

**Production dynamics  
of the mysid *Neomysis awatschensis* (Brandt, 1851)  
in the Yura River estuary, central Sea of Japan**

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## **Dedication**

To my wife Mrs. Ooga Anita Nyaboke and our children Ooga Justine Mageto and Ooga Rita Kemunto for their love during my studies.



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## Abstract

Mysids occur abundantly in estuarine and coastal waters, serving as major food items for many fish species of commercial importance. The mysid *Neomysis awatschensis* (Brandt, 1851) is a common crustacean abundantly occurring and distributed in brackish waters adjacent to the coastal zones in Japan, tolerant to acute salinity fluctuations and temperature changes predominant in estuaries. The current study was conducted in the Yura River estuary, which is characterized by freshwater discharges that transports huge amounts of nutrients to the estuary grounds while seawater intrusion deposits crucial phytoplankton in the estuarine ecosystem that initiates primary production. However, the importance of crustaceans such as mysid requires further investigations to understand their role in the estuary considering that they are key prey item for commercially important coastal fishes. This study focuses on the population dynamics of *N. awatschensis* in relation to estuarine environmental conditions in the salt-wedge estuary feeding habits and survival strategies.

### ***Production dynamics of N. awatschensis in the Yura River estuary***

Mysids play an essential role as the dominant prey species for coastal fishes. In Japan, the mysid *Neomysis awatschensis* is commonly distributed in estuarine and shallow coastal waters with high productivity due to supply of nutrients from the watershed. The present study investigates the distribution and life history of the mysid *N. awatschensis* in relation to biotic and abiotic factors by monthly sampling from March 2014 to July 2015 at stations along the Yura River estuary, the central Sea of Japan.

The Yura River physical conditions change seasonally and are affected by its discharge. Salt-wedge-intrusions were frequently observed and found up to 18 km from the river mouth from April

to December 2014 when river discharge was low, whereas in 2015 intrusions were less pronounced, possibly due to river mouth clogging. Clear seasonality was observed in mysid density, showing highest densities (up to 202 inds/m<sup>2</sup>) in spring and early summer in contrast with lowest densities (< 16 inds/m<sup>2</sup>) from late summer to winter. *Neomysis awatschensis* appeared to change its reproduction characteristics in response to changes in the environment, large-sized females occurred in spring to produce large numbers of eggs and larvae during the high primary production period. In late summer, the population declined due to increasing mortality of juveniles at high temperatures. In autumn and winter, high river discharge and low temperature suppressed reproduction and survival of *N. awatschensis*. It is notable that *N. awatschensis* occurred within a salinity range of 0–32 and no clear influence of salinity on the mysid density was found.

Those results suggest that reproduction is partially suppressed by variable and oligotrophic conditions in the Yura River estuary. Combined with the flexibility to seasonal changes in temperature and food availability, the great tolerance to acute salinity fluctuations allows *N. awatschensis* to maintain its population in the estuary.

### ***Feeding ecology of N. awatschensis in the Yura River estuary***

Spatial and seasonal variations in the diet composition of the hyperbenthic mysid *Neomysis awatschensis* were investigated. The mysid and its potential food sources were surveyed during the daytime along the Yura River estuary monthly from March 2014 to July 2015. The mysid dietary ecology was studied through optical and scanning electron microscopy and stable isotope analyses. The stomach content analysis revealed that benthic diatoms (e.g. *Bacillaria paxillifer* and *Navicula* spp.) dominated in the stomach content of both juveniles and adults regardless of the site and season, indicating that *N. awatschensis* frequently ingested benthic diatoms at least during daytime whereas planktonic diatoms and green algae contributed minimally to mysid diet. The

stable isotope analysis and subsequent Bayesian mixing model allowed an approximate estimate of food items of *N. awatschensis*: epilithic microalgae and marine particulate organic matter dominated under conditions of salt-wedge intrusion into the estuary, whereas without salt-wedge intrusion riverine particulate organic matter contributed significantly. Hence, the results indicate that *Neomysis awatschensis* is an herbivorous feeder, ingesting benthic–planktonic diatoms in addition to planktonic green algae in relation to prevailing environmental conditions in the Yura River estuary.

### ***Conclusion***

The study results revealed that *N. awatschensis* has succeeded in maintaining its population in the estuary due to its flexibility to food availability and tolerance to the fluctuating environmental conditions prevalent in the estuary.



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## Abbreviations and symbols

BMAel	Epilithic Benthic Microalgae
BMAep	Epipelagic Benthic Microalgae
CI	Credibility Interval
e. g.	For example
HCl	Hydrochloric acid
i.e.	That is
MEXT	Ministry of Education Culture Sports Science and Technology
MLIT	Ministry of Land, Infrastructure, Transport and Tourism
PCA	Principal Component Analysis
POMm	Marine Particulate Organic Matter made up of mainly phytoplankton
POMr	Riverine Particulate Organic Matter
SD	Standard Deviation
SIAR	Stable Isotope Analysis in R
sp	Species
°C	Degree Celsius
δ	Delta
‰	Per mille





**Brooding female *Neomysis awatschensis***



## Chapter 1

### General introduction

#### 1.1. Mysid ecology

Mysids are an important element in a variety of water bodies from completely saline environments in oceans to freshwater ecosystems all over the world (Mauchline, 1971; Mauchline and Murano, 1977; Mauchline, 1980). Some mysid species have large biomasses (David *et al.*, 2005; Rappé *et al.*, 2011) and play crucial roles in estuarine, coastal and marine ecosystems where they are major prey items of invertebrates and fishes, thereby having a key role in transporting large amounts of matter and energy from primary producers to higher trophic levels (Ohtsuka *et al.*, 1995; Sudo, 2003; Delgado *et al.*, 2013). Some mysids are used as feed for cultured fishes and as human food in Southeast Asia (Omori, 1978; Mantiri *et al.*, 2012). To elucidate the biological production mechanisms and survival strategies of this important constituent in aquatic ecosystems, it is necessary to understand the ecology of mysids in relation to regularly fluctuating biotic and abiotic environmental conditions of aquatic systems.

Mysids are referred to as “opossum” because the mature females have ventral brooding pouch where eggs develop, and the young ones develop to a morphological stage similar to adults at the time of release (Mauchline, 1980; Roast *et al.*, 1998). Mysids are generally known as omnivorous feeders capable of consuming detritus matter, algal fragments, diatoms, rotifers, copepods, amphipods, daphnia and other zooplankton (Mauchline, 1980; Takahashi, 2004). Mysids can

adjust their feeding patterns to resource availability and can feed selectively on species of different sizes and thus modify the composition of zooplankton and phytoplankton communities of ecosystems (Siegfried and Kopache, 1980; Kouassi *et al.*, 2006).

Reproduction in mysids shows a latitudinal pattern, such that tropical and subtropical species breed continuously whereas temperate mysids have discrete cohorts every year (Wooldridge and Bailey, 1982; Hanamura *et al.*, 2009). Fluctuations in physico-chemical parameters could be responsible for the distribution patterns and occurrence of mysid species (Mees *et al.*, 1994; Yamada *et al.*, 2007). In Japan, high mysid densities occur seasonally in open estuaries (Yamada *et al.*, 2007; Suzuki *et al.*, 2009).

#### *1.1.1. Mysid taxonomy and distribution patterns*

The order Mysida (Crustacea: Peracarida) consists of species inhabiting aquatic environments from oceans to inland waters that is, ranging from marine to freshwater environments (Mauchline and Murano, 1977; Deprez *et al.*, 2004; Petryashev and Daneliya, 2014). Currently there are 1065 mysid species that have been documented and described, with over 170 genera (Deprez *et al.*, 2004). The genus *Neomysis* (Czerniavsky, 1882) consists of 20 species out of which 11 species are confined to lagoon and estuarine environments (Mauchline, 1980). Five species of the genus *Neomysis* have been recorded in Japan, they include: *Neomysis japonica*, Sea of Japan (Ishikawa and Shima, 1951) *N. mirabilis*; *N. czerniawskii*, Saroma-ko estuary, Okhotsky Sea (Takahashi *et al.*, 2015), *N. spinosa*, Niida River estuary Pacific Ocean (Chihara and Murano, 1997) and *N. awatschensis* (Nanami *et al.*, 2005; Yamada *et al.*, 2007) (Table 1-1).



Table 1-1. Example of the distribution of 11 species of the genus *Neomysis* confined to lagoons and estuaries.

No	Species	Location	Reference
1	<i>N. americana</i> (Smith, 1873)	Delaware River estuary, USA	(Hulburt, 1957)
2	<i>N. awatschensis</i> (Brandt, 1851)	Kasumigaura lagoon, Japan	(Toda <i>et al.</i> , 1982)
3	<i>N. czerniawskii</i> (Derzhavin, 1913)	Akkeshi-ko lagoon, Japan	(Yamada <i>et al.</i> , 2007)
4	<i>N. integer</i> (Leach, 1814)	Gironde River estuary, Belgium	(Fockedey and Mees, 1999)
5	<i>N. japonica</i> (Nakazawa, 1910)	Chikugo River estuary, Japan	(Suzuki <i>et al.</i> , 2009)
6	<i>N. kadiakensis</i> (Ortmann, 1908)	San Fransisco estuary, USA	(Dean <i>et al.</i> , 2005)
7	<i>N. mercedis</i> (Holmes, 1897)	Sacramento River estuary, USA	(Siegfried and Kopache, 1980)
8	<i>N. mirabilis</i> (Czerniavsky, 1882)	Akkeshi-ko lagoon, Japan	(Yamada and Kumagai, 2012)
9	<i>N. nigra</i> (Nakazawa, 1910)	Ojo-ri embayment lagoon, S. Korea	(Bae <i>et al.</i> , 2016)
10	<i>N. spinosa</i> (Nakazawa, 1910)	Aomori, Japan	(Chihara and Murano, 1997)
11	<i>N. vulgaris</i> (Thompson, 1828)	Barnegat pool, Netherlands	(Hopkins, 1965)

Table 1-2. Distribution of *Neomysis awatschensis* in lagoons and estuaries, adjacent to the Japanese coast. Japan 1–14, South Korea - 15 and Russia - 16.

No	Geographical location	Prefecture/province	Reference
1	Abashiri lagoon	Hokkaido	(Yusa and Chiba, 2014)
2	Akkeshi-ko lagoon	Hokkaido	(Yamada <i>et al.</i> , 2007)
3	Chikugo River estuary	Fukuoka	(Suzuki <i>et al.</i> , 2009)
4	Gamo lagoon	Miyagi	(Tomiyaama and Omori, 2008)
5	Hinuma-ko lagoon	Ibaraki	(Nanami <i>et al.</i> , 2005)
6	Hiroura lagoon	Miyagi	(Katayama <i>et al.</i> , 2011)
7	Kasumigaura lagoon	Ibaraki	(Toda <i>et al.</i> , 1982)
8	Makoto lagoon	Hokkaido	(Yusa and Chiba, 2014)
9	Kitaura-ko lagoon	Ibaraki	(Endo <i>et al.</i> , 2015)
10	Nanakita River estuary	Miyagi	(Tomiyaama <i>et al.</i> , 2009)
11	Natori River estuary	Miyagi	(Tomiyaama <i>et al.</i> , 2009)
12	Saroma-ko lagoon	Hokkaido	(Takahashi <i>et al.</i> , 2015)
13	Tohutsu lagoon	Hokkaido	(Yusa and Chiba, 2014)
14	Yura River estuary	Kyoto	(Fuji <i>et al.</i> , 2016)
15	Jangho-ri	Gangwon (South Korea)	(Jo and Jeon, 2007)
16	Murav' inaya River estuary	Primorsky Krai (Russia)	(Lukyanova <i>et al.</i> , 2009)

The mysid *N. awatschensis* is an estuarine species, inhabiting from temperate to boreal waters on both western and eastern margins of the Pacific Ocean (Petryashev, 2007; Petryashev and Daneliya, 2014). On the western Pacific Ocean side, *N. awatschensis* is a common mysid in shallow waters adjacent to the Japanese coastal strip, from Hokkaido to western Honshu and coasts from South Korea to Primorsky in Russia (Table 1-2). Recently, Petryashev and Daneliya (2014) synonymized *Neomysis intermedia* (Czerniavsky, 1882) which occurs sympatrically with *Neomysis awatschensis* (Brandt, 1851) to *N. awatschensis*.

External morphology of *N. awatschensis* (Brandt, 1851) is critical in distinguishing among the different mysid species, the telson is trapezoidal with a wide truncated apex, bearing two pairs of spines two long terminal and two short medial (Banner, 1954; Mauchline, 1980; Petryashev and Daneliya, 2014). It is a hyperbenthic, euryhaline, and eurythermal species confined to brackish environments within estuaries (Mauchline, 1980; Yamada *et al.*, 2007). Previously, *N. awatschensis* has been reported in estuaries such as the Yura River estuary, Sea of Japan (Antonio *et al.*, 2010; Fuji *et al.*, 2016), the Chikugo River estuary, Ariake Sea (Suzuki *et al.*, 2009); and in sporadically opening/closing waters such as Kasumigaura lagoon (Toda *et al.*, 1987; Hanazato, 1990), Hiroura lagoon, Pacific Ocean (Katayama *et al.*, 2011); Akkeshi-ko (Yamada *et al.*, 2007), Lake Hinuma (Nanami *et al.*, 2005) and Saroma-ko lagoon, Sea of Okhotsk (Takahashi *et al.*, 2015). The density of *N. awatschensis* has been shown to exceed  $10^3$  individuals  $m^{-2}$  in temperate eutrophic lagoons but in boreal lagoons it remains around 20 individuals  $m^{-2}$  (Toda *et al.*, 1982; Yamada *et al.*, 2007; Yusa and Chiba, 2014).

### 1.1.2. Influence of habitat on mysid physiology and life-history

Physiological adjustment for example osmoregulation and tolerance to temperature and salinity fluctuations (Woodward *et al.*, 2005) influences an organisms spatial occurrence and

distribution (Schmidt-Nielsen, 1984; Woodward *et al.*, 2005). Estuarine habitats require large physiological modifications (Kaiser *et al.*, 2011) such as adjustments in circulation and respiration physiology, osmoregulation functions and the ability to sustain such energetic demands (Schmidt-Nielsen, 1997). A critical physiological process in aquatic animals is osmoregulation; changes in salinity and temperature will require organisms to change internal body conditions in relation to available metabolic energy (Kaiser *et al.*, 2011).

Temperate mysid have synchronized their life histories to changing seasonal environmental conditions (Atkinson, 1995; Kaiser *et al.*, 2011) because the limits of body physiological tolerance breaks down at given months of the year for example in winter when temperatures are harsh or at certain ontogenetic stages such as larvae when some mysid species are susceptible to adverse environmental conditions in the estuary (Mauchline, 1980; Kaiser *et al.*, 2011; Rappé *et al.*, 2011). Mysid species adapt to diverse strategies to overcome physiological challenges, for example breeding in favorable environmental conditions and production of large numbers of juveniles (Kaiser *et al.*, 2011; Rappé *et al.*, 2011). Mysid species can be successful in completing the life history stages by modifying their life stages in relation to the prevailing environmental conditions in estuaries (Toda *et al.*, 1987; Kaiser *et al.*, 2011; Rappé *et al.*, 2011).

