Assessing the role of demersal zooplankton in the food web of shallow coastal ecosystems using stable carbon and nitrogen isotopes

静岡大学博士論文

Assessing the role of demersal zooplankton in the food web of shallow coastal ecosystems using stable carbon and nitrogen isotopes

炭素・窒素の安定同位体を用いた沿岸浅海域生態系の食物網における底生動物プランクトンの役割の評価

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VU Manh Hung

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Abbreviations

Abbreviations	Denote for
ATP	Adenosine triphosphate
DON	Dissolve Organic Nitrogen
DZ	Demersal zooplankton
LG	Coral reef lagoon
MG	Mangrove
MCMC	Markov chain Monte Carlo
MPB	Microphytobenthos
PDB	Pee Dee Beleminate
SIA	Stable Isotope Analysis
SG	Seagrass
SG+CR	Seagrass mixed with Coral colonies
TEFs	Trophic Enrichment Factors

General Abstract

The coastal habitats exist somehow isolated from the marine environment. However, they keep significant interactions among interconnected coastal ecosystems. Mangroves, seagrass beds, and coral reef lagoons are examples. Mangroves distribute mainly on estuarine areas, which play a major role as a trap of sediments' runoff. Toward to the sea, seagrass distributes in the subtidal area; they act as a buffer area accumulating sediments and pollutants from runoff of terrestrial zones before reaching coral habitat. Meanwhile, coral reef lagoons protect inshore habitats from the wave action. Overall, mangroves, seagrass beds, and coral reefs lagoons are important marine coastal ecosystems that sustain high biodiversity and are highly efficient at transferring organic matter from primary producers to higher trophic levels. Although, these habitats accumulate a large quantity of non-living organic matter in their sediments in the form of detritus, leaf litter, and decomposed dead organisms; there are few species of consumers can utilize this food source directly. Thus, sediment detritivores and herbivores might be important for connecting these food webs by transferring energy from primary producers to higher trophic levels. Demersal zooplankton (DZ) emerge to the water column at night with high densities in shallow coastal ecosystems (i.e. seagrass, coral reefs, soft bottoms, and kelp beds). Therefore they might play a major role in linking small particles (detritus and primary producers attached to sediments) to planktivores (fishes and other suspended feeders). This study aims to clarify the role of DZ in three shallow coastal ecosystems, using stable isotope analysis in combination with stomach content analysis.

Determination of the proportional contribution of food sources to the diet of consumers by solely the stomach contents show serious limitation because the food is rapidly digested in comparison to the slow digestion of non-living organic matter derived from sediments. Natural stable isotope analysis is a powerful tool to determine the relationship between predators and their food sources. The stable isotope analysis in R (SIAR) isotopic mixing model based on dual stable carbon and nitrogen isotope signature was applied to calculate the proportion of food source in the diet of consumers. Stomach content analysis of fishes and invertebrates was used to find out indications of potential food sources. The combination of both methods was applied in this study. Moreover, the biomass and species composition of DZ was also studied in order to evaluate their contribution in each of the studied environments.

The experiment presented in chapter 3 was designed to find out the trophic enrichment factor between *Artemia salina* and its food source, the diatom *Nitzschia* sp. Natural stable carbon and nitrogen isotope of *Artemia salina* and its food source were measured at different stages of the life cycle of *Artemia salina*. This study demonstrated that isotopic signal of *Artemia salina* reached the equilibrium isotope value at day 25 after hatching and the trophic enrichment factor of *Artemia salina* was determined to mean (± SD) 0.0 ± 0.9 (‰) for $\Delta^{13}C$ and 1.0 ± 0.5 (‰) for $\Delta^{15}N$.

The trophic enrichment factor between zooplankton and its food sources that were determined in chapter 3 was applied to estimate the proportion of potential food sources in the diet of DZ. This study was presented in chapter 4

and conducted the reef lagoon at Bise, Okinawa, Japan, which is composed of a seagrass area and coral heads mixture with seagrass the reef lagoon. The result of stable isotope mixing model highlights the role of organic matter derived from seagrass, particularly seagrass detritus, influencing the abundance of DZ in each specific habitat. Phytoplankton and macro algae also play an important role as a food source for DZ in the lagoon. When comparing the importance of potential food sources, it suggests that DZ prefer on main food source which depending on available sources and their migration among habitat in small spatial scales.

Finally, in Chapter 5, the role of DZ as a food source for higher trophic levels in an estuarine area was discussed, especially with respect to the food preference and size selection of their consumers. The lowest DZ biomass was recorded in mangroves and mainly dominated by smaller organisms because their consumers in this habitat prefer large-sized prey. The $\delta^{13}C$ and $\delta^{15}N$ signatures showed that, in mangroves, DZ constituted a higher proportion of the diet of fishes than in lagoon habitats; however, DZ did not play a significant role in the diet of fishes and macroinvertebrates in the lagoon. Consistency among biomass, stomach contents, and the proportions of DZ of all size classes in the diet of mangrove fishes indicated that DZ serve as a major food source. In contrast, fishes in lagoon habitats consumed more crabs, shrimps, and mollusks than DZ. We found that role of DZ as a food source was different in the different habitats.

CHAPTER 1

GENERAL INTRODUCTION

Chapter 1

General Introduction

1.1 The interconnection between mangrove, seagrass and coral reef habitats

The shallow coastal habitats can exist in isolation because there is a diversity of interaction among habitats, mangrove distributed on coastal zone and estuary areas, which play as a trap sediment. Toward to the sea, seagrass distributes in the subtidal area, they play as a buffer area and accumulate other pollution runoff from terrestrial before they reach coral habitat. Meanwhile, coral protects inshore habitats from wave action from the open sea. Mangrove, seagrass, and coral habitats have long been known for their high diversity, vast productivity and provide various ecosystem services (Nagelkerken, 2009). Although these coastal ecosystems exist in isolation area, they usually occur together in a large-scale which considerable interaction may occur (Mumby and Hastings, 2008; Ogden and Zieman, 1977; Sheaves, 2005; Valentine et al., 2008). According to Ogden and Zieman (1977), cross-ecosystem interactions can mostly be subdivided into biological, chemical, and physical. Previous studies considered the exchange of animals among habitats, mostly feeding migration by fishes among ecosystems (Mcfarland et al., 1979; Ogden and Zieman, 1977; Weinstein and Heck, 1979), spawning and ontogenetic migration by fishes and decapods (Mumby and Hastings, 2008; Nakamura et al., 2007) were also studied. Recently studies also provided clear evidence of

animal exchange among habitats using advanced techniques; they have studied at different temporal and spatial scales, example the tidal migration, time scales (daytime, nighttime, and seasons or years) and distance scales (Mumby and Hastings, 2008; Valentine et al., 2008). However, the food sources which main supply for the animal during their migration and ontogenetic stages in each habitat were not well understood.

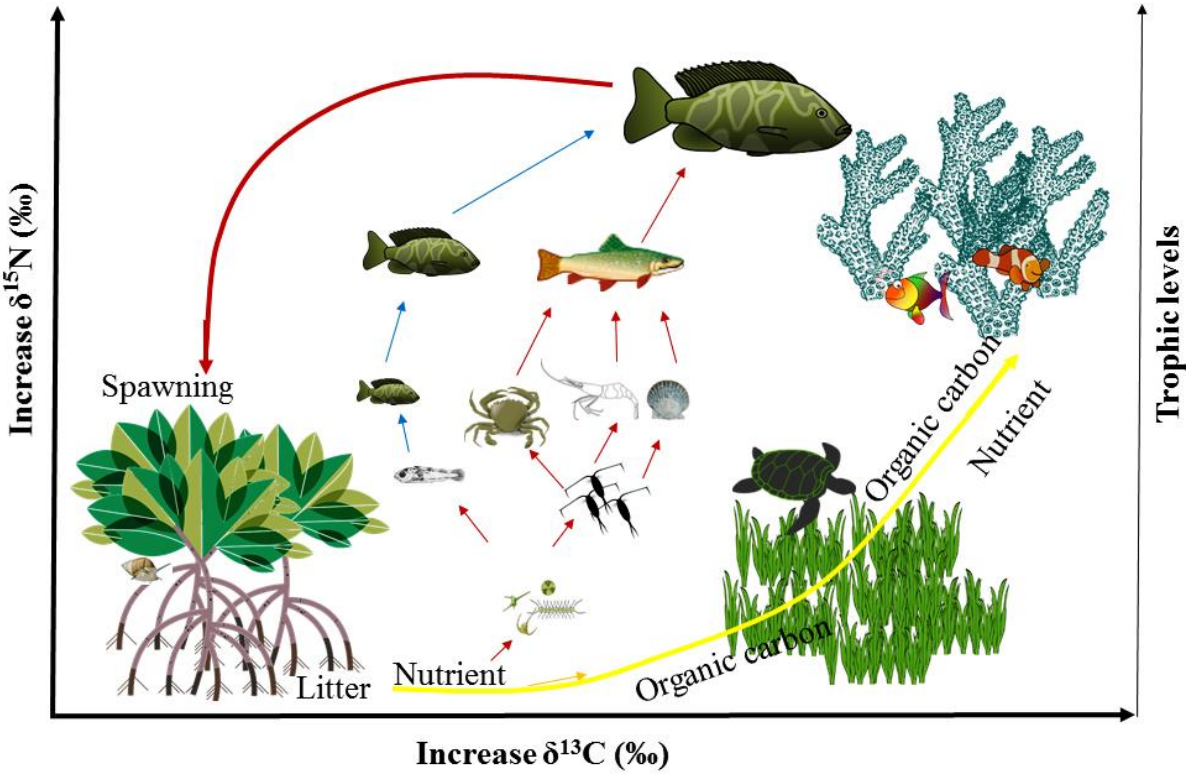

Figure 1. 1 Interconnection between habitats among shallow coastal ecosystem

1.2 Stable isotope application in aquatic food web research

The traditional methodology approach to assess aquatic food web including gut and stomach contents analysis, fecal analysis (identify the item contents), observation of the feeding preference of predator both in the field and laboratory (through the behavior of consumers), the dietary items of consumer analysis using DNA barcoding (reference from DNA database),

radiotracer (using radio-label as tracer to a food source and following the label through the food chain), immunological (extract antisera which are taxon-specific from organism), and fatty acid application (Kainz et al., 2004; Meier-Augenstein, 2002). Although these methods are useful and have helped to understand the food web structure, they also show various limitations, such as some prey are rapidly digested, whereas another, particularly hard part of preys are slowly digested and remain in the stomach of consumers. Moreover, the gut contents identification is difficult and requires a good taxonomic knowledge of all present organisms and other material. Therefore, comparisons base on gut contents between species may not be accurate and do not reflect clearly the consumer's preference (Michener and Lajtha, 2008). The DNA barcoding method is difficult to identify accuracy of diet qualification because DNA database is limit for all present organisms and DNA consumer is also normally present in the diet sample (King et al., 2008). The radio-labeling has a disadvantage as the statistically significant number of labeled species are difficult to recover and need secure permission to use radioactive isotopes (Michener and Lajtha, 2008). The immunological method cannot be used for large species and is an expensive method with strictly qualitative (Michener and Lajtha, 2008). The ratio of fatty acids in the food items and predator tissue have enjoyed success using and potential to be a major competitor to isotopic analysis in many cases (Michener and Lajtha, 2008).

Stable isotope analysis application in the studies aquatic food web to determine the relationship between predators and their food sources have been increasing and are frequently used to examine the food sources in aquatic food

webs (Nakamura et al., 2008; Pasquaud et al., 2010; Tue et al., 2013; Vinagre et al., 2012). Base on the natural isotopic fractionation between consumers and their prey, this method is a useful tool for aquatic food web analysis, especially in the marine ecosystem. Stable isotope analysis provides both, source information with sulfur and carbon isotopic analysis, and trophic level information using stable nitrogen isotope (Michener and Lajtha, 2008). The oxygen and hydrogen also have applied for studies of aquatic food webs, but uncommonly (Zhang et al., 2016). Here we focus on the application of stable carbon and nitrogen isotope to resolve estuarine food web structure.

Stable isotope mixing model uses the mixture isotopic signature to estimate the proportion of sources using mathematical mixing model. Application of isotope mixing model for estimating food source percentage in the aquatic food web is increasingly used for evaluation of the distribution of different potential food sources. Some other existing models such as IsoError, Isocons, and IsoSource can also be coped with multiple sources. However, they cannot incorporate uncertainty and variation (Parnell et al., 2010). Bayesian inference offers the way to reduce the limitations indicated above (Parnell et al., 2013, 2010). Bayesian fashion is formalized in the models MixSIR (Jackson et al., 2009; Moore and Semmens, 2008) and SIAR (Parnell et al., 2010). The SIAR model is a minor difference with MixSIR. SIAR use standard Markov chain Monte Carlo (MCMC) with Metropolis-Hastings steps to update the dietary proportions (Parnell et al., 2013) while MixSIR uses Sample Importance Resampling and SIAR including an overall residual error term lacking from MixSIR (Jackson et al., 2009; Parnell et al., 2010). In the order of

our objectives, we apply SIAR V4 an open package (Parnell and Jackson, 2013) of R software (R Core Team, 2015) to calculate the proportion of total diet that was contributed by different food sources, based on the stable isotope values of consumers, the mean and standard deviation (SD) stable isotope values of food sources, and the TEFs of food sources.

1.3 Demersal zooplankton (DZ) in the coastal shallow food web

Demersal zooplankton reside or hide near the substrate during the daytime, and they emerge and spend a short time in the water column during the night-time and return to the substratum before sunrise (Alldredge and King, 1980; Hobson and Chess, 1979; Melo et al., 2010). Studies on demersal zooplankton have shown high abundances emerging at night from seagrass, coral reefs, soft bottoms, and kelp beds (Jayabarathi et al., 2012; Mascart, 2010; Youngbluth, 1982), suggesting that DZ might play a role as a link between lower to higher trophic levels. DZ have high turnover rate and short life cycle (weeks to months). Thus they are quickly responding to organic matter input and close to primary production food sources (Escaravage et al., 1989; Heip et al., 1985; Lebreton et al., 2012). Their secondary production rate was estimated as 9.0 to 29.4 g C m^{-2} $year^{-1}$ (Escaravage et al., 1989). Moreover, DZ was found to be common food items in stomach content of planktonic fishes and shrimps (Coull, 1999; Hyndes and Lavery, 2005). Thus, they may serve as a significant food source for planktivores in shallow water habitats (Melo et al., 2010). However, few studies were dedicated to understanding the

role of DZ in these habitats. Thus, the diet of DZ, who are their consumers and how much they are consumed is still unknown.

1.4 Research objectives and thesis layout

The study presented in this thesis aims to clarify the function of demersal zooplankton in the coastal shallow water ecosystem focusing on (1) role of demersal zooplankton as a food source for higher trophic level and (2) food sources of demersal zooplankton to highlight their function in the linkage from lower to higher trophic levels. The main questions, which motived our objectives in this work are as follows:

1. How demersal zooplankton serve as a food source into the estuarine aquatic food web?
2. Do consumers prefer demersal zooplankton as a major food source?
3. How much proportion of demersal zooplankton contributes in compared against another potential food source in the diet of consumers?
4. Does consumer prefer on different body size fraction of demersal zooplankton?
5. What is main food source of demersal zooplankton?
6. Is there interconnection among habitats in shallow water influencing the food source of demersal zooplankton?

This thesis was represented in 5 chapters to demonstrate the role of demersal zooplankton in the ecosystem shallow water ecosystem in the Southern part of Japan.

Chapter 1 gives an overview of the interconnections among connected habitats in the shallow coastal ecosystem, particularly mangrove forest,

seagrass bed, and coral reef habitat. This chapter provides basic information about the application of Stable Isotope in the study of aquatic food webs. This chapter also gives the reader an overview about demersal zooplankton in the coastal shallow food web.

Chapter 2 talks about methodology, which includes a brief description of the studied site, detail description about the procedure of sample collection, sample treatments and technical information on stable isotope measurement. Moreover, this chapter introduces into the equation and stable isotope mixing model application for demersal zooplankton biomass calculation and estimation of the proportion of food source in the diet of consumers, respectively.

Chapter 3 is a study on stable isotope fractionation of zooplankton in a mono-feeding experiment. This study was conducted to find out the trophic enrichment factor between zooplankton and its food using a feeding experiment with *Artemia salina* and the diatom *Nitzschia* sp. The finding of this study will be applied to estimate the proportion of potential food source in the diet of demersal zooplankton.

Chapter 4 was designed to elucidate the food sources of demersal zooplankton in the shallow water ecosystem, particularly in a reef lagoon at Bise (Okinawa, Japan), which is composed of seagrass bed mixing with coral heads.

Chapter 5 gives a complete picture of the role of demersal zooplankton in the aquatic food web. The role of DZ as a food source for higher trophic levels in an estuarine area was discussed. This study combines stable isotope analysis and stomach content of consumers to clarify the proportional contribution of

DZ and other food sources to the diet of fishes and macroinvertebrates in the mangrove and lagoon habitats at Fukido Estuary, Japan. This study demonstrated that demersal zooplankton was important to the diet of fishes in mangrove habitats, with larger size classes being preferred, but did not have a significant role compared to other food sources in the diet of fishes and macroinvertebrates in the lagoon habitat. This difference is attributed to the fact that mangrove habitat is an important nursery and provide a wide range of food for supporting juvenile fish during ontogenetic stages compared to the lagoon habitat.

CHAPTER 2

METHODOLOGY

Chapter 2

Methodology

2.1 Study sites

The mono-feeding experiment for estimating stable isotope fractionation between zooplankton and its food source was conducted at Laboratory of Biogeochemical, Shizuoka University, Japan (Chapter 3). The study on the dietary proportion of demersal zooplankton in the coastal shallow environment was done at Bise, Okinawa Islands, Japan (Chapter 4). The study on the role of demersal zooplankton in estuarine food web was carried out in the Fukido Estuary, Ishigaki Island, Japan (Chapter 5).

2.1.1 Experiment in Bise, Okinawa Islands, Japan

Seagrasses and corals coexist in reef lagoon at Bise, Okinawa Island (Fig. 2.1). Seagrass bed extends in the shallow nearshore zone of Bise beach and is dominated by *Thalassia hemprichii* with sand and coral rubber substrates. Seagrass habitat (SG) distributes at a distance of 50 m from the beach and from that toward the middle of lagoon about 150 to 200 m seagrass mixtures with coral colonies (mainly branching coral *Montipora digitata*). The coexistence of seagrass and coral with a high density of seagrass and healthy coral is an interesting habitat in Bise reef lagoon; therefore in the present study, this area was selected as the seagrass mixture with coral habitat (SG+CR). Coral habitat (CR) distributes without the presence of seagrass from the middle of the lagoon to reef barrier. Seagrass in the shallow shoreline of the beach acts as a trap of

sediments and accumulates other pollutants from human activity near the beach and terrestrial runoff, which helps to make a clean environment for coral habitat. Meanwhile, coral protects lagoon habitats from the wave's action and currents from the open sea.

Figure 2. 1 Map of the study area at Bise Beach, Okinawa Island, Japan. SG: Seagrass bed habitat, SG+CR: Seagrass and Coral mixture habitat, and CR: Coral habitat

2.1.2 Experiment in Fukido Estuary, Ishigaki Island, Southern of Japan

Fukido estuary is located on the Itona Coast of Ishigaki Island, at the southern tip of Japan (Fig. 2.2A). We focused on the mangrove (MG) habitat at the mouth of Fukido River and the adjacent coral reef lagoon (LG) habitat, which is dominated by shallow seagrass (SG) followed by a zone where seagrass is mixed with coral colonies (SG+CR). The MG habitat extends from the mouth of the Fukido River (mouth is 10–40 m across) and continues 300 m upstream, covering an area of about 18.7 ha (Kurosawa, 2003). *Rhizophora*

stylosa, Bruguiera gymnorrhiza, Kandelia candel, and *Lumnitzera racemose* dominate the mangrove forest. In the center of the river mouth, the water depth ranges from 0.5 to 1 m at low tide and 1 to 2 m at high tide, where mangrove prop roots are alternately inundated and exposed during the tidal cycle (Nakamura et al., 2008; Shibuno et al., 2008). The seagrass bed (water depth range of 0.5–1 m at low tide and 2–3 m at high tide) extends for about 2.5 km along the coast at a distance of 30 to 120 m offshore and is dominated by *Thalassia hemprichii* (Shibuno et al., 2008). Toward the middle of the lagoon, seagrass mixes with some coral colonies. Branching corals (mainly *Montipora* spp.) and massive corals (especially *Porites* spp.) dominate in this area of coexistence. During the flood tide, seawater passes over a large sand sill across the mouth of the river (Fig. 2.2C) and flows backward, inundating the mangrove forest. During the ebb tide, the water flows out through a small canal across the sand sill to the lagoon area. This sill completely separates mangrove water from lagoon water (Kurosawa, 2003; Nihei et al., 2002).

