

Phytoplankton as a principal diet for callianassid shrimp larvae in coastal waters, estimated from laboratory rearing and stable isotope analysis

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ABSTRACT: The field diet of meroplanktonic decapod crustacean larvae is poorly known, despite standard use of microzooplankton as food in laboratory culture. Using callianassid shrimp *Nihonotrypaea harmandi* larvae collected from a 65 m deep inner-shelf location off mid-western Kyushu, Japan, between June and August 2012 and 2013 and mass-reared in the laboratory, a phytoplankton-based diet through larval development (zoeae I–VI to decapodid) was demonstrated. When the pure-cultured diatom *Chaetoceros gracilis* was fed to zoeae, survival rate to decapodids was 3.4 to 3.9% in 26 to 40 d at 22°C, which was comparable to previous rearing results for zoeae fed microzooplankton. Trophic enrichment factors (TEFs) from stable isotope (SI) analysis of zoeal whole-body tissue in the laboratory were 2.0‰ for $\delta^{13}\text{C}$ and 1.9‰ for $\delta^{15}\text{N}$. In the field water column, diatoms dominated the nano- to micro-sized plankton, accounting for 38 to 81% of the biovolume, followed by heterotrophic protists. The trophic position (TP) estimated from amino acid-specific $\delta^{15}\text{N}$ values for the field-collected zoeae VI was 2.1 ($\text{TP}_{\text{Glu/Phe}}$) or 2.7 ($\text{TP}_{\text{Ala/Phe}}$), suggesting that those zoeae fed on mixtures of phytoplankton and heterotrophs including protists. Bulk SI analyses were performed for particulate organic matter (POM; proxy for phytoplankton), microzooplankton (mainly calanoid copepods), and shrimp zoeae to elucidate the diet of larvae in the water column. A shift in SI from fresh to degraded POM was determined through the incubation of field-collected POM. Based on this shift during degradation and larval TEFs, phytoplankton and their sinking detritus with heterotrophic protists were estimated to be the principal diet for those larvae residing mostly below the chlorophyll maximum layer.

KEY WORDS: *Nihonotrypaea harmandi* · Zoea · Rearing · Diatom · Isotopic trophic enrichment factor · Amino-acid- $\delta^{15}\text{N}$ -based trophic position · Phytodetritus · Protists

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INTRODUCTION

Meroplanktonic larvae of macrobenthos are a seasonally significant component of zooplankton assemblages in tropical, temperate, and boreal estuarine and coastal waters. The successful acquisition of food by planktotrophic larvae is one critical process in their normal development and survival (Thorson

1950, Day & McEdward 1984, Olson & Olson 1989, Metaxas & Saunders 2009). Decapod crustaceans are a common component of both benthic macro-invertebrate communities and meroplanktonic assemblages in these nearshore waters. The potential food sources for planktotrophic decapod larvae comprise bacteria, phytoplankton ranging from pico- to micro-sized groups, zooplankton ranging from nano- to meso-

sized groups, and detritus and fecal pellets (Lebour 1922, Stickney & Perkins 1981, Factor & Dexter 1993, Mascetti & Wehrmann 1996, Lehto et al. 1998, Anger 2001, Schwamborn et al. 2006, Fileman et al. 2014). In laboratory rearing of decapod larvae, brine shrimp *Artemia* spp. nauplii and rotifers (e.g. *Brachionus* spp.), which are microzooplankton never encountered by those larvae in their natural environment, have most frequently been used for complete larval development from newly hatched to post-larval stages (McConaughy 1985, Harvey & Epifanio 1997, Anger 2001, Calado et al. 2010). Such success in larval culture, often with excess supply of those food items, does not necessarily mean that microzooplankton are a principal food source for natural larval populations, as individual density, microscale patchiness, filterability (e.g. non-motile phytoplankton by filter-feeding larvae) or catchability (e.g. motile phytoplankton or zooplankton by raptorial larvae), digestibility, and nutritional value are likely to play their roles in natural diets. Principal diets of decapod crustacean larvae under natural conditions thus remain to be determined.

Successful rearing of planktotrophic larvae of species in 1 of the 2 decapod suborders, Pleocyemata, to the decapodid (= post-larval) or juvenile stage solely using phytoplankton has been achieved in only a few studies on brachyuran crabs (Atkins 1955, Bousquette 1980, Harms & Seeger 1989). In the latter study, larvae of a majid fed a diatom species showed a longer developmental duration and lower survival rate than those fed *Artemia* sp. nauplii. In other studies, phytoplankton are regarded as partially valid foods, typically compared with 2 control rearing treatments, either fed *Artemia* spp. nauplii (complete) or starved (invalid) (Table 1). For zoeal stages in pe-

naeid shrimp (suborder Dendrobranchiata), phytoplankton (mainly diatoms) are considered a fully effective food source (Preston et al. 1992, Yano 2005).

Even if phytoplankton are a principal diet of meroplanktonic decapod crustacean larvae to complete their development in the field, examination alone of ingested food species contained in larval digestive tracts or fecal pellets, including algal cell walls, photosynthetic pigments, and DNA-identified amorphous material, will not suffice to reach a convincing conclusion. In addition to live forms, phytodetritus can be a potential food source for decapod larvae (Meyer-Harms & Harms 1993, Kiørboe 2011, Turner 2015). Although carbon and nitrogen stable isotope ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) analysis is one promising way to estimate diets of those larvae, the number of actual isotope studies is scarce. In a study area ranging from a mangrove shore to an offshore shelf in northeastern Brazil, the contribution of mangrove carbon to decapod larval nutrition was regarded as negligible, and pelagic primary producers were suggested as principal diet sources (Schwamborn et al. 1999, 2002). However, the estimation of the trophic position (TP) and food sources for target species based on such 'bulk' isotope analysis has uncertainties. First, it is impossible to cover all potential food sources in the field, where a variety of primary producers and consumers exist. Second, isotope values of primary producers are variable in space and time due to the variability of their species composition, and dissolved inorganic carbon and nitrogen sources. Finally, as enrichment of ^{13}C and ^{15}N in biological tissues across trophic levels is variable depending on the species involved, commonly used trophic enrichment factors (TEFs) (ca. 1‰ for ^{13}C and 3 to 4‰ for ^{15}N ; e.g. DeNiro & Epstein 1978, Minagawa & Wada 1984) in

Table 1. Partially valid phytoplanktonic food items for the development of reared larvae in groups of the suborder Pleocyemata

Food items	Groups in Pleocyemata	Reference(s)
Diatoms	Brachyurans and/or anomurans	Hartman & Letterman (1978), Harms & Seeger (1989), Paul et al. (1989), Harms et al. (1991, 1994), Konishi et al. (1997)
Diatoms	Carideans	Stickney & Perkins (1981), Emmerson (1985)
Diatoms and dinoflagellates	Brachyurans	Incze & Paul (1983), Meyer-Harms & Harms (1993)
Dinoflagellates	Brachyurans	Perez & Sulkin (2005), Burnett & Sulkin (2007), Shaber & Sulkin (2007)
Dinoflagellates	Brachyurans and/or anomurans	Hinz et al. (2001)
Dinoflagellates and green alga	Brachyurans	Sulkin et al. (1998)
Dinoflagellates, green alga, and seagrass detritus	Brachyurans	Lehto et al. (1998)
Haptophyte alga	Brachyurans and/or anomurans	Mascetti & Wehrmann (1996)

place of species-specific ones (e.g. Yokoyama et al. 2005) may mislead the interpretation of food-chain structure. In addition to bulk analysis, $\delta^{15}\text{N}$ of amino acids (AAs) have been widely used to estimate more accurate TPs (McClelland & Montoya 2002, Ohkouchi et al. 2017 and references therein). Among the AAs found in organisms, 2 types have been identified in terms of their ^{15}N -enrichment across trophic levels. One is 'trophic amino acids', the $\delta^{15}\text{N}$ of which significantly increase from one trophic level to the next, while the other, 'source amino acids', show only slight shifts in $\delta^{15}\text{N}$ between trophic levels. Therefore, the analysis of $\delta^{15}\text{N}$ in a set of AAs covering these 2 types (mainly phenylalanine for source AA and glutamic acid for trophic AA) for a target species potentially enables the identification of accurate TPs (Chikaraishi et al. 2009). Furthermore, it has recently been reported that one trophic AA, alanine, is effective at more correctly estimating a trophic pathway including both protistan and metazoan consumers (Gutiérrez-Rodríguez et al. 2014, Décima et al. 2017, Landry & Décima 2017). Therefore, the use of alanine as trophic AA would be more adequate for food chain analyses in coastal oceans as well as pelagic waters where trophic transfers through protistan grazers are not negligible.

Callianassid shrimp or ghost shrimp (Pleocyemata: Axiidea) play key roles as ecosystem engineers and community organizers in estuarine and coastal marine sedimentary habitats (Pillay & Branch 2011, Dworschak et al. 2012). Planktotrophic larvae of callianassids go through 3 to 7 zoeal stages to reach the decapodid stage (Kubo et al. 2006, Pohle et al. 2011, Kornienko et al. 2015). The survival rate of those larvae significantly affects the population dynamics of adults, which could further exert an impact on the community and ecosystem surrounding that population. Following the release of zoeae I from the shore, those larvae grow in the inner-shelf coastal ocean (Johnson & Gonor 1982, Yannicelli et al. 2006, Tamaki et al. 2010).