## **1.2. Importance of estuaries**

Estuaries are among the most valuable ecosystems in the world (Costanza *et al.*, 1997) serving millions of people since the prehistoric times (Lotze *et al.*, 2006), also providing nursery and feeding habitats for crustaceans and fishes with high biological productivity (Beck *et al.*, 2001; Elliott *et al.*, 2007; Shimizu *et al.*, 2014). An estuary is an inlet of the sea reaching into a river valley as far as the upper limit of tidal rise, usually divisible into three sectors: (1) lower, free

connection with the open sea; (2) middle, subject to strong salt and freshwater mixing ; (3) upper, characterized by fresh water but subject to daily tidal actions (Kaiser *et al.*, 2011).

Globally, there is a wide range of estuary types, demonstrating large differences in geomorphology, tidal range, sea water-intrusion and salinity regimes (Eyre, 1998; Kaiser *et al.*, 2011). Estuaries are characterized by a wide range of tidal amplitude than other marine ecosystems, ranging from less than a meter in many tropical systems such as Vellar estuary, India (Satheeshkumar *et al.*, 2012) to over 16 m in the Bay of Fundy, Nova Scotia, Canada (Leleu *et al.*, 2009). Estuaries occur in the tropics such as Matang mangrove estuary, Malaysia (Ramarn *et al.*, 2015); warm temperate estuaries such as St. Lucia estuary, South Africa (Carrasco and Perissinotto, 2010), and cold temperate estuaries for example Gironde estuary, France (Fockedey and Mees, 1999). The scale of water movement into estuarine systems can be very large and four categories of estuaries have been suggested depending on the tidal range: microtidal (< 2 m), mesotidal (2–4 m), macrotidal (4–6 m) and hypertidal (> 6 m). Tidal movement is a prime indicative descriptor of the world estuaries (Kaiser *et al.*, 2011).

#### *1.2.1. Environmental variability in estuaries*

Estuaries are characterized by fluctuating physical and chemical characteristics which subject estuarine communities to considerable physiological and ecological stress (Ysebaert and Herman, 2002; Martineau *et al.*, 2004). The ecosystem communities among the estuaries are variable because of contrasting gradients of physico-chemical parameters, which are controlled by fresh-water inputs and periodically opening and closing of estuary mouths due to the presence of sandbar at the mouth can restrict movement of species habitually migrating between fresh-water and marine habitats, as well as recruitment of coastal and marine breeders (Morales *et al.*, 2001; Perissinotto *et al.*, 2002). Classically, salinity is considered to be the dominant influence on the

distribution, survival and structure of the estuarine communities, and controls the presence and absence of many animals (Kaiser *et al.*, 2011; Rappé *et al.*, 2011). In brackish lagoons, short to medium term spatial variation may be limited accompanied with the usual eutrophic conditions, but drastic changes in salinity and temperature may suddenly occur when systems become open to the sea (Roy *et al.*, 2001). In oligotrophic estuaries, spatial gradient of temperature, salinity and nutrients are subject to changes with the river discharge and the tidal cycle giving rise to very complex and dynamic conditions within short periods of time (Kaiser *et al.*, 2002; Kasai *et al.*, 2010). Species inhabiting both estuarine types, require eco-physiological mastery of such highly fluctuating environmental conditions. Therefore, depending on its physiological flexibility, it may require different life-history strategies to survive and reproduce in different niches (Mauchline, 1980; Toda *et al.*, 1987; Yamada *et al.*, 2007). Even though many zoological and ecological studies have been conducted in estuaries, it is necessary to compare species survival at different estuarine conditions and localities (Yamada *et al.*, 2007; Vilas *et al.*, 2009).

World estuaries including those in Japanese temperate regions are susceptible to effects of climate change and anthropogenic disturbances (Kaiser *et al.*, 2011; Yanagi, 2015). However, eutrophication problems such as oxygen-deficient water masses and red tides have become major environmental problems in Japanese estuaries (Yanagi, 2015). In addition, global warming events such as global temperature increase by 2 °C and subsequent sea level rise by up to 58 cm by 2100 in the region (Kurihara *et al.*, 2005; Ishii *et al.*, 2006) are likely to create complex situations in estuaries.

### **1.3. Reason for selecting the Yura River estuary**

Previous studies on *N. awatschensis* life history and production were mainly conducted in lagoons and brackish lakes in the Pacific Ocean side, which are intermittently opening/closing

systems (Toda *et al.*, 1982; Yamada *et al.*, 2007) and usually eutrophic (Oyama *et al.*, 2009; Yoshimura *et al.*, 2012). In contrast, the Yura River estuary which is characterized by microtidal, salt-wedge and relatively oligotrophic environmental conditions is located on the Sea of Japan side (Kasai *et al.* 2010; Watanabe *et al.* 2014). The present study was conducted in the Yura River estuary to understand the influence of the prevailing environmental conditions on the production dynamics and feeding habits of *N. awatschensis*.

### 1.3.1. The study area

The Yura river is the longest river in Kyoto Prefecture (Negoro and Gotoh, 1983). With a total length of 146 km (Fig. 1-1), located in the northern part of Kyoto Prefecture, the river flows from Mt. Mikuni which is 776 m above sea level (Ministry of Land, Infrastructure, Transportation and Tourism, 2017). The river has a watershed area of 1880 km<sup>2</sup> with an annual average discharge of 50 m<sup>3</sup>/s flowing into the Tango Bay located at the western Wakasa Bay area (Kasai *et al.*, 2010; Watanabe *et al.*, 2014). The watershed area has 81% forest cover with natural forest remaining in the headwaters (T. Yoshioka, personal communication) and secondary forest and riparian vegetation are found along the river. The downstream area is characterized by a flat riverbed.

The north-westerly winter winds off the Asian continent bring heavy precipitation from the Sea of Japan to the watershed area. The area is subject to cyclonic rain depressions in summer. The river discharge is therefore high in winter to early spring because of snow melting and generally low from late spring to autumn with sporadic increases caused by cyclonic rain storms (Kasai *et al.*, 2010; Watanabe *et al.*, 2017). Hydrographic conditions in the study area changed seasonally due to intrusions of seawater, and were mainly determined by river discharge and the sea level (Kasai *et al.*, 2010).

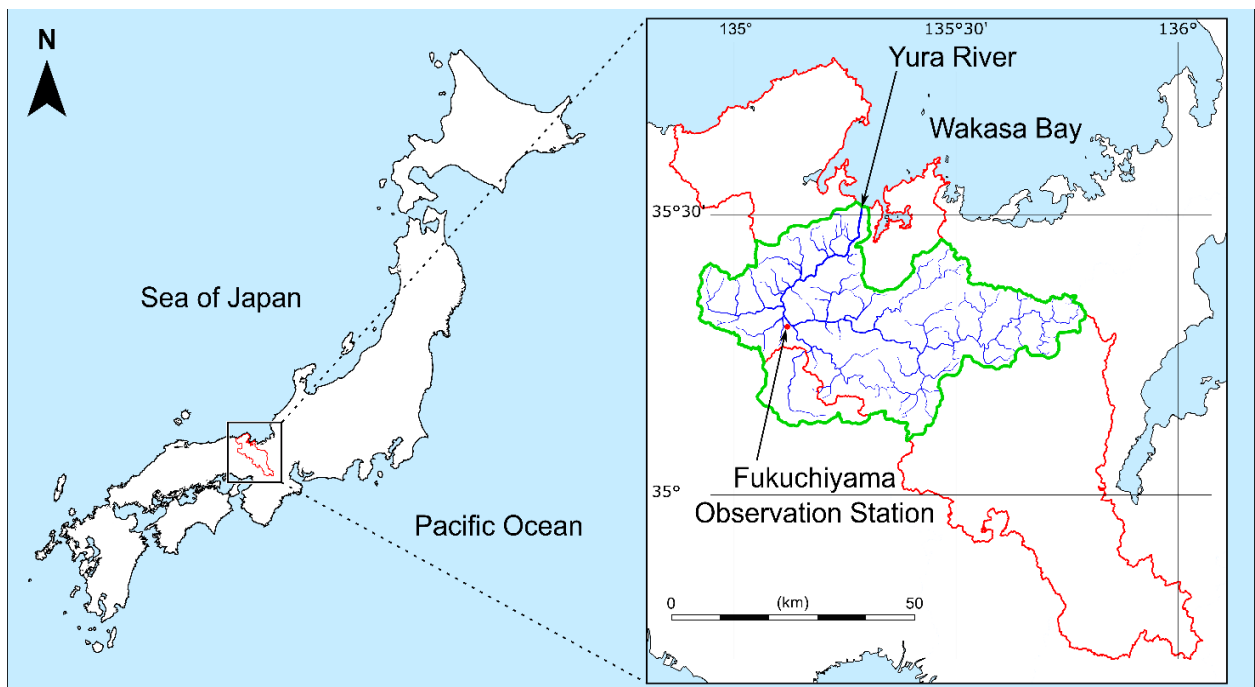


Fig. 1-1 Map of the Yura River watershed in Kyoto prefecture.  
The green outline represents the Yura River watershed. The red outline represents the Kyoto prefecture.

The present study was carried out from the river mouth to 18 km upstream. The lower-most 10 km of the Yura River estuary is a strait which is 100–500 m wide and 3–5 m deep with a nearly flat riverbed resulting in a shallow sill at the mouth. Seawater easily intrudes into the river, since the riverbed is lower than the sea surface until about 20 km upstream from the river mouth (Watanabe *et al.*, 2014). As the typical tidal range in the estuary is less than 0.5 m, the effects of tidal currents on the physical conditions are negligible. Therefore, the estuary is classified as a microtidal estuary (Kasai *et al.*, 2010; Funahashi *et al.*, 2013).

### *1.3.2. The main objective and significance of this study*

The overall objective of this study is to clarify the production dynamics of the dominant mysid species *Neomysis awatschensis* in the Yura River estuary. The specific objectives are to:

- 1) investigate the population structure of *N. awatschensis* in relation to seasonal changes in environmental parameters and river discharge in the Yura River estuary, and
- 2) describe the feeding habits of *N. awatschensis* in the Yura River estuary, including spatial-temporal variations.

Information on the life history in relation to environmental conditions and the feeding habits of the mysid *N. awatschensis* will deepen the understanding on the ecology of this species and its role in the ecosystem of the Yura River estuary.



## Chapter 2

### Production dynamics of *Neomysis awatschensis* in the Yura River estuary

#### 2.1. Introduction

Mysids are common benthic invertebrates in temperate coastal estuaries (Mauchline, 1980; Allen, 1984), the biology and ecology of many estuarine mysid species has been studied (Jo and Jeon, 2007; Yamada *et al.*, 2007; Rappé *et al.*, 2011; Bae *et al.*, 2016) including species life history (Cooper *et al.*, 1992; Mees *et al.*, 1994). Life history characteristics of estuarine mysid can vary considerably even among members of the same genus (Mauchline, 1980). Some member of the genus *Neomysis* are common in brackish waters adjacent to the Japanese coastal areas (Chihara and Murano, 1997; Yamada *et al.*, 2007; Takahashi *et al.*, 2015).

Several studies have reported ecological aspects of *Neomysis awatschensis* in coastal estuaries of Japan: abundance and reproductive seasons in the Kasumigaura Lake (Toda *et al.*, 1982), distribution and production in Akkeshi-ko Lake (Yamada *et al.*, 2007), vertical distribution in Hiroura lagoon (Katayama, *et al.*, 2011) and horizontal distribution in Chikugo River estuary (Suzuki *et al.*, 2009). Although, the population dynamics of *N. awatschensis* has been well documented (Toda *et al.*, 1982; Yamada *et al.*, 2007), those studies focused only on brackish lakes. Estuaries such as the Yura River estuary, characterized by larger fluctuations in environmental variables and lower reaches of rivers and adjacent waters might drive the population dynamics of *N. awatschensis* in a different way than in brackish lakes.

The present study was designed to clarify the population dynamics of *N. awatschensis* in relation to the environmental conditions in the Yura River estuary; a microtidal estuary flowing into the Sea of Japan and serving as a nursery for many estuarine and coastal fish species (Fuji *et al.*, 2010; Aoki *et al.*, 2014; Fuji *et al.*, 2016). The specific objectives were to depict (1) spatiotemporal changes in the mysid population structure and (2) the influence of environmental factors on the mysid population dynamics in the Yura River estuary.

## **2.2. Materials and methods**

### *2.2.1. Sampling methods*

Field sampling was conducted every month from March 2014 to July 2015 at five stations (R1, R2, R3, R4 and R5, Fig. 2-1). Since *N. awatschensis* is distributed close to the bottom at day time (Toda *et al.*, 1982; Katayama *et al.*, 2011), mysids were sampled during the day using a sledge net (a square-conical net of 0.3 m width, 0.2 m height, 2 m long, 0.5 mm mesh). The sledge net was towed along a 10 m transect at 0.5, 0.75 and 1 m depths parallel to the river bank at each sampling station. Because *N. awatschensis* is distributed from 0.5 to 3 m with a peak density at 1–2 m in depth throughout the year in the Yura River estuary (KW. Suzuki, personal communication), samples collected from 0.5 to 1 m depth were considered adequately representative. Mysid density was calculated as the total catch divided by the swept area at each depth ( $0.3 \times 10 \text{ m}^2$ ). Samples were fixed in 10% buffered formaldehyde solution and later preserved in 70% ethanol.