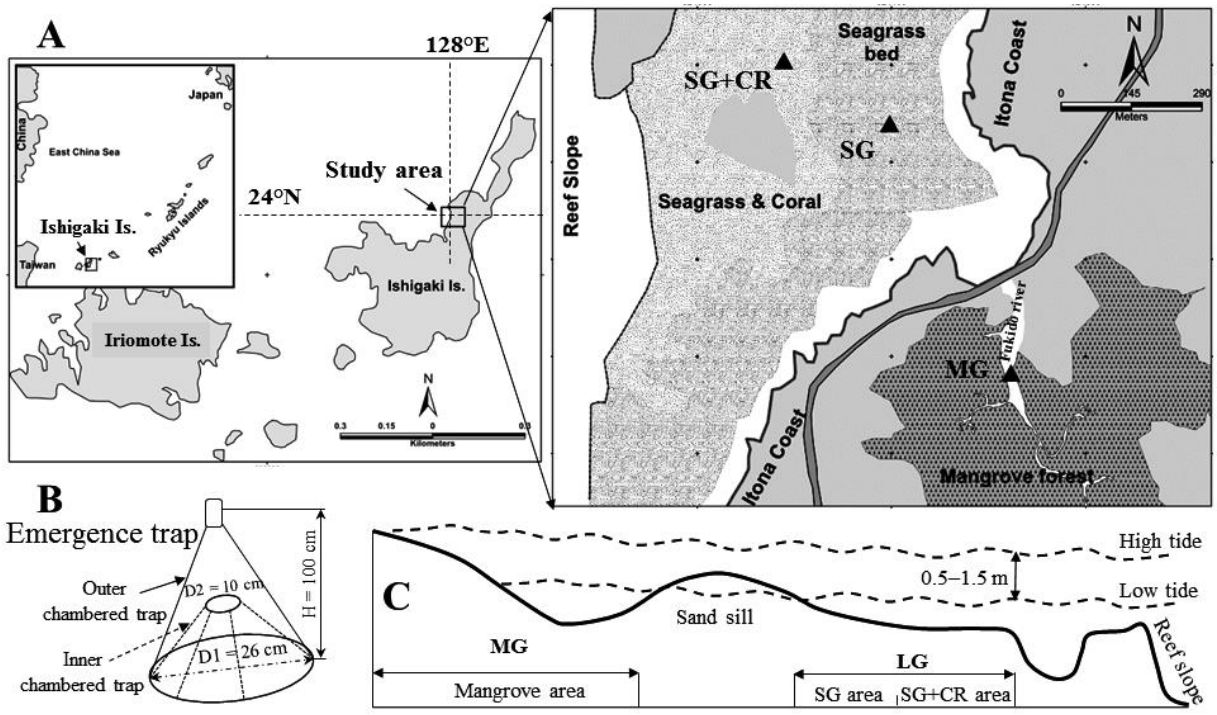

Figure 2. 2 A: Map of Fukido Estuary, Ishigaki, Okinawa, Japan; **B**: Emergence trap with a mesh size of 73 µm. **C**: Vertical section of the sampling area (LG: Lagoon).

Note: ▲ indicates the position of trap set-up and sampling areas; MG: Mangrove area; SG: Seagrass area and SG+CR: Seagrass mixed with Coral area.

2.2 Sample collection

DZ was collected with a modified "emergence trap" of conical shape, according to Hobson and Chess (1979), using 2 chambered traps with a mesh size of 73 µm (Fig. 2.2B). Three emergence traps were set-up at SG, SG+CR and CR in Bise reef lagoon (Fig. 2.1) and MG, SG, and SG+CR in the Fukido estuary (Fig. 2.2A). The mouth of each trap was fixed at 5 cm under the sediment surface using soil anchors and was set up from 18:00 to 07:00 of the next day. DZ samples were separated into fractions using sieves; these fractions were: 73 µm (73 to 100 µm), 100 µm (100 to 250 µm), 250 µm (250 to 500

µm), 500 µm (500 to 1000 µm), 1000 µm (1000 to 2000 µm), and greater than 2000 µm (> 2000 µm). Then the fractions were divided into two sub-samples: one for identification, for which zooplankton was fixed in 5% formalin, and one for stable isotope analysis (SIA). SIA samples were examined under the stereomicroscope (SMZ 1000, Nikon Inc., Tokyo, Japan) to remove detritus so as to avoid contaminating the isotopic signal. The SIA samples were then stored at -20 °C until isotopic analysis.

Two bait traps (10 m long and 6 mm mesh size) were set overnight in each area to collect fishes and macroinvertebrates. The muscle tissues of fishes and macroinvertebrates were used for stable isotope analysis because the constant isotopic value of this tissue reflects the isotopic value of food sources utilized over extended periods of time (i.e., several weeks to months) (Herzka, 2005).

The leaves of 1 mangrove species (*Rhizophora stylosa*) and two seagrass species (*Thalassia hemprichii* and *Halodule pinifolia*) were collected by hand in the mangrove and seagrass areas, respectively. Mangrove and seagrass leaves were washed with MQ pure water (Arium® 611VF, Sartorius Stedim Biotech GmbH, Germany) to remove all detritus before it was stored at -20 °C until further treatment.

Phytoplankton samples were collected using a plankton net. In each habitat, 100 L of seawater was pre-filtered using a mesh with a pore size of 73 µm to remove large material (e.g., detritus, zooplankton). Phytoplankton was captured using a mesh size of 10 µm. Retained phytoplankton were filtered onto pre-combusted Whatman GF/F filters and were stored at -20 °C.

Aliquots of the surface sediment (upper 2 cm) were sampled to study primary microbial producers. These aliquots were homogenized and stored in Corning tubes (50 ml) at -20 °C.

Microphytobenthos (MPB) may include micro-algal and cyanobacteria, which were collected by scraping the surface of coral rubble from surface sediment (upper 2 cm) near the DZ collection locations. Sediment was washed by MQ pure water and pre-filtered using a mesh with a pore size of 100 µm to remove infauna. MPB was retained on a mesh size of 20 µm, then filtered onto GFF pre-combustion and stored at -20° C.

Three sample replicates of epiphytes were sampled on the seagrass leaf blade at SG and SG+CR habitats. Three seagrass leaf blades were collected by hand, then careful transferred in Corning tube (50 ml) immediately after pickup under water. Samples were mixed for 30 s in an MS1 Minishaker at 1000 rpm. The samples were then pre-filtered by a mesh with a pore size of 73 µm to remove large material (e.g., detritus, sponge). Epiphytes were filtered onto GFF pre-combustion and stored at -20° C.

In each habitat, 10 L seawater was filtered onto GFF pre-combustion and stored at -20° C. Particulate organic matter (POM) retained on GFF filter was kept at -20° C.

2.3 Determining the abundance of DZ

DZ was identified and counted under a stereomicroscope. The abundance of DZ taxa was calculated as the number of individual m^{-2} (ind. m^{-2}) on the bottom surface area, based on the mouth area of the emergence trap" (see Fig. 1B).

2.4 Sample treatment

Sediment was dried at 60 °C in an oven to a constant weight (around 20 h) and was then passed through a sieve with a pore size of 63 μm to separate out large particles (coarse gravel, mangrove detritus, seagrass roots, and mollusk shells). Sediment powder was homogenized for sediment SIA, and might have contained bacteria, small primary producers, and fine detritus. The litter that remained on the sieve (< 200 μm) that was derived from mangrove and seagrass was ground to a fine powder for detritus stable isotope analysis. The other SIA samples were dried at 60 °C until a constant weight was reached. Then, the samples were ground to a fine powder.

The homogeneous powder of the SIA samples was divided into two fractions for $\delta^{15}N$ and $\delta^{13}C$ analysis. The samples for $\delta^{15}N$ were stored in a dry box until analysis without acid treatment to prevent acidification affecting the $\delta^{15}N$ values (Bunn et al., 1995; Mateo et al., 2008). The samples for $\delta^{13}C$ analysis were further treated to remove the lipid content and carbonates. In brief, the lipid content in animal tissue can alter the results and conclusions of $\delta^{13}C$ analysis in the aquatic food web and migration studies (Bunn et al., 1995; Focken and Becker, 1998). Thus, in the present study, animal samples were treated to remove lipids following a method modified from Logan et al. (2008). Specifically, dried powder samples were placed in centrifuge tubes to which a solvent with a 2:1 ratio of chloroform: methanol was added, with a volume 3 to 5 times larger than the sample size. Samples were mixed for 30 s in an MS1 Minishaker (IKA® Works (Asia) Sdn. Bhd., Malaysia) at 1000 rpm. The samples were then left undisturbed for about 20 min, after which they were

centrifuged at 2500 × *g* for 10 min. The supernatant containing the solvent and lipids was discarded. This process was repeated until there was an entirely clear supernatant. After removing the lipids, the animal tissues were subjected to an acidification procedure to remove carbonates. The samples (animal tissues, sediment, and seagrass leaves) were acidified by dropping a solution of 1 N HCl onto the sample until bubbling ceased (Jacob et al., 2005). Then, the samples were washed three times with Milli-Q water before being dried at 60 °C to a constant weight and ground to a fine powder. DZ samples and phytoplankton trapped on GF/F were fumed with concentrated 12 N HCl in a glass desiccator for 12 h to remove carbonates, and were then dried at 60 °C to a constant weight and ground to a fine powder. All powder samples were stored in Eppendorf tubes inside a dry box until analysis.

2.5 Stable isotope measurement

Stable carbon and nitrogen isotopes were analyzed at the Laboratory of Aquatic Animal Ecology, School of Marine Biosciences, Kitasato University, Japan. The samples were dried in an electric oven at 60 °C for six hours before analysis. Subsamples of 0.5 ± 0.07 mg (Mean ± SD) dry weight for animal tissues and 2.0 ± 0.08 mg (Mean ± SD) dry weight for DZ were placed in ultra-pure tin capsules, and the samples were burned in an elemental analyzer (Flash EA, Thermo Fisher Scientific, Waltham, MA, USA). Combustion gasses continuously moved through a flow controller (ConFlo, Thermo Fisher), and then the stable carbon and nitrogen isotope compositions were detected with a mass spectrometer (Delta^{plus}XP, Thermo Fisher). L-alanine was used as the

working standard. Repeated measurements of the standard showed a standard deviation of 0.2 ‰ or less. Stable isotope ratios were expressed in δ notation (part per thousand, ‰) as deviations from international standards according to the following equation:

$$\delta X = (R_{sample}/R_{standard} - 1) \times 1000$$

Where X represents ^{13}C or ^{15}N, and R represents isotope ratios $^{13}C/^{12}C$ or $^{15}N/^{14}N$, respectively. R_{sample} and $R_{standard}$ are the isotope ratio of the sample and working standard, respectively.

2.6 Estimation of proportional contribution from food sources

The SIAR package (Parnell and Jackson, 2013) of R software (R Core Team, 2015) was used to calculate the proportion of total diet that was contributed by food sources, based on the stable isotope values of consumers, the mean and standard deviation (SD) stable isotope values of food sources, and the trophic enrichment factor (TEF) of food sources.

2.7 Statistical analysis

The Shannon diversity index (H′) was calculated to estimate the diversity of the DZ community. One-way ANOVA was performed to test the differences ($p < 0.05$) in the isotopic values of DZ and their proportion in the diets of consumers. The similarity of DZ community between components habitats in reef lagoon was calculated based on species composition and their abundance using PRIMER V6.

CHAPTER 3

DETEMINATION OF THE STABLE ISOTOPE FRACTIONATION OF ZOOPLANKTON USING FEEDING EXPERIMENT WITH *ARTEMIA SALINA* AND THE DIATOM *NITZSCHIA* SP.

Chapter 3

Determination of the stable isotope fractionation of zooplankton using a feeding experiment with *Artemia salina* and the diatom *Nitzschia* sp.

Abstract

The isotopic fractionation from food source to consumer is the cause of difference isotopic ratio between consumer and their prey, which is called Trophic Enrichment Factor (TEF). TEF can apply to calculate the trophic position, food chain length, energy flow between sources and organism production. This study was designed to estimate the TEF between herbivorous zooplankton and its food source using *Artemia salina* and a monoculture of the diatom *Nitzschia* sp. as a food source. *Artemia salina* was hatched in the laboratory under control condition in a growth chamber. *Nitzschia* sp. was cultured to feed *Artemia salina.* Natural stable carbon and nitrogen isotope of *Artemia salina* and its food source were measured at a different stage of *Artemia salina* in their life cycle. This study demonstrated that isotopic signals of *Artemia salina* reached to equilibrium with its food source at day 25 after hatching and the trophic enrichment factor of *Artemia salina* was determined to mean (± SD) 0.0 ± 0.9 (‰) for $\Delta^{13}C$ and 1.0 ± 0.5 (‰) for $\Delta^{15}N$. Our results provide a basic tool for studying on zooplankton food web.

3.1 Introduction

Stable isotope analysis is a powerful tool in studies on migration, food web and feeding behavior (Dionne et al., 2016; Fry, 2007; Michener and Kaufman, 2008). Stable isotopes are atoms, which have the same number of protons, and

electrons but have different masses (i.e. different neutrons). Thus, heavier isotopes react more slowly in chemical reactions than light isotopes. Depending on the reaction, heavy and light isotopes accumulate in different ratios in the reactive or the products, a phenomenon called isotopic fractionation (Dionne et al., 2016), and it changes according to the relative abundance of isotopes with a different mass. Two kinds of isotope fractionation effects are equilibrium and kinetic (Peterson and Fry, 1987). An equilibrium isotope fractionation occurs in a reversible system where one isotope concentrate in one component, then the component is commonly referred to the concentrated isotope. The kinetic fractionation utilizes energy for the isotopic fractionation. Thus, kinetic isotopic fractionation in nature tends to prefer lighter isotopic to heavier isotopic, because "energy costs" are lower (De Carvalho et al., 2009; Fry, 2007; Vander Zanden et al., 2015; Wikipedia, n.d.).

The isotopic fractionation from food source to consumer is the cause of difference isotopic ratio between consumer and their prey, which is called Trophic Enrichment Factors (TEFs). TEFs can be estimated by feeding an animal for a period long enough to allow its tissues to renew themselves with the elements from the food source (i.e. their isotopic turnover rate), and then by comparing the isotopic ratios between the food source and the animal tissues (Fry & Arnold, 1982; Tieszen et al., 1983; Hobson & Clark, 1992). TEFs are applied to calculate trophic position, food chain length; energy flows between sources and organism production (Vander Zanden and Rasmussen, 2001). TEFs are necessary before the use of stable isotope analyses in ecological studies. TEFs correspond to the difference between the stable isotope ratios of a

consumer and its food source, resulting in the discrimination of stable isotopes due to the physiology and behavior of the consumer (Caut et al., 2009; Boecklen et al., 2011; Philipps et al., 2014). Previous literature reviewed the mean of TEFs variation ranging from -0.7 to 9.2 ‰ for $\Delta^{15}N$ with an overall mean (±1 SD) of 2.92 ± 1.78 ‰, and $\Delta^{13}C$ value range from -2.1 to 2.8 ‰ with an overall mean (±1 SD) of 0.47 ± 1.23 ‰ among habitats, taxon, type of body part and reason (see summary Vander Zanden and Rasmussen, 2001). In recent studies, the fixed TEFs have been used to examine food web, estimate the diet proportion of consumers and trophic level of aquatic ecosystem ranging from 2.6 to 3.4 ‰ and 0 to 1 ‰ for $\delta^{15}N$ and $\delta^{13}C$, respectively (França et al., 2011; Fry and Ewel, 2003; Le Loc'h et al., 2008; Lebreton et al., 2012; Nakamura et al., 2007; Post, 2002; Tue et al., 2013).

In order to estimate the dietary proportion of DZ, we may apply the TEFs between zooplankton and its food sources. However, the TEFs value mention above were used to large consumers such as bird, fishes, and mammals. Assessing the isotopic discrimination of zooplankton and their prey is difficult because of they have a life cycle of a few weeks to months, and food sources supplement rapid changed by a physical factor (i.e. currents, tide and season, etc.). Thus, their isotopic turnover rate shows significant variation relies on many factors. Moreover, their body size is small therefore using traditional methods (as stomach content) to elucidate the potential food source is impossible. For this reason, this chapter aims to estimate the isotopic discrimination between zooplankton and its food source to find out the TEFs of zooplankton. For this purpose, grazing experiment using the diatom *Nitzschia*

sp. as a food source for *Artemia salina* was developed in our laboratory. The dual stable carbon and nitrogen isotope of *Artemia salina* and *Nitzschia* sp. were measured during the life cycle of *Artemia salina* to estimate the TEFs of zooplankton.

3.2 Experimental design

3.2.1 Microalgae culture for preparation food source

Filtered seawater was prepared from surface water of Suruga Bay (Shizuoka, Japan) by using GFF. A Solution for diatom culture enriched by adding 0.1 ml Marine Algae Culture Medium KW21 (Aquatic Enterprise Co., Malaysia) and 0.45 mg Sodium Meta-Silicate into 1 L filtered seawater (Salinity: 33.6 ‰ and pH: 8.2) was prepared in Nalgene bottles (1.5 L). To this solution, 2 ml *Nitzschia* sp. from mother culture (15 to 18 x 10^5 Cells/ml) was added to culture solution and continuously mixed by a magnetic stirrer. *Nitzschia* sp. was cultured under controlled condition by setting the light intensity at 0.5 ± 0.1 μmol photons.m^{-2}.s^{-1} for nighttime (12 h) and 21.4 ± 2.8 μmol photons.m^{-2}.s^{-1} for daytime and temperature at 20.3 ± 0.2°C. We observed the variation of *Nitzschia* sp. abundance in a closed culture system under a microscope to determine appropriate timing (exponential growth face) for transferring aliquots to new bottle for continuous culture. Based on the abundance result, *Nitzschia* sp. was transferred to new bottle (3 bottles) every four days. By that interval timetable, we could keep *Nitzschia* sp. monoculture with the same abundance and the stages of their life cycle among three culture bottles for feeding *Artemia salina*.

3.2.2 Preparation Artemia nauplii

For the preparation of free nauplii, dry eggs of *Artemia salina* (Japan Pet Drugs Co., Ltd.) were soaked in 1 L filtered seawater (Salinity: 33.6 ‰ and pH: 8.2) containing in Nalgene bottle for hatching at 20.3 ± 0.2 °C inside an incubator. *A. salina* hatched after four days with 80 % of hatching. *Artemia* nauplii were separated covering of eggs under a microscope then *Artemia* nauplii were kept in 0.5 L filter seawater for Monoculture feeding experiment until the second generation, eggs were produced and hatched in the laboratory, reached maturity.

3.2.3 Mono-feeding culture experiment of Artemia salina

Active young *Artemia* collected for initial samples after hatching without feeding, about 20 nauplii individuals were kept on GFF filter with three replicates. Samples were washed three times with MQ water before dry in an electric oven at 60 °C until constant weight.

Mono-feeding using *Nitzschia* sp. to feed *A. salina* every day until reaching adult stage of next generation. *Nitzschia* sp. abundance in culture bottle ranged from 2000 to 3000 cells x ml^{-1} to ensure food supply for *A. salina* is always enough.

Artemia salina individual was picked to a new bottle containing filtered seawater before one-day sample collection, to allow the necessary time for food digestion in gut contain and therefore avoid the influence of isotopic signals from *Nitzschia* sp.

We collected *A. salina* sample after hatching at day one (without feeding), day 5, day 15, day 25, day 35 and mating time (day 40 – 42). Under our observation, that times were corresponding to *A. salina* stage consist of early nauplii (day 1), Nauplii (day 5), juvenile (day 15 to 25), small eggs occurred hereafter called early adult (day 35), Adult stage (mating time) and adult stage of the next generation (day 40 – 42). About 20 individual were kept on GFF filter with three replicates. Meantime, *Nitzschia* sp. sample (n = 3 for each) were filtered by GFF filter. GFF containing samples were washed three times with MQ water before dry in an electric oven at 60 °C until constant weight.

Further treatment and stable isotope analysis for samples collected onto GFF were described in Chapter 2 (Methodology).

3.2.4 Diet-consumer discrimination factors calculation

The diet-consumer discrimination factors also, called TEFs, was estimated based on the isotopic signature of predator and their prey following the equation: $\Delta X = \delta_{consumer} - \delta_{prey}$ where X is ^{13}C or ^{15}N.

3.3 Results

3.3.1 Abundance of Nitzschia sp. *in a closed culture system*

Figure 3.1 presents the abundance of *Nitzschia* sp. in a closed culture system. Abundance increased rapidly in an exponential way until day 4 and reached stable state on day 6. After day 6, the abundance decreased rapidly.

3.3.2 The isotopic discrimination of **Artemia salina** *during Mono-feeding rearing*

Figure 3.2 showed the value of dual stable carbon and nitrogen isotope of *Nitzschia* sp. and *A. salina* during mono feeding incubation. The $\delta^{13}C$ and $\delta^{15}N$ signature of *Nitzschia* sp. showed a small variation between different feeding times. Mean (± SD) of $\delta^{13}C$ and $\delta^{15}N$ were -14.9 ‰ (± 1.0) and -2.8 ‰ (± 0.0), respectively.

The nitrogen isotopic signature of *Artemia salina* was significantly depleted from eggs and early nauplii to day 25 after hatching. These values ranged from 9.7 to -1.9 ‰. Meanwhile, the $\delta^{13}C$ enriched from -20.7 to -14.9 ‰. Overall, both isotopic value of *A. salina* tended to approach the isotopic signature of their food source (*Nitzschia* sp.). However, the $\delta^{13}C$ and $\delta^{15}N$ of *A. salina* from 35 days to adult stages showed ad inverse tendency to the values of previous stages from eggs to nauplii at day 25 (see Fig 3.2).

Figure 3.3 shows the variation of stable carbon and nitrogen isotope signatures and the carbon to nitrogen atom ratio of *A. salina* and *Nitzschia* sp. during the experiment using the mono-feeding system. The C/N ratio of *A. salina* ranged from 4.1 to 5.4 (mol/mol). The $\delta^{13}C$ is a strongly correlated with $\delta^{15}N$, and C/N ratio, their correlation coefficient (r) were -0.94 and -0.96, respectively (Appendices - 3.1). Meanwhile, the relationship between $\delta^{15}N$ and C/N ratio was positive linear of correlation, and their correlation coefficient was 0.94 (Appendices - 3.1).

The C/N ratio and both stable isotope signatures of *Nitzschia* sp. were minor variation. It indicated that *Nitzschia* sp. is a stable food source and available for the estimation of isotopic fractionation between zooplankton consumer (*A. salina*) and its mono food source (*Nitzschia* sp.).

3.3.3 Trophic fractionation between zooplankton and their food in Mono-feeding experiment

Table 3.1 showed isotopic fractionation of *A. salina* and its mono food source (*Nitzschia* sp.) in the Mono-feeding incubation and *A. salina* body length at different stages. The $\Delta^{13}C$ increased from -5.4 ‰ (5 days) to 0 ‰ (25 days), but these value decreased from 35 days (-0.7 ‰) after hatching to -2.3 ‰ in adult stages of next generation. In the opposite direction, the $\Delta^{15}N$ significant decreased from 5 days after hatching (9.8 ‰) to 25 days (1.0 ‰) and increased to 4.4 ‰ in adult stages of next generation. While *A. salina* body total length grew quickly from 5 days to 25 days and their body length reached maximum (4.4 to 4.6 mm) from 35 days to adult stages (Table 3.1).