In midwestern Kyushu, Japan, a population of the callianassid shrimp *Nihonotrypaea harmandi* (Bouvier 1901) is distributed on intertidal sandflats in a region ranging from estuary (Ariake Sound) to inner-shelf coastal ocean (Amakusa-nada) (Tamaki et al. 1999) (Fig. 1). Around the western edge of the region, the largest local population in the region exists on the intertidal sandflat in Tomioka Bay (Tomioka sandflat; 32.521°N, 130.037°E) (Tamaki & Harada 2005, Tamaki & Takeuchi 2016). Tomioka Bay forms a coastal boundary layer with water depths ≤ 30 m. Larvae released from the sandflat are transported across

Tomioka Bay to Amakusa-nada, where the main nursery ground for larvae lies 10 to 20 km north and west of the sandflat (Tamaki & Miyabe 2000, Tamaki et al. 2010, 2013). Complete rearing of *N. harmandi* larvae has been achieved in the laboratory using the rotifer *Brachionus rotundiformis*, and newly hatched *Artemia* sp. nauplius (Konishi et al. 1999). By contrast, examination of both the mouthpart morphology and digestive tract content of field-caught *N. harmandi* larvae suggests that they are herbivorous, mainly feeding on diatoms (Somiya et al. 2014). But there are no records of complete larval rearing solely with phytoplankton.

The objective of the present study was to test whether phytoplankton and/or phytoplankton-derived detritus (phytodetritus) are the principal food source for zoeal larvae of *N. harmandi* in the coastal ocean. First, larvae of *N. harmandi* were mass-reared with pure-cultured phytoplankton as a single food source in the laboratory. Second, the taxonomic composition of nano- and micro-plankton over the water column in the field was determined to support the analysis of food sources for the zoeal larvae. Finally, stable isotope values of bulk and individual AAs were determined for field samples to estimate the actual food source. The TEFs for the bulk $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in zoeal larvae were determined for a sample from the above rearing experiment. The shift in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values associated with microbial degradation of phytoplankton was obtained by a laboratory incubation of particulate organic matter in order to incorporate phytodetritus into the food-chain diagram as a potential food source. It was concluded that mixtures of phytoplankton and heterotrophs including protists were the most likely diet for field larvae of *N. harmandi*.

MATERIALS AND METHODS

Biology of ghost shrimp adults and larvae

Using the TEFs for juveniles of *Nihonotrypaea harmandi* reared with pure-cultured diatoms *Chaetoceros gracilis* in the laboratory (Yokoyama et al. 2005), the diet of adults collected from the Tomioka sandflat was estimated to be phytoplankton and benthic microalgae (Shimoda et al. 2007). The reproductive season for the *N. harmandi* population spans June through October. Larval development consists of 6 zoeal (up to the zoea VI) and 1 decapodid stages (Konishi et al. 1999, Somiya et al. 2014). Maximum total length for zoeae I and VI is ca. 3 and 7 mm,

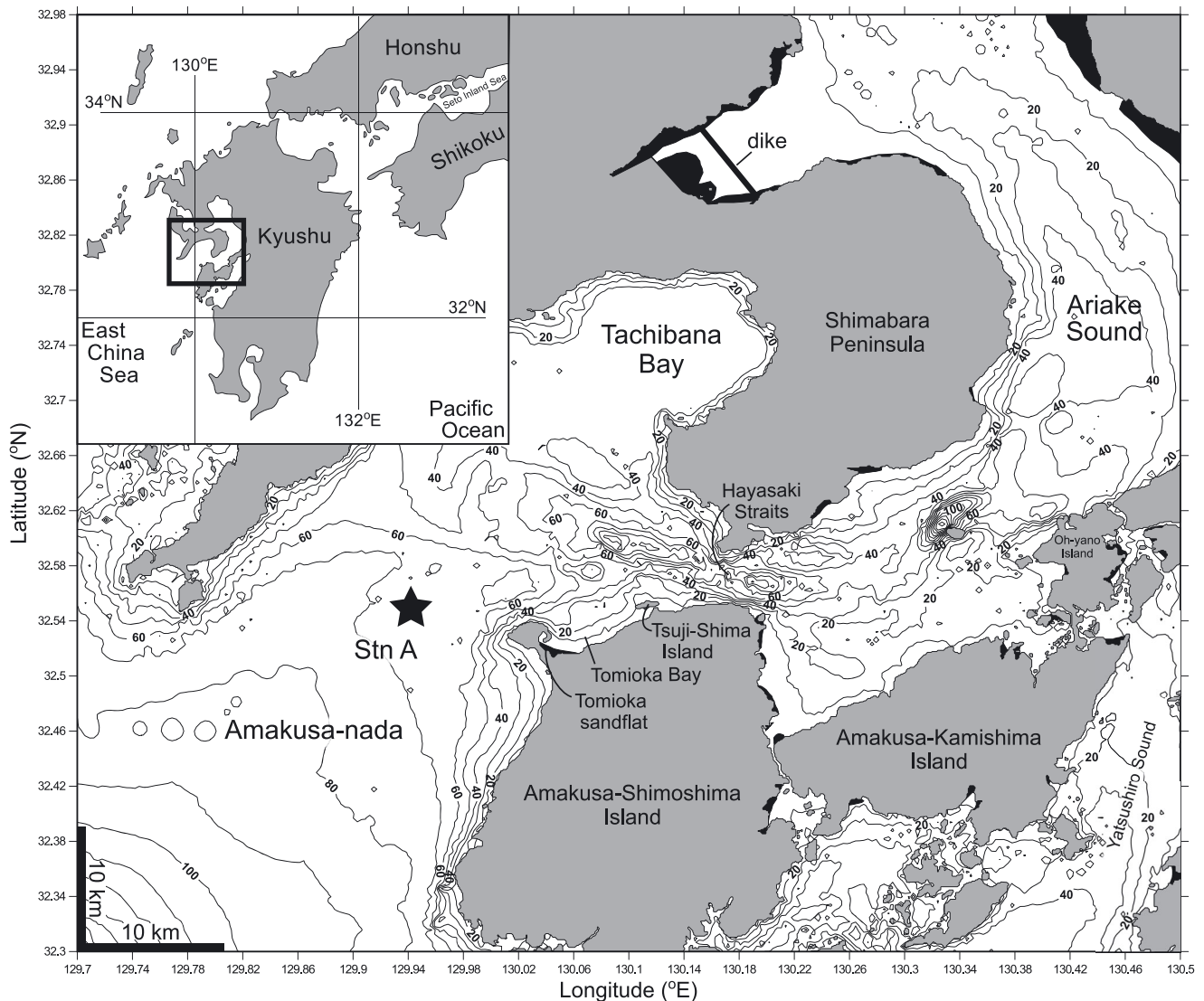


Fig. 1. Study region and location of tidal flats along the shoreline in mid-western Kyushu, Japan. Water-depth isopleths every 10 m were made by contouring (Surfer®8; Golden Software) point data provided by the Hydrographic and Oceanographic Department, Japan Coast Guard. Tidal flat areas, including the Tomioka sandflat, are indicated in black. Stn A in Amakusa-nada was regularly visited for water and plankton sampling

respectively. Within the water column at a 65 m deep location 10 km west of the sandflat in Amakusa-nada (Stn A in Fig. 1; 32.545° N, 129.942° E), the mean vertical positions of most zoeal stages were at 30 to 40 m depths with prevailing temperatures of 21 to 22°C and salinities of 33.5 to 34 during the end of July to early August 2006 (Tamaki et al. 2010). In the laboratory at 21°C, it took 30 d for most of the newly hatched zoeae reared with *Brachionus rotundiformis*, and newly hatched *Artemia* sp. nauplii to reach the decapodid stage (Tamaki et al. 2013). Starved zoeae I survived for up to 5 d and never proceeded to the zoea II stage (Y. Saitoh & A. Tamaki unpubl. data).

Setup for ghost shrimp larval rearing in the laboratory

Larvae of *N. harmandi* were mass-reared in 2 black, semi-cylindrical polyethylene tanks (rearing tanks 1 and 2) with a 30 l capacity during July and August 2013. Surface water from a coastal ocean area, filtered through a 10 µm mesh (TOCEL; JFE Advantech), was stored and used for the rearing experiment. The water temperature in the tanks was kept at around 22°C (which corresponded to the field water-column conditions for the larvae) using 2 water baths (see Section 1.1 in the Supplement at www.int-res).

com/articles/suppl/m592p141_supp.pdf). The seawater temperature and salinity in each tank were recorded every 1 min with a Compact-CT (JFE Advantech). Gentle aeration and slow water circulation in each tank was made with a stream of tiny air bubbles passed through 2 air stones with 20 mm Ø, which kept only food particles (i.e. not deposits) suspended. During rearing, the room lights were kept off as a rule, except during water exchanges, food supply, and retrieval of larvae. Furthermore, the tank tops were covered with fine-mesh black sheets, which was intended to approximate faint daylight conditions at 30 to 40 m water depths in Amakusa-nada (Tamaki et al. 2010).

Collection of ghost shrimp ovigerous females and initiation of larval rearing

During daytime low tide on the Tomioka sandflat on 7 July 2013, 57 females of *N. harmandi* with well-developed embryos were collected. These embryos were expected to hatch during the immediate nighttime as determined by their large eyes and transparent bodies due to a minimum amount of remaining yolks. In the laboratory, the females were kept in containers with filtered seawater until their larvae were released. Synchronized mass-release events occurred twice, at 02:20 and 04:00 h on 8 July. Each time, swimming larvae (newly hatched zoeae I) were collected with a nylon net and gently transferred to a transparent container containing 1.5 to 3.0 l filtered seawater. From each container, 12 samples of 5 ml water were randomly taken with a measuring pipette. After counting the number of larvae in each sample, they were returned to the container for re-sampling. The mean (\pm SD) numbers of larvae sample⁻¹ (12.0 ± 4.0 and 3.7 ± 1.2 in the first and second events, respectively) were used to estimate the total numbers of larvae, which were approximately 7200 and 1100 larvae in the first and second events, respectively. These 2 batches of larvae were mixed in a cup with filtered seawater and split into 2 cups each with a known volume of water. It was estimated that 4500 and 3800 larvae were contained in these cups. In this order, the cup contents were gently poured into rearing tanks 1 and 2, respectively, each of which contained a sufficient quantity of pure-cultured diatoms (*C. gracilis*; Bivalve Culture Institute, Sasebo City). All the above procedures were completed by 05:00 h. The live diatom stock (approximately 5×10^8 cells ml⁻¹ in a bottle of 1000 ml seawater) can be

stored at between 0 and 5°C in the refrigerator for at least 50 d (see Yokoyama et al. 2005 and Tamaki et al. 2013 for successfully rearing decapodids and juveniles of *N. harmandi* with this product).