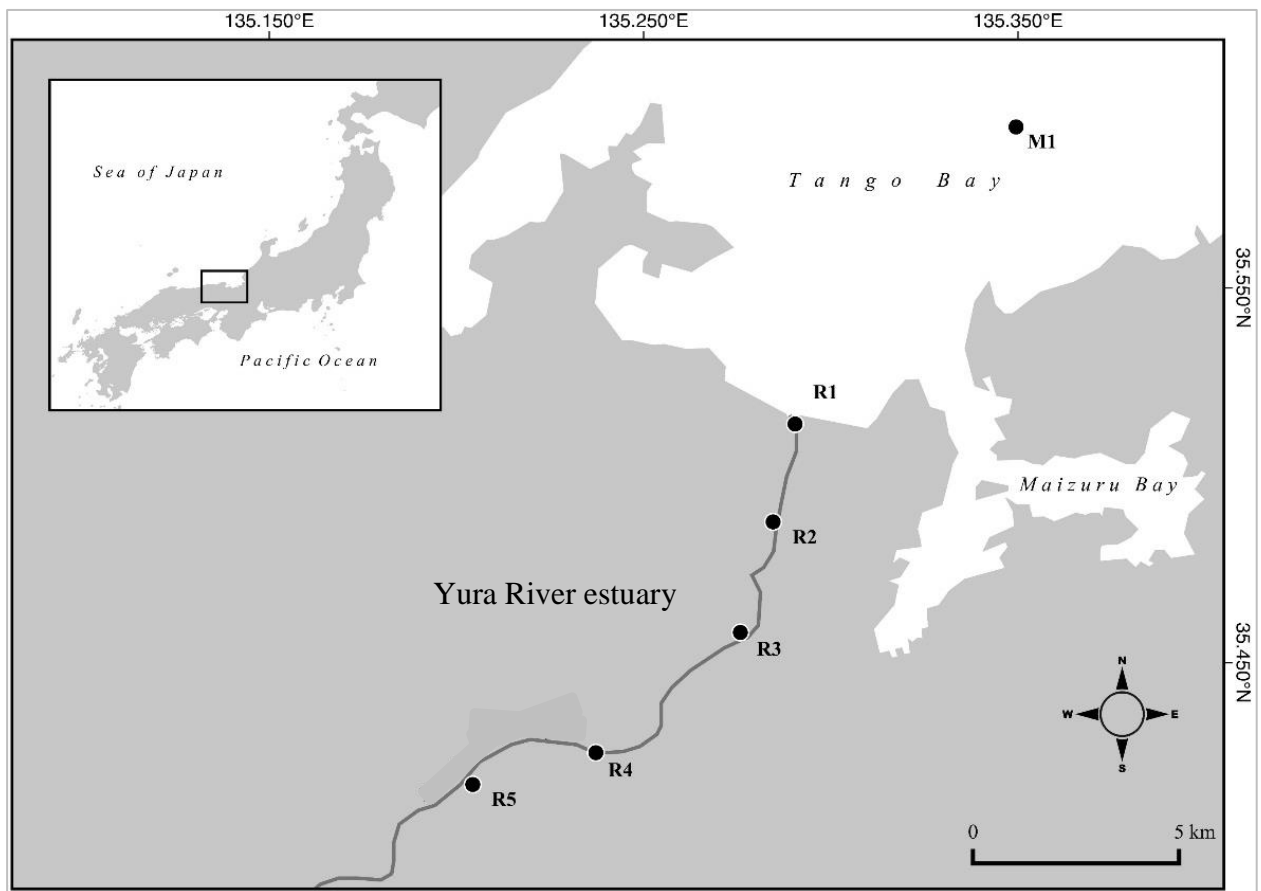


Fig. 2-1 Location of the study area.

The map indicates the sampling stations from R1 to R5 along the Yura River estuary and the offshore station M1 in Tango Bay. Sampling was conducted monthly from March 2014 to July 2015.

Water temperature, salinity (measured in practical salinity units) and chlorophyll fluorescence (in the present study, chlorophyll fluorescence is used as a unit-less indicator of primary production, as calibration with chlorophyll-*a* concentration was not conducted in the Yura River estuary) were measured using a water quality profiler (AAQ-RINKO, AAQ170, JFE Advantech). To represent each station, duplicate measurements were made at 0.1 m above the river bed and the results were averaged. River discharge data (measured at the Fukuchiyama Observation Station, 37 km upstream from the river mouth) were obtained from the Ministry of Land, Infrastructure, Transport and Tourism (MLIT, 2016). Despite the distance between this station and our sampling stations, it provided useful information on the seasonality of river discharge. An average of two weeks' river discharge before each sampling day was used for analysis in this study.

### 2.2.2. Laboratory procedures

Mysid species were identified based on Chihara and Murano (1997). *Neomysis awatschensis*, which accounted for 98% of the total mysid catches, was enumerated for each tow and categorized into four life-cycle stages: (1) juveniles, individuals without secondary sexual characteristics; (2) males, individuals with secondary sexual characteristics distinguished by their elongated 4<sup>th</sup> pleopods and their lobus masculine at the antennule peduncle, (3) females, individuals with marsupium developed but not yet filled with egg or larva; and (4) brooding females, individuals with eggs and/or larvae present in the marsupium adapted from Mauchline (1980).

The carapace length (CL: the distance from the apex of the rostrum to the posterior lateral edge, in mm) for randomly selected 100 individuals per tow was measured to the nearest 0.01 mm, using a calibrated microscope (Olympus™ SZX10). All individuals were measured, if the catch did not exceed 100. Length frequency histograms were made with 0.25 mm size-class intervals following Sturges's rules (Sturges, 1926; Bae *et al.*, 2016).

All brooding females were separated from the collected samples. The number of eggs/larvae in the marsupium (hereafter referred as brood size) were counted. “Larva” refers to a post-hatching individual in the marsupium. Adult refers to combined males, females and brooding females.

As from previous studies it was observed that brooding female crustaceans lose eggs/larvae from marsupium during net collection, preservation and laboratory sorting (Murtaugh, 1989), it was therefore difficult to estimate the correct brood size. Thus, a relationship was estimated from maximum and mean brood sizes against the corresponding brooding female carapace lengths. The egg production rate [ $BS_{max}/D_e$ ] is the number of individuals produced by a brooding female per day at a specific temperature on each sampling day.  $BS_{max}$  is the maximum brood size and  $D_e$  is the incubation time (days) which was estimated by the equation,  $D_e = 408 (T-2.9)^{-1.33}$  where T is the average temperature of the stations in each sampling day (Toda and Takahashi, 1985).

According to Toda and Takahashi (1985), juvenile mortality [ $\ln(R_e/R_j)/D_j$ ] was estimated from the number of individuals released from marsupia per day ( $R_e$ ) with that passing through 1 mm of carapace length per day ( $R_j$ ). The carapace length of a newly released juvenile mysid is 0.25 mm, as previously reported by Sturges (1926). A 1 mm threshold was arbitrarily selected to indicate the start of the juvenile growth stage.  $R_e$  and  $R_j$  are calculated as follows:  $R_e = N_e/D_e$ , and  $R_j = N_j/D_j$  where  $N_e$  is the number of egg/larvae per square meter and  $N_j$  is the number of juveniles of less than 1 mm carapace length per square meter on each sampling day, and  $D_e$  (days) and  $D_j$  (days) are durations from egg laying to releasing and from 0.25 mm to 1 mm juvenile carapace length, respectively. Using the average temperature of the five sampling stations in each month,  $D_e$  and  $D_j$  were estimated from nonlinear relationship between stage duration and ambient temperature (Toda and Takahashi, 1985). The calculation of mortality was done under the assumption that there was a constant egg laying rate over both stages (egg/larva and juvenile stages), no mortality during both stages. Mortality occurs only between stages.

### 2.2.3. Statistical analysis

The density of *N. awatschensis* was expressed as the number of individuals per square meter to facilitate comparison with other studies. Brood density was computed as the number of all eggs/larvae at a station per square meter. The standardization over a surface area was preferred over a volume of water as mysids are known to occur in a water layer just above the bottom. Moreover, as no sampling was conducted at night, possible vertical migration behavior in mysid did not affect the sampling campaign. Results of the three tows were averaged to represent density at each station in each sampling month.

Principal Component Analysis (Abdi and Williams, 2010) was conducted to reduce the dimensionality of the four environmental variables: temperature, salinity, chlorophyll fluorescence and river discharge. The newly produced variables were then related to the density of *N. awatschensis* at each life-cycle stage, carapace length of brooding females and brood sizes.

The monotonous increase/decrease was judged by the Spearman's rho correlation coefficient that might be observed between density of *N. awatschensis*, brooding female carapace lengths and brood sizes with PC1 and PC2 respectively. These statistical tests were performed, and graphs generated in the R program (R Core Team, 2017) and the MINITAB program version 17.

## 2.3. Results

### 2.3.1. Environmental variability

Water temperature changed with season; it increased gradually from March to August before decreasing from September to February (Fig. 2-2a). The highest temperature was 27 °C in August

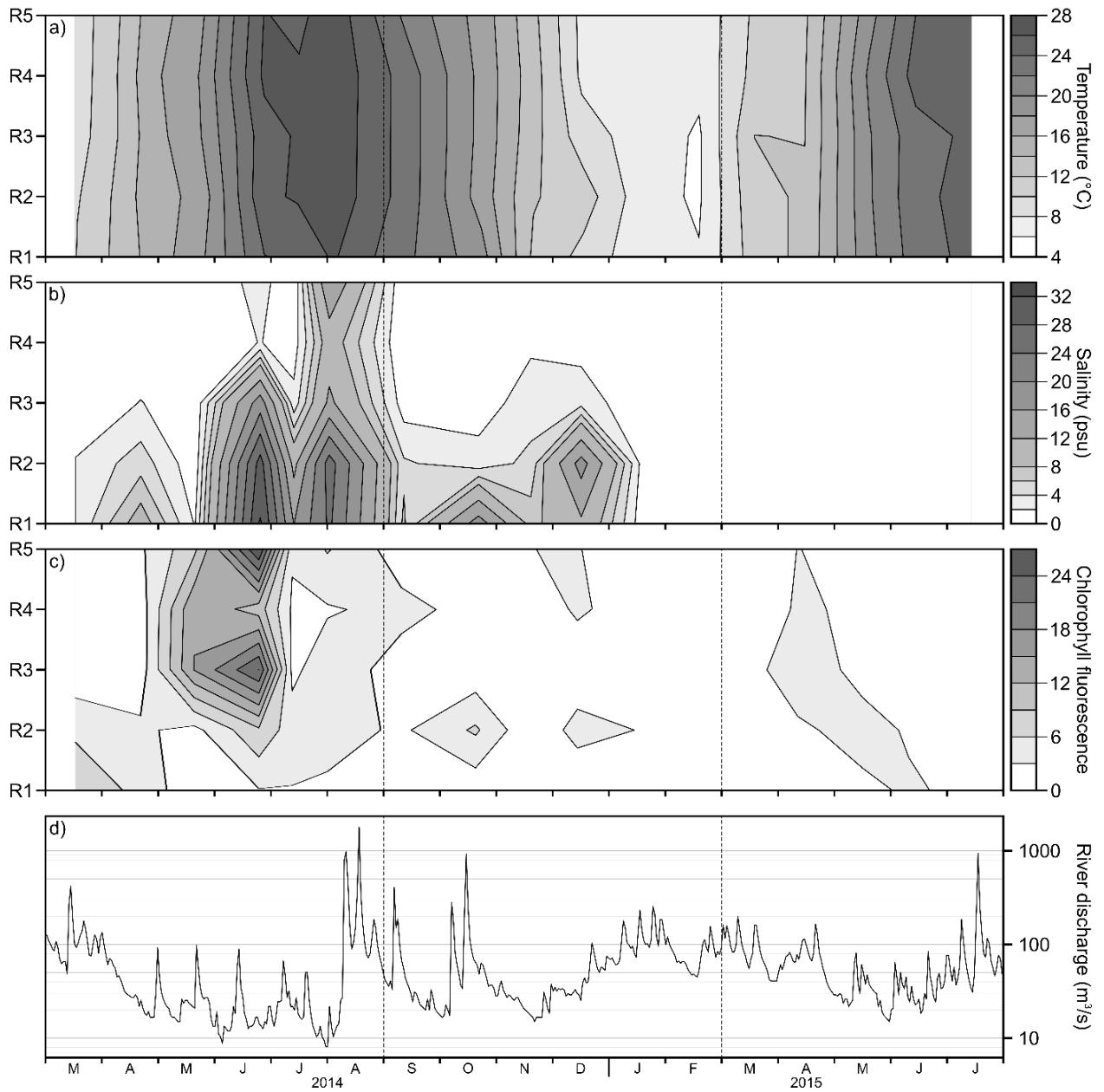


Fig. 2-2 Seasonal variability in environmental parameters.

Seasonal variability in temperature (a) salinity (b) and chlorophyll fluorescence (c) from stations R1 to R5 in the Yura River estuary and seasonal variability in the daily river discharge monitored at the Fukuchiyama weather station (d). The vertical dotted lines separate seasons: spring–summer 2014, autumn–winter 2014 and spring–summer 2015.

2014 at all sampling stations and lowest 5 °C in February 2015 at stations R2 and R3. In contrast with the clear seasonal changes in temperatures, the spatial differences were negligible in each sampling month.

Bottom salinity varied between 0 and 32 at the three lower stations (R1, R2 and R3), whereas at the two upper stations (R4 and R5) it was always close to zero except in August 2014 (Fig. 2-2b). Conversely, salinity remained below 6 at all sampling stations from January through July 2015. High salinities ( $> 10$ ) were observed only in 2014, between April and December. Chlorophyll fluorescence gradually increased from March to June in 2014, it decreased after June and then remained low throughout the latter part of the sampling duration (Fig. 2-2c).

River discharge fluctuated daily and monthly (Fig. 2-2d), but there was a tendency of being high in winter and early spring, and low in other seasons except for cyclonic heavy rain events. High daily river discharge events occurred in August 2014 with a peak of 1788 m<sup>3</sup>/s and October 2014 with a peak of 922 m<sup>3</sup>/s, while the lowest river discharge events were recorded in June and in July 2014 with minimum of 8 m<sup>3</sup>/s. A median of 38 m<sup>3</sup>/s recorded throughout the sampling duration.

Based on temporal trends on environmental parameters, the following three periods were established: spring–summer period in 2014 (from March to August 2014) distinguished by steadily increasing temperatures, highest salinity ranges and maximum chlorophyll fluorescence. The autumn–winter period (from September 2014 to February 2015) was characterized by gradually declining temperatures, low salinity and chlorophyll fluorescence. The spring–summer period in 2015 (from March to July 2015), was characterized by progressively increasing temperatures, minimal salinity and moderate chlorophyll fluorescence with a slight increase in April 2015.