3.4 Discussions and Conclusions

Stable isotopes normally exist in the natural. The isotopic fractionation is different among organic and inorganic matters (Fry, 2007; Michener and Kaufman, 2008). Previous studies demonstrated that the $\delta^{13}C$ and $\delta^{15}N$ fractionation between consumers and their food sources could be used to determine assimilated food and estimate the proportional contribution of food sources in the diet of consumers. The contribution of food sources is essential information for improving our knowledge on studies of aquatic food webs,

conservation, managements and aquaculture (Caut et al., 2009; Matley et al., 2016; Watanabe et al., 2013). In the current study, the isotopic fractionation between a monoculture of the diatom *Nitzschia* sp. and the crustacean *A. salina* was determined by mono feeding system using the $\delta^{13}C$ and $\delta^{15}N$ signatures.

The selected food used for estimation the TEFs should have the following characteristics: 1) a potential food source with a stable amount of food supplement to feed the consumer during the experiment; 2) food source should be distinguishable isotopically with the consumer. Because the food source is a complement of nutrient for the consumer, thus if the food supply shows major variations in different stages of the consumer (in this case *A. salina),* it will be affected the food assimilation during the growth rate of *Artemia* (Prusińska et al., 2015). Moreover, *Nitzschia* sp. is a micro diatom which usually is used in aquaculture and applied as a food source in the specific experiment of *Artemia* (Toi et al., 2013). However, *Nitzschia* sp. was cultured in a closed system, where the nutrient concentration may change in the culture environment. Therefore it can affect the growth rate of *Nitzschia,* and the resulting isotopic signature of *Nitzschia* sp. may vary at different growth stages. In the present study, we followed *Nitzschia* sp. growth curve, and we selected diatoms reaching the stable growth stage (from day 4 to day 6) as a food source to *A. salina*. The means of a stable isotope of Nitzschia were -14.9 ‰ (± 1.0) for $\delta^{13}C$ and -2.8 ‰ (± 0.0) for $\delta^{15}N$. There was a significant difference between isotopic signatures of *A. salina* and *Nitzschia* sp. (Fig. 3.2 & 3.3), which was expected to show the distinct fractionation between consumer and its food source. Overall, *Nitzschia* sp. is an appropriate food source for the estimation the TEFs of a zooplankton

using *A. salina* experiment in the mono feeding system.

Although the $\delta^{15}N$ value of *Nitzschia* sp. was negative (-2.8 ‰), which is unusual in natural, our result is in agreement with Watanabe et al. (2013) who reported that the $\delta^{15}N$ value of diatom (cultured under nutrient supplement conditions) ranged from -1.0 ‰ (*Chaetoceros calcitrans*) to -7.5 ‰ (*Navicula ramossisima*). The negative $\delta^{15}N$ value of *Nitzschia* sp. may be due to the enrichment of inorganic nitrogen from the medium culture (Watanabe et al., 2013).

Previous studies demonstrated that the isotopic fractionation in different body parts of an organism is different. They also compared the whole body from large animals (bear, dolphin, fishes) to small organism (Mysid), showing differences due to the different fraction of components (i.e. lipid, protein, carbon hydrate) and turnover rate (Deniro and Epstein, 1981; Gorokhova and Hansson, 1999; Matley et al., 2016; Tieszen et al., 1983). In this study, we considered the whole body of *Artemia* discrimination from food isotopic to achieve TEF for zooplankton community in the difference fraction (Chapter 4).

Although this study did not assess the effect of lipid content to isotopic fractionation, we examined the time exchange of $\delta^{13}C$ and $\delta^{15}N$ between consumer and its food during their life cycle in the mono feeding chamber experiment. Our results in a dual stable isotope of *Artemia* showed clearly the equilibrium of isotopic fractionation, they had a tendency towards the isotopic value of *Nitzschia* sp. from Nauplii (day 5) to the Juvenile stage (day 25), and these value reached isotope equilibrium at day 25 after hatching (Fig. 3.2 & 3.3). The turnover process was continued after isotope equilibrium (day 25), the

isotopic values tend to be in the opposite direction to those of food value, i.e. the $\delta^{13}C$ of *A. salina* depleted while $\delta^{15}N$ enriched compare to the isotopic signature of *Nitzschia* sp. (Fig. 3.2). The exchange of isotopic value might due to changes in components during the different growth phase of *A. salina.* In our observation, the *A. salina* body length increased and reached maximum (4.4 – 4.6 cm) at early adult stage (day 35) and adult stage (day 40 – 42) (Table 3.1) when small eggs of *A. salina* started to occur and mate. The result indicated that *A. salina* might be prepared and accumulated energy for mating and eggs producing from day 25 to the adult stage. Normally that process requires lipid accumulation in organisms. Moreover, *Artemia* eggs were reported to contain high essential fatty acid (Lavens et al., 1989; Ruiz et al., 2007). Our result on C/N ratio supported this evidence. C/N ratio decreased from nauplii to the juvenile stage (day 25) may due to *A. salina* accumulated enrichment nitrogen of food source for renewable and build new protein during growth (Fig. 3.3). However, C/N ratio increased from day 35 to adult indicating that increase of carbon might be due to lipid accumulation process. On the other hand, the $\delta^{13}C$ value is depleted in lipid fraction more than other components (e.g. protein) (DeNiro and Epstein, 1997; Tieszen et al., 1983). Thus, stable carbon isotope fractionation was affected by lipid content of *Artemia* from day 35 to adult periods.

Nitrogen isotope ratio of the consumer is known to be enriched over its diet; our result is consistent with other reports (França et al., 2011; Fry and Ewel, 2003; Le Loc'h et al., 2008; Lebreton et al., 2012; Post, 2002; Vander Zanden and Rasmussen, 2001). However, the $\Delta^{15}N$ increased of *A. salina* reached isotope equilibrium (25 days). That value increasing from 35 days may be

explained due to the formation of eggs containing non-essential amino acid (Helland et al., 2000) which is a cause of ^{15}N enrichment (Matley et al., 2016; Pinnegar and Polunin, 1999).

Overall, the TEFs between *A. salina* and its mono diet (*Nitzschia*) should be considered at 25 days after hatching, i.e. the mean (1±SD) of $\Delta^{13}C$ and $\Delta^{15}N$ are 0.0 ±0.9 ‰ and 1.0 ±0.5 ‰, respectively. This result of $\Delta^{15}N$ is lower than normally TEFs value of animal which was applied in previous studies to estimate the trophic level and proportion of animals' diet in the aquatic food web, i.e. $\Delta^{15}N$ ranged from 2.6 to 3.4 ‰. However, our finding on $\Delta^{13}C$ is similar to previous reports i.e. $\Delta^{13}C$ ranged from 0 to 1 ‰ (França et al., 2011; Fry and Ewel, 2003; Le Loc'h et al., 2008; Lebreton et al., 2012; Nakamura et al., 2007; Post, 2002; Tue et al., 2013; Vander Zanden and Rasmussen, 2001).

In conclusion, based on the result of dual stable carbon and nitrogen isotope fractionation of *A. salina* during their life cycle we determined the TEFs of *A. salina* from its diet using mono feeding growth experiment. The isotopic values of *A. salina* reached equilibrium with its food (*Nitzschia* sp.) at day 25 after hatching. Our finding provides a basic tool for studying on zooplankton food web.

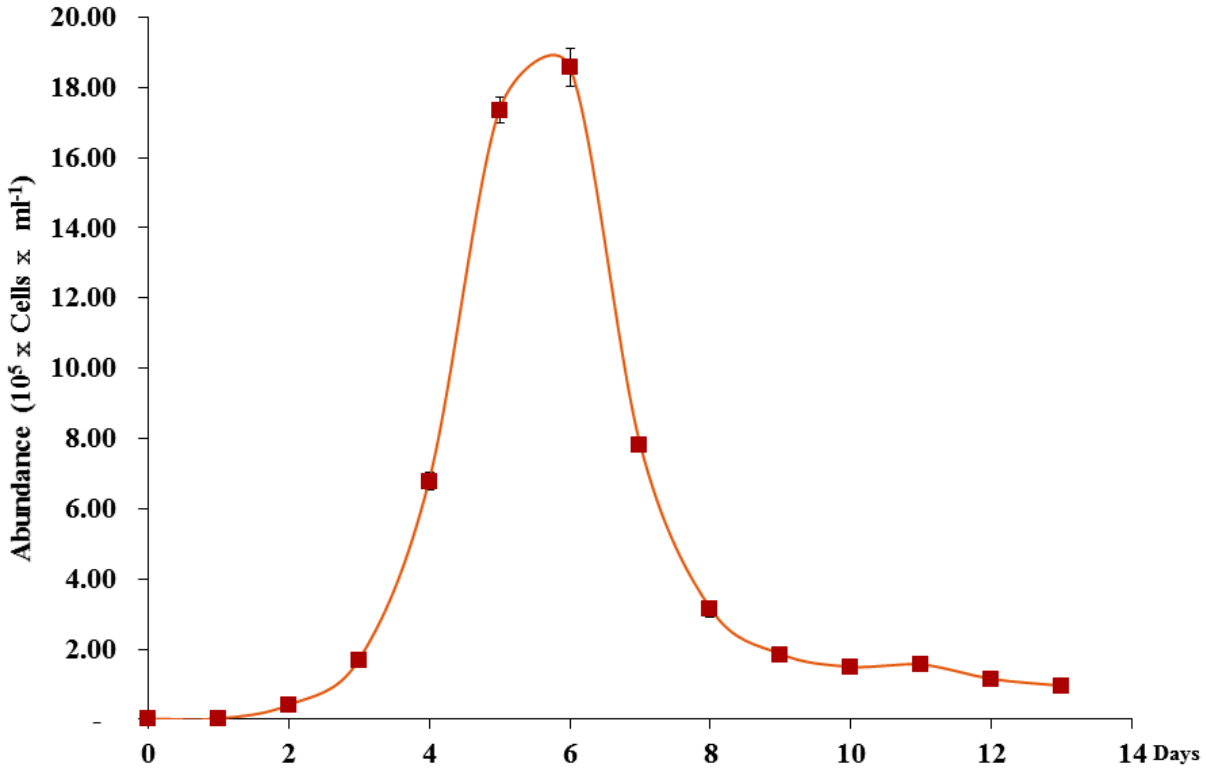

Figure 3. 1 Abundance variation of *Nitzschia* sp. in a closed system

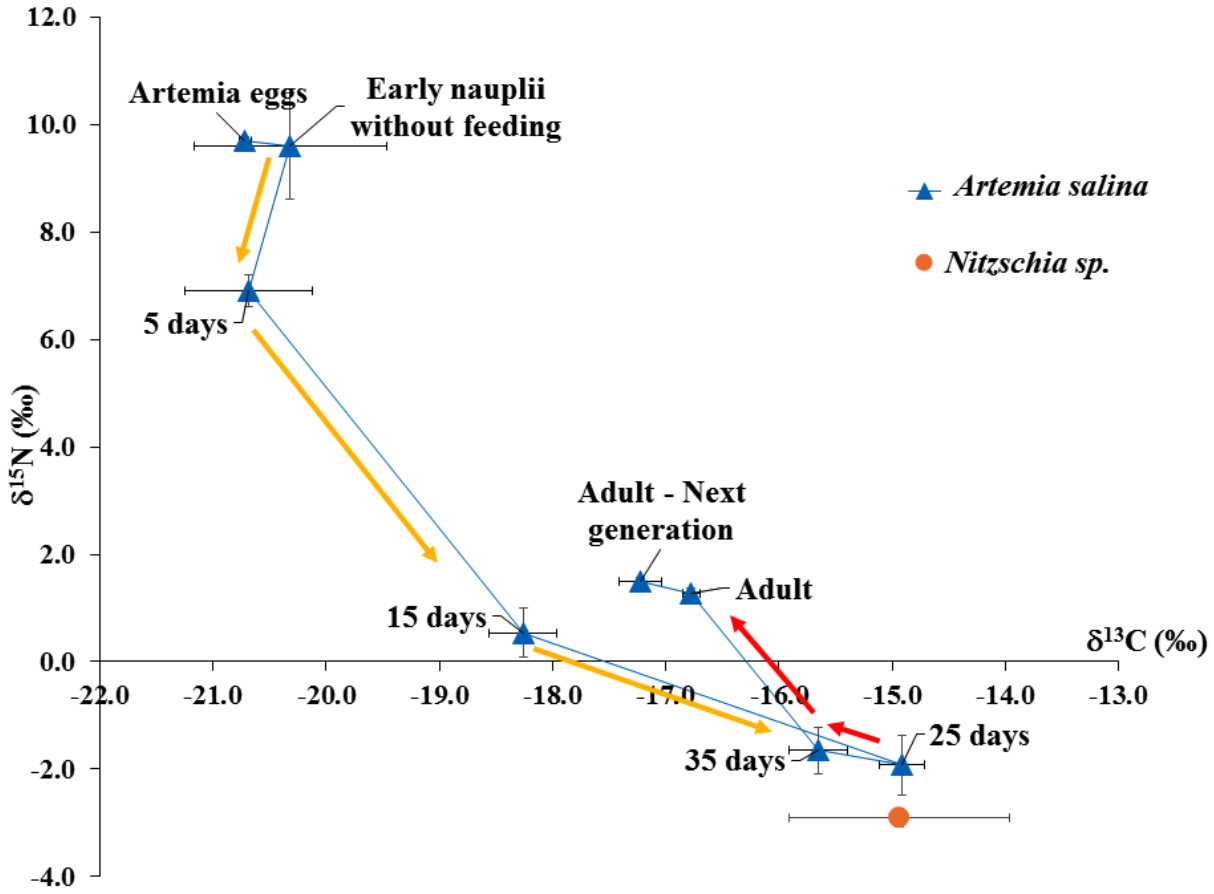

Figure 3. 2 Mean (± SD) of dual stable carbon and nitrogen isotope of *Artemia salina* and *Nitzschia* sp. in the Mono-feeding experiment

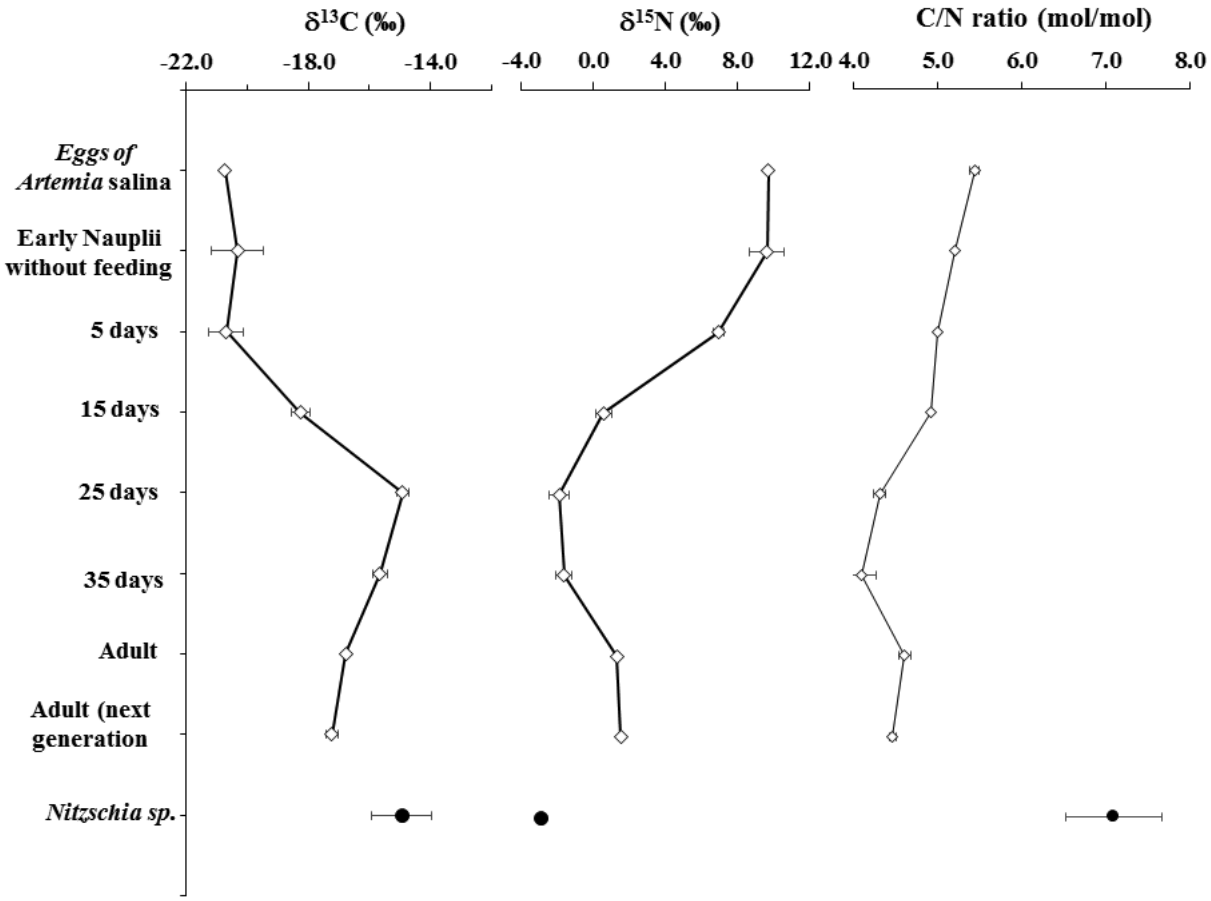

Figure. 3 Discrimination of isotopic between *Artemia salina* and their food source

Table 3. 1 Mean (± SD) isotopic fractionation of *Artemia salina* and *Nitzschia* sp. in the Mono-feeding incubation and body length of *A. salina* during their life cycle

Time following hatching (days)	**Stages of *Artemia salina***	**$\Delta^{13}C$ (‰)**	**$\Delta^{15}N$ (‰)**	**Total length (mm)**
5	Nauplii	-5.4 ± 1.0	9.8 ± 0.2	0.9±0.1
15	Juvenile	-3.3 ± 1.0	3.4 ± 0.2	1.6±0.1
25	Juvenile	0.0 ± 0.9	1.0 ± 0.5	3.2±0.7
35	Early adult	-0.7 ± 0.9	1.3 ± 0.4	4.4±0.3
40	Adult	-1.8 ± 0.9	4.2 ± 0.2	4.6±0.3
42	Adult (2^{nd} generation)	-2.3 ± 0.9	4.4 ± 0.2	4.5±0.4

CHAPTER 4

FOOD SOURCES OF DEMERSAL ZOOPLANKTON IN THE REEF LAGOON AT BISE, OKINAWA, JAPAN

Chapter 4

Food sources of demersal zooplankton in the reef lagoon at Bise, Okinawa, Japan

Abstract

Demersal zooplankton play an important role as the linkage between primary producers and small organic matter (mainly detritus) to higher trophic levels. However, the food sources of DZ were not well studied until now due to their small size, high turnover rate, and short life cycle. This study was conducted at Bise (Okinawa Island, Japan), which is composed of seagrass, seagrass mixture coral colonies, and coral in reef lagoon. The proportion of the potential food sources were estimated, using the mixing model based on the natural stable isotope of DZ and its food sources to clarify the food sources of demersal zooplankton in this site. The potential food sources were classified into three groups based on the difference of $\delta^{13}C$ values. The results of this study highlight the role of organic matter derived from seagrass, particularly seagrass detritus, influencing the abundance of DZ in each specific habitat. Phytoplankton and macro algae also play an important role as a food source for DZ in the lagoon. When comparing the importance of potential food sources, it was clear that DZ feed on food sources, which are available in their habitat and their migration among habitats in small spatial scale.

4.1 Introduction

Production of marine primary producers accounts for about 40 % of the primary production on the earth, and coastal marine ecosystem contributes to

highest rates to the primary carbon production (Duarte and Cebrián, 1996; Mascart, 2010). Main photosynthetic carbon products in marine coastal ecosystems are generated by mangroves, marsh plants, seagrasses, macroalgae, coral reef algae, coastal phytoplankton, and microphytobenthos (Duarte and Cebrián, 1996). Seagrass beds and coral reefs in shallow coastal water are among the most important ecosystems, providing a wild range of potential food sources (such as seagrass leave, epiphytes, detritus matter, and macro algae, microphytobenthos) for a high diversity of consumers, (Jaxion-Harm et al., 2012; Larkum et al., 2007). However, these food sources show seasonal variations and show different pattern and level of digestion (Cebrian, 1999; Duarte, 1989; Larkum et al., 2007). Some invertebrates and vertebrates can consume directly the food sources derive from seagrass and macroalgae, but most of that food sources are decomposed by bacteria to be smaller organic matter and easily digestible (i.e. DOM, POM), then that organic matter sources can be utilized by smaller consumers.

Demersal zooplankton distribute with high densities (~ 10^6 individual x m^{-2}) in shallow coastal waters such as seagrass beds, estuaries and coral reefs, kelps and soft bottoms (Coull, 1999; Jayabarathi et al., 2012; Mascart, 2010; Youngbluth, 1982). Thus, demersal zooplankton might play a role as the linkage between primary producers and higher trophic levels. DZ have high turnover rate and short life cycle (weeks to months). Thus they are quickly responding to organic matter input and closed to primary production food sources (Escaravage et al., 1989; Heip et al., 1985; Lebreton et al., 2012). Their secondary production rate was estimated from 9.0 to 29.4 g C m^{-2} $year^{-1}$

(Escaravage et al., 1989). Moreover, DZ was found to be common food items in the stomach content of planktonic fishes and shrimps (Coull, 1999; Hyndes and Lavery, 2005).

Previous studies had hypothesized that DZ might play a key role in benthic energy flows, as a converter from primary production to an available food source for higher trophic levels (Lebreton et al., 2012, 2011; Melo et al., 2010). Nevertheless, understanding about the food sources of DZ was not well explored (Lebreton et al., 2012) mainly because their size are small and accounts for a short life cycle. In seagrass bed area, detritus derived from seagrass litter, macro algae which were decomposed by bacteria to small practical and re-suspended in the water column may represent a possible food source for DZ (Muller-Solger et al., 2002). Microphytobenthos (MPB) occupying the upper layer of sediment surface, show high abundances in seagrass beds (Kaldy et al., 2002), and they show easily digestibility (Duarte and Cebrián, 1996). Thus MPB is an available food source for consumers that live near the bottom such as DZ. Epiphytes often exhibit high production in seagrass bed; their ubiquitous member includes bacteria, fungi, and protozoa (Borowitzka et al., 2006) which may be a food source for DZ (Danovaro, 1996; Moncreiff et al., 1992). Phytoplankton is known as a food source for zooplankton, they provide a large primary production and represent a major food source for the food web in seagrass beds and reef lagoons (Moncreiff et al., 1992). Thus phytoplankton may also be an important potential food source for DZ. Macroalgae also represent a food source of DZ in seagrass bed and coral reef (Macko et al., 1982).