Routine procedures for ghost shrimp larval rearing and retrieval, and data analysis

Starting from 09:00 h on 8 July 2013 (Day 0), the daily routine work for each of the 2 tanks was completed between 09:00 and 11:00 h. Carcasses, feces, and exuviae of *N. harmandi* larvae, and *C. gracilis* flocs deposited on the tank bottom were removed with pipettes. Three-fourths of the tank water was gently siphoned off, with a 63 µm mesh nylon net covering the siphon entrance to prevent larvae from being inhaled, and replaced with new filtered seawater stored in the room. A fixed number of new diatoms was spread over the tank water, poured with a small quantity of filtered seawater in which the pre-dilution of concentrated diatoms had been made. The daily ration of *C. gracilis* per ml in the tank water was approximately 5.0 to 6.7×10^3 cells. These rations were determined by comparing the total volume of diatoms, as a product of cell diameter (5 to 7 µm)-based sphere volume \times total number, with that of rotifers and/or brine shrimp nauplii, the supply of which provided in excess to similar initial numbers of zoeae I had led to substantial decapodid yields (Tamaki et al. 2013).

At some date intervals, to check whether the larval stages proceeded as in the previous rearing experiments with rotifers and brine shrimp nauplii (Konishi et al. 1999, Tamaki et al. 2013), small numbers of larvae up to the zoea IV stage were collected with a pipette and fixed with 5% neutralized seawater formalin during the above routine work (in total, 39 and 56 larvae from rearing tanks 1 and 2, respectively). No sampling for the zoeae V and VI was done to obtain a maximum number of decapodids. All decapodids that emerged daily, which were mostly moving horizontally close to the tank bottom, were collected with a pipette. They were kept frozen at -20°C for later stable isotope (SI) analysis. On Day 40, all larvae including those still at the zoeal stages were collected and fixed (decapodids) or stored for SI analysis (zoeae).

Usually, when rearing *N. harmandi* larvae, decapodids occur in 2 cohorts over time, forming the corresponding normal-distribution groups in the daily emerging individuals (A. Tamaki unpubl. data). Following Aizawa & Takiguchi (1999), with a modifica-

tion to Hasselblad (1966), such a composite normal-distribution group was separated into the component distributions.

Size-frequency distributions for ghost shrimp embryos and newly hatched zoeae I

The multiple cohorts of decapodids of *N. harmandi* occurring through the rearing dates might reflect those cohorts that could exist already at the stages of newly fertilized embryos and newly hatched zoeae I, with dimension differences between the cohorts. The subsequent developmental durations can vary accordingly. To detect the presence of such initial cohorts, females with (1) newly fertilized embryos and (2) embryos about to hatch were collected from the Tomioka sandflat during daytime low tides on 24 and 26 August and 10 September 2014. Determination of these embryonic states is detailed in Section 1.2 in the Supplement. The females with embryos-(1) were individually fixed with 10% neutralized seawater formalin. Those with embryos-(2) were brought alive to the laboratory.

Under a light microscope in the laboratory, the above embryonic-state-(1) was confirmed as the stage before reaching the embryonic nauplius. In total, 73 females with those embryos were obtained. Ten embryos were randomly removed from each female, and the longer and shorter axes of the ellipse of each embryo in plane aspect were measured to 0.01 mm with an eyepiece micrometer attached to the microscope at 100× magnification. The mean longer and shorter axis lengths were used to calculate the volume of a spheroid embryo with its longer axis as the axis of revolution. Using data for all females combined, the embryonic volume-frequency distribution was made. The females with embryonic-state-(2) were kept individually in small containers with filtered seawater until their larval release during the immediate nighttime. In total, 61 females released larvae, which were collected and fixed with 5% neutralized seawater formalin. For 10 randomly chosen larvae from each female, the mid-dorsal total length in lateral aspect, from rostral spine to telson tips, was traced with a camera lucida of a stereomicroscope at 30 to 50× magnification. The curve length was measured to 0.01 mm using ImageJ 1.48v (<http://imagej.nih.gov/ij/>). Using the mean larval total length from every female, a total-length-frequency distribution group was made. Each composite normal-distribution group was separated into the components as previously.

Sampling diatoms and larvae reared in the laboratory for SI analysis

The liquid with concentrated diatoms (*C. gracilis*) for the rearing of *N. harmandi* larvae was sampled for SI analysis on Days 0, 9, 15, and 28, and immediately processed (see Section 1.3 in the Supplement).

The numbers of *N. harmandi* larvae retrieved for SI analysis (combined from the 2 rearing tanks) consisted of approximately 50 to 60 for zoeae I immediately after hatching (retrieved separately from those larvae subsequently used for the rearing), 9 for zoeae IV on Day 15, 52 for decapodids (7 to 16 each on Days 28 and 30 to 33), and all zoeae remaining on Day 40 (20 zoeae V and 37 zoeae VI). After their defecation, these specimens were kept frozen at -20°C until analysis. The TEF associated with the larval feeding on diatoms was calculated as the difference in SI values between zoeal whole-body tissues and diatoms as follows, when the larval SI values became steady during the rearing: $\text{TEF} (\Delta\delta^{13}\text{C} \text{ or } \Delta\delta^{15}\text{N}) = \text{SI value (larvae)} - \text{SI value (diatoms)}$.

Field sampling for SI and plankton composition analyses

Water and biological sampling for chemical analyses were carried out at Stn A in Amakusa-nada (Fig. 1) during 2 cruises in 2012 (10 to 12 July and 7 to 10 August) and 3 cruises in 2013 (27 to 29 June, 10 to 12 July, and 7 to 9 August) onboard the training vessel (T/V) 'Kakuyo-Marui' (Nagasaki University) equipped with 12 Niskin bottles (5 l) mounted on a rosette multiple sampler (RMS; General Oceanics) with the CTD probe (SBE 9/11; Seabird Electronics). Regarding the potential food sources for *N. harmandi* larvae, water samples for particulate organic matter (POM) to be used for SI analyses were collected, 2 or 3 times in each cruise, from the chlorophyll (chl) maximum layers and 1 or 2 other layers 5 to 10 m above or below each chl maximum layer (10 to 25 m depth except for August 2012 [30 to 50 m], as detected with the CTD probe), which was expected to contain high content of fresh phytoplankton. Chl *a* concentrations in the water column ($\mu\text{g l}^{-1}$) were calculated from chl fluorescence at each layer, and conversion factor was obtained at Stn A during the shared cruises in August and September 2012 (S. Takeda pers. comm.). Each 5 to 10 l sample of seawater was filtered through a 200 μm nylon mesh and a pre-combusted filter (GF/F; 47 mm \varnothing), and then the sample was gently washed with a few ml of distilled

water. Samples were kept frozen at -20°C until analysis and processed in the same manner as for the diatom sample (see Section 1.3 in the Supplement).

Zoeae of *N. harmandi* (no decapodids were obtained) and their potential food sources, zooplankton (mostly composed of calanoid copepods), were collected for bulk SI analyses by vertical towing with a Norpac Net (0.45 m mouth diameter, 1.8 m length, and 330 μm mesh size) from 5 m above the seabed to the surface at Stn A at both low- and high-tide times. These plankton samples were immediately fixed with neutralized formalin solution (5% final conc.) and brought to the laboratory. Under a stereomicroscope, larval samples were separated into the 6 stages (zoea I to VI) both in 2012 and 2013, while zooplankton were collected only in 2012 and separated into 6 body-size fractions (3.5–2.5, 2.5–2.0, 2.0–1.5, 1.5–1.2, 1.2–0.8, and 0.8–0.3 mm). Prior to the SI analysis of these field-collected samples, the effect of preservation in formalin solution on the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for zoeal tissues (i.e. difference in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values between the zoeal samples with and without formalin treatment) was tested for several zoeal stages and the values used for the calibration of the SI values for both zoeae and other zooplankton.

Zoeae of *N. harmandi* for $\delta^{15}\text{N}$ analysis in amino acids were collected on 10 August 2012 from 30 m depth by horizontally towing a ‘fish-larvae’ net (1.3 m mouth diameter, 4.5 m length, and 330 μm mesh size) at a speed of 1.5 knots for 10 min. The sample was immediately brought to the laboratory alive, and zoeae VI were selected under a stereomicroscope and kept frozen at -20°C until analysis. About a 12 h lag from sampling to freezing was enough for those zoeae to evacuate their digestive tract contents, suggesting no contamination of zoeal tissues with gut contents for SI analysis.

Water samples for the taxonomic composition analysis of nano- to micro-sized plankton were collected from 4 depth layers (0, 20, 40, and 60 m) with a bucket (0 m) or Niskin bottles on 7 August 2012. Sampled water was immediately fixed with acid Lugol's solution (2% in final conc.) and stored in cool and dark conditions. In the laboratory, 3.1 to 4.4 ml of water sample was set in a Sedgewick–Rafter chamber (Guillard 1978) for each layer, so that >300 of those plankton were included. All individuals larger than 4 μm in equivalent spherical diameter (i.e. eukaryotic unicellular organisms and filamentous cyanobacteria) were observed and identified to varying levels from class to suborder under an inverted biological microscope equipped with a 60 \times objective lens (Olympus IX71). Plankton biovolume was indi-

vidually estimated assuming approximate geometrical figures such as spherical, ellipsoidal, cylindrical, and conical shapes.

POM degradation experiment

To check for the potential alteration of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in POM by microbial degradation in the water column during suspension and sinking into deeper layers, laboratory incubation experiments were conducted in June and July 2013. To prepare POM presumably with a high content of phytoplankton, seawater samples collected from the chl maximum layer at Stn A (preceding section) were filtered sequentially through 200 and 20 μm nylon meshes. The organic matter trapped on the 20 μm nylon mesh was re-suspended in filtered seawater (through GF/F) in duplicate 200 ml bottles. The sample bottles were immediately stored in a portable incubator (CN-25C; Mitsubishi Electric) on-board and incubated at a temperature of 22°C in the dark for 1 wk. Duplicate POM samples were collected on pre-combusted GF/F filters (25 mm \varnothing) on 3 dates (Days 0, 2 or 3, and 5 or 7), and processed in the same manner as for the diatom samples in the larval rearing (see Section 1.3 in the Supplement).