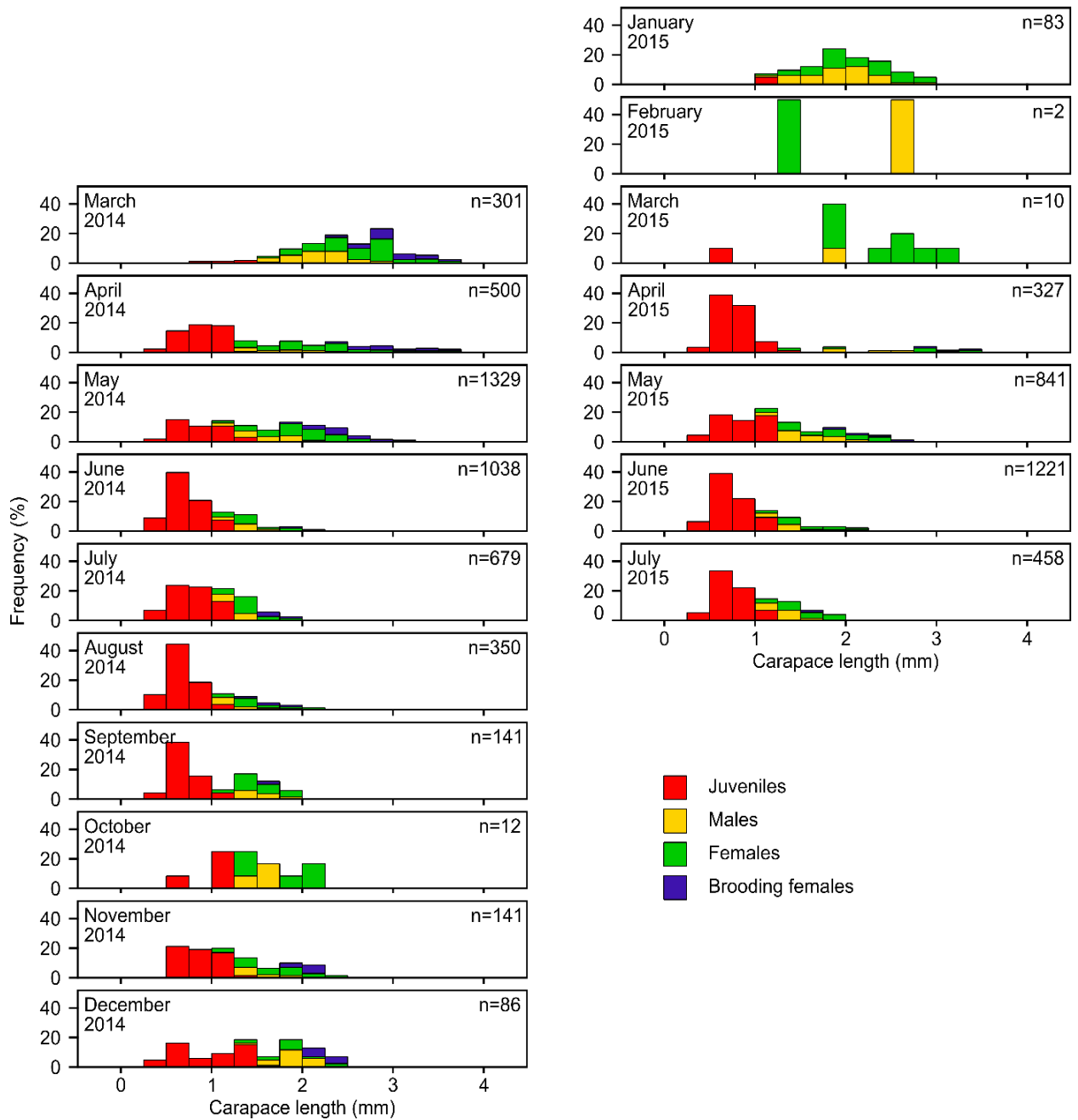


Fig. 2-3 Life-cycle composition and carapace length frequency distribution.

Life-cycle composition and carapace length frequency distribution of the mysid *Neomysis awatschensis* collected monthly from stations R1 to R5 in the Yura River from March 2014 to July 2015.

### 2.3.2. *The length frequency distribution and life-cycle stages*

Juveniles were the most frequent group from April to December 2014 and from April to July 2015 (Fig. 2-3), indicating high juvenile recruitment. However, juveniles were scanty from January to March 2015. Males and females were recorded in all the sampling months. Brooding females were not collected in October 2014 and from January to March 2015.

Mysid carapace lengths changed seasonally (Fig. 2-3), the largest carapace length (4 mm) was found in April of both 2014 and 2015. Large-sized individuals (> 2 mm) dominated the adult generation from March to May, though from June to December small-sized individuals replaced them.

### 2.3.3. *Spatiotemporal occurrence*

The mean total density of mysid changed seasonally (Fig. 2-4a), showing highest densities during the spring–summer periods with peaks of 202 (R2) and 150 (R3) inds/m<sup>2</sup> in May 2014 and June 2015, respectively. In contrast, the mysid total density remained below 10 inds/m<sup>2</sup> throughout the autumn–winter period 2014 (Fig. 2-4b). The clear seasonality observed in the total density was largely attributable to seasonal changes in the density of juveniles (Fig. 2-4d), which accounted for > 63% of the total density in the spring–summer period in 2014 as well as 2015 and 54% in the autumn–winter period 2014. Adults presented highest densities of 108 (R2) and 31 (R2) inds/m<sup>2</sup> during the spring–summer period in 2014 and 2015, respectively (Fig. 2-4c). The lowest density of 5 inds/m<sup>2</sup> (R2) was found during the autumn–winter period 2014. The brood density showed notable seasonal variation (Fig. 2-4e), being high in spring with highest value of 319 inds/m<sup>2</sup> (R1) in May and scarce during the autumn–winter period 2014 with less than 18 inds/m<sup>2</sup>. Moreover, eggs were not observed in October 2014 and from January to March 2015. In terms of space, there were no clear trend of occurrence in any life-cycle stages, although higher densities of adults and juveniles were found at stations R2 and R3.

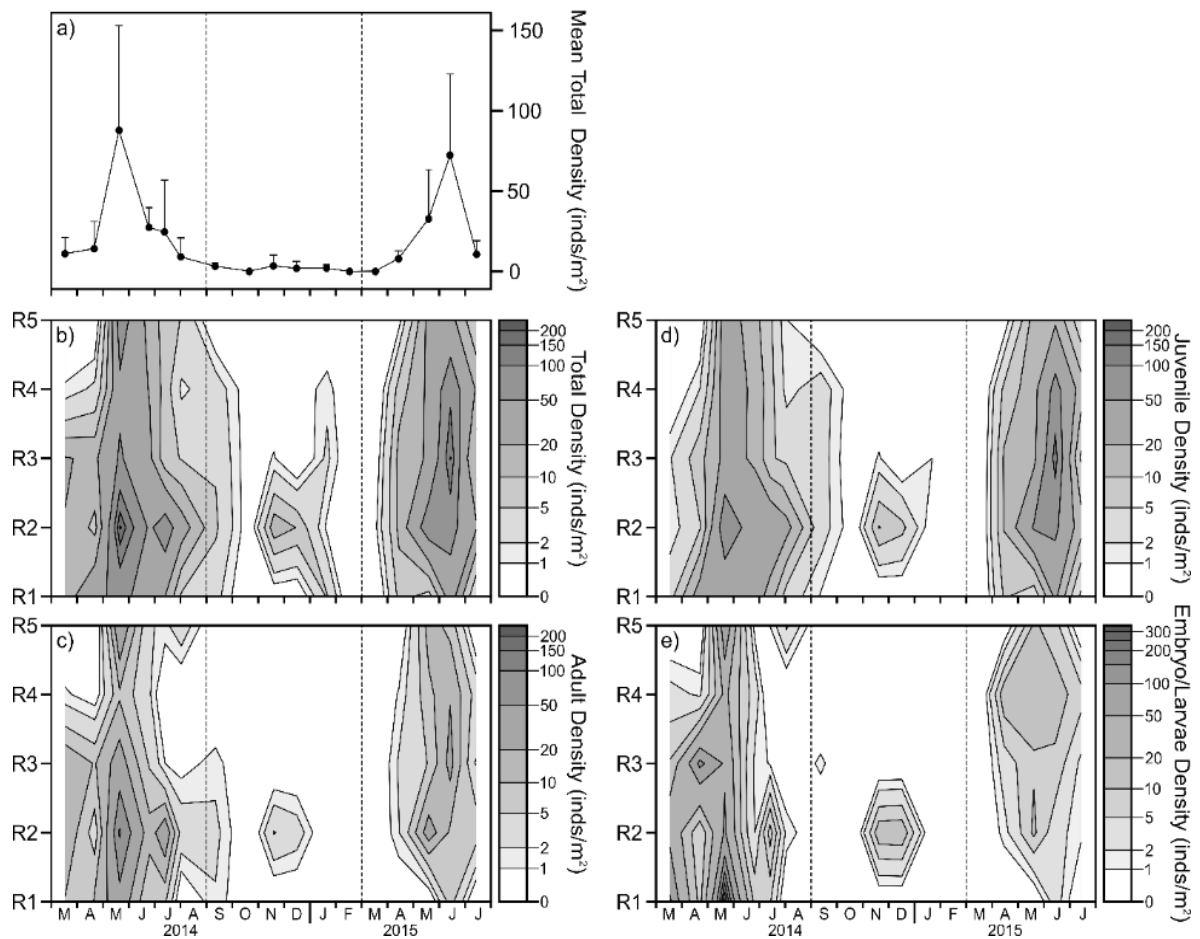


Fig. 2-4 Seasonal variability in density of *Neomysis awatschensis*.

Seasonal variability of mean total density of the total density *Neomysis awatschensis* + standard deviation (a) and seasonal variability of total mysid density (b), adult density (c), juvenile density (d) and brood density (e) from station R1 to R5 in the Yura River estuary. The vertical dotted lines separate seasons: spring–summer 2014, autumn–winter 2014 and spring–summer 2015.

#### *2.3.4. Principal Component Analysis of the environmental factors*

The first two principal components (PC) of the Principal Component Analysis on the four environmental factors accounted for 76% of the total variance (Fig. 2-5a). PC1 largely accounted for the explained variance whereas PC2 contributed little (Figs 2-5a and 2-5b). PC1 axis correlated positively with; temperature, salinity, chlorophyll fluorescence and negatively with river discharge. Temperature and river discharge are the variables contributing the most weight to the PC1 axis. Temperature and river discharge being nearly orthogonal to PC2 are therefore not correlated to PC2. The PC1 axis largely separated the spring–summer 2014 period from the autumn–winter and spring–summer 2015 periods (Fig. 2-5b), whereas the PC2 axis contributed little to the separation of the three periods. The spring–summer 2014 period was characterized by higher temperature, higher salinity, higher chlorophyll fluorescence and low river discharge.

#### *2.3.5. Density and reproduction traits in relation to principal components*

In all the life-cycle stages, the mysid density remained close to zero at negative PC1 values, whereas at positive PC1 values mysid density varied widely, with peak densities observed at PC1 value range of 0 to 1 (Figs 2-6a–d). The mysid density was lower in the autumn–winter period 2014 than in the spring–summer period both in 2014 and 2015 even under the same PC1 value ranges. The mysid density at all the life-cycle stages showed no clear relationship with PC2 values (Figs 2-6e–h).

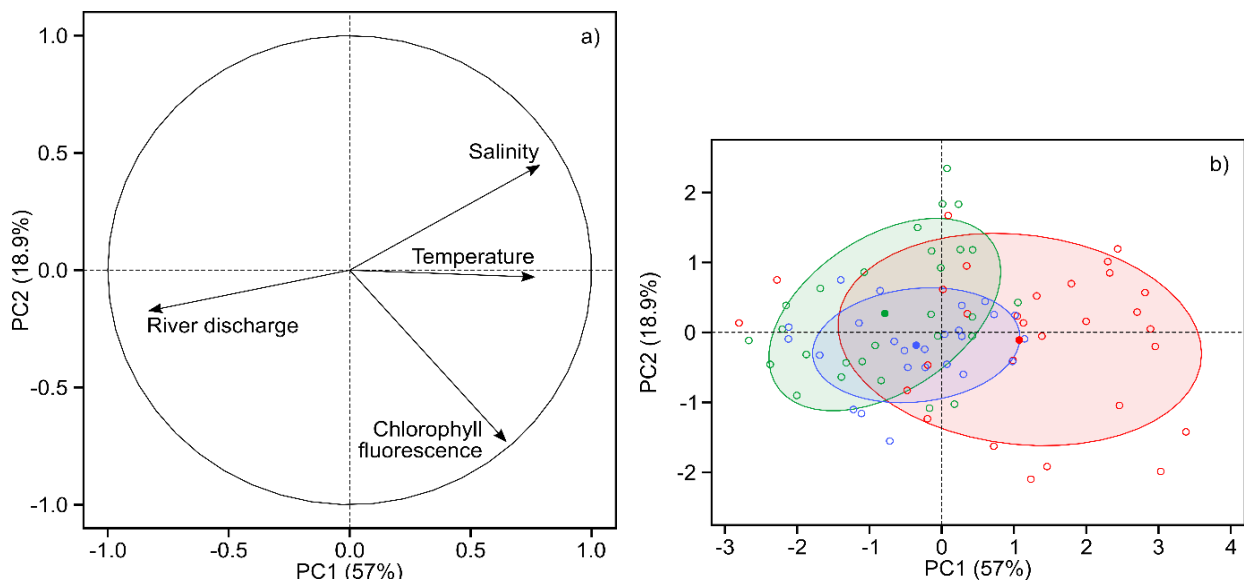


Fig. 2-5 PCA biplot showing loadings of the environmental variables.

PCA biplot showing loadings of the environmental variables; temperature, salinity, chlorophyll fluorescence and river discharge (a) and site scores (b) from stations R1 to R5 in the Yura River estuary from March 2014 to July 2015. Colors are used to differentiate the three periods. Red open circles and ellipse represent the spring–summer 2014, green open circles and ellipse represent the autumn–winter 2014 and blue open circles and ellipse represent the spring–summer 2015. Ellipses represent standard deviations and closed circles represent the centroid of the respective ellipses.