The discrimination of stable isotope between consumer and their prey item is called Trophic Enrichment Factor (TEF), TEF was determined by laboratory experiment or field experiment and fixed value in an estimation of trophic level or identification of the relationship between end member of the food web (Fry, 2007; Michener and Kaufman, 2008). Base on the discrimination between consumer and its food source, stable isotope analysis become a powerful tool and bio tracer to identify the food source that was selected by consumers (Layman et al., 2012). On the other hand, stable isotope analysis requires a small mass of sample (less than 100 μg) (Carman and Fry, 2002).Thus, this method is able to measure the stable carbon and nitrogen isotope value of DZ and its potential preys.

This study aims to clarify the food sources of DZ in the reef lagoon at Bise, Okinawa. We examined the DZ community structure in specifically studied habitats and estimated the proportional contribution of potential food sources in the diet of DZ using natural dual stable carbon and nitrogen isotope to evaluate the food sources that available in the reef lagoon. The study is expected to provide new understandings about the food sources of DZ in shallow lagoon ecosystem.

4.2 Experimental design

Bise lagoon is composed of three specific habitats including seagrass bed, seagrass mixture coral colonies, and corals in the reef lagoon. In each habitat, 2 “emergence traps” were set up from 18:00 to 07:00 of the next day for DZ collection.

Microphytobenthos (MPB) was collected on coral rubble and sediment surface at near DZ sampling place.

POM and phytoplankton were sampled near DZ sampling at 19:00 and 22:00 in each habitat.

Estimation of proportional contribution from food sources

To estimate the proportional contribution of potential food source in the diet of DZ in each habitat using stable isotope mixing model SIAR V4, we applied the TEF (± SD) for $\delta^{13}C$ and $\delta^{15}N$ as 0.0 ± 0.9 ‰ and 1.0 ± 0.5 ‰, respectively (see Chapter 3).

4.3 Results

4.3.1 Demersal zooplankton community

In Bise reef lagoon, 40 taxa of DZ were identified to the lowest possible taxon (Table 4.1). Table 4.1 also shows the size range and relative abundance of DZ in each habitat. The dominant groups in the CR habitat included Polychaeta (larvae) (26.5 %), followed by Isopoda (10.3 %), Harpacticoida (nauplius) (9.2 %). In SG habitat, Polychaeta (larvae) contributed 17.4 %, followed by the copepod *C. thompsoni* (16.7 %) and Isopoda (16.1 %). In SG+CR habitat, Polychaeta (larvae) contributed 30.3 %, followed by Isopoda (12.3 %) and *C. thompsoni* (9.9 %).

The number of taxa and diversity index (H′) of DZ at each habitat were similar. Total taxa in CR, SG and SG+CR were 29, 28 and 29 taxa, respectively (Table 4.1) and H′ was 2.5, 2.6 and 2.7 in CR, SG, and SG+CR, respectively (Table 4.2). However, the abundance of DZ in SG and SG+CR habitats were

significantly higher than in CR habitat, i.e. the DZ abundance in SG and SG+CR were 17.6 ± 2.3 (Ind. m^{-2}) and 18.8 ± 6.8 (Ind. m^{-2}), meanwhile DZ abundance in CR habitat was 6.8 ± 2.0 (Ind. m^{-2}) (Table 4.2). The result of the assessment of the similarity base on the abundance and composition of DZ between habitats showed that the similarity between SG and SG+CR was high (80.1 %), while the similarity between CR to SG and SG+CR were 69.1 % and 68.5 %, respectively.

4.3.2 Dual stable carbon and nitrogen isotope of demersal zooplankton and its potential food sources

Figure 4.1 shows the stable carbon and nitrogen isotope signatures of DZ and its potential food sources. The $\delta^{15}N$ value of DZ ranged from 3.9 to 7.3 ‰, and their $\delta^{13}C$ ranged from -19.7 to -14.4 ‰. The isotopic values of DZ were different among habitats. In SG habitat, the $\delta^{15}N$ value of DZ ranged from 5.4 to 7.3 ‰, and for $\delta^{13}C$ of DZ almost all value ranged from -17.1 to -14.4 ‰, excepted DZ size class 500 to 1000 μm (-19.7 ‰). In CR habitat, the $\delta^{13}C$ of DZ showed no significant difference among size fraction; its value ranged from -18.2 to -17.1 ‰. However, the $\delta^{13}C$ of different DZ size fractions showed a wide range from -19.2 to -15.5 ‰.

The $\delta^{13}C$ of food sources can be divided into three group, with their $\delta^{13}C$ value showing significant differences ($p<0.05$). The $\delta^{13}C$ of group 1 (phytoplankton and *Ulva* sp.) ranged from -19.3 to -18.7 ‰. Group 2 includes POM and SG litter; their $\delta^{13}C$ value ranged from -17.6 to -15.9 ‰. Moreover, group 3 consists of Epiphytes and MPB had $\delta^{13}C$ value ranged from -13.8 to -12.0 ‰.

4.3.3 Proportional contribution of food sources in the diet of demersal zooplankton

Table 4.3 shows the proportion of the potential food sources in the diet of DZ in each habitat. In SG habitat, smaller DZ size classes (73 to 100 μm) tend to prefer on epiphytes and MPB more than another food sources i.e. epiphytes and MPB contributed 17.2 % and 17.4 % to the diet of DZ <73 μm, and 20.9 % and 21.1 % in diet of DZ of 73 to 100 μm, respectively. Lager DZ size classes (250 to 4000 μm) seem to prefer on food sources derive from SG litter, *Ulva* sp., and phytoplankton, example in the diet of DZ 250 to 500 μm, *Ulva* sp. contributed 18.5 %, followed by SG litter (18.4 %) and Phytoplankton (17.4 %). The percentage of these food sources in the diet of larger DZ showed the similar tendency of DZ 250 to 500 μm (Table 4.3).

In SG+CR habitat, DZ < 73 μm preferred on epiphytes (18.7 %) and MPB (17.5 %). Moreover, DZ < 73 μm also consumed high percentage on food source derived from SG litter (17.6 %). Although DZ of 73 to 100 μm in SG+CR preferred on epiphytes with a high percentage (17.1 %). However, they seemed to consume the food source derived from SG litter (18.3 %) and *Ulva* sp. (17.0 %). DZ of larger size classes seem to prefer on phytoplankton such as 24.3 % for DZ 250 to 500 μm, 17.9 % for DZ 500 to 1000 μm, 20.1 % for DZ 1000 to 2000 μm, 20.6 % for DZ 2000 to 4000 μm, and 22.2 % for DZ ≥ 4000 μm. In the diet of DZ larger size classes (250 to 4000 μm) in SG+CR habitat, following the proportion value of phytoplankton by food source derived from *Ulva* sp., SG litter and POM (Table 4.3).

In CR habitat, the mixing model results showed that DZ seem to prefer more on phytoplankton in all size fraction of DZ (73 to 4000 μm), followed by POM and *Ulva* sp. (Table 4.3). The proportional distributions of food sources were difference compare to other studied habitats. DZ small size fraction (73 to 100 μm) accumulated on epiphytes and MBP smaller than another food source. Meanwhile, in SG and SG+CR habitats, they seemed to prefer on these food sources more than other. Moreover, the SG litter food source contributed less percentage compare to other studied habitats (Table 4.3).

4.4 Discussions and Conclusions

4.4.1 Stable isotope signatures and the potential food sources

The $\delta^{13}C$ values of the potential food sources are well discriminated (Fig. 4.1), based on $\delta^{13}C$ values the potential food sources were divided into three groups. The similarity of $\delta^{13}C$ value between the component foods sources in each group indicates that they might serve in similar proportion in the diet of consumers. The $\delta^{13}C$ fractionation between consumers and its food sources remains in the small range from 0 to 1 ‰ (Vander Zanden and Rasmussen, 2001). Therefore $\delta^{13}C$ usually is used to trace carbon pathway from food sources to consumer (Bouillon et al., 2008). The $\delta^{13}C$ values of POM were similar to those value of SG litter, but their values were significantly different to that of phytoplankton (Fig. 4.1). It indicated that carbon source of POM mainly derived from SG detritus. Seagrass leaves were buried in sediment or floating on the surface water or suspended in the water column (Larkum et al., 2007). This plant material is decomposed by bacteria in the process of decay and converted into a smaller fraction (POC) and dissolved organic carbon

(DOM). Therefore, organic carbon derives from seagrass production contributes mainly in the lagoon to the particular seagrass leaves detritus (Larkum et al., 2007). The $\delta^{13}C$ of primary producers potential food sources of DZ in this study were well discriminated into two groups. The $\delta^{13}C$ of phytoplankton (-19.3 to -19.0) and *Ulva* sp. (-18.7 ‰) were lighter than Epiphytes (-12.5 to -12.0 ‰) and MPB (-13.8 to -12.7 ‰) (Fig. 4.1). The $\delta^{13}C$ signatures of these food sources are in agreement with the range of previous observations in the seagrass bed (Kang et al., 1999; Lebreton et al., 2012, 2011) and others environments (Boschker et al., 2000; Kharlamenko et al., 2008; Schaal et al., 2008). The discrimination of $\delta^{13}C$ between food sources is necessary to compare the proportion of food sources in the diet of consumers (Bond and Diamond, 2011; Healy et al., 2016; Hopkins and Ferguson, 2012; Parnell and Jackson, 2013).

4.4.2 Potential food sources of demersal zooplankton

Role of Epiphytes and MPB as DZ food sources

Epiphytes account for over 50% of the standing stock in seagrass meadows (Borowitzka et al., 2006) and MPB algae can represent up to 54% of seagrass production (Lebreton et al., 2011). They are important primary producers in seagrass bed and have a significant contribution to food web (Borowitzka et al., 2006). Our mixing model result showed that epiphytes and MPB seem to contribute in high percentage in the diet of small DZ (73 to 100 μm) in SG and SG+CR habitats, and higher than other food sources (Table 4.3). This result can be explained since the smaller size DZ show less motion and swim near the bottom (Alldredge and King, 1985), therefore they prefer on diatom and micro

algae which are the main component of epiphytes and MPB that can be easily found on seagrass leaves, rhizomes and surface of sediment (upper 2 cm) (Borowitzka et al., 2006; Lebreton et al., 2011). Although, the distance between sampling points between SG+CR and CR was only 150 m (Fig 2.1), however, epiphytes did not show a significant contribution in the diet of DZ in CR habitat (Table 4.3). This result indicates that the migration DZ from habitat to other habitat was limited and their migration occurs in small spatial scale. The percentage of MPB contributed in DZ diet was smaller than other food sources in CR habitat. In the CR habitat, although sunlight can more penetrate to the bottom than in SG and SG+CR, which are shaded by SG canopy, however, currents (mainly tidal currents) are frequently removing the bottom of the CR habitat. Therefore, the small organisms attached on sediment such as micro algae show low density and biomass. Moreover, the numbers of individuals of DZ of smaller size classes in CR were smaller than SG and SG+CR (Table 4.1 and Appendices – 4.1).

Role of POM and SG litter as food sources of DZ

High biomass of seagrass detrital matter is stored in sediment layer that is an important organic matter source for the deposit feeders (Lebreton et al., 2012). The Mixing model results showed that SG litter highly contributed to the diet of DZ in SG and SG+CR habitats, particularly 250 to 1000 μm of DZ (Table 4.3). Moreover, DZ species belonging to that size classes contributed with the highest abundance in SG and SG+CR habitats (Table 4.2 and Appendices 4.1). However, the contribution of SG litter as a food source was

not significant in CR habitat to compare to other food sources. In SG habitats, SG detritus can be stored on the bottom of, meanwhile, the wind, currents, and waves usually influence in CR habitat where SG litter cannot easily sink and store in CR habitat. On the other hand, the abundance of DZ in SG and SG+CR habitats were significantly higher than CR habitat (Table 4.2). These results indicated that organic matter derives from seagrass detritus might is a major food source influencing the abundance of DZ.

POM contributed with the highest proportion as a food source in the diet of DZ in almost of size class fractions in CR habitat (Table 4.3). Meiobenthic community in seagrass bed was mainly relying on POM (Lebreton et al., 2012). In this study, POM was mainly composed of organic matter derived from SG litter as shown in above discussion (see 4.4.1 section). Overall, this finding highlights the role of organic matter derived from seagrass detritus as an important food source in the reef lagoon.

Role of Phytoplankton and Ulva sp. as DZ food sources

Phytoplankton is an important food source for zooplankton in the aquatic food web. However, the role of phytoplankton as a food source in shallow water, particularly in the diet of DZ is poor understanding. Because they spend almost time in their life cycle on the bottom and emerge to water column for a short period during dark hours (Alldredge and King, 1980; Hammer, 1981). Therefore, they might prefer more on food source available on the bottom than in the water column (i.e. phytoplankton). Nevertheless, DZ were reported as an opportunistic feeder in a seagrass bed (Hyndes and Lavery, 2005; Leduc et al.,

2009). Thus they are quickly responding to the available food source during their life cycle. Moreover, the mixing model showed that phytoplankton contributed with a high proportion in the diet of DZ at three studied habitats according to size fractions. This result indicated that the phytoplankton might be a good food source as it is provided from the open ocean into the lagoon by to tidal currents. Therefore, the water exchange between reef lagoons is a major factor influence to food sources of DZ. Macroalgae *Ulva* sp. distribute widely in Bise lagoon that was reported as a food source for marine amphipod and meiofauna (Leduc et al., 2009; Macko et al., 1982). The result of mixing model support for this evidence since *Ulva* sp. was found to contribute with high percentages in the diet of DZ in CR and SG+CR.

In conclusion, this study highlights the role of organic matter derived from seagrass, particularly seagrass detritus influencing the abundance of DZ in each specific habitat. Phytoplankton and macro algae are also important food sources for DZ in the lagoon. When comparing the importance of potential food sources, it is suggested that DZ prefer on the food source, which is easily available on a small spatial scales.

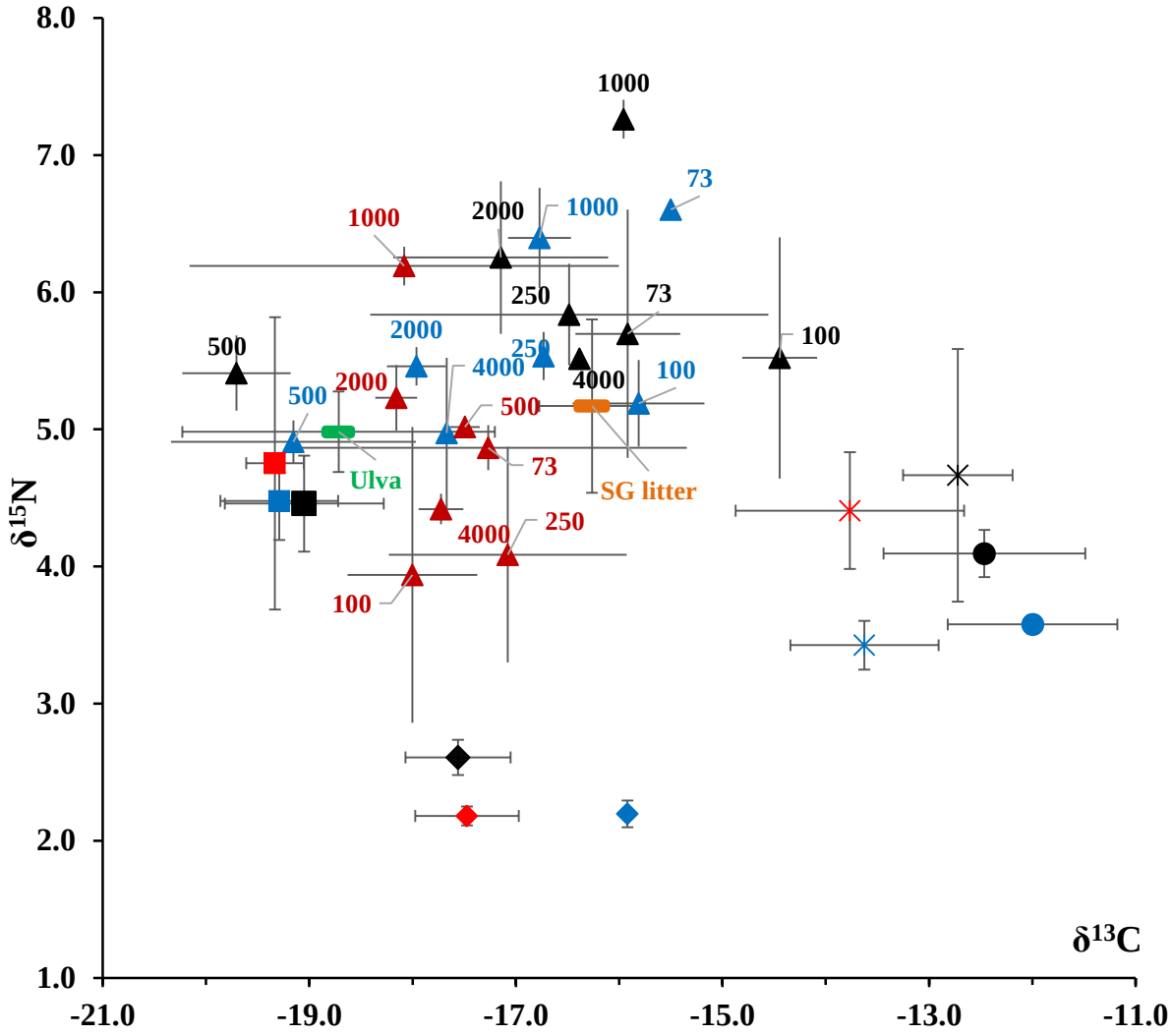

Figure 4. 1 Carbon and nitrogen isotopic signatures (mean ± SD) of demersal zooplankton, their consumers, and other food sources in Fukido Estuary.

The symbols indicate demersal zooplankton (Δ), POM (◊), Phytoplankton (□), Epiphytes (○), and MPB (x). Solid black, green and dark red filled symbols denote organisms collected in the SG, SG+CR, and CR. The numbers indicate the upper value of DZ size classes.

Table 4. 1 Composition, size range, and relative abundance (%) of demersal zooplankton captured by emergence traps.

Abbreviations: CR, SG, and SG+CR denote the coral, seagrass and seagrass mixture coral habitats, respectively.

Taxa	Size range [μm]	CR	SG	SG+CR
SARCOMASTIGOPHORA				
Granuloreticulosea				
Foraminiferida		0.6	1.0	
NEMATODA			0.3	
MOLLUSCA				
Gastropoda				
Gastropoda larva		1.9	1.0	1.2
ANNELIDA				
Polychaeta				
Polychaeta (larvae)	300 - 9000	26.5	17.4	30.3
ARTHROPODA				
Maxillopoda				
Ostracoda		0.6	0.3	
Acartia japonica		0.6		0.3
Acartia (copepodite)		0.3		
Undinula vulgaris			0.3	
Calanidae (copepodite)				0.3
Paracalanidae (copepodite)	250 - 500	0.6		
Calanopia minor		1.1	0.3	0.6
Calanopia thompsoni	2200 - 2500	1	16.7	9.9
Pontellidae (copepodite)				0.3
Oithona oculata	650 - 800	2.5	5.8	4.5
Oithona rigida		0.3	1.3	
Oithonidae (copepodite)	300 - 600	3.3	3.2	2.4
Cyclopoida	300 - 650	4.5	2.9	2.4
Cyclopoida (copepodite)		2.8	1.0	1.5

Microsetella (copepodite)			0.3	
Harpacticoida	300 - 900	6.7	5.8	7.5
Harpacticoida (copepodite)	200 - 600	9.2	3.2	6.0
Corycaeidae (copepodite)		0.3		
Oncaea zernovi				0.3
Poecilostomatoida	700 - 800	0.8	1.6	1.8
Poecilostomatoida (copepodite)		2.2	2.3	0.9
Monstrilloida (copepodite)				0.3
Copepoda (nauplius)	100 - 450	6.4	4.5	3.9
Facetotecta (nauplius)		0.3		
Cirripedia (nauplius)		0.8	1.0	1.2
Cirripedia (cypris)			1.0	
Malacostraca				
Mysidacea			0.3	0.9
Cumacea		0.6	2.6	0.3
Isopoda	400 - 1000	10.3	16.1	12.3
Gammaridea	1000 - 3000	2.8	2.9	2.7
Anomura (zoea)		0.3		0.3
Anomura (glaucothoe)			0.3	
Brachyura (zoea)	800 - 2200	1.4	1.9	1.5
Decapoda (zoea)	1100 - 3000	1.1	3.2	3.3
Decapoda (mysis)	1000 - 3000	1.4	1.9	2.4
CHORDATA				
Osteichthyes				
Osteichthyes (larvae)				0.3
Total taxa		**29**	**28**	**29**
Total individual		**3590**	**9330**	**9990**

Table 4. 2 Demersal zooplankton diversity, abundance and the similarity among habitats in Bise lagoon, Okinawa Islands.

H′ is Shannon Index, the abbreviation SG, SG+CR and CR denote seagrass, seagrass mixture coral, and coral habitats, respectively.

Habitats	**H′**	**Abundance of DZ Mean (±SD) x 10^4 (Ind. m-2)**	**Similarity (%)**	
			SG	**SG+CR**
SG	2.6	17.6 ±2.3	100.0	
SG+CR	2.7	18.8 ±6.8	80.1	100.0
CR	2.5	6.8 ±2.0	69.1	68.5

Table 4. 3 Proportional diets of demersal zooplankton in the reef lagoon at Bise, Okinawa, Japan.

The mean and the 90% credibility intervals (5% and 95%) of the proportions are reported for each potential food source in the demersal zooplankton diet. DZ, MPB, Phyto-, and POM denote the demersal zooplankton, microphytobenthos, phytoplankton and practical organic matter. A number of 73, 100, 250, 500, 1000, 2000 and 4000 are the size classes of 73 to 100 µm, 100 to 250 µm, 250 to 500 µm, 500 to 1000 µm, 1000 to 2000 µm, 2000 to 4000 µm and larger than 4000 µm, respectively.