AA extraction and purification

Roughly powdered zoeal whole-body tissues of *N. harmandi* (zoea VI, 2 sets of about 20 ind., ca. 4 mg dry weight collected at Stn A in August 2012) were pre-treated prior to $\delta^{15}\text{N}$ analysis of individual AAs. The subsequent procedures followed the protocol given in Ishikawa et al. (2014), with some modifications (see Section 1.4 in the Supplement).

SI measurement of bulk and AA samples

For the zooplankton samples (including *N. harmandi* larvae) collected at Stn A in Amakusa-nada in 2012 and 2013, and from the rearing experiment for the larvae, approximately 0.3 to 0.5 mg of the sample in dry weight (i.e. 15 to 20 ind. for zoea I, 11 to 13 for zoea II, 9 to 11 for zoea III, 6 to 8 for zoea IV, 3 to 5 for zoea V, 3 each for zoea VI and decapodid, and 10 to 50 ind. for zooplankton depending on body-size groups, randomly selected from the sample stock) was transferred to silver capsules (SÄNTIS Analytical) and dried at 60°C after acidification with a few

drops of 1 N HCl to remove inorganic carbon. The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for diatoms (diet in the rearing experiment), POM (field sample and degradation experiment), and zooplankton samples were measured with an elemental analyzer and an isotope-ratio mass spectrometer (FLASH 2000-Conflo IV-Delta V Advantage; Thermo Fisher Scientific). Instrument precision was checked with a calibration standard (L-alanine) every 5 samples (standard deviation is $<0.12\%$ for $\delta^{13}\text{C}$, $<0.14\%$ for $\delta^{15}\text{N}$). The $\delta^{15}\text{N}$ values for the samples with lower N contents ($<20\ \mu\text{g}$) were eliminated from the results (e.g. N at POM degradation experiment), because the accuracy of the measured values can be low, despite the calibration with standards, due to a mass-dependent shift in $\delta^{15}\text{N}$ values in small-quantity samples (Ogawa et al. 2010).

The $\delta^{15}\text{N}$ values of the 3 kinds of AAs (alanine [Ala], glutamic acid [Glu], and phenylalanine [Phe]) were determined by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) using a Delta V plus isotope ratio mass spectrometer (Thermo Fisher Scientific) coupled to a gas chromatograph (GC7890A; Agilent Technologies) via a modified GC-Isolink interface consisting of combustion and reduction furnaces. The AA derivatives were injected into the GC column using a Gerstel PTV injector in solvent vent mode. The programs on temperature, retention time, and carrier gas flow rate in each process (i.e. injection, combustion, reduction, and separation in GC) followed the methods given in Ishikawa et al. (2014). The CO_2 generated in the combustion furnace was removed using a liquid N trap. Standard mixtures of 8 kinds of AAs ($\delta^{15}\text{N}$ ranging from -25.9 to $+45.6\%$) were analyzed with zoael sample in turn to confirm the reproducibility of the isotope measurements. Standard deviations of the standards were better than 0.8% with a sample quantity of $5\ \text{nmol}\ \mu\text{l}^{-1}\ \text{N}$.

It was anticipated that protozoan consumers potentially contributed to the diet of *N. harmandi* larvae (cf. Fileman et al. 2014); there is a certain amount of biomass of protozoa such as ciliate and heterotrophic dinoflagellate in coastal waters, and sinking phytodetritus with heterotrophs could be a candidate for the diet. Therefore, both the TP-estimate based on the $\delta^{15}\text{N}$ values of Glu and Phe with canonical parameters for the metazoan food chain (i.e. $\text{TP}_{\text{Glu/Phe}}$; Chikaraishi et al. 2009) and the TP-estimate based on the $\delta^{15}\text{N}$ values of Ala and

Phe with another set of parameters for the metazoan food chain potentially including a protozoan pathway (i.e. $\text{TP}_{\text{Ala/Phe}}$; Décima et al. 2017) were calculated for the targeted samples using the following equations, respectively:

$$\text{TP}_{\text{Glu/Phe}} = (\delta^{15}\text{N}_{\text{Glu}} - \delta^{15}\text{N}_{\text{Phe}} - 3.4) / 7.6 + 1 \quad (1)$$

$$\text{TP}_{\text{Ala/Phe}} = (\delta^{15}\text{N}_{\text{Ala}} - \delta^{15}\text{N}_{\text{Phe}} - 3.2) / 5.5 + 1 \quad (2)$$

RESULTS

Size-frequency distributions for ghost shrimp embryos and newly hatched zoeae I

The newly fertilized embryo-volume-frequency distribution and newly hatched zoea I-total length-frequency distribution of *Nihonotrypaea harmandi* in 2014 are shown in Fig. 2. Each frequency distribution comprised 2 cohorts (cohorts 1 and 2), to which normal distributions were fitted. The mean diameters of embryos (per female) ranged from 0.44 (short axis) to 0.52 mm (long axis). The estimated embryo volume ranged from 0.37 to 0.59 mm^3 . Cohort 1 comprised 77 % of all embryos, with mean ($\pm\text{SD}$) of $0.42 \pm 0.02\ \text{mm}^3$. For cohort 2, the mean embryo volume was $0.50 \pm 0.04\ \text{mm}^3$, which was 1.19 times greater than for cohort 1. The total length of zoea I ranged from 2.56 to 2.95 mm. Cohort 1 comprised 54 % of all zoeae I, with mean of $2.70 \pm 0.06\ \text{mm}$. For cohort 2, the mean total length was $2.87 \pm 0.06\ \text{mm}$. The (mean total length)³ was 1.20 times greater than that in cohort 1, where the cube is a measure for larval volume.

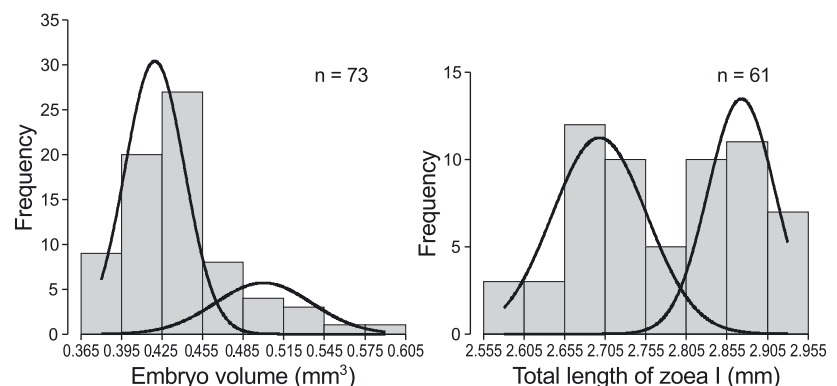


Fig. 2. Newly fertilized embryo-volume-frequency distribution and newly hatched zoea I-total length-frequency distribution of *Nihonotrypaea harmandi* specimens collected from late August to early September 2014. The 2 normal distributions were fitted to each frequency distribution

Water temperature and salinity in laboratory rearing tanks for ghost shrimp larvae

The time series of seawater temperature and salinity in the 2 rearing tanks for *N. harmandi* larvae through the whole period (05:00 h on 8 July to 09:00 h on 17 August in 2013) are shown in Fig. S1 in the Supplement. In rearing tank 1, the water temperature ranged from 20.9 to 23.1°C, with mean (\pm SD) of $22.2 \pm 0.25^\circ\text{C}$. Salinity ranged from 32.5 to 34.1, with mean of 33.5 ± 0.2 . In rearing tank 2, the water temperature ranged from 21.3 to 23.0°C, with mean of $22.3 \pm 0.1^\circ\text{C}$. Salinity ranged from 32.3 to 33.8, with mean of 33.4 ± 0.2 .

Development and survival of ghost shrimp larvae in laboratory rearing

At the time of daily exchange of water in the rearing tanks, the 'old' water was brownish in color, suggesting an excess ration of *Chaetoceros gracilis*. The daily occurrence of decapodids of *N. harmandi* in rearing tanks 1 and 2 is shown in Fig. 3. During the rearing period in each tank, there were 2 major cohorts (cohorts 1 and 2), to which normal distribution curves were fitted. Decapodids emerged first on Day 26 in both tanks. In rearing tank 1, a total of 175 decapodids occurred until Day 40, giving a survival rate of 3.9% of the initial number of zoeae I from which the number of advanced-stage zoeae retrieved on the way to the decapodid stage was subtracted. Cohort 1 comprised 30% of all decapodids, with mean (\pm SD) occurrence in day number being 28.7 ± 1.0 . For cohort 2, the mean occurrence as day number was 34.2 ± 2.6 . In rearing tank 2, a total of 127 decapodids occurred until Day 40, giving a survival rate of 3.4%. Cohort 1 comprised 35% of all decapodids, with mean occurrence in day number being 28.8 ± 1.4 . For cohort 2, the mean occurrence as day number was 35.3 ± 2.3 .