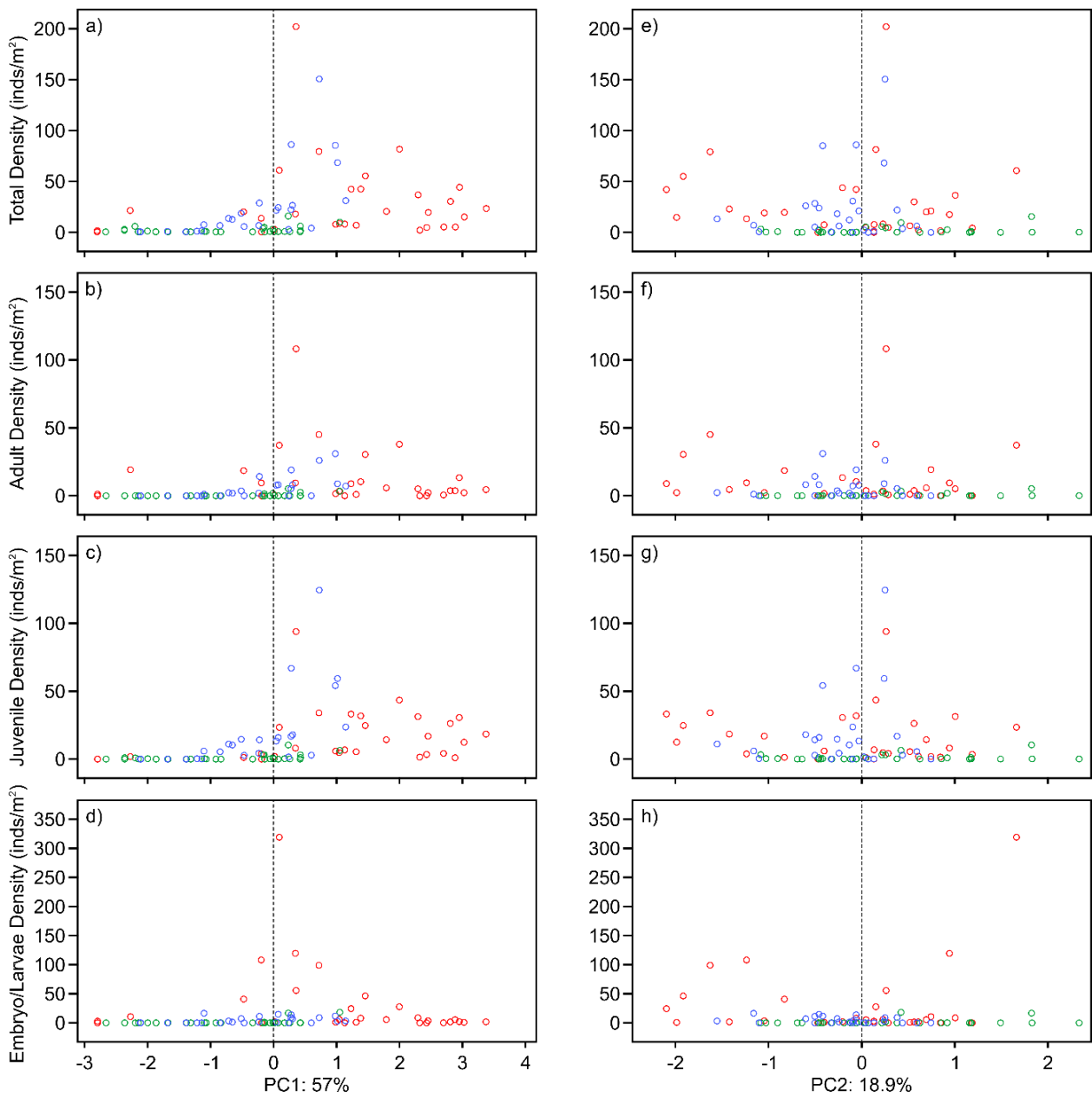


Fig. 2-6 Densities of *Neomysis awatschensis* in relation to PC1 and PC2.

Total density (a, e), adult density (b, f), juvenile density (c, g) and brood density (d, h) of *Neomysis awatschensis* in relation to PC1 and PC2 observed in the Yura River estuary. Red circles represent spring–summer 2014, green circles represent autumn–winter 2014 and blues circles represent spring–summer 2015.

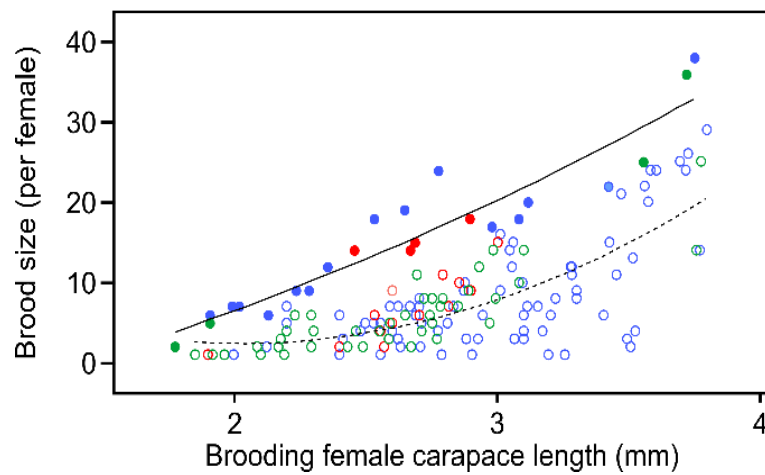


Fig. 2-7 Relationship between brooding female carapace length and brood size. Colors are used to differentiate the sampling duration into three periods; the blue open circles represent the spring–summer 2014, red open circles represent autumn–winter 2014 and the green open circles represent the spring–summer 2015. Closed circles represent maximum brood sizes. The solid line represents the maximum brood size quadratic regression curve. The dotted line represents the average brood size quadratic regression curve.

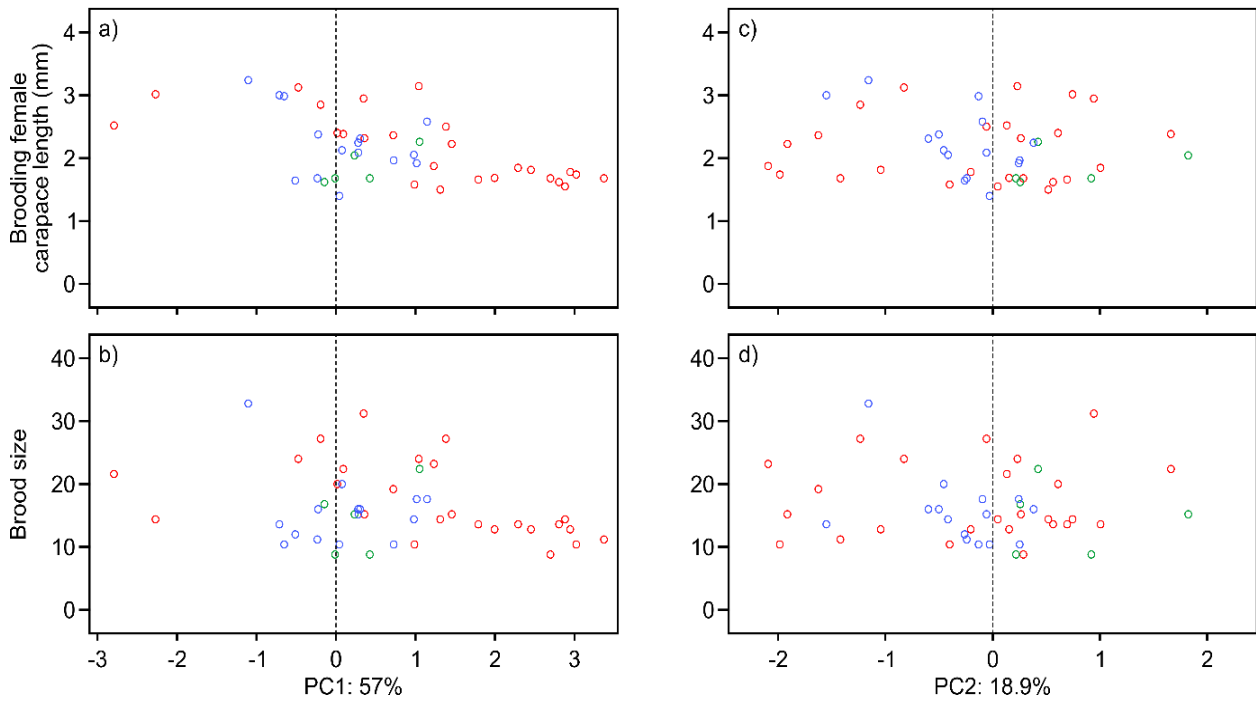


Fig. 2-8 Brooding female carapace length and brood size in relation to PC1 and PC2. Brooding female carapace length (a, c) and brood size (b, d) of the mysid *Neomysis awatschensis* in relation to PC1 and PC2 observed in the Yura River estuary. Red circles represent spring–summer 2014, green circles represent autumn–winter 2014 and blues circles represent spring–summer 2015



Significant positive relationships between brooding female carapace lengths and brood sizes were found and represented by the following equations (Fig. 2-7):

$$BS = 0.18 \times BFCL^{3.39} (R^2 = 0.57, n = 155, p < 0.05)$$

$$BS_{\max} = 0.47 \times BFCL^{3.30} (R^2 = 0.85, n = 23, p < 0.05)$$

Where BS is the brood size (brooding per female), BFCL is brooding female carapace length, and  $BS_{\max}$  is the maximum brood size. Larger brooding females had more eggs/larvae than smaller brooding females. Furthermore, brood sizes were higher during the spring–summer periods (< 40) than during the autumn–winter period (< 20).

The carapace length of brooding females varied considerably between 1.5–3.2 mm at intermediate PC1 values (-1 to 1), beyond which it remained close to 1.5 mm (Fig. 2-8a). Brood size showed a similar pattern: large variation (1–30) at PC1 values in contrast with small sizes (< 10) at higher PC1 value range (Fig. 2-8b). Data were scarce at negative PC1 values, because brooding females were hardly found in such conditions. Neither the brooding female carapace length nor the brood size showed any correlation with the PC2 value ranges (Figs 2-8c and 2-8d).

### 2.3.6. Egg production and juvenile mortality

The daily egg production rate was always high in the spring–summer periods in 2014 and 2015, but low or negligible in the autumn–winter period (Fig. 2-9a). Highest daily egg production rate of 3.27 was recorded in May 2014. Juvenile mortality displayed an increasing trend in the spring–summer period 2014, mortality increased from 0.1 per day in March 2014 to 0.6 per day in August 2014 (Fig. 2-9b). Although juvenile mortality remained 0.3 per day in November and December 2014, it increased from 0.1 to 0.5 per day in the spring–summer period 2015. juvenile mortality was unavailable in several months. Because eggs/larvae and/or juveniles were not found.

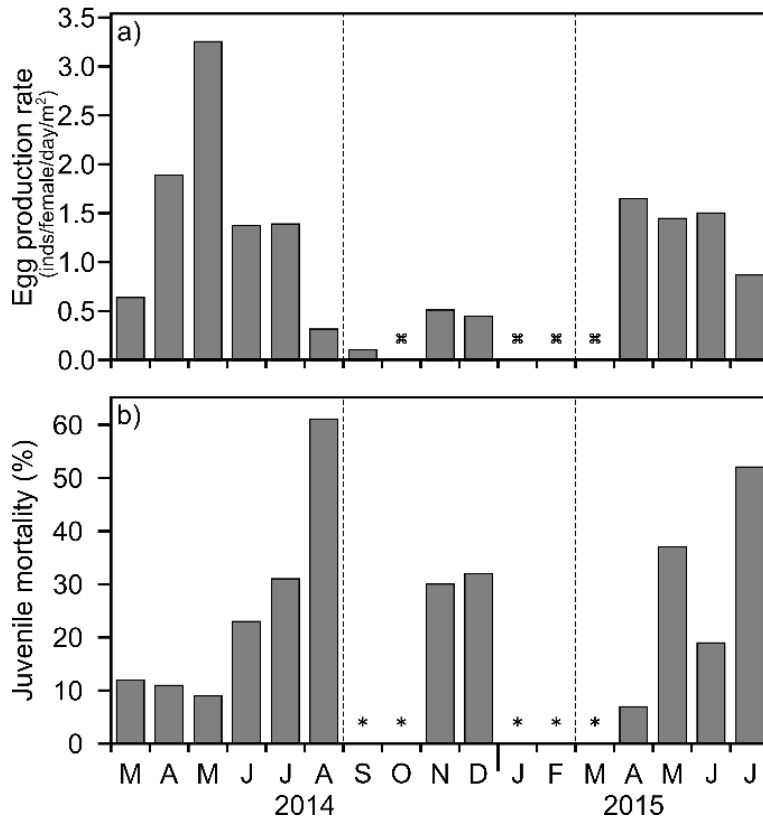


Fig. 2-9 Daily egg production and juvenile mortality of *Neomysis awatschensis*.

Daily egg production (a) and juvenile mortality (b) of the mysid *Neomysis awatschensis* in the Yura River estuary collected from March 2014 to July 2015. Crosses and asterisks indicate the unavailability of daily egg production and juvenile mortality respectively. The Vertical dotted lines separate seasons: spring–summer 2014, autumn–winter 2014 and spring–summer 2015.

## 2.4. Discussion

### 2.4.1. Environmental variability

The spring–summer period 2014 was characterized by increasing temperatures as well as high salinity and high chlorophyll fluorescence. In contrast, the autumn–winter period 2014 was distinguishable by decreasing temperatures, mid-lower levels of salinity and chlorophyll fluorescence. In the spring–summer period 2015, although temperatures gradually increased, chlorophyll fluorescence was low, and salinity remained close to zero throughout the sampling stations.

The seasonal change in chlorophyll fluorescence observed in 2014 is well supported by previous studies ( Kasai *et al.*, 2010; Watanabe *et al.*, 2014, 2017). In the Yura River estuary, riverine nutrient fluxes are high from February to July at the same time when a salt-wedge intrudes into the estuary, thus allowing brackish and marine phytoplankton to bloom and form chlorophyll maximum layer below the halocline. In contrast, large river discharge flushes out the salt-wedge water which discourages phytoplankton production within the estuary in the winter and the rainy seasons(Kasai *et al.*, 2010; Funahashi *et al.*, 2013).

Characterized by low levels of salinity and chlorophyll fluorescence, the spring–summer period 2015 was different from the usual spring–summer conditions in the Yura River estuary. There were no clear differences in river discharge between 2014 and 2015 during spring–summer periods. However, sand accumulated at the river mouth from February to June 2015 (Fukuchiyama Office of Rivers and National Highways, MLIT, personal communication) potentially blocking salt-wedge intrusions into the estuary during this period. Low chlorophyll fluorescence in spring–summer period in 2015 was probably the consequence of reduced inflow of marine phytoplankton. Generally, morphological changes in a river mouth substantially affect environmental conditions in the estuary (Morales *et al.*, 2001; Viet *et al.*, 2006).

#### 2.4.2. Influence of temperature and salinity on the biological characteristics

The present results suggest that water temperature was the prime factor affecting the biological characteristics of *Neomysis awatschensis*. Although temperature, salinity and chlorophyll fluorescence combined to contribute positively to the density of *N. awatschensis*, in general, highest densities were not found under highest levels of the three environmental factors. This indicates optimum ranges and/or combinations of the three environmental factors are responsible for high densities of *N. awatschensis* in the Yura River estuary. Details about the strong dependence of this species on temperature have been documented from both field and laboratory studies (Toda *et al.*, 1982, 1983, 1984; Toda and Takahashi, 1985; Toda *et al.*, 1987). Temperature dependency is a general characteristic for many other mysid taxa including the genus *Neomysis* (Rudstam *et al.*, 1999; Baldó *et al.*, 2001; Nanami *et al.*, 2005). In the Yura River estuary, *N. awatschensis* inhabited within a wide salinity range of 0–32 and no clear influence of salinity on the mysid density and distribution was found. Furthermore, all the life-cycle stages of *N. awatschensis* occurred concurrently throughout the estuary. The present results agree with earlier studies, in which *N. awatschensis* was found within broad salinity ranges (Nanami *et al.*, 2005; Yamada *et al.*, 2007). In some other mysid species, such as *N. integer* (Mees *et al.*, 1994), *Mesopodopsis slabberi* (David *et al.*, 2005), and *Schistomysis spiritus* (Rappé *et al.*, 2011), densities were correlated mainly with salinity and those species occur in well-defined salinity zones. It has also been reported that different life-cycle stages in mysid species can be spatially segregated along the salinity gradient (Parkera and West, 1979; Baldó *et al.*, 2001; Suzuki *et al.*, 2009). The differences in findings may be attributed not only to variations in salinity tolerance in mysid species but also to distinct physical settings and circulation modes among the estuaries. Spatial variability in salinity is considered more gradual in brackish lagoons and macrotidal well-mixed estuaries (Wooldridge, 1986; Mees *et al.*, 1994; Baldó *et al.*, 2001) than in microtidal salt-

wedge estuaries, where fresh water and sea water are not mixed but stratified (Kasai *et al.*, 2010; Funahashi *et al.*, 2013). It is certain that the great tolerance for salinity fluctuations contributes to the maintenance of the *N. awatschensis* densities in the Yura River estuary.