Habitats	**DZ size classes (µm)**	**Epiphytes**	**MPB**	**POM**	**SG litter**	**Phyto-**	***Ulva* sp.**
Seagrass	73 – 100	17.2 (2.6 - 31.8)	17.4 (2.7 - 32.1)	16.4 (2.2 - 31.2)	16.9 (2.0 - 33.1)	16.3 (2.1 - 31.4)	15.8 (2.0 - 30.5)
	100 – 250	20.9 (3.5 - 38.1)	21.1 (3.8 - 38.6)	13.6 (1.4 - 29.3)	16.7 (1.9 - 33.1)	13.6 (1.2 - 29.1)	14.2 (1.3 - 30.2)
	250 – 500	15.6 (1.7 - 31.1)	16.7 (2.1 - 32.7)	13.3 (1.3 - 29.2)	18.4 (2.6 - 35.1)	17.4 (2.2 - 33.6)	18.5 (2.7 - 35.1)
	500 – 1000	15.4 (1.7 - 31.3)	15.1 (1.5 - 30.6)	16.0 (2.4 - 30.3)	16.9 (2.1 - 32.0)	18.2 (2.5 - 34.6)	18.4 (2.7 - 34.5)

	1000 – 2000	16.9 (2.6 - 31.5)	17.6 (2.8 - 32.3)	15.6 (1.8 - 30.8)	17.5 (2.4 - 33.1)	16.3 (2.2 - 31.2)	16.1 (2.0 - 31.2)
	2000 – 4000	13.4 (1.3 - 28.9)	14.8 (1.5 - 30.8)	15.4 (1.5 - 31.8)	18.2 (2.3 - 34.9)	18.8 (2.8 - 35.3)	19.5 (3.3 - 36.2)
	> 4000	16.4 (1.9 - 32.3)	16.6 (1.9 - 32.4)	16.2 (2.0 - 32.2)	16.8 (2.1 - 32.9)	17.0 (2.1 - 33.1)	17.0 (2.0 - 33.0)
Seagrass mixture coral	73 – 100	18.7 (3.6 - 32.5)	17.5 (2.6 - 32.6)	15.6 (1.8 - 31.0)	17.6 (2.4 - 33.8)	15.2 (2.0 - 30.1)	15.4 (1.9 - 30.6)
	100 – 250	17.1 (2.7 - 31.5)	16.7 (2.2 - 32.0)	14.1 (1.8 - 28.9)	18.3 (3.2 - 33.5)	16.7 (2.3 - 31.6)	17.0 (2.4 - 32.2)
	250 – 500	11.2 (1.2 - 26.6)	13.7 (1.5 - 28.5)	11.1 (0.8 - 27.7)	19.9 (4.4 - 35.9)	24.3 (4.3 - 56.2)	19.8 (4.7 - 35.4)
	500 – 1000	15.5 (1.6 - 31.4)	16.0 (1.7 - 31.7)	17.3 (3.0 - 31.2)	15.8 (1.9 - 30.8)	17.9 (2.5 - 34.0)	17.4 (2.6 - 32.6)
	1000 – 2000	12.8 (1.1 - 27.9)	14.2 (1.4 - 29.9)	15.2 (1.5 - 31.9)	18.4 (2.6 - 35.5)	20.1 (3.8 - 35.4)	19.4 (3.4 - 35.1)
	2000 – 4000	12.5 (0.9 - 28.6)	14.3 (1.2 - 30.5)	16.8 (2.5 - 32.1)	16.4 (1.9 - 32.3)	20.6 (3.5 - 38.1)	19.4 (3.3 - 35.9)

	> 4000	11.6 (0.9 - 27.5)	12.8 (1.1 - 28.7)	12.1 (1.1 - 27.4)	18.9 (2.7 - 35.2)	22.2 (4.0 - 42.0)	22.3 (4.4 - 40.0)
Coral	73 – 100	15.9 (1.9 - 31.1)	15.5 (1.8 - 30.8)	20.6 (4.8 - 34.5)	15.3 (1.6 - 30.9)	16.3 (1.9 - 31.9)	16.4 (2.1 - 31.8)
	100 – 250	13.3 (1.0 - 29.9)	13.9 (1.2 - 29.9)	19.6 (2.9 - 37.2)	15.8 (1.7 - 32.0)	19.1 (2.7 - 36.6)	18.3 (2.4 - 35.3)
	250 – 500	15.0 (1.5 - 30.2)	15.1 (1.5 - 30.6)	19.5 (2.9 - 36.3)	15.6 (1.6 - 31.6)	17.5 (2.3 - 33.4)	17.1 (2.0 - 33.4)
	500 – 1000	12.8 (1.1 - 28.1)	13.7 (1.4 - 28.9)	20.3 (5.0 - 34.3)	15.5 (1.8 - 30.9)	18.7 (3.1 - 34.3)	19.0 (3.2 - 34.4)
	1000 – 2000	14.2 (1.2 - 30.6)	15.7 (1.8 - 31.7)	13.5 (1.1 - 29.7)	18.6 (2.5 - 35.7)	19.0 (2.6 - 36.2)	19.0 (2.8 - 36.1)
	2000 – 4000	13.1 (1.1 - 29.2)	14.2 (1.2 - 30.1)	17.3 (3.0 - 31.2)	16.7 (2.3 - 32.2)	19.2 (3.1 - 36.3)	19.4 (3.1 - 35.9)
	> 4000	12.9 (1.1 - 29.5)	13.0 (1.1 - 29.1)	23.7 (4.6 - 43.6)	14.1 (1.3 - 30.3)	19.1 (2.6 - 35.9)	17.3 (2.1 - 34.2)

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CHAPTER 5

ROLE OF DEMERSAL ZOOPLANKTON AS A FOOD SOURCE FOR HIGHER TROPHIC LEVELS AT FUKIDO ESTUARY, ISHIGAKI ISLAND, OKINAWA, JAPAN

Chapter 5

Role of demersal zooplankton as a food source for higher trophic levels at Fukido Estuary, Ishigaki Island, Okinawa, Japan

Abstract

Demersal zooplankton (DZ) appear in the water column at night, and are highly abundant in mangrove, seagrass, and coral reef habitats; however, few studies have discussed their role in aquatic food webs, considering different consumers and their preferences on different DZ' size classes. This study elucidates the role of DZ as a food source for higher trophic levels in an estuarine area, particularly with respect to the food preference and size selection of their consumers. The study was conducted in the mangrove forest of Fukido Estuary and an adjacent reef lagoon (with seagrass-dominated and seagrass-coral mixture areas) on Ishigaki Island, Japan. The abundance of demersal zooplankton was 4.0, 5.4, and 11.3 × 10^4 ind.m^{-2} for seagrass, mangrove, and seagrass-coral mixture habitats, respectively. The lowest DZ biomass was recorded in mangroves and mainly dominant by smaller organisms because their consumers in this habitat prefer large-sized prey. The $\delta^{13}C$ and $\delta^{15}N$ signatures showed that, in mangroves, demersal zooplankton constituted a higher proportion of the diet of fishes than in lagoon habitats; however, demersal zooplankton did not have a significant role in the diet of fishes and macroinvertebrates in the lagoon. Consistency among biomass, stomach contents, and the proportions of DZ of all size classes in the diet of mangrove fishes indicated that DZ serve as a major food source. In contrast,

fishes in lagoon habitats consumed more crabs, shrimps, and mollusks than DZ. In conclusion, our analytical approach allowed us to demonstrate that DZ of different body sizes serves as food sources for different consumers in different habitats of the estuarine ecosystem.

5.1 Introduction

Mangroves, seagrasses, and coral reefs are important marine coastal ecosystems because they sustain high biodiversity (Marguillier et al., 1997; Zieman et al., 1984) and are highly efficient at transferring organic matter from primary producers to higher trophic levels (Nagelkerken, 2009). These well-structured ecosystems provide stable nursery sites and a wide range of food sources for diverse fishes and invertebrates (Beck et al., 2001; Melo et al., 2010; Touchette, 2007). These habitats accumulate a large quantity of non-living organic matter in their sediments in the form of detritus, leaf litter, and decomposed dead organisms (Bouillon and Connolly, 2009; Kristensen et al., 2008). However, few species of consumers are able to utilize this food source directly. Thus, sediment detritivores and herbivores might be important for connecting these food webs by transferring energy from primary producers to higher trophic levels (Mascart, 2010; Sogard, 1984). Previous studies have reported that demersal zooplankton (DZ) are important for linking small particles (detritus and primary producers attached to sediments) to planktivorous fishes (Boltovskoy, 1999; Morgan, 1990).

DZ reside (or hide) near the substrate during the daytime, emerge at night when they spend a short time in the water column, and return to the substratum

before sunrise (Alldredge and King, 1980; Hobson and Chess, 1979; Melo et al., 2010). According to Alldredge and King (1980), the night-time migration of DZ has several advantages, including feeding (on small organisms, such as pico- and nano- size plankton or smaller DZ), reproduction (polychaetes spawn at the surface, amphipods mate in the water column), escape from predation by benthic invertebrates, ecdysis, and dispersal to potentially more favorable locations to reduce competition for food and space. Studies have shown that large numbers of DZ emerge from the seagrass, coral reefs, soft bottoms, and kelp beds at night (Jayabarathi et al., 2012; Mascart, 2010; Youngbluth, 1982). Thus, they might serve as an important food source for planktivores (Melo et al., 2010). However, few studies (Chew et al., 2012; Smith et al., 1979) have investigated how DZ are linked to their consumers in the aquatic food web of coastal ecosystems.

In the food webs of natural systems, it is difficult to determine the proportional contribution of food sources to the diet of consumers on the basis of stomach contents, because the food is rapidly digested in comparison to the slow digestion of non-living organic matter derived from sediments (Fry and Ewel, 2003). As a result, there has been an increasing number of studies using dual stable carbon and nitrogen isotopes to determine the relationship between predators and their food sources (Nakamura et al., 2008; Pasquaud et al., 2010; Tue et al., 2013; Vinagre et al., 2012; Fry, 2007). The $\delta^{15}N$ in the tissues of a consumer is typically 2.6‰ to 3.4‰ richer than that in their prey; thus, $\delta^{15}N$ studies are often conducted to estimate the trophic position of species in the food web (Deniro and Epstein, 1981; Fry, 2007; Post, 2002). The $\delta^{13}C$ of a consumer

increases by 0‰ to 1‰ with respect to their food sources (Michener and Lajtha, 2008; Tue et al., 2013). Therefore, the $\delta^{13}C$ signature could be used to trace the carbon pathway when the $\delta^{13}C$ value of food sources differs across prey items (Bouillon et al., 2008). The stable isotope analysis in R (SIAR) isotopic mixing model (Parnell and Jackson, 2013) is increasingly being applied to estimate the proportions of various food sources ingested by a consumer, on the basis of the isotopic signatures of consumers, food sources, and the trophic enrichment factor (TEF) (Tue et al., 2013). The SIAR model is an open-source package that uses Bayesian inference to address natural variation and the uncertainty of stable isotope data to calculate the probability of food source contributions as percentages of the total diet (Pacella et al., 2013).

In the present study, we hypothesized that DZ contribute in major proportion to the diet of higher trophic levels at Fukido estuarine aquatic food web, and these consumers preferentially feed on DZ of different size classes. We tested this hypothesis by (1) analyzing the abundance and biomass of DZ in a mangrove and an adjacent lagoon (seagrass dominated and seagrass-coral mixture) habitat, to assess the potential amount of DZ that serves as a food source, and (2) determining the proportion of DZ of different size classes in the diets of fishes and macroinvertebrates distributed in mangrove and reef lagoon area, by combining the isotopic mixing model (SIAR) with stomach content analysis. This study is expected to provide new insights on the role and importance of DZ as a food source for consumers at the three sub-environments that compose the Fukido estuary.

5.2 Experimental design

Sampling was carried out at MG, SG, and SG+CR in March 2015 and 2016.

Determining the abundance and biomass of DZ

DZ was identified and counted under a stereomicroscope (SMZ 1000, Nikon Inc.). The abundance of DZ taxa was calculated as the number of individual m^{-2} ($ind.m^{-2}$) on the bottom surface area, based on the mouth area of the "emergence trap" (see Fig. 1B). The carbon biomass of DZ was calculated as the carbon content in µg C ind^{-1}, based on the taxonomic level and the average size class, using the regression equation given by Heidelberg et al., (2010); LN (Copepod biomass) = 1.82 × log (L) + 1.28 (r^2 = 0.893, df = 16, F = 125; sig. = 1.12×10^{-8}) and LN (Other taxa biomass) = 1.46 × LN (L) + 1.03 (r^2 = 0.733, df = 16, F = 80.7; sig. = 3.47×10^{-7}), where LN is the natural logarithm and L is the average size in mm.

Analysis of fish stomach content

The stomach and gut were dissected from the fish body and preserved with 90% ethanol. The stomach content was observed under a stereomicroscope (SMZ 1000, Nikon Inc.), and the percentage of items present was estimated. The food sources were identified to the lowest possible taxon.

Estimation of proportional contribution from food sources

To compare the percentage of DZ with other food sources at each of the selected habitats, we estimated the contribution of food sources to the diets of consumers (fishes, macroinvertebrates) in each habitat using the mean of the

TEF (± SD) for $\delta^{13}C$ and $\delta^{15}N$ as 0.4 ± 1.3‰ and 3.4 ± 1.0‰, respectively (Post, 2002). By the results of the stomach contents analysis, we compared DZ against other food sources (including crabs, shrimps, mollusks and, detritus) in the diet of fishes. In the diet of macroinvertebrates, we compared DZ against the plant (mangrove or seagrass) leaves, phytoplankton, detritus, and sediment.

5.3 Results

5.3.1 Composition of DZ communities

In the present study, 18 DZ taxa were identified (Table 5.1). The species groups that had high-frequency distributions and higher abundance in all three habitats were Harpacticoida (copepodite), Foraminifera, and Gammaridea (Table 5.1). In the MG habitat, Harpacticoida (copepodite) contributed 43.2%, followed by Nematoda (24.3%) and the nauplii of Copepoda (10.8%). In the SG habitat, Foraminifera contributed 27.8%, followed by Isopoda (22.2%). In the SG+CR habitat, *Oithona rigida* had the highest abundance (25.7%), followed by the nauplii of Copepoda (22.9%) and Harpacticoida (copepodite) (12.9%).

The diversity index (H′) increased from MG (1.6) towards SG+CR (2.2) habitat (Fig. 5.1A). DZ abundance was highest in SG+CR (11.3×10^4 ind.m^{-2}), followed by MG (5.4×10^4 ind.m^{-2}) and SG (4.0×10^4 ind.m^{-2}) (Fig. 5.1A). When analyzing the biomass of the different size classes, we observed that the larger size classes (i.e., 500 to 1000 µm, 1000 to 2000 µm, and >2000 µm) contributed more to the total biomass of SG and SG+CR compared to MG (Fig. 5.1B). The biomass contributions of the smaller size classes (73 µm, 100 µm,

250 µm) were similar in both MG and SG habitats. Small organisms mainly dominated the total DZ biomass in the MG habitat. Moreover, the total biomass in MG (205.2 × 10^3 µg C m^{-2}) was lower than the total biomass in SG (341.1 × 10^3 µg C m^{-2}) and SG+CR (709.0 × 10^3 µg C m^{-2}) (Fig. 5.1B). Thus, the MG habitat contained smaller organisms, with the lower abundance dominating the total biomass. This abundance was lower than the total biomass of DZ in SG and SG+CR.

5.3.2 Stomach contents of fishes

Table 5.2 shows the stomach contents of 11 fish species collected in Fukido Estuary. The preferred foods of fishes in MG were zooplankton, which are components of DZ (Table 5.1). Fishes from MG primarily consumed copepods (5–15%) and polychaetes (5–25%), followed by amphipods (3–10%), nematodes (5–10%), and foraminifers (2–5%). Some fishes, such as *Y. cringer* (2%), *C. punctata* (5%), *F. amboinensis* (7%), and *Lutjanus* sp. (20%) consumed crabs. Shrimp and mollusks were consumed by larger fishes, such as *Lutjanus* sp. (10%) and *A. semipunctata* (10%), respectively (Table 5.2). However, the main stomach content of some fish specimens in MG was detritus (*G. oyena* [80%] and *A. semipunctata* [50%]) and fine soil and sand grains (*F. amboinensis* [40%] and *Z. dunckeri* [30%]) (Table 5.2). Fish specimens collected in the LG habitat mainly consumed crabs (10–15%), mollusks (10–30%), and shrimp (10%). Moreover, some fishes consumed DZ species, such as amphipods (20–30%), copepods (10%), and polychaetes (5%), in the lagoon habitat (Table 5.2).

5.3.3 Isotopic signature of consumers, DZ, and other food sources

Figure 5.2 shows the stable carbon and nitrogen isotope signatures of DZ, other food sources (mangrove leave, seagrass leave, detritus, sediment, and phytoplankton), and their consumers (fishes, crabs, shrimps and mollusks) in the aquatic food web of Fukido Estuary. The $\delta^{13}C$ values of DZ ranged from -24.9 to -20.4‰ and -20.5 to -15.4‰ in the MG and LG areas, respectively. The $\delta^{15}N$ signatures of DZ in both habitats were not significantly different (ANOVA, $p = 0.479$), ranging from 2.7 to 4.6‰ (Fig. 5.2).

The $\delta^{13}C$ and $\delta^{15}N$ values of other food sources (mangrove and seagrass leaves, phytoplankton, detritus, and sediment) ranged from -29.4 to -25.9‰ and 0.6 to 2.1‰, respectively, in MG; and from -21.3 to -10.3‰ ($\delta^{13}C$) and 0.7 to 1.8‰ ($\delta^{15}N$) in the LG habitat. The $\delta^{13}C$ and $\delta^{15}N$ values of consumers ranged from -26.7 to -18.8‰ and 4.3 to 9.3‰ in MG, respectively. In comparison, these signatures ranged from -14.2 to -11.0‰ ($\delta^{13}C$) and from 4.2 to 9.7‰ ($\delta^{15}N$) in the lagoon habitat (Fig. 5.2).

5.3.4 Proportional distribution of DZ in the diets of higher trophic levels

The contributions of the potential food sources of the fishes and macroinvertebrates in Fukido Estuary are presented in Table 5.3 and Table 5.4, respectively. Table 5.3 shows that DZ of larger size classes (500 to 2000 μm) contributed more to the diet of MG fishes than the DZ of smaller size classes (73 to 250 μm). In the diet of some mangrove fish species, the proportional contribution of DZ from the larger size classes (500 to 2000 μm) was higher than that of other food sources (e.g., crabs, shrimp, mollusks, and detritus). *A. semipunctata* consumed large quantities of DZ large than 2000 μm (15.0%),

followed by 500–1000 μm DZ (13.4%) and 1000–2000 μm DZ (12.9%). DZ contributed 11.1–12.5% to the diet of *Y. criniger*. DZ are larger than 2000 μm contributed the highest proportion (12.9%) to the diet of *Z. dunckeri*, followed by 1000–2000 μm DZ (12.6%) and 500–1000 μm DZ (12.1%). Other food sources contributed 4.3–11.7%, 7.1–10.5%, and 7.3–9.7% to the diet of *A. semipunctata*, *Y. criniger*, and *Z. dunckeri*, respectively. In comparison, some mangrove fish species consumed more crabs and shrimps than DZ. For instance, *F. amboinensis* consumed the highest proportion of crabs (19.0%), followed by shrimps (15.8%). The diet of *C. punctate* contained 13.1% crabs and 11.7% shrimps. DZ contributed just 6.2–7.8% (73–250 μm size classes) and 8.7–13.6% (500–2000 μm) to the diet of *F. amboinensis*, and 8.8–9.6% (73–250 μm) and 10.1–11.6% (500 – 2000 μm) to the diet of *C. punctata*.

Lutjanus sp. and *G. oyena* consumed similar quantities of DZ (500–2000 μm) and other crustaceans (such as crabs and shrimps) in MG habitat; however, *Lutjanus* sp. collected in the LG habitat consumed more crabs (12.2%) and shrimps (11.2%) than DZ (9.8–10.1%). Other lagoon fishes also consumed more on crabs, shrimps, and mollusks than DZ (Table 5.3).

The results of the mixing model of the macroinvertebrates diet items and their proportions in Fukido Estuary are presented in Table 5.4. Mangrove crabs (*Scylla serrata* and *Charybdis* sp.) consumed more DZ of bigger size classes (500 to 2000 μm) than smaller size classes (73–250 μm) or other food sources (i.e., those derived from mangrove leaves, detritus, sediment, and phytoplankton). In comparison, some macroinvertebrates (such as *E. japonica* and *C. septemspinosa*)

consumed more DZ of smaller size classes (73–250 μm) than larger size classes (500–2000 μm). Almost all macro-invertebrates (except *Charybdis* sp. and *S. serrata*) from both habitats preferentially consumed food sources derived from detritus, sediment, plants, and phytoplankton compared to DZ.

5.4 Discussions and Conclusions

5.4.1 Role of DZ as a potential food source in an estuarine food web

Mangroves, seagrasses, and coral reefs support and provide shelter for a large number and high diversity of fish and invertebrates (Larkum et al., 2007; Zieman et al., 1984). The average size ratio of predator to prey is usually around 10 to 1, while the abundance ratio is typically the inverse (Chen and Terry, 2014; Litchman et al., 2013). Therefore, DZ must contribute substantially to the system, as a large reserve of highly abundant food source with a wide range of body dimensions. Previous studies reported that DZ are highly abundant in shallow habitats (Melo et al., 2010). DZ usually reside in the top 3 cm of the sediment, emerging in large numbers at night and occupying the water column up to 30 cm from the bottom (Alldredge and King, 1985). They only remain in the water column for a short time to avoid predators that use vision to locate their prey (Alldredge and King, 1985). In the present study, DZ abundance ranged from 4.0 to 11.3 × 10^4 ind.m^{-2} (Fig. 5.1A), with a wide range of body sizes being detected (75 to 9100 μm) (Table 5.1). At Fukido Estuary, the abundance of DZ was higher than that previously reported in other geographical areas. For instance, Melo et al. (2010) reported comparatively lower DZ abundance (5 × 10^3 ind.m^{-2}) in a seagrass area in the southwestern Atlantic Ocean. In comparison, the abundance of DZ in Onslow

Bay (North Carolina, USA) ranged from 1 to 6 × 10^4 ind.m^{-2} depending on substrate structure and season (Cahoon and Tronzo, 1992).