TEF associated with diatom-feeding by ghost shrimp larvae

The SI values of the diet (diatom: *C. gracilis*) in the *N. harmandi* larval rearing experiment were almost constant through the study period (mean \pm SD, $n = 17$ values of $\delta^{13}\text{C} = -22.7 \pm 0.3\text{‰}$ and $\delta^{15}\text{N} = -1.9 \pm 0.3\text{‰}$; Fig. 4), and these mean values were used for the calculation of TEFs. The SI values in the zoal whole-body tissues gradually shifted, with development,

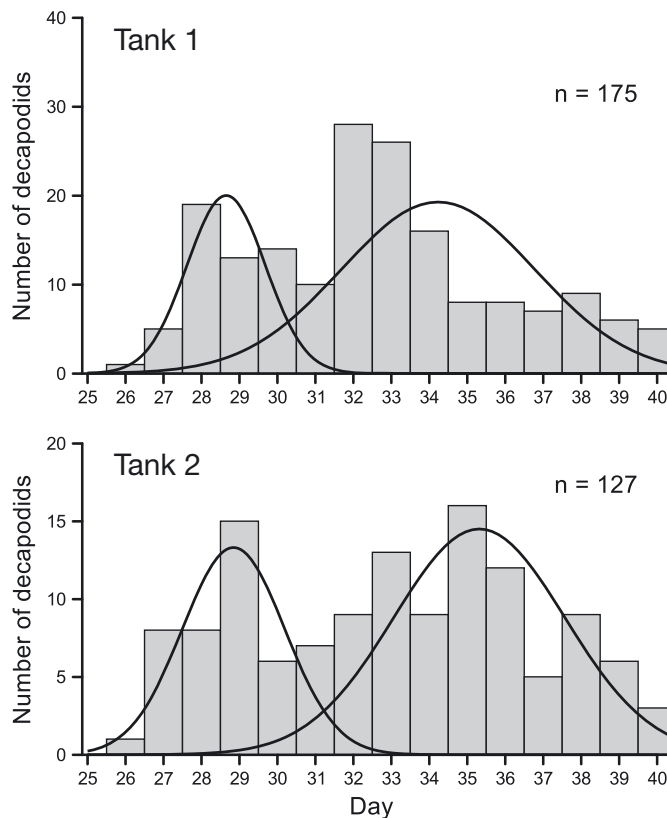


Fig. 3. Daily occurrence of decapodids of *Nihonotrypaea harmandi* in the 2 rearing tanks between 3 August (Day 26) and 17 August (Day 40) 2013. Decapodids emerged first on Day 26. The 2 normal distributions were fitted to each frequency distribution

from those in zoea I ($\delta^{13}\text{C} = -18.1 \pm 0.1\text{‰}$ and $\delta^{15}\text{N} = 7.1 \pm 0.02\text{‰}$, $n = 3$) to the asymptotic values closer to that for diatoms. The SI values in the body tissue were -20.7‰ for $\delta^{13}\text{C}$ and 1.0‰ for $\delta^{15}\text{N}$ ($n = 1$) at the zoea IV on Day 15 and reached a steady state at the decapodid during Day 28 to Day 33 ($\delta^{13}\text{C} = -20.7 \pm 0.3\text{‰}$ and $\delta^{15}\text{N} = 0.0 \pm 0.3\text{‰}$, $n = 15$). The SI values for the larvae that still remained at the zoal stages (V and VI) in the tanks on Day 40 ($\delta^{13}\text{C} = -20.5 \pm 0.3\text{‰}$ and $\delta^{15}\text{N} = 0.0 \pm 0.3\text{‰}$, $n = 3$) were similar to those steady values. Thus, the TEFs associated with the feeding of diatoms by *N. harmandi* zoeae was calculated as 2.0‰ for $\delta^{13}\text{C}$ and 1.9‰ for $\delta^{15}\text{N}$.

Measurement for shift in phytoplankton SI values with degradation

During the degradation experiment in June 2013, the mean (\pm SD) SI values for the POM (fraction between 20 and 200 μm) from the chl maximum layers gradually decreased from Day 0 ($\delta^{13}\text{C} = -20.0 \pm 0.4\text{‰}$,

$n = 3$ and $\delta^{15}\text{N} = 5.6$ to 5.7‰ , $n = 2$) to Day 7 ($\delta^{13}\text{C} = -20.9$ to -20.3‰ , $n = 2$ and $\delta^{15}\text{N} = 4.9\text{‰}$, $n = 1$) (Fig. 5). A similar decreasing tendency was observed in July 2013. The SI values for the POM decreased from Day 0 ($\delta^{13}\text{C} = -19.4$ to -19.1‰ , $n = 2$ and $\delta^{15}\text{N} = 6.2$ to 6.4‰ , $n = 2$) to Day 5 ($\delta^{13}\text{C} = -19.6$ to -19.5‰ , $n = 2$ and $\delta^{15}\text{N} = 5.2$ to 5.6‰ , $n = 2$). The average daily decreasing rates (‰ d^{-1}) were -0.09 for $\delta^{13}\text{C}$ and -0.11 for $\delta^{15}\text{N}$ during the 7 d in June, and -0.07 for $\delta^{13}\text{C}$ and -0.18 for $\delta^{15}\text{N}$ during the 5 d in July.

Estimation of TP for ghost shrimp larvae based on AA $\delta^{15}\text{N}$

The AA-specific $\delta^{15}\text{N}$ values of 2 sets of the *N. harmandi* zoea-VI samples were 2.3 and 2.9‰ for Phe, 14.7 and 15.2‰ for Ala, and 14.4 and 14.5‰ for Glu,

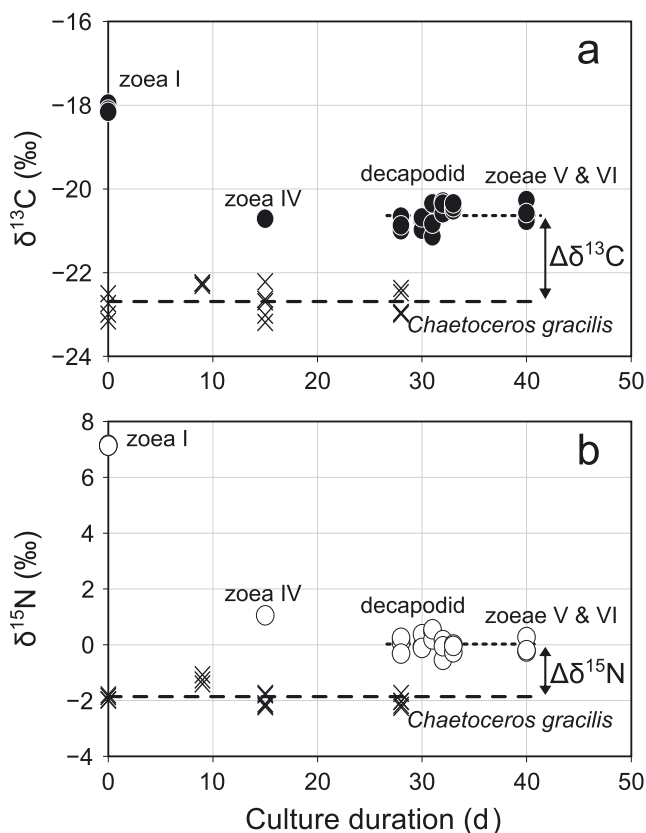


Fig. 4. Shift in (a) $\delta^{13}\text{C}$ and (b) $\delta^{15}\text{N}$ values (solid and blank circle plots, respectively) for the whole-body tissue of *Nihonotrypaea harmandi* zoeae and decapodids in relation to that for food (diatom, *Chaetoceros gracilis*; crosses) during the feeding experiment. See text for sample numbers (n_s). The samples retrieved on Day 40 for stable isotope analysis consisted of zoeae V and VI only. Dotted line and broken line in each panel: mean values for *N. harmandi* and *C. gracilis*, respectively. $\Delta\delta^{13}\text{C}$ and $\Delta\delta^{15}\text{N}$ designate the trophic enrichment factors (TEFs)

respectively. The calculated mean TPs for ghost shrimp larvae were 2.1 for $\text{TP}_{\text{Glu/Phe}}$ and 2.7 for $\text{TP}_{\text{Ala/Phe}}$.

Taxonomic composition and standing crop of small-sized plankton in Amakusa-nada

Total abundance and total biovolume of nano- to micro-sized plankton in the 4 depth layers from the surface to 60 m at Stn A on 7 August 2012 ranged from 7.0×10^4 to $1.2 \times 10^5 \text{ ind. l}^{-1}$ and from 1.5×10^8 to $2.4 \times 10^8 \mu\text{m}^3 \text{ l}^{-1}$, respectively, with values tending to decrease with depth (Fig. 6). Diatoms were dominant through the water column, both in abundance (47.3 to 55.3%) and biovolume (37.7 to 80.7%). Specifically, centric diatoms composed of Coscinodiscineae, Rhizosoleniineae, and Biddulphiineae accounted for 70.7 to 90.0% in abundance and 93.6 to 97.2% in biovolume of the diatoms. Dinoflagellates (11.0 to 27.7% in abundance and 9.5 to 23.1% in biovolume, mainly composed of Gymnodiniales) or ciliates (5.8 to 12.2% in abundance and 3.8 to 33.2% in biovolume, mainly composed of aloricate type) were subordinate.