There were clear seasonal changes in reproductive traits, stage composition and body size in *N. awatschensis* in the Yura River estuary. High egg production rate and low mortality in spring contributed to high mysid density in early summer. Egg production rates were previously reported by Toda *et al.*(1984). As summer temperatures increased, egg production rate of *N. awatschensis* decreased as well as the brood sizes. Large-sized females occurred in winter and produced large numbers of broods in spring of 2014 and 2015. Previously, small-sized adult mysids have been collected in higher temperature conditions (Sudo *et al.*, 2011; Delgado *et al.*, 2013), other mysid species with similar results include *N. americana* (Pezzack and Corey, 1979) and *N. integer* (Mees *et al.*, 1994). Moreover, body size at maturation decreases as temperature increases, resulting in smaller brood sizes at higher temperatures (Toda *et al.*, 1984).

No brooding female was caught in the Yura River estuary from January to March 2015, the reproduction of *Neomysis awatschensis* appeared to be stopped in winter. In *N. awatschensis*, the disappearance of brooding females was recorded in the Kasumigaura Lake from December to February (Toda *et al.*, 1982) and Akkeshi-ko Lake from October to March (Yamada *et al.*, 2007). These results suggest that *N. awatschensis* does not reproduce at low temperatures (< 10°C). The mysid *Rhopalophthalmus terranatalis* was also reported to almost disappear in the winter season from the Sunday estuary which is a well-mixed estuary (Wooldridge, 1986). On the other hand, Toda *et al.*(1984) reported that daily egg reproduction rate was higher at higher temperatures (up to 25 °C) in *N. awatschensis* in the laboratory. This is explained by the fact that a sharp decrease in the brooding duration overcame the gradual decrease in brood size at higher temperatures (Toda *et al.*, 1987). Smaller brood sizes are also reported in summer for *Neomysis integer* (Mees *et al.*, 1994; Rappé *et al.*, 2011), *Neomysis mirabilis* (Yamada *et al.*, 2007; Takahashi *et al.*, 2015) and

*Neomysis nigra* (Toda *et al.*, 1987; Bae *et al.*, 2016) determined that the allocation of assimilated carbon to growth (including reproduction) substantially exceeded that to respiration at 15–20 °C in *N. awatschensis*. Higher temperatures raise the growth rate in *N. awatschensis* (Toda *et al.*, 1984; Toda and Takahashi, 1985). Higher mortality at higher temperatures would be responsible for the population decline observed in the late summer season. Because higher temperatures would raise mortality rates in *N. awatschensis* (Toda and Takahashi, 1985).

#### 2.4.3. The population dynamics

In the Yura River estuary, *N. awatschensis* marked a density peak (up to 202 inds/m<sup>2</sup>) in early summer, specifically in May 2014 and in June 2015. A similar pattern was reported from Akkeshiko Lake, where *N. awatschensis* marked a density peak (up to 3,350 inds/m<sup>2</sup>) in mid-summer (Yamada *et al.*, 2007). However, two density peaks (over 10,000 inds/m<sup>2</sup>) in spring and autumn seasons were recognized in the *N. awatschensis* population of Kasumigaura Lake (Toda *et al.*, 1982, 1983). Although all density peaks were found at temperature ranges of 15–25 °C, the figures of density peaks differed considerably among the study sites. In the Yura River estuary, large river discharge is likely to flush the *N. awatschensis* population out of the estuary and possibly decrease phytoplankton production in the estuary. On the other hand, the Kasumigaura Lake is famous for eutrophication, as high nutrient concentrations support high phytoplankton production in the Lake throughout the year (Soma *et al.*, 1995; Fukushima *et al.*, 1996; Yoshimura *et al.*, 2012). Stable and eutrophic conditions might have contributed to the extremely high densities of *N. awatschensis* in the Kasumigaura Lake. In contrast, highly variable and oligotrophic conditions would limit the high densities of the *N. awatschensis* population in the Yura River estuary even in early summer.

The density peak of *N. awatschensis* was observed in the Yura River estuary almost at the same timing of the year in both 2014 and in 2015, although salinity and chlorophyll fluorescence

differed significantly between the two years. Given the great tolerance for wide salinity ranges (0–32), it is unlikely that salinity conditions affect *N. awatschensis* populations. However, though information on osmoregulation in mysid is lacking, changes in salinity may require a substantial amount of energy to maintain the mysid osmotic pressure at a constant level (Baldó *et al.*, 2001; Verslycke and Janssen, 2002; Schiariti *et al.*, 2006). Food availability might have been lower in 2015 than 2014, as chlorophyll fluorescence is an indicator of phytoplankton production. Food items for *N. awatschensis*, however, include phytoplankton, benthic microalgae and zooplankton (Hanazato, 1990; Toda and Wada, 1990; Antonio *et al.*, 2010). Moreover, *N. awatschensis* is an opportunistic omnivorous feeder that can flexibly change feeding behavior in response to the environment (Hanazato, 1990). The gut of *N. awatschensis* collected in the Yura River estuary in 2015 consisted mainly of benthic diatom species and green algae (See chapter 3). Although densities of benthic microalgae were not studied, the *N. awatschensis* population might have depended on benthic microalgae rather than phytoplankton in 2015. In the Yura River estuary, the population growth of *N. awatschensis* is seemingly triggered by the seasonal relaxation of harsh winter conditions, such as high river discharge, low temperatures and low food availability.

## **2.5. Conclusion**

The high potentiality of *N. awatschensis* to grow and reproduce (Toda *et al.*, 1982; Yamada *et al.*, 2007) is partially suppressed by the variable and oligotrophic conditions in the Yura River estuary. Combined with the flexibility to seasonal changes in temperature and food availability, the great tolerance to acute salinity fluctuations allows *N. awatschensis* to maintain its population in the estuary..





## Chapter 3

### Feeding habits of *N. awatschensis* in the Yura River estuary

#### 3.1 Introduction

Mysids are common zooplankton crustacean in diverse aquatic habitats (Mauchline, 1980). The distribution of the genus *Neomysis* (Crustacea: Mysidae) in coastal lagoons and estuaries (Czerniavsky, 1882; Yamada *et al.*, 2007; Petryashev and Daneliya, 2014) can be attributed to its strong capabilities to withstand and adapt to highly changing environments e.g. *Neomysis* tolerance to a wide range of salinities (Pezzack and Corey, 1979; Omweri *et al.*, 2017) and water temperatures (Mauchline, 1980; Roast *et al.*, 1998). The estuarine hyperbenthic mysid, *Neomysis awatschensis* (Brandt, 1851) occurs abundantly in lagoons and estuaries in Japan (Yamada *et al.*, 2007; Suzuki *et al.*, 2009; Takahashi *et al.*, 2015). Feeding habits of *N. awatschensis* have been previously studied in the hyper-eutrophic Kasumigaura lagoon to find seemingly conflicting results. A strong dependence on phytoplankton throughout the year was indicated by stable isotope analysis (Toda and Wada, 1990), whereas possible predation on cladocerans, rotifer and copepods was hypothesized by Hanazato (1990) through observations on temporal changes in zooplankton densities.

The Yura River ends in a salt-wedge estuary that flows into Tango Bay and the larger Wakasa Bay, Sea of Japan (Kasai *et al.*, 2010; Funahashi *et al.*, 2013). The mysid *N. awatschensis* occur seasonally in the estuary with maximum densities during the spring–summer period (Omweri *et*

*al.*, 2017). This species is the main prey item for the juveniles of Japanese temperate seabass *Lateolabrax japonicus* (Antonio *et al.*, 2010, 2012; Fuji *et al.*, 2010, 2016) and other fishes (Aoki *et al.*, 2014) that use estuaries as a nursery ground. Although *N. awatschensis* is an essential link species between lower and higher trophic levels in the estuary, its feeding habits remained understudied. The variable and oligotrophic Yura River estuary would provide food sources to *N. awatschensis* differently from the stable and eutrophic freshwater Kasumigaura lagoon (Soma *et al.*, 1995; Yoshimura *et al.*, 2012), where the ecology of *N. awatschensis* has been studied in detail (Toda *et al.*, 1982, 1983; Toda and Takahashi, 1985; Toda and Wada, 1990).

The quantitative examination of stomach contents can provide useful information about the recently ingested food items such as species identification, abundance and size (Storch, 1989; Ramarn *et al.*, 2015; Takahashi *et al.*, 2015) and therefore on the feeding ecology of the consumers. However, stomach content analysis is not always capable of identifying food items that are assimilated into animal tissues (Johannsson *et al.*, 2001; Lehtiniemi *et al.*, 2009). The stable carbon isotope ratio ( $\delta^{13}\text{C}$ ) is commonly used to estimate the proportional contribution of food sources within a mixture (consumer tissues), and thereby make inferences about the composition of the animal's assimilated diet (Peterson and Fry, 1987; Parnell *et al.*, 2010). The stable nitrogen isotope ratio ( $\delta^{15}\text{N}$ ) is used to determine the trophic levels of producers and consumers (Minagawa and Wada, 1984; Peterson and Fry, 1987). In contrast to stomach content analysis, stable isotope technique integrates feeding information over a longer time frame (Vander Zanden *et al.*, 1999; Galván *et al.*, 2012).

The current study aims to determine the feeding habits of *N. awatschensis* in the Yura River estuary. The specific objectives include: (a) the identification of stomach contents using both optical and scanning electron microscopes, (b) the estimation of the relative contribution of potential food sources to the diet of *N. awatschensis* using stable isotope analysis.

## 3.2. Materials and methods

### 3.2.1. Field survey

Mysid and its potential food sources were collected monthly from March 2014 to July 2015 from five stations (R1, R2, R3, R4 and R5, Fig. 2-1). A sledge net (See chapter 2) was towed parallel to the river bank at each sampling station and catches were divided into two sub-samples. One sub-sample was fixed in 10% formaldehyde solution and then preserved in 70% ethanol for stomach content examinations. The other sub-sample was frozen for stable isotope analyses. *Neomysis awatschensis* specimens were identified and categorised into juvenile or adult stages according to the absence or presence of secondary sexual characteristics (Mauchline, 1980; Chihara and Murano, 1997).

The potential food sources for *N. awatschensis* might include epilithic and epipelagic benthic microalgae (BMAel and BMAep, respectively), and riverine and marine particulate organic matter (POMr and POMm, respectively) (Toda and Wada, 1990; Takahashi, 2004; Takahashi *et al.*, 2015, 2017). BMAel were collected by gentle brushing submerged stones at stations R1–R5. BMAep were collected by scrapping the upper 2 mm of sediment at the stations R3 and R5 where mudflats were available. Sediment samples were brought back to the laboratory within six hours to extract the carbon and nitrogen elements following the procedure of Couch (1989) modified by Yokoyama and Ishihi (2003) before further stable isotope analysis. To collect POMr - consisting mainly of freshwater phytoplankton, terrestrial plant and inorganic particles-water was sampled from the river surface at the uppermost station R5 (salinity < 0.05). To collect POMm - consisting mainly of marine phytoplankton - water was sampled from the chlorophyll maximum depth at the offshore station M1 (latitude 35.6067 °N, longitude 135.3465 °E) in Tango Bay (Fig. 2-1).

### 3.2.2. Stomach content

#### 3.2.2.1. Sample preparation for optical microscopy

Relative quantitative analysis on the diet composition of *N. awatschensis* was conducted by optical microscopic observations of the stomach contents. Each mysid specimen was dehydrated according to Seinhorst (1959). To obtain microscopic slides of the stomach contents, gradual dehydration series from ethanol to glycerine solution was used. The carapaces were removed and the stomach was separate from the intestine by an incision. The stomach contents were extracted and placed in a drop of glycerine on a microscopic slide. In each sampled month, lower estuary specimens (station R2) were examined separately from the ones from the upper estuary (station R4). Under an optical microscope (Olympus™ BX 50, magnification  $\times 400$ ), stomach contents from 3–10 individuals per station per month were pooled and spread on a glass slide for identification up to the species level. The relative percent of covered area for each food item in the visual field was determined for each species (Hynes, 1950; Takahashi and Kawaguchi, 1998) and used as an index of contribution of each species to the diet of *N. awatschensis*. Unidentified amorphous matter including sand was not included in the determination of contribution ratios, although it was sometimes dominant in the stomach of the specimens. This process was conducted for juveniles and adults, which were sampled in April, July, September, November and December 2014 and January, April and July 2015.

For convenience, diatoms were sorted into benthic and planktonic species according to Kasim and Mukai (2006). For species not included in Kasim and Mukai (2006) additional references were used (Medlin, 1991; Potapova and Charles, 2003; Cantonati and Lange-Bertalot, 2009; Park *et al.*, 2012; Benito *et al.*, 2015). Difficulty and uncertainty in the classification of benthic and planktonic diatoms are discussed later. Planktonic green algae were sorted according to Bhosale *et al.* (2010).

### 3.2.2.2. *Sample preparation for scanning electron microscopy*

A total of 18 specimens were selected from stations R2 and R4 to represent the lower and upper part of the estuary respectively for the examination of their stomach content under Scanning Electron Microscope (SEM) (Storch, 1989; Lehtiniemi *et al.*, 2009). At station R2, five specimens were selected in November 2014 and five specimens in May 2015. At station R4 five specimens were selected in January 2015 and three specimens in May 2015. In each sampling month one juvenile was randomly selected and the remainder were all adults also randomly selected. All specimens were treated following the protocol for paraffin processing of tissue (Protocols Online, 2010). In summary, specimens were fixed and decalcified in a solution of picric acid, formaldehyde and acetic acid for six hours. After dehydration through series of ethanol, xylene and paraffin, the specimens were transferred to holding slides, then cooled down to let the paraffin solidify. The paraffin blocks containing the specimens were sliced using a 10 µm microtome to expose mysid stomach contents. The slices were placed on glass slides. To remove excess paraffin, the glass slides were passed through a series of xylene, ethanol and finally t-butly alcohol then freeze dried. The specimens on the glass slides were coated using platinum (E-1030, ion sputter, Hitachi™) to avoid charging-effect of the samples. Stomach contents were examined, identified and micrographs taken using a SEM (Hitachi™, S-4800). SEM observations were conducted to supplement optical microscopy observations and confirm the occurrence of diatoms.