The biomass of DZ in the 1000–2000 μm and > 2000 μm size classes were lower than those smaller size classes in MG compared to that in the other habitats (Fig. 5.1B). Compared to that in the other habitats in our study, the abundance of DZ in MG was intermediate, with smaller biomass. Sultana et al. (2016) reported that the sediment in mangrove habitat supports high primary production. Thus, DZ might be present in higher abundance and biomass than that detected in our study. However, our results showed that smaller DZ contributed the most to DZ biomass, indicating that higher trophic level organisms preferentially consume larger DZ (Fig. 5.1). Therefore, DZ with lower biomass and of smaller size might remain in the mangrove habitat. This suggestion is supported by the result of the mixing model used to estimate the proportion of DZ in the diet of fishes (Table 5.3). This model showed that the proportional contribution of larger DZ (500–2000 μm) were higher than that of smaller DZ (73–250 μm) in the diet of almost all mangrove fishes. Thus, consumers in the mangrove area might actively feed on more DZ than previously thought (Nakamura et al., 2008; Tue et al., 2013). This hypothesis is consistent with the stomach contents of fishes, which showed that the mangrove fishes primarily fed on DZ compared to other food sources (Table 5.2).

5.4.2 Proportional contribution of DZ in the diet of consumers

Analyzing stomach contents traditionally performs estimation of the proportional contributions of prey in the diet of consumers in coastal ecosystems. This method is convenient because it can be carried out rapidly

and economically, providing a direct estimation of prey contents (Hyslop, 1980; Pasquaud et al., 2010; Winemiller et al., 2011). However, stomach content analysis has some limitations, because living organisms digest food more rapidly than non-living organic matter (Fry and Ewel, 2003). The SIAR mixing model is based on the isotopic signatures of prey and consumers and has been increasingly used to determine the proportional contributions of food sources (Parnell et al., 2010). In this study, we combined both methods (stomach content and mixing model) to clarify and highlight the contributions of DZ to the diet of fishes and macroinvertebrates at Fukido Estuary.

The mixing model showed that DZ contributed more to the diet of mangrove fishes than another food source in the MG habitat, particularly for *A. semipunctata*, *Y. criniger*, and *Z. dunckeri* (Table 5.3). In particular, these fish species consumed larger DZ (of 500 to 2000 μm), which were dominated by nematodes, polychaetes (larvae), copepods, amphipods, and gammarids (Table 5.1). The results of the stomach content analysis supported this observation (Table 5.2). Our results were also consistent with those of previous reports (Masuda et al., 1984; Myers, 1999; Rainboth, 1996). In comparison, crabs and shrimp contributed to the diet of *F. amboinensis* and *C. punctata* more than DZ. Other studies also reported that *F. amboinensis* primarily consumes small crustaceans (Masuda et al., 1984), whereas *C. punctata* preferentially feeds on small benthic invertebrates and small crabs (Knapp, 1999). However, our stomach content analyses of these fishes showed that they preferentially fed on nematodes and copepods compared to crabs and shrimps (Table 5.2). This discrepancy might be explained by the fact that stomach contents only reflect

feeding events, rather than assimilated material; consequently, this type of analysis is biased by the differences in digestibility of prey items (Polunin and Pinnegar, 2002). Thus, the proportions of food sources based on isotopic analysis probably provide a more accurate picture of feeding behavior than stomach contents. Our results showed that mangrove fishes predominantly fed on DZ compared to other food sources. In comparison, lagoon fishes consumed more crabs, shrimps, and mollusks than DZ. These differences were supported by both the results of the mixing model (Table 5.3) and the stomach contents analysis (Table 5.2). Lagoon fishes of large body size might preferentially feed on larger food sources. This suggestion was supported by the results of the feeding preferences of *Lutjanus* sp., which is a migratory fish that frequents Fukido Estuary (Nakamura et al., 2008). Juvenile stage *Lutjanus* sp. are relatively small (around 30–125 cm) (Nakamura and Tsuchiya, 2008; Shibuno et al., 2008), and they inhabit MG as a nursery. Juvenile *Lutjanus* sp. consumed similar proportions of DZ (500–2000 μm) as crabs and shrimps in MG (Table 5.3), perhaps because the *Lutjanus* sp. might prefer DZ than crustaceans that have hard exoskeletons. As adults, when their body size becomes greater than 150 cm, *Lutjanus* sp. migrate to the lagoon area (Nakamura et al., 2008), where they switch their feeding preference to crustaceans (crabs and shrimps) during ontogenetic migration (Table 5.2 and Table 5.3). Thus, DZ might be an important food source for fishes in mangrove habitats compared to other studied habitats.

Benthic macroinvertebrates are distributed in all coastal ecosystems (mangrove, seagrass and coral reef, etc.), and inhabit areas on or near the

seabed (Attrill, 1998; Barnes, 2013; Castro et al., 2008; Kumar and Khan, 2013). This group exhibits diverse ecological niches and invests in a variety of feeding behaviors. For instance, there are deposit feeders (mainly polychaetes and some mollusks), detritivores (some echinoderms and crustaceans), predators (echinoderms and crustaceans), filter feeders (mainly bivalves and crustaceans), among others. Our result showed that DZ contributed more than other food sources to the diet of 2 crab species (*Scylla serrata* and *Charybdis* sp.) (Table 5.4). Other studies have also reported that these two crab species preferentially feed on fishes, crustaceans, mollusks, and polychaetes in mangrove habitats (Sara et al., 2007; Wikipedia, n.d.). The stable carbon and nitrogen isotope signature of *S. serrata* are comparable to that of some mangrove fishes (*Z. dunckeri, A. semipunctata, and F. amboinensis*) (Fig. 5.2); thus, these crabs probably feed on food sources similar to fishes, preferentially selecting large-sized DZ. This suggestion was supported by the results of the mixing model on the diet of S. serrata, which showed that they feed on larger rather than smaller DZ (Table 5.4). In contrast, some species (such as *E. japonicas* and *C. septemspinosa*) primarily consumed food sources that were derived from detritus, sediment, and plant debris rather than DZ (Table 5.4). This result supports that obtained by the previous report (Kolpakov et al., 2012). Other macroinvertebrates (except *Scylla serrata* and *Charybdis* sp.) appeared to consume similar quantities of small (73–250 μm) and large DZ in the lagoon habitat, but preferentially fed on smaller DZ in mangrove habitat (Table 4).

In conclusion, this study demonstrated that DZ are an important food source in Fukido Estuary. The mangrove habitat supported the lowest DZ

biomass and was mostly made up of small-sized DZ. Thus, higher trophic level organisms might consume large-sized DZ, resulting in the smaller sizes remaining (unconsumed) in the mangrove habitat. This suggestion supports the proportions of DZ size classes detected in the stomach contents of mangrove fishes. Large percentages of DZ were detected in the stomachs of mangrove fishes, confirming their importance as a food source. In contrast, DZ did not have a significant role in the diet of fishes in lagoon habitats compared to other food sources. The combined results of abundance and biomass with the proportions of DZ in the diet of consumers highlight the important role of DZ in the diet of active feeders in mangrove habitats compared to that in other habitats. To our knowledge, this study is the first attempt to elucidate the role of DZ within the food web of an estuary, using the stable isotope mixing model of size class and biomass combined with direct observations of stomach contents. In conclusion, we showed that the suggested approach (combining stable isotope mixed models with stomach content observations) was reliable, demonstrating that DZ of different body sizes serves as food sources for different consumers in different habitats.

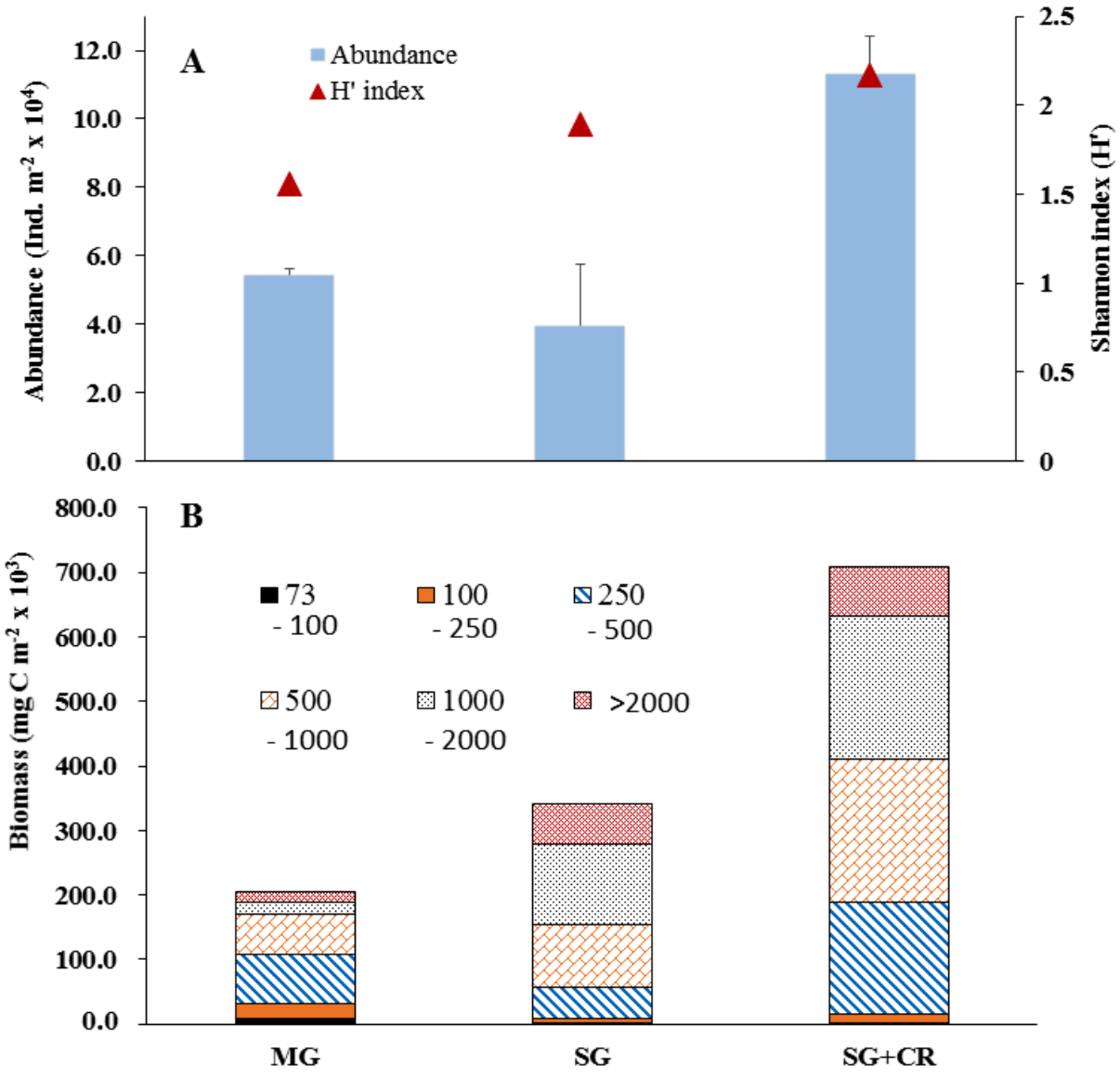

Figure 5. 1 A: Abundance and Shannon index: ind. m^{-2} (mean ± SD), Shannon diversity index (H′) and **B**: Biomass within size classes of demersal zooplankton at Fukido Estuary.

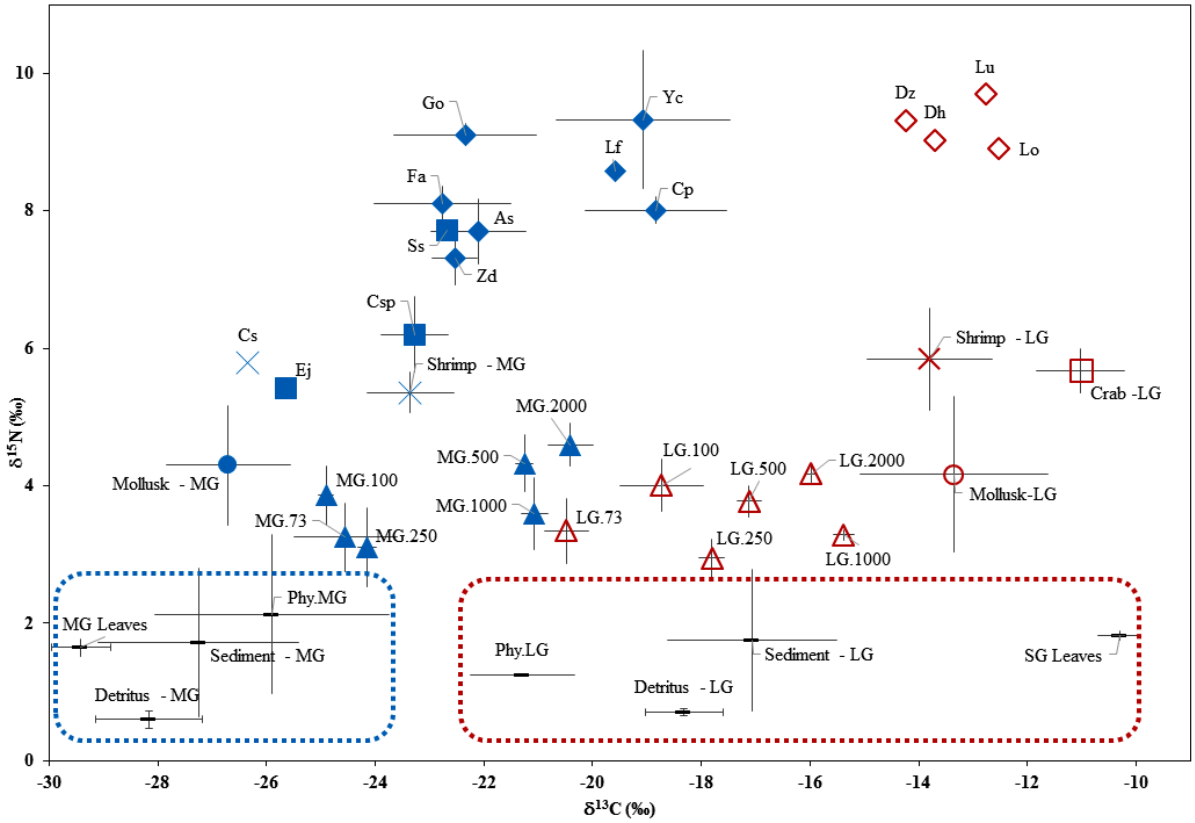

Figure 5. 2 Carbon and nitrogen isotopic signatures (Mean ± SD) of demersal zooplankton, their consumers, and other food sources in Fukido Estuary.

The symbols indicate demersal zooplankton (Δ), fishes (◊), crabs (□), mollusks (○), and shrimps (x). Solid black filled symbols denote organisms collected in the mangrove, and open symbols denote samples collected from the lagoon (SG and SG+CR). Dotted lines indicate other food sources derive from mangrove (MG) and lagoon (LG) habitats. Label abbreviations are shown in Table 5.3 and Table 5.4.

Table 5. 1 Composition, size range, and relative abundance (%) of demersal zooplankton captured by emergence traps.

Abbreviations: MG, SG, and SG+CR denote the mangrove, seagrass and seagrass mixture coral habitats, respectively.

Taxa	Size range [μm]	MG (%)	SG (%)	SG+CR (%)
SARCOMASTIGOPHORA				
Foraminiferidae	75–250	8.1	27.8	7.1
NEMATODA	300–1200	24.3	11.1	-
MOLLUSCA				
Gastropoda (larvae)	75–350	5.4	-	-
ANNELIDA				
Polychaeta (larvae)	250–1500	2.7	-	-
ARTHROPODA				
Ostracoda		-	8.3	-
Bestiolina similis		-	-	2.9
Calanoida (copepodite)		-	-	1.4
Oithona rigida	700–800	-	-	25.7
Oithonidae (copepodite)	400–700	-	-	4.3
Cyclopoida (copepodite)	200–400	-	-	1.4
Harpacticoida	400–1000	-	5.6	2.9
Harpacticoida (copepodite)	200–700	43.2	11.1	12.9
Poecilostomatoida (copepodite)	250–600	-	-	4.3
Copepoda (nauplius)	80–250	10.8	-	22.9
Cumacea	1200–9100	-	2.8	-
Isopoda	300–1500	-	22.2	4.3
Gammaridea	900–3800	5.4	11.1	4.3
Zoea of Brachyura	500–1300	-	-	5.7
Total taxa		**7**	**8**	**13**

Table 5. 2 Fish in Mangrove and Lagoon habitats and the average percentage of the prey items recorded in their stomach contents in Fukido Estuary.

Cr: crabs (crab + hermit crab), Sh: shrimps, Mo: Mollusks, Demersal zooplankton (Ne: nematodes, Co: copepods, Am: amphipods, Iso: isopods, Po: polychaetes, Fo: Foraminifers), Sg: seagrass, Se: sediment, De: detritus, Other: small material and unidentified foods. TL = total length; n = number of samples

Taxa	n	TL (±SD) (mm)	Stomach contents (%)												
			Cr	Sh	Mo	Ne	Co	Am	Iso	Po	Fo	Sg	Se	De	Other
Mangrove habitat															
Asterropteryx semipunctata	5	128.0 ± 17.9			10	5	10			15	5			50	5
Yongeichthys criniger	2	76.0 ± 33.9	2			10	10	3		25				40	10
Fibramia amboinensis	10	66.3 ± 10.3	7	3		8	5				2		40	30	5
Gerres oyena	3	70.3 ± 17.9												80	20
Lutjanus sp.	1	120.0	20	10				10		5				10	45
Cociella punctata	3	112.7 ± 4.6	5			10	15	5		5				40	20
Zenarchopterus dunckeri	3	125.7 ± 13.2					10			20	5		30	15	20
Lagoon habitat															
Lutjanus sp.	1	228.2	15		10			10	10					5	50
Lethrinus ornatus	1	120.0					10					8		10	72
Diodon holocanthus	1	204.0	10		20			20	10	5	5	5		20	5
Dendrochirus zebra	1	295.0		10	30									5	55

Table 5. 3 Proportion of demersal zooplankton and other food sources in the diet of fishes in Fukido Estuary.

The Mean and the 90% credibility intervals (5% and 95%) of the proportions are reported for each potential food source in the fish diet. DZ.73, DZ.100, DZ.250, DZ.500, DZ.1000, and DZ.2000 denote the demersal zooplankton size classes of 73 to 100 µm, 100 to 250 µm, 250 to 500 µm, 500 to 1000 µm, 1000 to 2000 µm, and larger than 2000 (µm), respectively.

Fishes	Label	Animal	DZ.73	DZ.100	DZ.250	DZ.500	DZ.1000	DZ.2000	Crabs	Shrimps	Mollusks	Detritus
Mangrove habitat												
A. semipunctata	As	Fish	8.7	8.5	8.8	13.4	12.9	15.0	11.7	9.6	7.1	4.3
			(0.8–19.6)	(0.7–19.2)	(0.8–19.6)	(2.1–25.8)	(2.1–24.5)	(3.2–27.4)	(1.9–22.0)	(1.0–20.4)	(0.6–17.2)	(0.3–11.1)
Y. criniger	Yc	Fish	8.8	9.9	9.8	11.3	11.1	12.5	10.5	10.2	8.8	7.1
			(1.0–18.8)	(1.1–19.8)	(1.1–19.8)	(1.2–21.3)	(1.1–21.1)	(1.2–22.4)	(1.3–20.7)	(1.3–20.1)	(1.0–18.8)	(0.9–17.9)
F. amboinensis	Fa	Fish	6.6	7.8	6.2	11.6	8.7	13.6	19.0	15.8	8.0	2.6
			(0.6–16.3)	(0.7–18.7)	(0.6–15.5)	(1.6–23.2)	(0.9–19.7)	(2.3–25.6)	(7.5–30.6)	(3.9–28.0)	(0.8–18.1)	(0.2–6.8)
G. oyena	Go	Fish	9.3	9.4	9.2	11.1	10.8	11.9	11.8	10.8	9.0	6.7
			(0.8–19.6)	(0.9–19.9)	(0.8–19.5)	(1.4–21.6)	(1.2–21.3)	(1.7–22.8)	(1.6–22.9)	(1.2–21.5)	(0.9–19.4)	(0.5–16.3)
Lutjanus sp.	Lf	Fish	8.8	10.0	9.9	10.8	10.6	11.2	10.6	10.0	9.5	8.6
			(1.0–18.8)	(1.0–20.0)	(1.1–19.8)	(1.1–20.8)	(1.1–20.6)	(1.1–21.2)	(1.1–20.6)	(1.0–20.0)	(1.0–19.5)	(1.0–18.5)
C. punctata	Cp	Fish	8.8	9.6	8.8	11.0	10.1	11.6	13.1	11.7	9.7	5.6
			(0.8–19.0)	(1.0–20.1)	(0.9–18.8)	(1.4–21.8)	(1.1–20.7)	(1.5–22.3)	(2.6–23.1)	(1.7–22.3)	(1.0–20.1)	(0.4–14.6)
Z. dunckeri	Zd	Fish	9.8	9.2	10.1	12.1	12.6	12.9	9.7	8.6	7.6	7.3
			(1.1–20.2)	(0.9–19.4)	(1.0–20.5)	(1.9–22.5)	(2.2–22.7)	(2.6–23.3)	(1.2–19.3)	(0.8–18.3)	(0.7–17.3)	(0.8–16.1)
Lagoon habitat												
Lutjanus sp.	Lu	Fish	8.6	9.9	9.9	9.8	9.9	10.1	12.2	11.2	10.1	7.7
			(1.0–18.6)	(1.0–19.9)	(1.0–19.9)	(1.1–19.8)	(1.0–19.9)	(1.1–20.1)	(1.1–22.2)	(1.2–21.2)	(1.1–20.3)	(1.0–17.7)
L. ornatus	Lo	Fish	9.8	9.9	8.9	9.9	10.1	10.1	11.2	11.1	10.1	8.9
			(1.1–19.7)	(1.1–19.7)	(1.1–18.7)	(1.0–19.7)	(1.1–20.2)	(1.1–19.9)	(1.1–21.5)	(1.2–21.1)	(1.2–20.0)	(1.1–18.9)
D. holocanthus	Dh	Fish	8.7	8.9	8.9	10.0	9.9	11.1	12.4	11.2	10.2	8.7
			(1.0–18.6)	(1.1–18.9)	(1.0–18.8)	(1.0–20.1)	(1.0–19.9)	(1.1–21.1)	(1.2–22.5)	(1.1–21.3)	(1.2–20.2)	(1.0–18.8)
D. zebra	Dz	Fish	9.6	8.9	9.9	9.9	10.0	10.4	11.5	10.8	10.4	8.6
			(1.0–19.6)	(1.0–18.9)	(1.0–19.9)	(1.0–19.9)	(1.1–20.0)	(1.1–20.4)	(1.2–21.6)	(1.2–20.8)	(1.2–20.3)	(1.0–18.7)

Table 5. 4 Proportion of demersal zooplankton and other food sources in the diet of macroinvertebrates in Fukido Estuary. The Mean and the 90% credibility intervals (5% and 95%) of the proportions are reported for each potential food source in the macroinvertebrate diet. DZ.73, DZ.100, DZ.250, DZ.500, DZ.1000, and DZ.2000 denote demersal zooplankton size classes of 73 to 100 µm, 100 to 250 µm, 250 to 500 µm, 500 to 1000 µm, 1000 to 2000 µm, and larger than 2000 (µm), respectively. Phyto- is phytoplankton, Plant is mangrove leaves and seagrass leaves in mangrove and lagoon habitats, respectively.