Bulk SI values for ghost shrimp larvae and their potential food sources

The salinity averaged over the surface to 40 m depth at Stn A (Fig. 1) from CTD data in each cruise

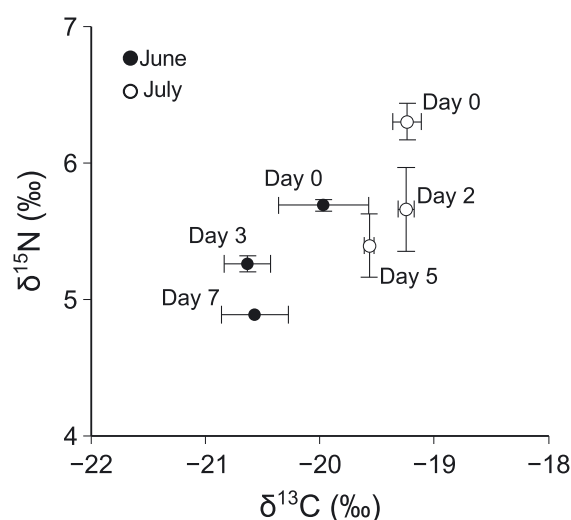


Fig. 5. Shift in range or mean ($\pm\text{SD}$) $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for particulate organic matter (POM) with microbial degradation under dark conditions in the laboratory in June and July 2013. See text for sample numbers (n_s)

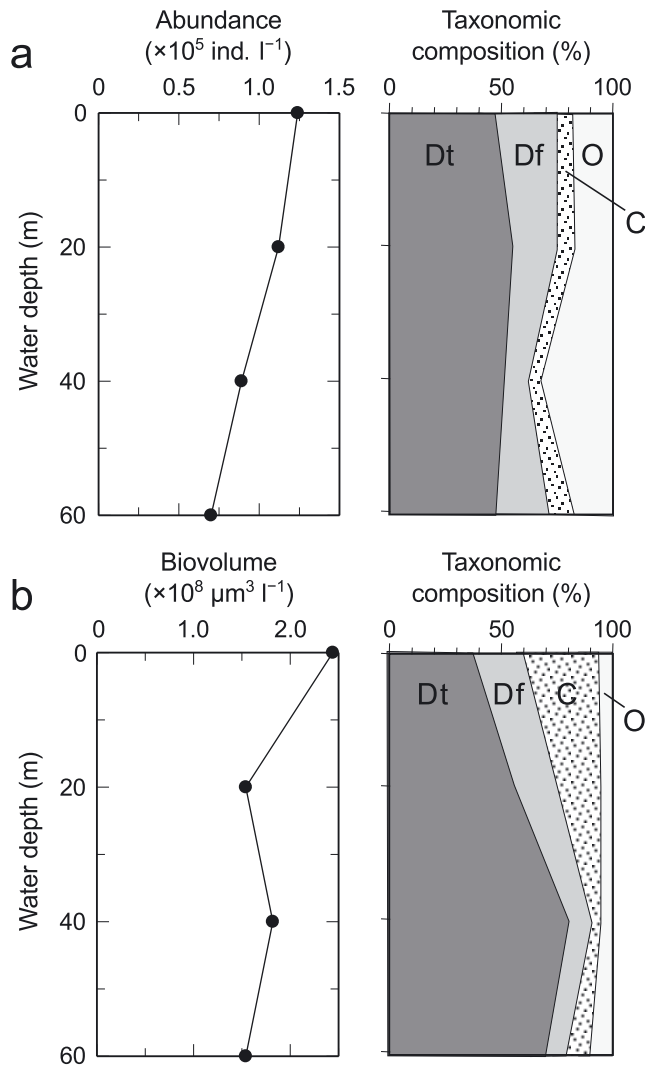


Fig. 6. Vertical profiles of (a) abundance and (b) biovolume of nano- and micro-sized plankton and their taxonomic compositions at Stn A in Amakusa-nada off mid-western Kyushu on 7 August 2012. Dt: diatom; Df: dinoflagellate; C: ciliate; O: other group including filamentous cyanobacteria and unidentified organisms

was lower in 2012 (mean \pm SD = 33.0 ± 0.5 in July and 32.6 ± 0.3 in August) than in 2013 (33.7 ± 0.2 in June, 33.6 ± 0.2 in July, and 33.4 ± 0.1 in August). Irrespective of the year, around the chl maximum layers at Stn A, higher chl *a* concentrations ($>10 \mu\text{g l}^{-1}$) were observed at 10 to 20 m depths in June and July, while lower peaks of 2 to $8 \mu\text{g l}^{-1}$ were observed at 30 to 50 m depths in August.

The SI values of biological samples and POM from Amakusa-nada were compiled separately for each of 2012 and 2013 (Fig. 7). The $\delta^{13}\text{C}$ in *N. harmandi* zoeal whole-body tissues preserved in formalin ($\delta^{13}\text{C}_{\text{formalin}}$)

had lighter values than those in frozen (= 'intact') samples ($\delta^{13}\text{C}_{\text{frozen}}$) (i.e. mean $\Delta\delta^{13}\text{C}$ [$\equiv \delta^{13}\text{C}_{\text{formalin}} - \delta^{13}\text{C}_{\text{frozen}}$] = $-1.0 \pm 0.2\text{‰}$, $n = 9$), while $\delta^{15}\text{N}_{\text{formalin}}$ had heavier values than those in $\delta^{15}\text{N}_{\text{frozen}}$ (i.e. $\Delta\delta^{15}\text{N}$ [$\equiv \delta^{15}\text{N}_{\text{formalin}} - \delta^{15}\text{N}_{\text{frozen}}$] = $0.5 \pm 0.3\text{‰}$, $n = 9$). Therefore, the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for the zoeal whole-body tissues given in Fig. 7 are those after the correction for the effect of formalin preservation by subtracting the above mean Δ -values from $\delta^{13}\text{C}_{\text{formalin}}$ or $\delta^{15}\text{N}_{\text{formalin}}$. The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for other zooplankton whole-body tissues also designate those compensated ones.

In 2012, the mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in *N. harmandi* zoeal whole-body tissues at stages I and II inclusive were $-18.3 \pm 0.2\text{‰}$ and $6.0 \pm 0.2\text{‰}$ ($n = 7$), respectively, and those values at stages III and IV inclusive had similar values ($\delta^{13}\text{C}$: $-18.5 \pm 0.3\text{‰}$; $\delta^{15}\text{N}$: $6.3 \pm 0.2\text{‰}$, $n = 6$) (Fig. 7a). Most of the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in the zoeae at stages V and VI inclusive showed lighter values (i.e. $\delta^{13}\text{C}$: $-20.2 \pm 0.3\text{‰}$; $\delta^{15}\text{N}$: $5.7 \pm 0.2\text{‰}$, $n = 5$), which were sufficiently distant from those at the earlier stages, though several latest-stage specimens (2 ind. of zoea V and 1 ind. of zoea VI) took values closer to the earlier-stage values. The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for POM around the chl maximum layer showed large variations ranging from -22.8 to -18.7‰ ($-20.5 \pm 1.3\text{‰}$, $n = 10$), and from 4.8 to 6.2‰ ($5.6 \pm 0.4\text{‰}$, $n = 10$), respectively. The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in the smaller zooplankton (0.3 to 1.2 mm in size) were $-19.9 \pm 0.3\text{‰}$ ($n = 3$) and $5.5 \pm 0.2\text{‰}$ ($n = 3$), respectively, while the larger zooplankton (1.2 to 3.5 mm in size) had relatively heavier values ($-19.5 \pm 0.2\text{‰}$, $n = 8$ for $\delta^{13}\text{C}$ and $6.3 \pm 0.3\text{‰}$, $n = 8$ for $\delta^{15}\text{N}$).

In 2013, the mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in *N. harmandi* zoeal whole-body tissues at stages I and II inclusive were -17.6 and 6.1‰ ($n = 1$ for June and July each), respectively, and those values gradually shifted to lighter ones with larval development; at stages III and IV inclusive ($\delta^{13}\text{C}$: $-18.3 \pm 0.3\text{‰}$; $\delta^{15}\text{N}$: $6.0 \pm 0.2\text{‰}$, $n = 6$) and at stages V and VI inclusive ($\delta^{13}\text{C}$: $-18.7 \pm 0.2\text{‰}$; $\delta^{15}\text{N}$: $5.7 \pm 0.3\text{‰}$, $n = 10$) (Fig. 7b). As in 2012, the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for POM in the chl maximum layer in 2013 showed large variations ranging from -22.1 to -19.4‰ ($-20.5 \pm 1.0\text{‰}$, $n = 5$), and from 4.1 to 5.6‰ ($5.0 \pm 0.6\text{‰}$, $n = 5$), respectively. The values for POM in 2012 and 2013 inclusive were near to the previously reported values at 5 m depth on a location 7.5 km east of Stn A in May and June 2004 ($\delta^{13}\text{C} = -21.3 \pm 0.2\text{‰}$ and $\delta^{15}\text{N} = 5.9 \pm 0.3\text{‰}$; Shimoda et al. 2007).