### 3.2.3. *Sample preparation for stable isotope analyses*

The whole body of *N. awatschensis* specimens was dipped in 1.2N HCl for 10–30 secs to dissolve all carbonates and rinsed thoroughly with distilled water to wash off dissolved carbonates before being dried at 60 °C for six hours. Water samples for BMAel, BMAep, POMr, and POMm

samples were filtered on pre-combusted Whatman GF/F glass fiber filters, acidified in 1.2N HCl to dissolve all carbonates and neutralised by 1.2N NaOH and then dried at 60 °C for twelve hours. Each sample was wrapped in a tin capsule and then analysed for stable carbon and nitrogen isotope ratios (i.e.  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) using a mass spectrometer (Thermo Fisher™ Delta V Plus connected with Elemental Analyzer EA). The ratios of  $^{13}\text{C}/^{12}\text{C}$  and  $^{15}\text{N}/^{14}\text{N}$  are expressed as relative differences to a standard by the following equation:

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

Where  $X$  is  $^{13}\text{C}$  or  $^{15}\text{N}$  and  $R$  is the corresponding ratio of  $^{13}\text{C}/^{12}\text{C}$  or  $^{15}\text{N}/^{14}\text{N}$ . Analytical errors ( $\pm$  SD of the standard) were 0.2‰ both in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ . The amino acid DL-alanine was used as a standard for all isotopic measurements (Yoshii *et al.*, 1999; Tayasu *et al.*, 2011). To characterize the properties of the four potential food sources, the molar base ratio of organic carbon and total nitrogen (C:N ratio) was calculated.

#### 3.2.4. Statistical analyses

The five sampling stations along the estuary were divided into two groups: the lower estuary (R1 and R2) and the upper estuary (R3, R4 and R5) characterised by regular and sporadic influences of sea water intrusions, respectively (See chapter 2). Considering the seasonal changes in temperature, salinity and chlorophyll fluorescence, four seasons were defined: spring (March–May), summer (June–August), autumn (September–November) and winter (December–February) (Omweri *et al.*, 2017).

Differences in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  of *N. awatschensis* among seasons and between upper and lower parts of the estuary were tested by using the Kruskal-Wallis Test / Conover *post-hoc* Test and the Wilcoxon Signed-Rank Test, respectively. Stable isotope ratios of potential food were at all sites

only for BMAel. Isotopic values for the other potential food sources (e.g. BMAep, POMr and POMm) were used in both upper and lower parts of the estuary. The SIAR version 4 (Stable Isotope Analysis in R program), the Bayesian multiple mixing model (Parnell *et al.*, 2010; Hopkins III and Ferguson, 2012; Parnell and Jackson, 2013) was used to elucidate the proportionate contribution of each potential food source to the diet of both juvenile and adult *N. awatschensis* on a spatial-seasonal dimension. The analysis assumes that all isotopes are assimilated equally. All statistical analyses were conducted and graphs were generated using the R program platform (R Core Team, 2017) and Minitab version 17.

### 3.2.5. Sensitivity analysis

The SIAR model results were subjected to a sensitivity analysis to ascertain the contribution ratios of potential food sources to the diet of *N. awatschensis* when uncertainty is high and species-specific trophic enrichment factors are unclear and variable (Parnell *et al.*, 2010; Galván *et al.*, 2012). The high uncertainty scenarios were selected for the lack of appropriate trophic enrichment factors (TEF) for  $\delta^{13}\text{C}$  in the genus *Neomysis*. Scenario 1 adopted the TEF for  $\delta^{13}\text{C} = -0.73 \pm 1\text{‰}$  from other aquatic organisms such as the ghost shrimp *Nihonotrypaea japonica* and *N. harmandi* (Toda and Wada, 1990; McCutchan *et al.*, 2003; Yokoyama *et al.*, 2005). Scenario 2 adopted TEF  $\delta^{13}\text{C} = 0.5 \pm 1\text{‰}$  from other mysid species *Acanthomysis thailandica* and *Mesopodopsis orientalis* (Toda and Wada, 1990; Ramarn *et al.*, 2015). The  $\delta^{15}\text{N}$  trophic enrichment factor was reported to be  $3.2 \pm 1\text{‰}$  for *N. awatschensis* (Toda and Wada, 1990). The standard deviation was set at  $\pm 1\text{‰}$  (Minagawa and Wada, 1984). The sensitivity analyses results are reported as 95% credibility intervals (CI) and mode values.

### 3.3. Results

#### 3.3.1. Water temperature and salinity variability

Water temperature changed seasonally, ranging from 5 °C in February 2015 to 27 °C in August 2014 (Fig. 3-1a). No clear differences in temperature between the upper and the lower parts of the estuary was observed. In 2014, salinity was always higher in the lower estuary (2–32) than in the upper estuary (0–18), whereas in 2015, salinity was constantly below 2 across the entire estuary (Fig. 3-1b).

#### 3.3.2. Stomach content analysis through optical microscopy

Diatoms were observed in the stomach of all juveniles (Figs 3-2a–b, Table 3-1). Benthic diatoms (e.g. *Bacillaria paxillifer* and *Navicula* spp.) accounted for 60–100% of the stomach contents (relative coverage area) in the upper estuary site and 45–100% in lower estuary site. However, no planktonic diatom was observed in the stomach of juveniles. Unidentified diatom fragments accounted for up to 30% of the stomach contents in the upper estuary (July 2015) and 10–20% in the lower estuary. Planktonic green algae (e.g. *Scenedesmus arcuatus* and *Staurastrum* spp.) were mostly observed in the upper estuary and accounted for 15–25% of the stomach contents. Zooplanktons (e.g. *Brachionus* spp and oligochaeta) were hardly observed, but only in July 2015, fragments of crustacean species accounted for maximum of 30% of the stomach contents in the lower estuary.



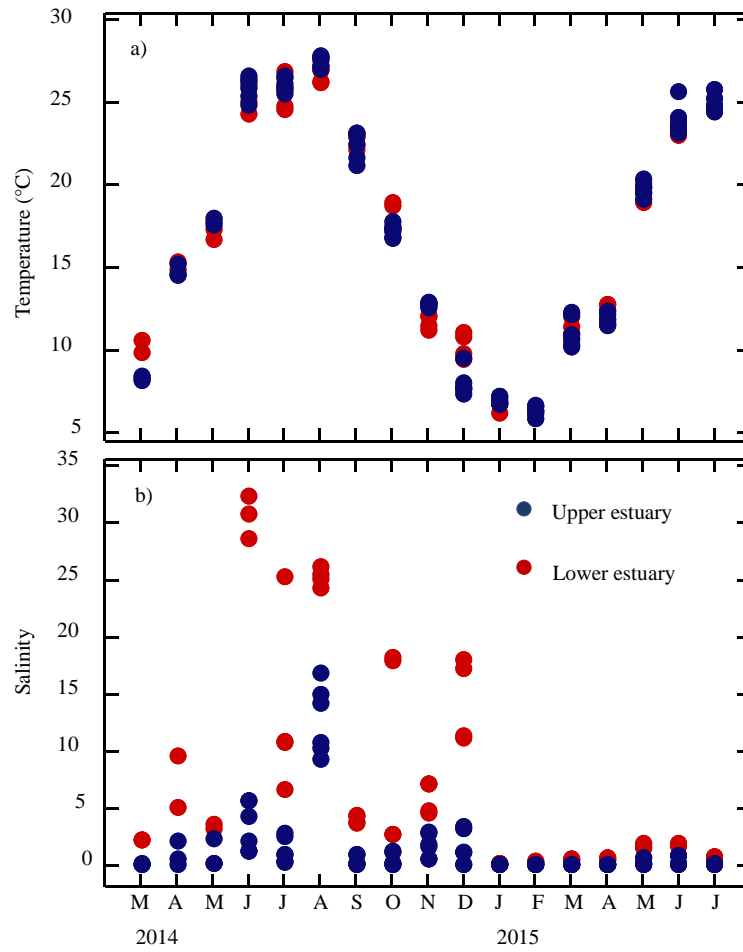


Fig. 3-1 Temporal changes in bottom temperature and salinity  
 Temporal changes in bottom temperature (a) and salinity (b) in the upper estuary (dark blue closed circles) and the lower estuary (red closed circles).

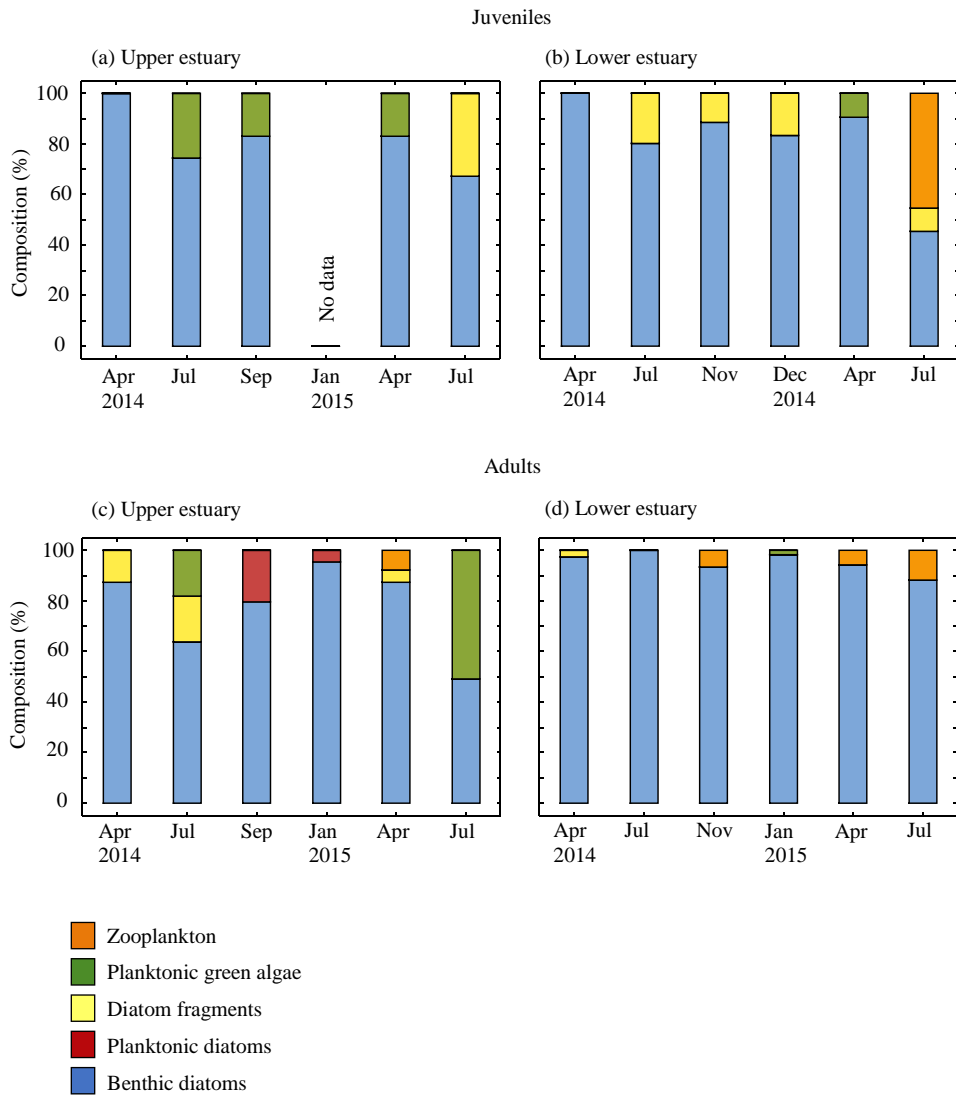


Fig. 3-2 Composition of the stomach contents of *Neomysis awatschensis*.

Table 3-1. Composition of the stomach content of juvenile *Neomysis awatschensis* (in % of coverage area). Letters represent sampling month: a: April 2014, b: July 2014, c: September 2014, d: December 2014, e: April 2015, f: July 2015. *n* is the sample size.

Taxon <i>n</i>	Upper estuary						Lower estuary					
	a	b	c	d	e	f	a	b	c	d	e	f
	3	10	4	-	9	8	7	10	6	10	10	10
Diatoms (benthic)												
<i>Fragilaria</i> spp.	0	0	17	-	0	0	0	0	0	0	0	0
<i>Cymbella</i> sp.	0	0	0	-	17	0	0	0	0	0	0	0
<i>Diploneis</i> spp.	0	0	0	-	0	0	0	0	0	9	0	0
<i>Gophonema</i> spp.	0	0	0	-	17	0	0	0	0	0	0	0
<i>Navicula</i> spp.	17	0	0	-	17	17	40	20	44	25	36	0
<i>Achnantheidium</i> spp.	0	0	0	-	0	0	0	0	0	0	9	0
<i>Cocconeis placentula</i>	17	0	0	-	0	0	0	0	0	0	0	0
<i>Cocconeis</i> spp.	0	0	0	-	0	33	0	10	0	0	0	0
<i>Planothidium</i> spp.	0	0	0	-	0	0	30	10	0	0	9	0
<i>Bacillaria paxillifer</i>	66	75	66	-	32	17	30	40	44	50	36	46
Diatom fragments	0	0	0	-	0	33	0	20	12	16	0	9
Planktonic green algae												
Euglenophyceae	0	0	0	-	0	0	0	0	0	0	10	0
<i>Scenedesmus arcuatus</i>	0	0	17	-	0	0	0	0	0	0	0	0
<i>Scenedesmus</i> sp.	0	25	0	-	0	0	0	0	0	0	0	0
<i>Staurastrum</i> spp.	0	0	0	-	17	0	0	0	0	0	0	0
Zooplankton												
<i>Brachionus</i> spp. (rotifer)	0	0	0	-	0	0	0	0	0	0	0	36
Oligochaeta (bristles)	0	0	0	-	0	0	0	0	0	0	0	9
	100	100	100		100	100	100	100	100	100	100	100

Table 3-2. Composition of the stomach content of adult *Neomysis awatschensis* (in % of coverage area). Letters represent sampling month: a: April 2014, b: July 2014, c: September 2014, d: December 2014, e: April 2015, f: July 2015. *n* is the sample size.