Invertebrates	Label	Animal	DZ.73	DZ.100	DZ.250	DZ.500	DZ.1000	DZ.2000	Plant	Phyto-	Sediment	Detritus
Mangrove habitat												
Scylla serrata	Sc	Crab	9.9	9.8	10.4	13.3	12.3	15.3	6.4	8.0	8.1	6.5
			(1.1–19.9)	(1.1–19.7)	(1.1–20.6)	(1.2–23.3)	(1.2–22.1)	(1.2–25.3)	(1.0–16.4)	(1.1–18.0)	(1.0–18.1)	(1.0–16.5)
Charybdis sp.	Csp	Crab	9.6	9.2	10.3	12.3	14.2	13.1	6.0	8.2	8.3	8.9
			(1.0–20.2)	(0.7–18.3)	(1.2–21.0)	(2.2–22.6)	(2.8–25.2)	(2.7–23.2)	(0.5–14.8)	(1.0–19.4)	(0.8–18.2)	(1.0–18.7)
Eriocheir	Ej	Crab	10.2	10.4	10.3	8.7	8.7	8.4	10.4	11.2	11.3	10.4
japonicas			(1.1–20.2)	(1.0–20.4)	(1.1–20.3)	(1.0–18.8)	(1.0–18.8)	(0.9–18.4)	(1.2–20.3)	(1.2–21.2)	(1.2–21.5)	(1.2–20.5)
Crangon	Cs	Shrimp	10.2	10.1	9.9	8.6	8.7	8.6	10.2	10.5	11.9	11.3
septemspinosa			(1.1–20.2)	(1.0–19.9)	(1.0–19.9)	(0.9–18.6)	(1.0–18.8)	(1.0–18.6)	(1.2–20.2)	(1.1–20.5)	(1.1–21.8)	(1.3–21.3)
Unidentified		Mollusk	10.0	9.9	10.0	9.8	9.7	9.8	10.3	10.9	11.2	11.5
			(1.1–20.1)	(1.1–20.0)	(1.1–20.0)	(1.0–19.8)	(1.0–19.8)	(1.0–19.8)	(1.2–20.5)	(1.1–20.9)	(1.1–21.2)	(1.1–21.5)
Lagoon habitat												
Unidentified		Crab	9.5	9.1	9.9	9.4	9.8	9.3	11.0	10.4	10.4	11.1
			(1.0–19.5)	(0.9–19.1)	(1.1–19.9)	(1.0–19.3)	(1.1–19.7)	(0.9–19.1)	(1.4–20.8)	(1.2–20.1)	(1.3–20.2)	(1.6–20.8)
Unidentified		Shrimp	9.9	9.9	10.0	9.9	10.0	9.9	10.3	10.0	11.3	10.2
			(1.1–19.8)	(1.1–19.8)	(1.1–19.9)	(1.0–20.0)	(1.1–19.9)	(1.1–19.9)	(1.2–20.1)	(1.1–20.0)	(1.1–21.3)	(1.1–20.2)
Unidentified		Mollusk	9.9	9.9	9.9	10.0	10.1	10.0	10.3	9.9	10.0	10.1
			(1.1–19.8)	(1.1–19.7)	(1.0–20.0)	(1.0–19.8)	(1.2–20.2)	(1.1–19.9)	(1.2–20.5)	(1.0–20.0)	(1.0–19.7)	(1.1–20.1)

CHAPTER 6

GENERAL CONCLUSIONS

Chapter 6

General conclusions

6.1. Findings

This study provides a new result of stable carbon and nitrogen isotope combines with stomach content analysis to clarify the role of demersal zooplankton as a linkage from lower to higher trophic level in the shallow ecosystem.

In order to estimate the proportion of food source in the diet of demersal zooplankton in a shallow ecosystem. The trophic enrichment factor of zooplankton was determined by an experiment using *Artemia salina* and a monoculture of the diatom *Nitzschia* sp. as a food source. The result of natural stable carbon and nitrogen isotope of *Artemia salina* at a different stage in their life cycle and *Nitzschia* sp. shows that the TEFs of zooplankton were determined when the dual carbon and nitrogen isotopic reached to equilibrium isotope at day 25 after hatching with the discrimination between *Artemia salina* and its food source were mean (±SD): 0.0 ± 0.9 (‰) for $\Delta^{13}C$ and 1.0 ± 0.5 (‰) for $\Delta^{15}N$.

The trophic enrichment factors of zooplankton were applied to assess the food source of demersal zooplankton in a reef lagoon. This study was conducted in the reef lagoon at Bise in 3 selections habitat (SG, SG+CR, and CR). The result of a mixing model based on the natural stable carbon and nitrogen isotope highlights the role of organic matter derived from SG particularly SG detritus influencing the abundance of DZ in SG and SG+CR habitats. Phytoplankton and macro algae play an important role as a food source for DZ in the reef lagoon. Demersal zooplankton consume on available food sources near their living area.

Demersal zooplankton distribute in a shallow ecosystem with high abundance suggest that they may contribute in major proportion to the diet of higher trophic levels in an aquatic food web. Moreover, demersal zooplankton exhibits wide size range, therefore it is expected that consumers preferentially feed on different size classes of DZ. The study on the role of DZ as a food source in an aquatic food web at Fukido estuary combined stomach content analysis (to find out the potential food source of consumers) and stable isotope analysis (to assess the proportion of DZ compare against another food sources). The stable isotope mixing model result highlights the important role of DZ in the diet of active feeders in mangrove habitats compared to that in another studied habitats. The abundance and biomass of demersal zooplankton were consistent with DZ proportion in the diet of consumers. The consumers (fishes and macro invertebrate) prefer on larger size classes of DZ and remaining small size classes that mostly contribute in the biomass of DZ in mangrove habitat. In contrast, the larger size of DZ were composed in DZ biomass at lagoon habitat, there were consumers with larger body size prefer more on large-sized prey than DZ.

6.2. Further research

Although, this study demonstrated that demersal zooplankton play an important role as a linkage transferring that organic sources to higher trophic levels. However, there are many physical factors acting and biological influence on the shallow coastal environment. Therefore, we propose further research to understand the demersal zooplankton community and the changing in an aquatic food web in shallow coastal ecosystem including:

1) The community structure of demersal zooplankton versus net zooplankton and their relative contribution in the biomass of the shallow ecosystems.

2) The multi-factor (i.e. tide, season) impact to the community structure and the role of demersal zooplankton in an estuarine ecosystem.

3) Changing of the food web under the multiple environmental stress (elevated temperature, acidification, and salinity) in the shallow ecosystems.

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Appendices

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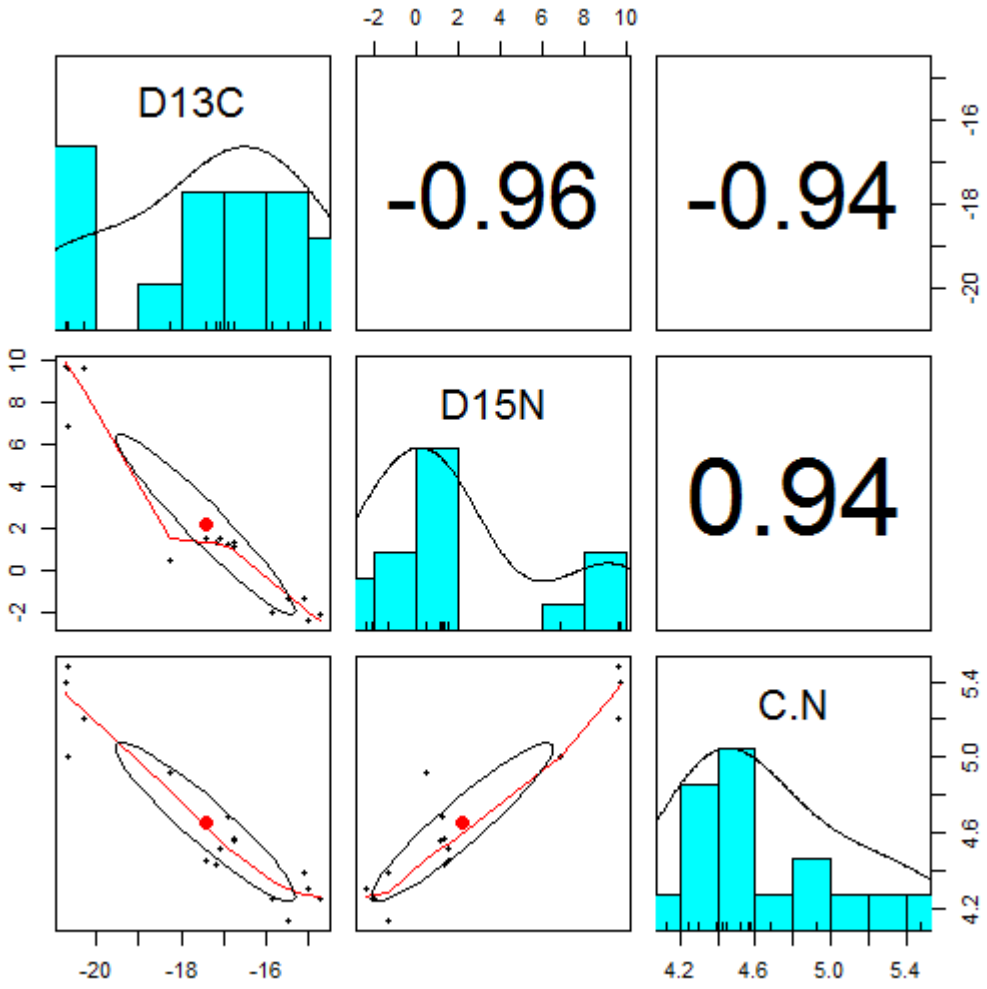
A – 3.1 The Correlation coefficient between ^{13}C, ^{15}N and C/N ratio of *Artemia salina* during their life cycle



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A – 4.1 Number courting of demersal zooplankton (individual) collected in reef lagoon at Bise

Taxa	Size range [μm]	CR	SG	SG+CR
SARCOMASTIGOPHORA				
Granuloreticulosea				
Foraminiferida		20	90	
NEMATODA			30	
MOLLUSCA				
Gastropoda				
Gastropoda larva	100 - 250	70	90	120
ANNELIDA				
Polychaeta				
Polychaeta (larvae)	300 - 9000	950	1620	3030
ARTHROPODA				
Maxillopoda				
Ostracoda		20	30	
Acartia japonica		20		30
Acartia (copepodite)		10		
Undinula vulgaris			30	
Calanidae (copepodite)				30
Paracalanidae (copepodite)	250 - 500	20		
Calanopia minor		40	30	60
Calanopia thompsoni	2200 - 2500	360	1560	990
Pontellidae (copepodite)				
Oithona oculata	650 - 800	90	540	450

Oithona rigida		10	120	
Oithonidae (copepodite)	300 - 600	120	300	240
Cyclopoida	300 - 650	160	270	240
Cyclopoida (copepodite)		2.8	100	90
Microsetella (copepodite)				
Harpacticoida	300 - 900	240	540	750
Harpacticoida (copepodite)	200 - 600	330	300	600
Corycaeidae (copepodite)		0.3	10	
Oncaea zernovi				30
Poecilostomatoida	700 - 800	30	150	180
Poecilostomatoida (copepodite)		2.2	80	210
Monstrilloida (copepodite)				
Copepoda (nauplius)	100 - 450	230	420	390
Facetotecta (nauplius)		10		
Cirripedia (nauplius)		30	90	120
Cirripedia (cypris)			90	
Molacostraca				
Mysidacea			30	90
Cumacea		20	240	30
Isopoda	400 - 1000	370	1500	1230
Gammaridea	1000 - 3000	100	270	270
Anomura (zoea)		10		30
Anomura (glaucothoe)			30	
Brachyura (zoea)	800 - 2200	50	180	150
Decapoda (zoea)	1100 - 3000	40	300	330
Decapoda (mysis)	1000 - 3000	50	180	240

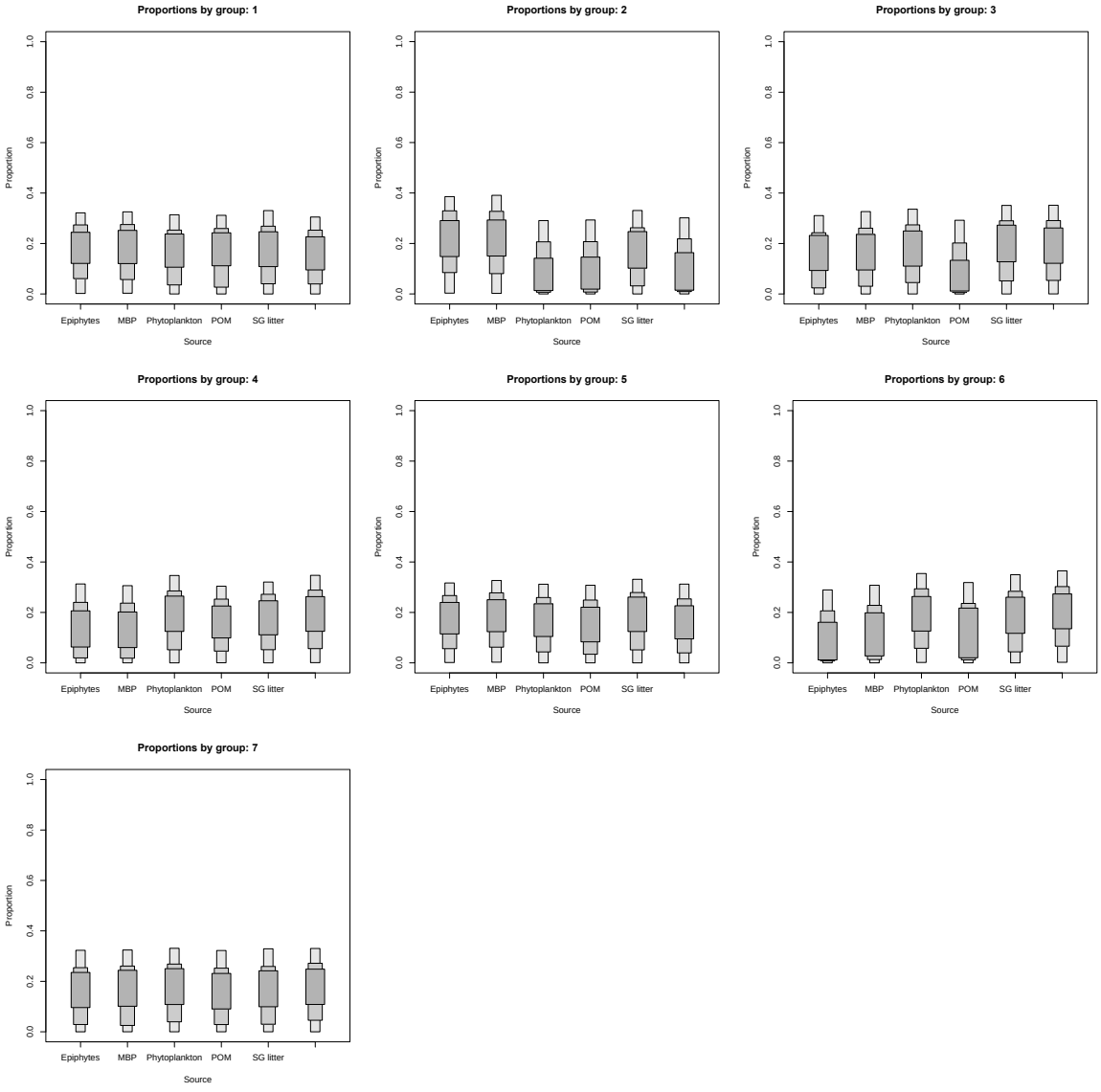
CHORDATA			
Osteichthyes			
Osteichthyes (larvae)			30
Total taxa	**29**	**28**	**29**
Total individual	**3590**	**9330**	**9990**

A – 4.3 The dominant species of demersal zooplankton in difference fraction size classes

Size classes	**Dominate of zooplankton group**
73 – 100	Nauplius of Copepoda
100 – 250	Nauplius of Copepoda, Harpacticoida, *Paracalanius* sp. (Copepoda)
250 – 500	Copepoda, Nauplius of Cirripedia, Isopoda, Paracalanius sp.
500 – 1000	Copepoda, Nauplius of Cirripedia, Isopoda, Zoea of Brachyura, Polychaeta larvae
1000 – 2000	Copepoda, Isopoda, Gammaridea (Amphipoda), Zoea of Brachyura, Zoea and Mysis of Decapoda, Polychaeta larvae
2000 - 4000	Gammaridea (Amphipoda), Mysidacea, Anomura, Cumacea, Zoea and Megalop of Brachyura, Zoea and Mysis of Decapoda, Polychaeta larvae, Shrimp larvae (Mysis)
> 4000	Amphipoda, Cumacea, Polychaeta, Anomura, Mysidacea, Shrimp larvae

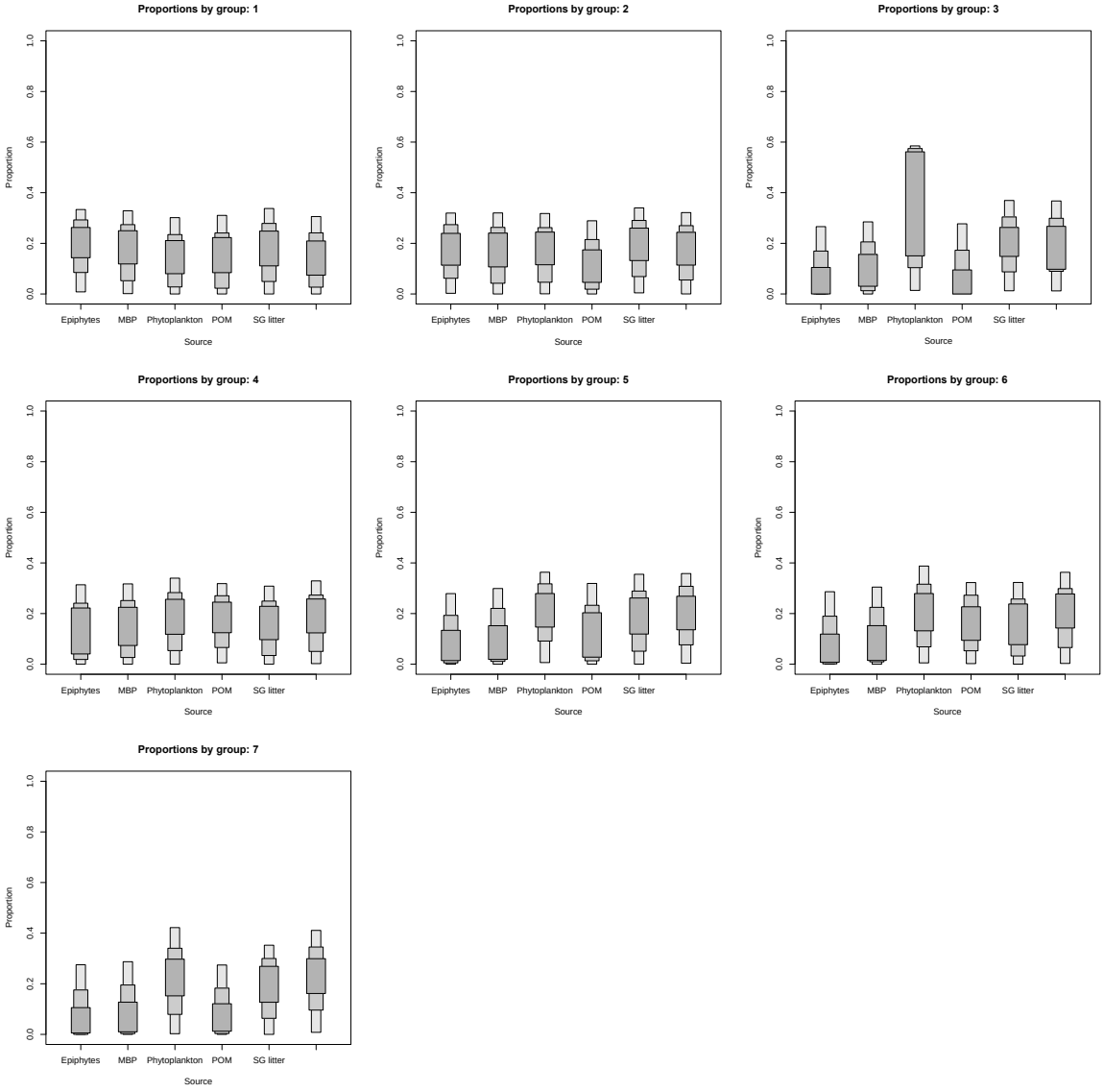
A – 4.3 Proportion of potential food sources in the diet of demersal zooplankton in seagrass habitat – Bise lagoon, Okinawa Islands.

Group 1, 2, 3, 4, 5, 6 and 7 denote demersal zooplankton size classes of 73, 100, 250, 500, 1000, 2000, and 4000 μm, respectively.