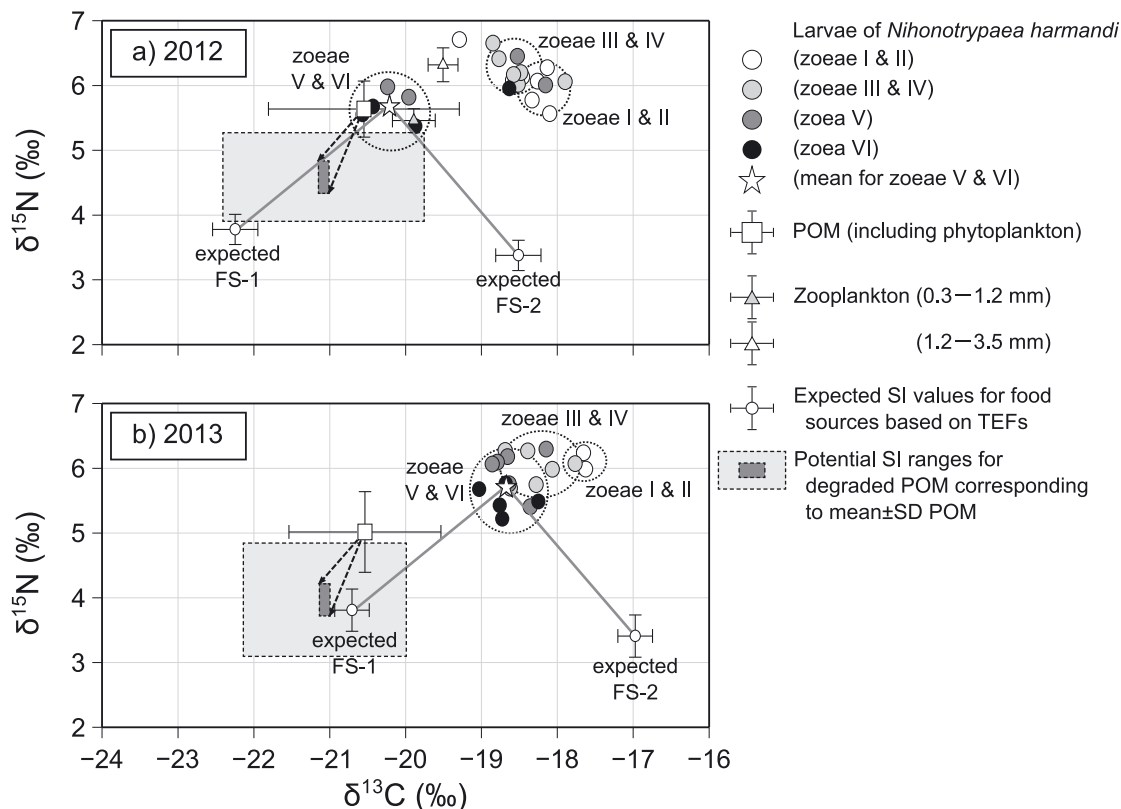


Fig. 7. Dual bulk stable isotope (SI) plots for particulate organic matter (POM), zooplankton, and zoeae I–VI of *Nihonotrypaea harmandi* collected from Stn A in Amakusa-nada off mid-western Kyushu in (a) 2012 and (b) 2013. Samples from multiple cruises during the summer were compiled for each year. Mean values for 2 successive zoeal stages inclusive (I and II, III and IV) or the single mean values for the zoeae V and VI for *N. harmandi* and the mean (\pm SD) values for the other components are indicated (see text for sample numbers, n_s). Star: mean values for zoeae V and VI inclusive (SDs are omitted for simplicity; see text for values). SI values for zooplankton in 2012 were distinctly lighter for the smaller 2 fractions (0.3–0.8 and 0.8–1.2 mm in size) than for the larger 4 fractions (>1.2 mm), so the zooplankton were separated into 2 groups at 1.2 mm. The mean SI values of the 2 expected food sources (FSs) corresponding to the mean values of the *N. harmandi* zoeae V and VI inclusive were calculated from respective trophic enrichment factors (TEFs): FS-1 from zoeal whole body-specific TEFs ($\Delta\delta^{13}\text{C} = 2.0\text{‰}$; $\Delta\delta^{15}\text{N} = 1.9\text{‰}$; Fig. 4) and FS-2 from *N. harmandi* juvenile whole body-specific TEFs, respectively (based on Fig. 5 in Yokoyama et al. 2005). Dark-shaded smaller box (rectangular area) and light-shaded larger box: expected smaller and larger ranges of SIs for the presumed degraded-POM, respectively, derived from the mean SIs for the POM and its SDs, respectively. The positions of 2 opposite corner points on the smaller box are indicated by 2 arrows, each subjected to microbial degradation of the POM at the corresponding daily decreasing rate in its mean SI values for 1 wk (Fig. 6)

DISCUSSION

The present study was initiated primarily to fill a gap between the universally standard laboratory diet (i.e. excess ration of a specific set of microzooplankton) for rearing decapod crustacean larvae and the absence, or low availability, of nutritionally equivalent microzooplankton in the coastal ocean water column containing plentiful phytoplankton and their detritus (phytodetritus).

The larvae of *Nihonotrypaea harmandi* mass-reared at a water temperature of 22°C, with a diet consisting solely of pure-cultured nano-sized dia-

toms *Chaetoceros gracilis* in an excess ration, reached the first peak in decapodid occurrence on Day 29 and the second one on Days 34 and 35, and survived at rates of 3.4 to 3.9% from the initially housed zoeae I (Fig. 3). Zoeal bulk SI equilibration with regard to diatom SIs (Fig. 4) excludes the possibility of ingesting deposited animal material. The above-mentioned values are comparable with those obtained from the 2 previous rearing experiments under similar conditions to the present study (Tamaki et al. 2013). There, about 6600 newly hatched zoeae were housed in a 30 l tank and reared with a combination of *C. gracilis* (targeted only for zoea I), *Brach-*

ionus rotundiformis (rotifer; provided for all zoeal stages), and *Artemia* sp. nauplii (targeted for zoeae III–VI), with each food item in an excess ration at 2 constant water temperatures. At 21°C, the first and second peaks in decapodid occurred on Day 30 (Tamaki et al. 2013) and Day 35 (A. Tamaki unpubl. data), respectively, with a combined retrieval rate of 7.35% (Tamaki et al. 2013). At 24°C, those peaks occurred on Day 30 (Tamaki et al. 2013) and Day 34 (A. Tamaki unpubl. data), with a combined retrieval rate of 4.7% (Tamaki et al. 2013). The 2 cohorts in the *N. harmandi* decapodids are likely to have originated from those cohorts in newly fertilized embryos and newly hatched zoeae I, for which a common ratio in the larger to smaller mean volumes existed (i.e. 1.2; Fig. 2). The larger eggs in *N. harmandi* occur around the time when females begin to participate in reproduction, and repeated egg laying with time brings about smaller eggs (A. Tamaki unpubl. data; cf. Kubo et al. 2006). One congeneric species, *N. japonica*, has a mean egg volume 1.5 to 2.0 times greater and a mean zoea I total length 1.11 times greater than those in *N. harmandi* (see Kubo et al. 2006 and Tamaki & Miyabe 2000, respectively); the difference in cubic total length is 1.37 times. The larval development of *N. japonica*, from zoea I through zoea II to zoea V to the first occurrence of decapodids, took 16 d in a laboratory rearing under similar conditions as in Tamaki et al. (2013) (Miyabe et al. 1998). Among decapod crustacean species, those with larger eggs tend to have greater yolk contents (Wear 1974), which would lead to shorter larval developmental durations, including callianassid shrimp species (Kubo et al. 2006). This might also be true for the intraspecific difference in egg volume and larval developmental duration.

Of the 3 available studies about the successful rearing of larvae of the decapod crustacean suborder Pleocyemata solely with phytoplankton, 2 were qualitative, in which nano- to micro-sized diatoms and dinoflagellates (Atkins 1955) or green alga and diatom (Bousquette 1980) were provided to pinnotherid brachyurans. The other one quantitatively tested the effect of 3 diatom species with different sizes (250, 40, and 10 μm in \varnothing) on the brachyuran *Hyas coarctatus*, with only the largest species (*Biddulphia sinensis*: micro-size) found to be a valid food but much less efficient than *Artemia* sp. nauplii (Harms & Seeger 1989). In that case, chain-forming in diatoms was regarded as essential for ingestion by the zoeae. In the digestive tract contents of *N. harmandi* zoeae from Amakusa-nada, micro-sized diatoms belonging to Rhizosoleniineae, Coscinodiscineae, Biddulphi-

ineae, and Pennales were found (Somiya et al. 2014). Each diatom frustule or piece was shorter than 25 μm in width and height, while the length was not limited; smaller or slender diatoms might be ingested whole, while larger or wider diatoms would be masticated by the mandible before ingestion. Cells of *C. gracilis* used for the present study were nano-sized and not chain-forming, and *N. harmandi* zoeae ingest them not individually but as a bolus accumulated by filter-feeding around the mouthparts (R. Somiya pers. comm.). Such ingestion of lumps was observed for conspecific decapodids and juveniles (Yokoyama et al. 2005, Tamaki et al. 2013). Under field conditions, intraspecifically, zoeae of Pleocyemata species ingest a wide range of non-motile (and motile) phytoplankton (and phytodetritus), from pico, through nano, to micro in size (Stickney & Perkins 1981, Paul et al. 1989, Meyer-Harms & Harms 1993, Fileman et al. 2014). Such indiscriminate ingestion of prey by those zoeae, including microzooplankton prey with limited mobility used for the laboratory rearing, can be understood by widely known filter-feeding habits of Pleocyemata larvae for any encountered prey of varying sizes (Rumrill et al. 1985, Factor & Dexter 1993, Crain 1999, Kjørboe 2011, Wirtz 2012).

The indiscriminate filter-feeding of decapod crustacean zoeae could support their growth and survival under non-motile prey-rich conditions in the dark (Harvey & Epifanio 1997, Hinz et al. 2001). In the water column of Amakusa-nada in the summer of 2006, most *N. harmandi* zoeae stayed below 20 m depth, where photon flux density was less than 2% of that at the surface, with the chl maximum layer at 22 to 24 m (Tamaki et al. 2010). In that water column in August 2012, diatoms were the predominant taxonomic group, accounting for 57 to 81% of the total biovolume of the potential prey assemblage between 20 and 40 m water depths (Fig. 6b). This tendency would be common in temperate coastal waters with higher nutrient concentrations during the summertime (Harvey et al. 1997, Furuya et al. 2003). Therefore, if *N. harmandi* zoeae indiscriminately ingest non-motile plankton by filter feeding in Amakusa-nada, diatoms and their sinking detritus would be the primary diet.

Although SI analysis can be used to provide strong evidence for the trophic status of meroplanktonic larvae of decapod crustaceans in the field, actual studies are scarce and limited to bulk analyses (Schwammborn et al. 1999, 2002). Of the present 2 TP estimates for *N. harmandi* zoeae from Amakusa-nada based on the $\delta^{15}\text{N}$ of amino acids (TP_{AAs}), one was 2.1 when using the most common measure suitable for meta-

zoan food-chain ($TP_{Glu/Phe}$), while the other was 2.7 when using a measure incorporating a possible protistan grazing pathway ($TP_{Ala/Phe}$). Combination of these 2 estimates suggest that contribution of metazoans to the diet of those zoeae is minimum ($TP_{Glu/Phe} \approx 2$) and that the primary diet is a mixture of phytoplankton and heterotrophs mainly including protozoa ($TP_{Ala/Phe} = 2$ to 3). The potentially mixed prey consisting of phytoplankton and protists was also reported for some suspension-feeding smaller holozooplankton based on the 2 TP_{AAs} (e.g. $TP_{Glu/Phe} \approx 2$ and $TP_{Ala/Phe} = 2.5$ to 3.0 for copepodite-stage individuals of *Calanus pacificus* and juveniles of *Euphausia pacifica* in Californian coastal waters; Décima et al. 2017); the body size of *N. harmandi* zoeae (3 to 7 mm) is larger than that of these zooplankton. Protists constituted a part of the diet for reared brachyuran larvae (Lehto et al. 1998, Hinz et al. 2001), and nano- and pico-eukaryotes and ciliates were contained in the digestive tract contents of wild-caught brachyuran and gebiidean larvae (Fileman et al. 2014). The setal structure and motion of mouthpart appendages of *N. harmandi* zoeae are inefficient in collecting sparse tiny cells and in grasping motile plankton such as free-living ciliates and dinoflagellates (Somiya et al. 2014, R. Somiya unpubl. data). Generally, phytodetritus is regarded as a complex aggregate with heterotrophs (Turner 2015), which can be large enough for decapod crustacean larvae to collect as well as large diatoms. Thus, phytoplankton, diatoms in particular (Fig. 6), and their detritus with associated heterotrophs including protozoa are estimated to be the most likely diet of *N. harmandi* zoeae.

