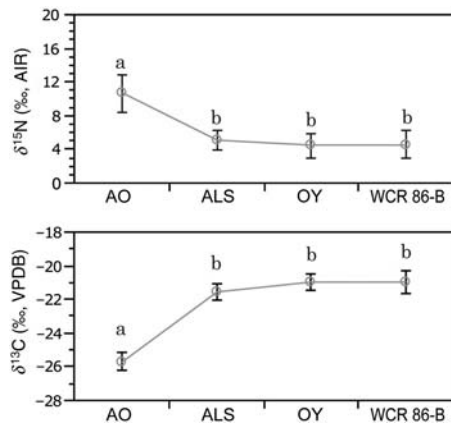


Fig. 7. Averaged $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ of samples from four oceanic regions (least mean squares fit \pm SE): Oyashio (OY), WCR 86-B, Antarctic Ocean (AO) and Gulf of Alaska (ALS). Within each panel, numbers with the same letter are not significantly different according to the Tukey's HSD *post hoc* test at $\alpha = 0.05$ applied to the results of ANCOVAs (Table IIIc and d).

temperature rather than by nutrient concentration, causing the low isotopic compositions (Wada *et al.*, 1987; Wada and Hattori, 1991), which may partially explain the difference between zooplankton from AO and those from other regions.

Based on the ANCOVA tests, we obtained common slopes (parallel relationships) with different intercepts for the four oceanic regions. This common relationship may result from the kinetic isotope effect inherent to the processes of amino acid synthesis as part of intermediary metabolism. It seems likely that the intercept of each parallel line depended on the isotopic composition of primary producers. The main metabolic pathways are common in almost all cells and organisms, and, in general, isotope ratios within organisms depend on the isotopic composition of reactants and on branch reactions within any *in vivo* metabolic pathway, such as carboxylation and some transamination reactions (Minagawa *et al.*, 1992). Additionally, Chikaraishi *et al.* (Chikaraishi *et al.*, 2009) proposed the use of the amino acid trophic level (ATL), with an emphasis on the occurrence of nitrogen isotope fractionation during amination and deamination processes. We therefore suspect that kinetic isotope effects are generated during the synthesis of amino acids (Macko *et al.*, 1986; Minagawa *et al.*, 1992). The composition of primary producers may also determine the intercept for each ecosystem on the $\delta^{15}\text{N}$ – $\delta^{13}\text{C}$ map (Wada and Hattori, 1991). In fact, significant linear relationships between carbon and nitrogen isotope effects at the protein synthesis level were observed in the organs of a cormorant that consumed the same type of food for 23 years (Mizutani *et al.*, 1991). According to the simple mass balance calculation, the

effect of this process does not alter the appearance of linearity on the isotopic map if the fractionation factor of the amino acid synthesis is constant throughout the food chain from primary producers to the animal at the highest TL. Consistent with this, the ANCOVA analyses suggested that the nitrogen and carbon isotope fractionation is constant irrespective of animal species in the present results. Considering these facts, the varying $\delta^{15}\text{N}/\delta^{13}\text{C}$ slopes might result from other factors that we have not analyzed, such as the availability of micronutrients for primary producers, *in vivo* amino acid metabolism and NH_4^+ excretion systems including the urea cycle and processing of tricarboxylic acid cycle (TCA cycle). The magnitude of the fractionation factor may vary depending upon the dynamic operation of energy production systems involving glycolysis, TCA cycle and oxidative phosphorylation, which are associated with the synthesis of the carbon skeleton of amino acids (Pecquerie *et al.*, 2010).

The detrital food chain and the grazing food chain occur simultaneously in places such as lagoons, where $\delta^{13}\text{C} \approx 0$ (e.g. Wada *et al.*, 1993). In this study, we did not directly observe the microbial loop. However, Kohzu *et al.* (Kohzu *et al.*, 1999) reported that wood-decomposing fungi completely exhaust wood nitrogen without nitrogen isotope fractionation under conditions of very low nitrogen availability ($<0.07\%$). We therefore expect low $\Delta\delta^{15}\text{N}/\Delta\delta^{13}\text{C}$ slope values for food chains supported mostly by microbial loops. Based on our results, we also expect that the $\Delta\delta^{15}\text{N}/\Delta\delta^{13}\text{C}$ slope of each food chain may reflect ecological factors, such as the anabolic–catabolic ratio of primary producers, differences in season and/or habitat depth, mixed diets of heterotrophs and differences in lipid content through the course of life cycles (Pecquerie *et al.*, 2010).

CONCLUSIONS

We investigated the relationship between nitrogen and carbon SIs throughout the year in the Oyashio region of the western North Pacific. Isotopic ratios of higher TLs, such as predatory and/or long-lived zooplankton, varied little with season, while the isotopic ratios of short-lived zooplankton varied with season on our $\delta^{15}\text{N}$ – $\delta^{13}\text{C}$ maps. Although it was not clear from our original data alone, analysis using additional data sets from other two oceanic regions suggests the existence of a common $\Delta\delta^{15}\text{N}/\Delta\delta^{13}\text{C}$ slope in oceanic food chains. We suspect that this common relationship most likely results from kinetic isotope effects in the processes of amino acid synthesis. However, we were only able to draw conclusions about regional differences in this

relationship by combining data from several sources. More extensive observations covering a range of TLs at each location will be needed to reach more detailed conclusions (e.g. concerning seasonal differences).

We expect lower $\Delta\delta^{15}\text{N}/\Delta\delta^{13}\text{C}$ slopes under oligotrophic conditions, increasing $\Delta\delta^{15}\text{N}/\Delta\delta^{13}\text{C}$ slopes as waters become more eutrophic, and very steep $\Delta\delta^{15}\text{N}/\Delta\delta^{13}\text{C}$ slopes in highly eutrophic coastal areas where $\Delta\delta^{13}\text{C} \approx 0$. However, the relationship between isotopic fractionation by branch reactions and trace metal availability remains poorly understood. Clarifying the role of trace metals or other controlling factors on isotopic fractionation *in vivo* is a promising topic for further research and would help to advance the understanding of the relationships among physical environments, physiology and ecology.

Our results suggest several promising ways of further exploiting the potential of SI ratios as tracers of biogeochemical cycles in both terrestrial and aquatic environments. By analyzing isotopic ratios of zooplankton at life cycle levels, we can understand variation in C and N isotope ratios of higher TLs, such as fish and seabirds. The relationship between SI ratios of primary producers and their immediate consumers has already been studied extensively, but the relationship could be examined at higher TLs and elucidated more precisely by exploiting regional differences in SI ratios. For example, Minami and Ogi (Minami and Ogi, 1997) investigated the migratory dynamics and dietary changes of the sooty shearwater in the Pacific using $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ of muscle tissue. Combining C and N SI ratios with other isotopes could allow for new developments in the field of traceability (Nakano, 2010). For example, Kennedy *et al.* (Kennedy *et al.*, 2002) reconstructed the life histories of migratory fish using Sr isotopes in otoliths of Antarctic salmon. In recent years, higher TL ecosystem models have been used to investigate population sizes and seasonal migration of fish using the results of lower trophic ecosystem models (e.g. Rose *et al.*, 2007; Okunishi *et al.*, 2009). SI data and models explicitly including SI could inform and validate such higher TL ecosystem models.

SUPPLEMENTARY DATA

Supplementary data can be found online at <http://plankt.oxfordjournals.org>.

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