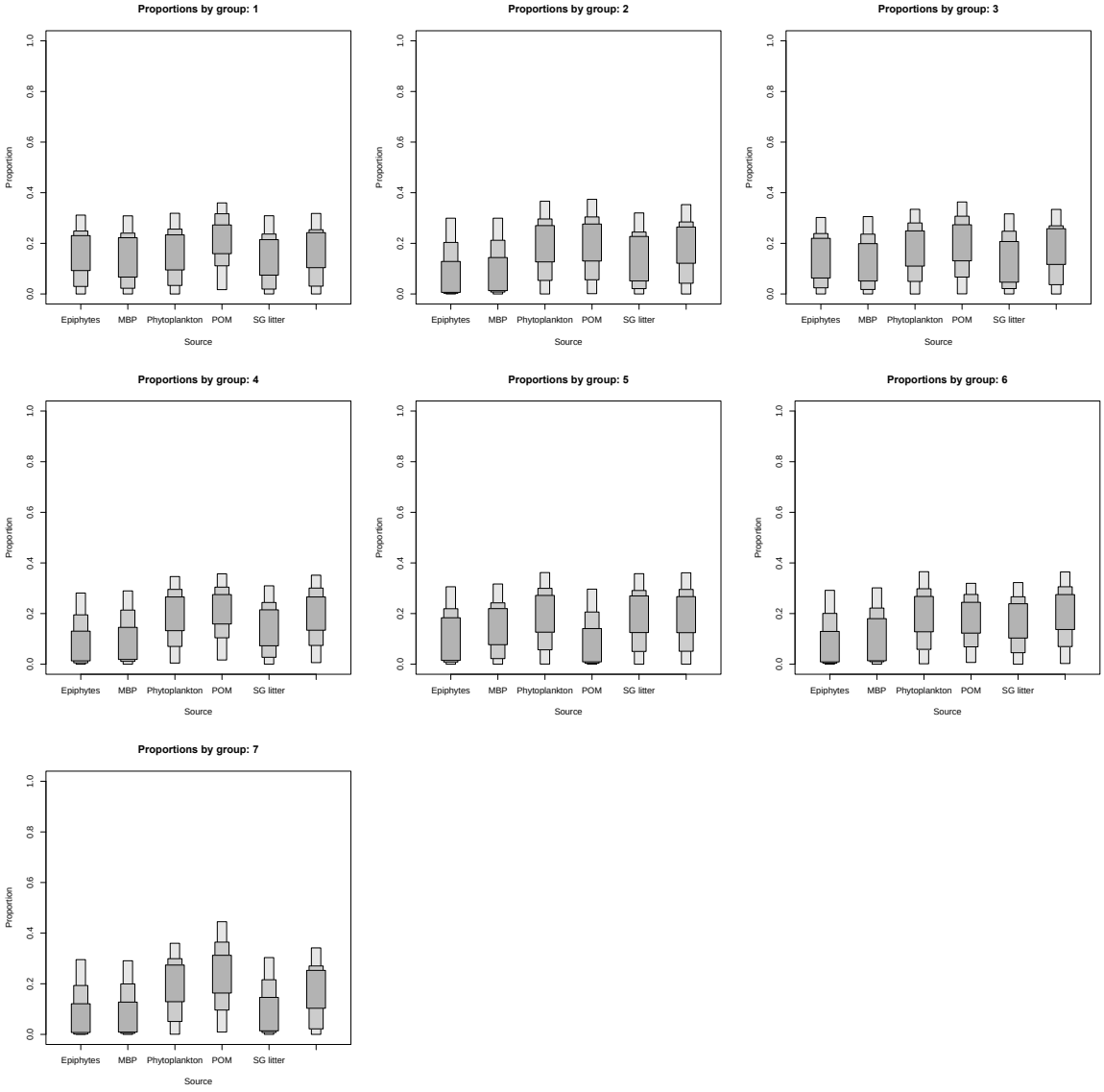
A – 4.4 Proportion of potential food sources in the diet of demersal zooplankton in seagrass mixture coral habitat – Bise lagoon, Okinawa Islands.

Group 1, 2, 3, 4, 5, 6 and 7 denote demersal zooplankton size classes of 73, 100, 250, 500, 1000, 2000, and 4000 μm, respectively.

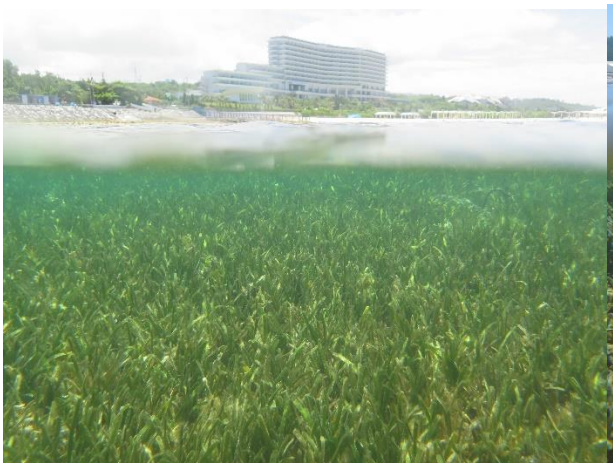


A – 4.5 Proportion of potential food sources in the diet of demersal zooplankton in coral habitat – Bise lagoon, Okinawa Islands.

Group 1, 2, 3, 4, 5, 6 and 7 denote demersal zooplankton size classes of 73, 100, 250, 500, 1000, 2000, and 4000 μm, respectively.

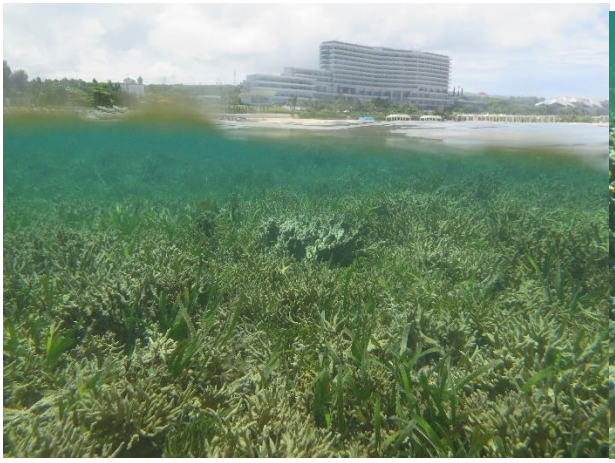


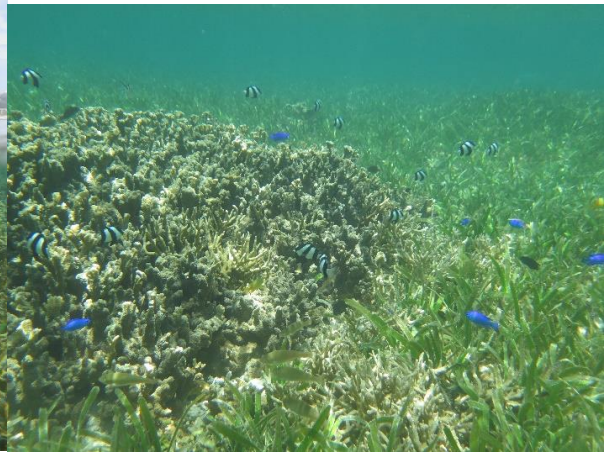
A – 4.6 Environment and specific habitats at Bise reef lagoon, Okinawa Island



Seagrass habitat

Coral habitat





Seagrass mixture coral habitat

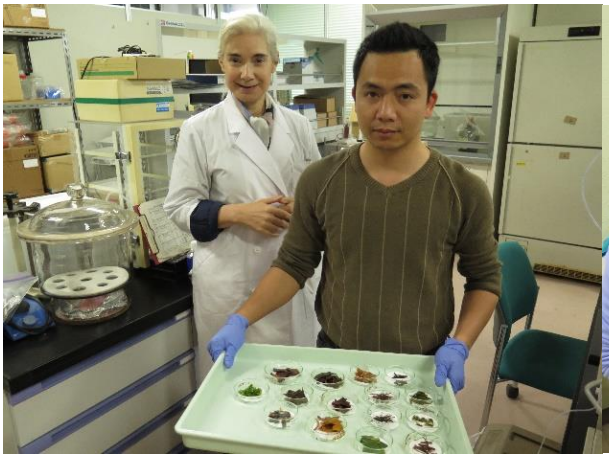
A – 4.8 Activities in Bise reef lagoon, Okinawa Island

Emergence traps set in each specific habitat in the reef lagoon

Some activities in laboratory

POM and phytoplankton collection

Microphytobenthod collection



Drying samples

Tissues of fishes and other consumer collection

Observe samples under microscope

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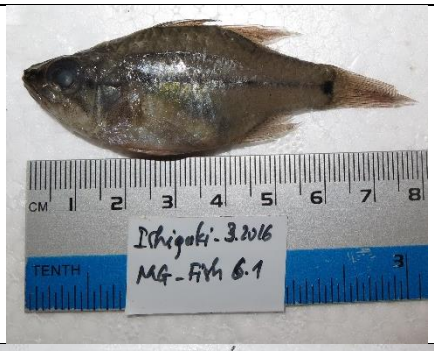
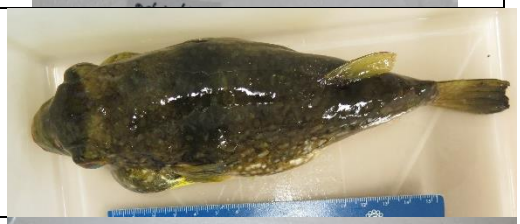
A-5.1 Fishes and other consumers collected in Fukido estuary

Family	N	Scientific Name	Images
Apogonidae	13	*Fibramia amboinensis* (Bleeker, 1853)	
Mugilidae	3	*Chelon subviridis* (Valenciennes, 1836)	
Zenarchopteridae	3	*Zenarchopterus dunckeri*	
Siganidae	4	*Siganus guttatus* (Bloch, 1787)	
Gobiidae	5	Asterropteryx semipunctata Rüppell, 1830	

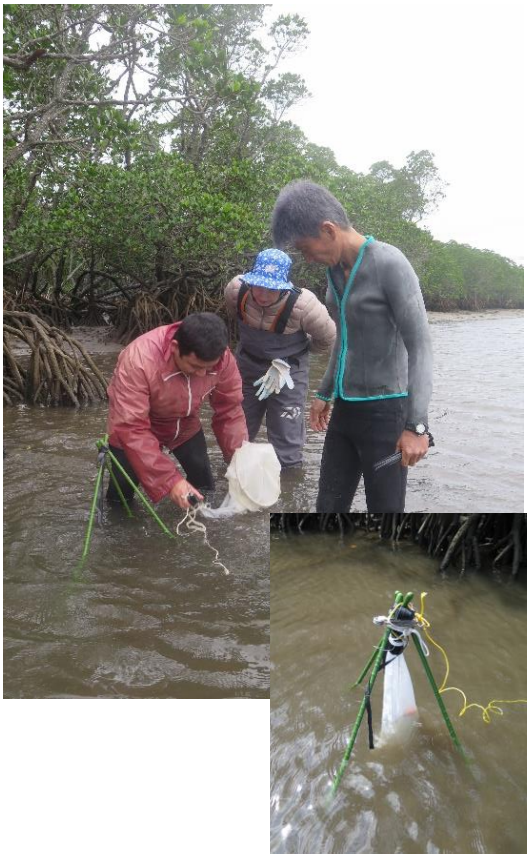
Gobiidae	2	Yongeichthys criniger (Valenciennes, 1837)	
Gerreidae	3	*Gerres oyena* (Forsskål, 1775)	
Lutjanidae	4	*Lutjanus* sp. (Forsskål, 1775)	
Platycephalidae	3	*Cociella punctata* (Cuvier, 1829)	
Tetraodontidae	2	*Tetraodon fluviatilis*	
Siganidae	1	*Siganus fuscescens* (Houttuyn, 1782)	

Scorpaenidae	1	*Dendrochirus zebra* (Cuvier, 1829)	
Lethrinidae	1	*Lethrinus ornatus* Valenciennes, 1830	
Diodontidae	1	*Diodon holocanthus* Linnaeus, 1758	
Crangonidae	3	Sand Shrimp (*Crangon septemspinosa*)	
Portunidae	8	*Scylla serrata*	

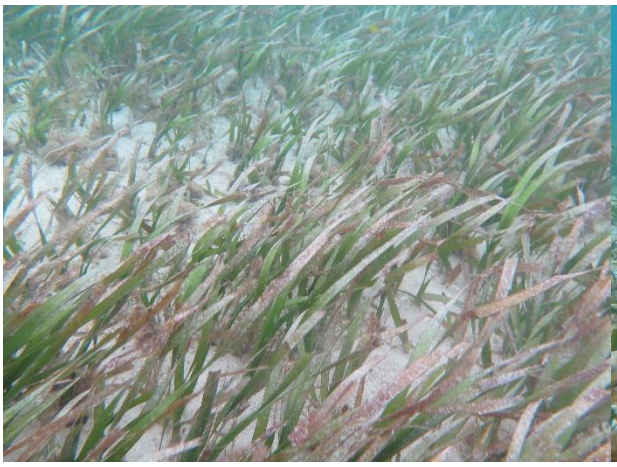
Portunidae	6	*Charybdis* sp.	
Grapsidae	3	*Eriocheir japonicus*	
Calappidae	1	*Calapa calapa*	
Littorinidae	4	*Littoria angulifera*	
	3	Hermit crab	

A – 5.3 Activities and specific habitats at Fukido Estuary, Ishigaki Island

Mangrove habitat



Lagoon habitat at Fukido Estuary, Ishigaki Island

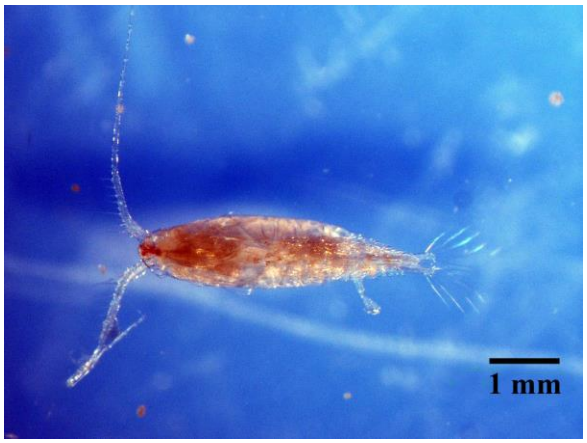


Demersal zooplankton identification of dominate taxa

1. Polychaeta

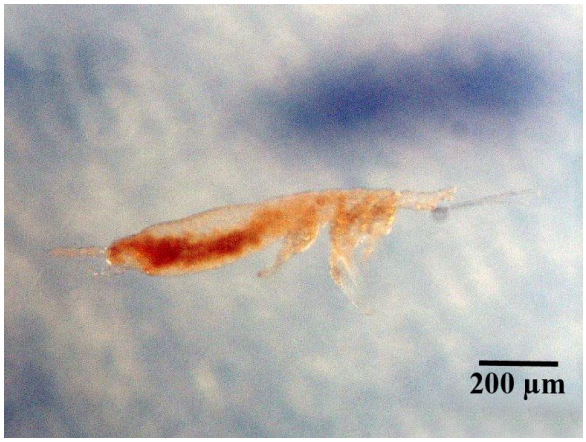
2. Copepoda

Calanoida



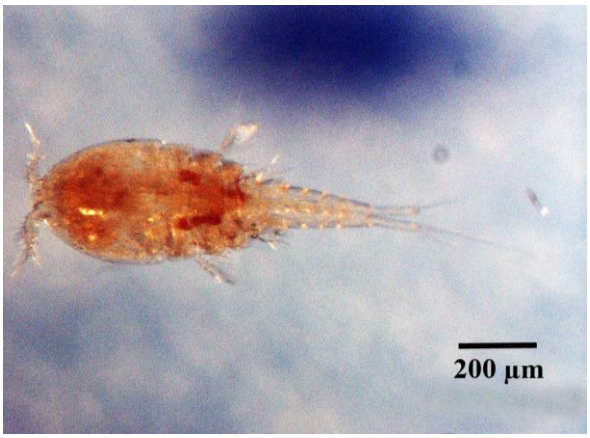
Harpacticoida

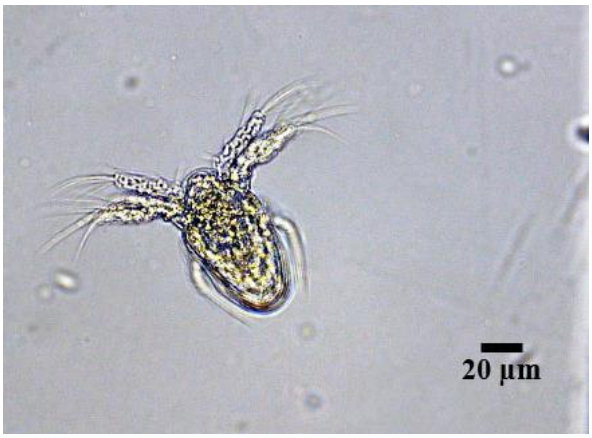
Monstrilloida



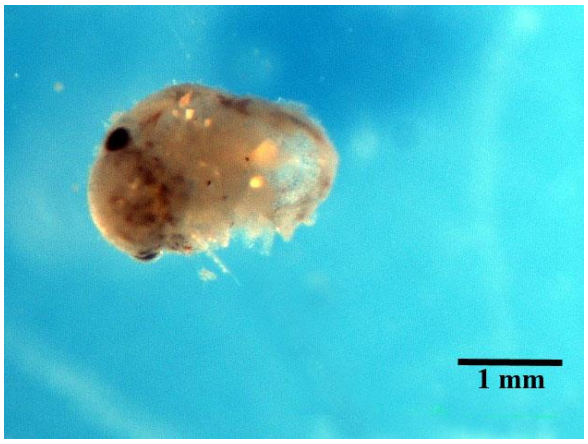
Poecilostomatoida

Cyclopoida **Nauplius of Copepoda**

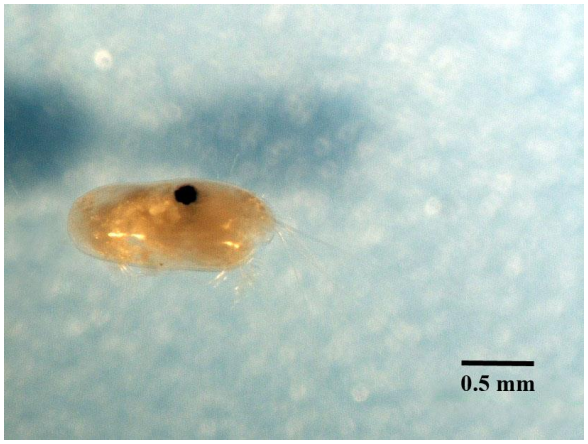




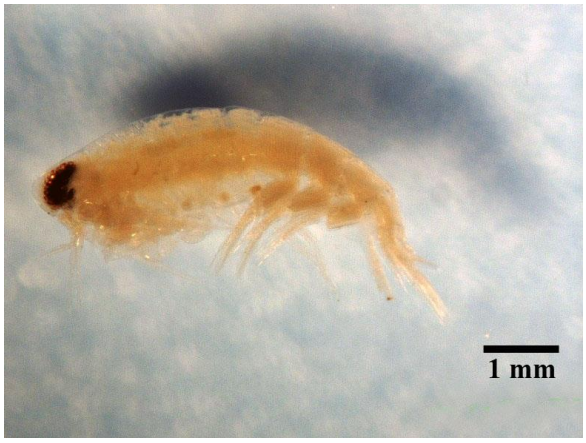
3. Isopoda



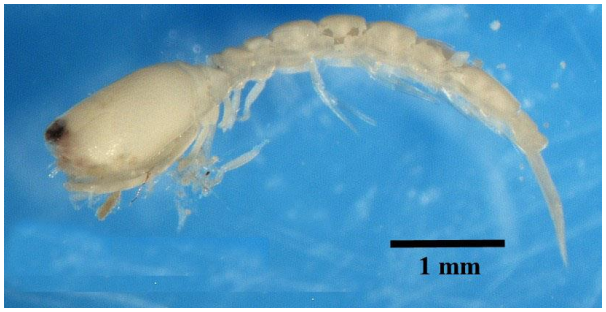
4. Ostracoda



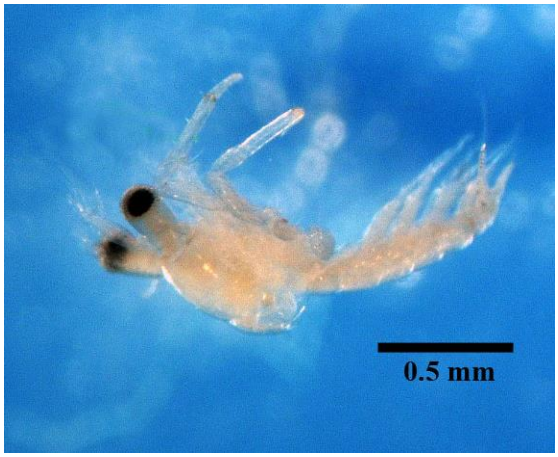
5. Amphipoda



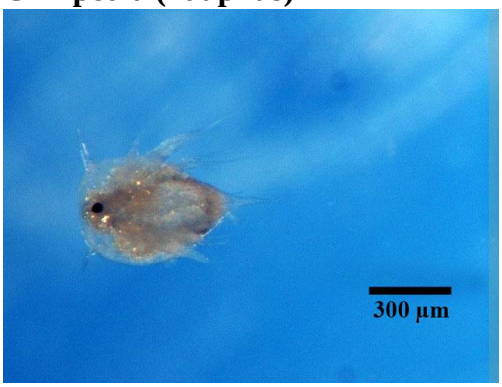
6. Cumacea

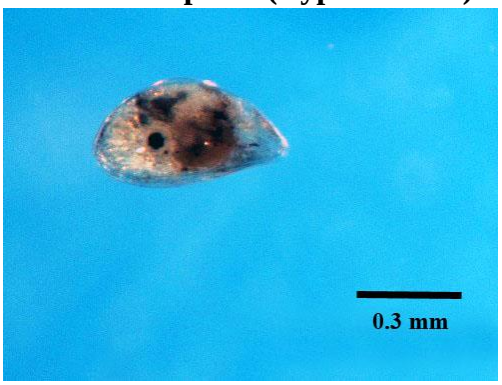


7. Anomura



8. Cirripedia (nauplius) **Cirripedia (Cypris larvae)**





9. Brachyura