The bulk SI analysis provided another clue to support the estimated contribution of phytodetritus to the *N. harmandi* larval diet. The bulk SI ($\delta^{13}C$ and $\delta^{15}N$) values for the latest-stage zoeae (V and VI) in Amakusa-nada are expected to have been equilibrated with those for their food sources, following the observation of the SIs in whole-body tissue of zoeae fed cultured diatoms and their stabilization around Day 20 (Fig. 4). In fact, in the dual bulk SI diagram comprising POM, zooplankton (only in 2012), and the zoeae, the SI values for the zoeae I and II shifted to the lighter ones as they grew to the zoeae V and VI both in 2012 and 2013 (Fig. 7). The mean (\pm SD) $\delta^{15}N$ values for zoeae V and VI in 2012 ($5.7 \pm 0.2\text{‰}$, excluding outliers) were similar to those of the smaller zooplankton ($5.5 \pm 0.2\text{‰}$), and even lighter than those of the larger zooplankton ($6.3 \pm 0.3\text{‰}$) (Fig. 7a). This suggests that copepod-dominated zooplankton (0.3 to 3.5 mm in size) were not the potential food source

for the *N. harmandi* zoeae. A similar configuration of microzooplankton (presumed primary consumers) and decapod crustacean zoeae in the dual bulk SI diagram was found for a plankton assemblage in the coastal ocean off a mangrove estuary in northeastern Brazil (Schwamborn et al. 1999, 2002). Unexpectedly in the present study, the mean $\delta^{15}N$ value for the POM ($5.7 \pm 0.5\text{‰}$) was not far below but rather close to the $\delta^{15}N$ value for the zoeae V and VI. This similarity poses a question on the status of POM (presumed phytoplankton) as the main food source for the zoeae, considering isotopic discrimination across trophic steps. Firstly, POM is not composed of phytoplankton only, but of a mixture of phytoplankton, phytodetritus, zooplankton debris, and fecal pellets (Lee 2002, Volkman & Tanoue 2002). With POM being such a complex mixture, the $\delta^{15}N$ value might be lighter for live phytoplankton per se than for POM. Secondly, even if the main constituent of POM from the chl maximum layers is live phytoplankton, its degraded form (i.e. phytodetritus including heterotrophs), with altered SI values, could be the principal diet of the *N. harmandi* zoeae. These larvae might selectively feed on sinking aggregates composed of degraded lumps of diatoms with heterotrophs, which could be more easily collected and ingested than loose cells.

The analyses of the dual bulk SI diagram indicated that phytodetritus was likely an important food source for *N. harmandi* zoeae, based on the SI shifts in POM during degradation and larval TEFs (Fig. 7). Those zoeae stay mainly at 30 to 50 m depths in Amakusa-nada (Tamaki et al. 2010), where sinking phytodetritus originated from the upper chl maximum layer (usually 10 to 20 m depths except for August, with 30 to 40 m depths) may be abundant. A potential daily shift in the SI values of POM derived from that layer, with microbial degradation, is estimated at -0.07 to -0.09‰ d^{-1} for $\delta^{13}C$ and -0.11 to -0.18‰ d^{-1} for $\delta^{15}N$, based on the 2 incubation experiments (Fig. 5). These alteration rates are comparable to those reported in Lehmann et al. (2002), in which diatom-dominated POM was incubated under different redox conditions (-0.06 to -0.13‰ d^{-1} for $\delta^{13}C$ and -0.10 to -0.12‰ d^{-1} for $\delta^{15}N$; calculated here from those for the initial 10 d of the original 111 d incubation). Assuming that live phytoplankton at the chl maximum layer in Amakusa-nada sink for a distance of 25 m in 7 d at an average speed of 3.5 m d^{-1} (cf. Kriest & Oschlies 2008), with the corresponding SI-value alterations ($\Delta\delta^{13}C = -0.5$ to -0.6‰ wk^{-1} ; $\Delta\delta^{15}N = -0.8$ to -1.3‰ wk^{-1}), the range of the SI values for the expected degraded POM will be located at the lighter position

than that for the measured POM with large variations. In 2012, the expected $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of the food source for *N. harmandi* zoeae V and VI inclusive based on their whole-body (including exoskeleton)-specific TEFs (2.0‰ for $\delta^{13}\text{C}$; 1.9‰ for $\delta^{15}\text{N}$; Fig. 4) lay close to the edge lines of the larger SI-box for the expected degraded-POM (expected FS [food source]-1 in Fig. 7a). In a previous companion rearing experiment for *N. harmandi* juveniles, Yokoyama et al. (2005) reported that TEFs for the whole body (−1.7‰ for $\delta^{13}\text{C}$; 2.3‰ for $\delta^{15}\text{N}$) were lower than those for the muscle, probably due to the effect of chitin in the exoskeleton; no lipid removal was made as in the present study. Based on these specific TEFs, there was no candidate organic matter for the zoeal food sources (expected FS-2). Thus, it appears indispensable to obtain the TEFs that are specific to the zoeal whole body, with its exoskeleton probably not so fully developed as in juvenile exoskeleton.

The configuration of POM, presumed degraded-POM, and *N. harmandi* zoeae in the dual bulk SI diagram for 2013 was largely similar to that for 2012, though the $\delta^{13}\text{C}$ values for zoeae V and VI inclusive in 2012 were lighter by about 1.5‰ (Fig. 7b). One reason for the latter might be a higher contribution of terrestrial dissolved inorganic carbon to primary production, with relatively lighter $\delta^{13}\text{C}$ values than those in seawater (e.g. Boutton 1991). The cumulative rainfall recorded at a meteorological observatory in Kumamoto Prefecture (32.813° N, 130.707° E), located near Ariake Sound, during the rainy season of Japan (June and July) was 3 times higher in 2012 than in 2013 (1200 vs. 430 mm; Japan Meteorological Agency Past Weather Data; www.data.jma.go.jp/obd/stats/etrn/index.php). The average discharge of the nearby largest river (Chikugo River emptying into the innermost Ariake Sound, with a 35 % watershed area in the sound; Iyama 2007) in this season were also 3 times higher in 2012 (493 vs. 163 m³ s^{−1} at Senoshita station; Ministry of Land, Infrastructure, Transport and Tourism Water Information System; <http://www1.river.go.jp/>). In accordance with this, the salinity in the water column at Stn A in Amakusana was lower in 2012 (see 'Results'). Although such river discharge may not have been clearly reflected in the $\delta^{13}\text{C}$ of POM in our limited survey results (i.e. 2 to 3 time water samplings for 1 d each month), its signature could have been manifested over time in the $\delta^{13}\text{C}$ of *N. harmandi* zoeae via primary production. Despite these yearly meteorological differences, the plot for the mean SIs of the expected food source for the latest-stage zoeae of *N. harmandi* (FS-1) lying close to the edge (in 2012) or inside (in 2013) of the

larger SI box for the presumed degraded-POM provides robust evidence for phytoplankton (especially diatom)-derived detritus including heterotrophs as the principal potential diet.

The bulk $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analysis for *N. harmandi* adults from the Tomioka sandflat revealed that their diet was phytoplankton (as POM) from the coastal ocean and benthic microalgae on the sandflat (Shimoda et al. 2007). Since the constant deposition of live microalgae into the shrimp burrow makes their density high, the SI signature of food sources in shrimp bodies appears to be unambiguous. In contrast, the density of live phytoplankton is lower in the water column, especially for herbivorous zooplankton staying below the chl maximum layer. At 10s of meters water depths in the inner-shelf coastal ocean, phytodetritus (enriched with heterotrophs) would serve as the seasonally most abundant food source for filter-feeding larvae of decapod crustaceans, probably being more dense than live phytoplankton (time-integrated vs. instantaneous resources).

CONCLUSIONS

The present study has demonstrated that the carnivorous feeding habit of decapod crustacean larvae preying on microzooplankton such as brine shrimp nauplii under laboratory culture is not necessarily applicable to their habit in the field, raising a possibility that phytoplankton-based diet is more common in meroplanktonic larvae of the suborder Pleocyemata than previously thought, especially in coastal oceans connected to estuaries with high primary production. In this demonstration, a combined approach (rearing with pure-cultured phytoplankton, field surveys of nano- to micro-sized plankton assemblage, TP analysis based on AA $\delta^{15}\text{N}$, determination of species-specific TEFs, and bulk SI analysis) proved useful to estimate diatom-dominated phytoplankton and their detritus with heterotrophs including protozoa as the principal diet of meroplanktonic larvae. The possible trophic status of those planktotrophic larvae as the consumer relying mainly on phytoplankton would be relevant to such aspects as (1) bottom-up effects of primary producers on the survival of those larvae (Thorson 1950, Stickney & Perkins 1981, Olson & Olson 1989, Shirley & Shirley 1989, Kirby et al. 2008), (2) reciprocal impact from those larvae to primary producers (Fileman et al. 2014), (3) vertical migration of those larvae in relation to phytoplankton and phytodetritus distribution in the water column and its consequent horizontal transport (Pearre 2003,

Woodson & McManus 2007, Tamaki et al. 2010), and (4) biogeography of plankto- and lecitho-trophic larval-type distribution with chlorophyll concentration distribution in the global sea surface (Thorson 1950, Marshall et al. 2012).

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