Taxon <i>n</i>	Upper estuary						Lower estuary					
	a	b	c	d	e	f	a	b	c	d	e	f
	5	3	3	8	10	3	6	6	5	7	10	4
Diatoms (benthic)												
Diatomaceae	0	0	0	5	0	0	0	0	0	0	0	0
<i>Fragilaria</i> spp.	0	0	0	5	0	0	0	0	0	0	0	0
<i>Amphora</i> spp.	0	0	0	0	3	0	3	28	0	0	0	0
<i>Caloneis</i> spp.	0	0	0	0	0	0	0	0	0	7	0	0
<i>Cymbella tumida</i>	0	0	0	0	3	0	0	0	0	0	0	0
<i>Cymbella turgidula</i>	0	0	0	0	3	0	0	0	0	0	0	0
<i>Diploneis</i> spp.	0	0	0	0	5	10	3	0	0	0	6	0
<i>Encyonema</i> spp.	13	0	0	0	10	0	0	0	0	0	6	0
<i>Fallacia</i> sp.	0	0	0	0	0	0	0	0	0	7	0	0
<i>Gomphonema</i> spp.	13	0	0	0	3	0	0	0	0	2	0	0
<i>Navicula</i> spp.	0	0	0	8	27	0	39	14	25	72	16	6
<i>Rhoicosphenia abbreviata</i>	13	0	0	0	3	0	0	0	0	0	6	0
<i>Rhoikoneis</i> sp.	0	0	0	0	0	0	0	0	0	0	6	0
<i>Achnanthydium</i> sp.	13	0	0	0	5	0	0	14	0	0	0	0
<i>Cocconeis placentula</i>	0	0	0	5	0	0	0	0	0	2	0	0
<i>Cocconeis scutellum</i>	0	18	0	0	0	0	0	0	0	0	0	0
<i>Cocconeis</i> spp.	0	0	0	0	0	0	0	14	6	0	6	0
<i>Planothydium</i> spp.	0	0	0	0	5	0	31	0	12	0	16	6
<i>Bacillaria paxillifer</i>	35	46	80	67	18	40	20	30	50	8	26	76
<i>Nitzschia</i> spp.	0	0	0	5	5	0	0	0	0	0	6	0
Diatoms (planktonic)												
Thalassiosiraceae	0	0	20	0	0	0	0	0	0	0	0	0
<i>Melosira varians</i>	0	0	0	5	0	0	0	0	0	0	0	0
Diatom fragments	13	18	0	0	5	0	3	0	0	0	0	0
Planktonic green algae												
<i>Lepocinlis salina</i>	0	0	0	0	0	10	0	0	0	0	0	0
<i>Lepocinlis salina</i>	0	0	0	0	0	10	0	0	0	0	0	0
<i>Monoraphidium</i> spp.	0	0	0	0	0	10	0	0	0	2	0	0
<i>Oedogonium</i> sp.	0	18	0	0	0	10	0	0	0	0	0	0
<i>Cosmarium</i> spp.	0	0	0	0	0	10	0	0	0	0	0	0
Zooplankton												
Nostocales	0	0	0	0	5	0	0	0	0	0	0	0
Polychaeta (bristles)	0	0	0	0	0	0	0	0	0	0	0	6
Oligochaeta (bristles)	0	0	0	0	3	0	0	0	6	0	6	6
	100	100	100	100	100	100	100	100	100	100	100	100

Although diatoms were observed in the stomach of adults, no apparent temporal trends in both parts of the estuary could be drawn (Figs 3-2c–d, Table 3-2). In the upper estuary, benthic diatoms (e.g. *Bacillaria paxillifer* and *Navicula* spp.) accounted for 40–90% of the stomach contents whereas they accounted for 80–100% in the lower estuary. Planktonic diatoms (i.e. Thalassiosiraceae and *Melosira varian*) were only observed in the upper estuary and accounted for 4–20% of the stomach contents. Unknown diatom fragments accounted for 5–20% of the stomach contents in the upper estuary and were scanty in the lower estuary.

Planktonic green algae (e.g. *Oedogonium* spp. and *Lepocinclis salina*) accounted for 18–50% in the upper estuary but were rarely observed in the lower estuary. Zooplankton were hardly observed and fragments of oligochaeta and polychaeta accounted for 7% of the stomach content in the upper estuary (April 2015) and 6–11% in the lower estuary.

### 3.3.3. Scanning electron microscopy analysis

Benthic diatoms (e.g. *Bacillaria paxillifer* and *Navicula* spp.) were observed in the stomach of adult mysid *N. awatschensis* for all seasons and across the whole estuary (Figs 3-3a–b). Planktonic diatoms (i.e. Thalassiosiraceae and *Melosira varian*) were observed only in the stomach of adults across the whole estuary (Figs 3-3c–d). Stomachs of both juveniles and adults were always full of food items, irrespective of the time of day, the sampling month or the site (Fig. 3-3e). Zooplankton such as polychaeta were scanty in the stomach of *N. awatschensis* (Fig. 3-3f).

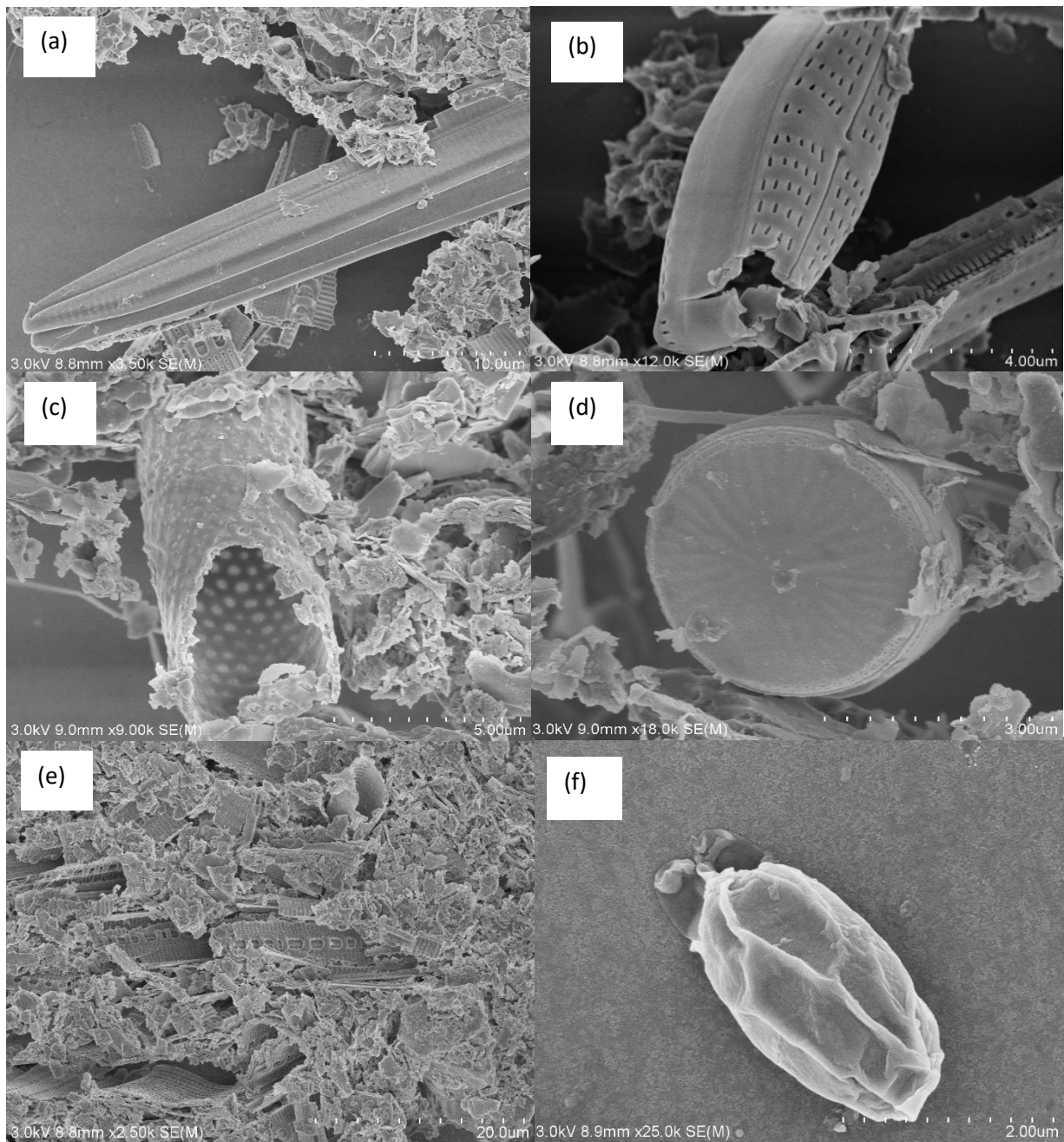


Fig. 3-3 Micrographs of the stomach content of *Neomysis awatschensis*.  
Micrographs of the stomach content of *Neomysis awatschensis* under a scanning electron microscope showing: - *Bacillaria paxillifer* (a), *Navicula* spp. (b), *Melosira varian* (c), Thalassiosiraceae (d), unidentified diatom fragment (e) and unidentified zooplankton (f).

### 3.3.4. Seasonal and spatial shifts in isotopic values of potential food sources

All the potential food sources presented year to year variations and large seasonal differences in terms of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values (Figs 3-4a-l). BMAel was the most enriched food source in terms of  $\delta^{13}\text{C}$  values across all seasons and sites, whereas POMr was the most depleted one with  $\delta^{13}\text{C}$  mean values of  $-28.7\text{‰}$  in spring 2014.

The  $\delta^{15}\text{N}$  mean values of BMAep were highly variable across all the seasons, having the most enriched value of  $9.9\text{‰}$  in spring 2014 and most depleted value of  $2.8\text{‰}$  in summer 2015. The  $\delta^{15}\text{N}$  values of BMAel ranged from 6 to  $8\text{‰}$  as those of POMm, and POMr remained in the range of  $3\text{--}7\text{‰}$  across all the seasons.

### 3.3.5. Variability in isotopic ratios of *N. awatschensis*

In the upper estuary, the  $\delta^{13}\text{C}$  mean values for juveniles were significantly different between summer 2014 ( $-24.0\text{‰}$ ) and summer 2015 ( $-25.9\text{‰}$ ) (Kruskal-Wallis test and Conover *post-hoc* test,  $p < 0.05$ , Fig. 3-5a). In the lower estuary, significant differences appeared among seasons (Fig. 3-5b). In the upper estuary, the  $\delta^{13}\text{C}$  mean values for adults were significantly different among seasons (Kruskal-Wallis test and Conover *post-hoc* test,  $p < 0.05$ , Fig. 3-5e). In the lower estuary, the  $\delta^{13}\text{C}$  mean values of adults were significantly enriched in spring 2014 ( $-18.4\text{‰}$ ) and depleted in summer 2015 ( $-24.0\text{‰}$ , Fig. 3-5f).

For juveniles the  $\delta^{13}\text{C}$  mean values were significantly different between the upper estuary and the lower estuary in both summer 2014 and 2015 (Wilcoxon Signed-Rank Test, Table 3-3). The same significant trend between the upper and the lower parts of the estuary was observed for the  $\delta^{13}\text{C}$  mean values for adults in all seasons.

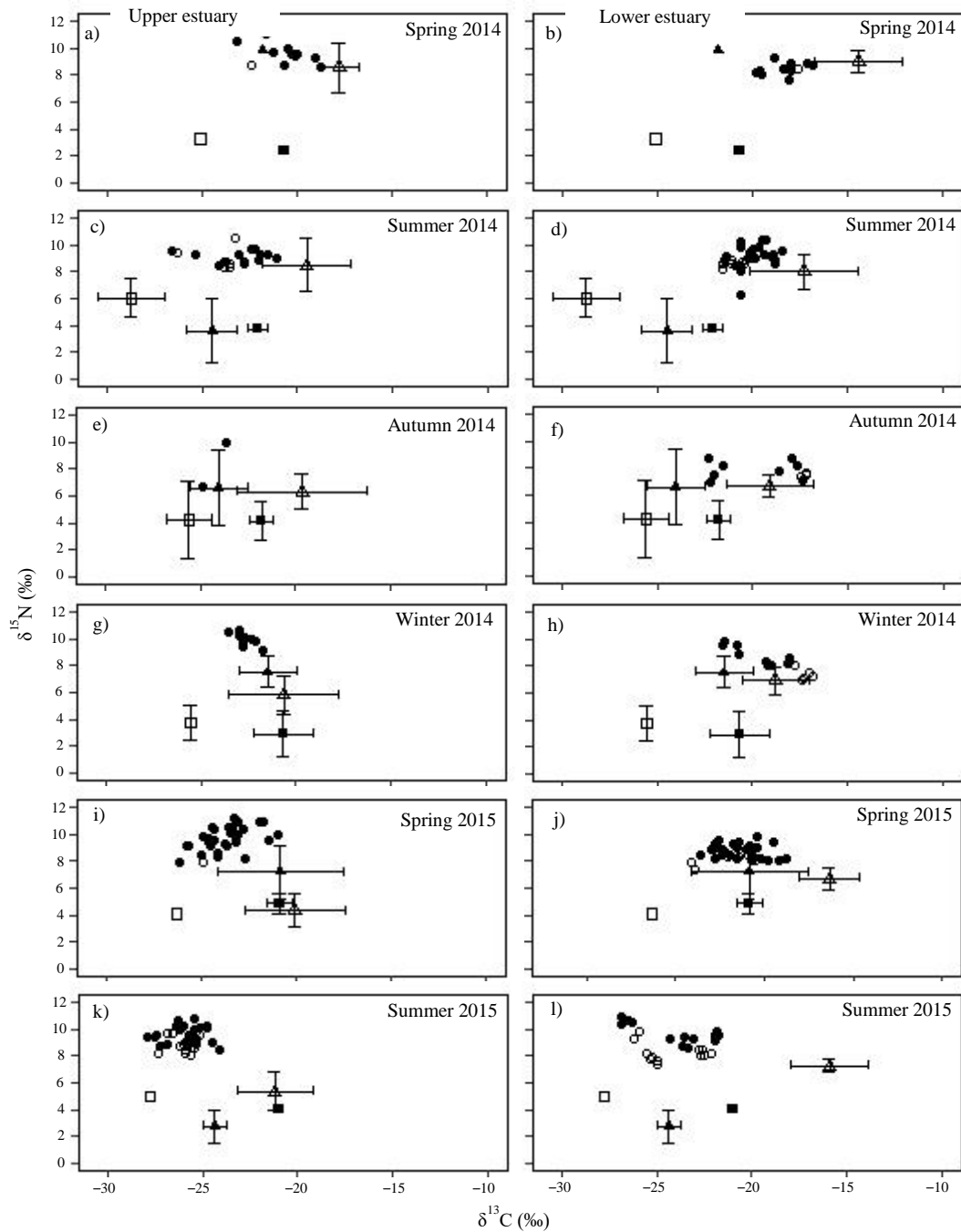


Fig. 3-4 Biplot for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  of *Neomysis awatschensis* and its potential food  
 Biplot for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  of *Neomysis awatschensis* and its potential food sources. Open circles represent juveniles, closed circles adults, open triangles epilithic microalgae, closed triangles epipellic microalgae, open squares riverine particulate organic matter and closed squares marine particulate organic matter. Error bar represent standard deviation



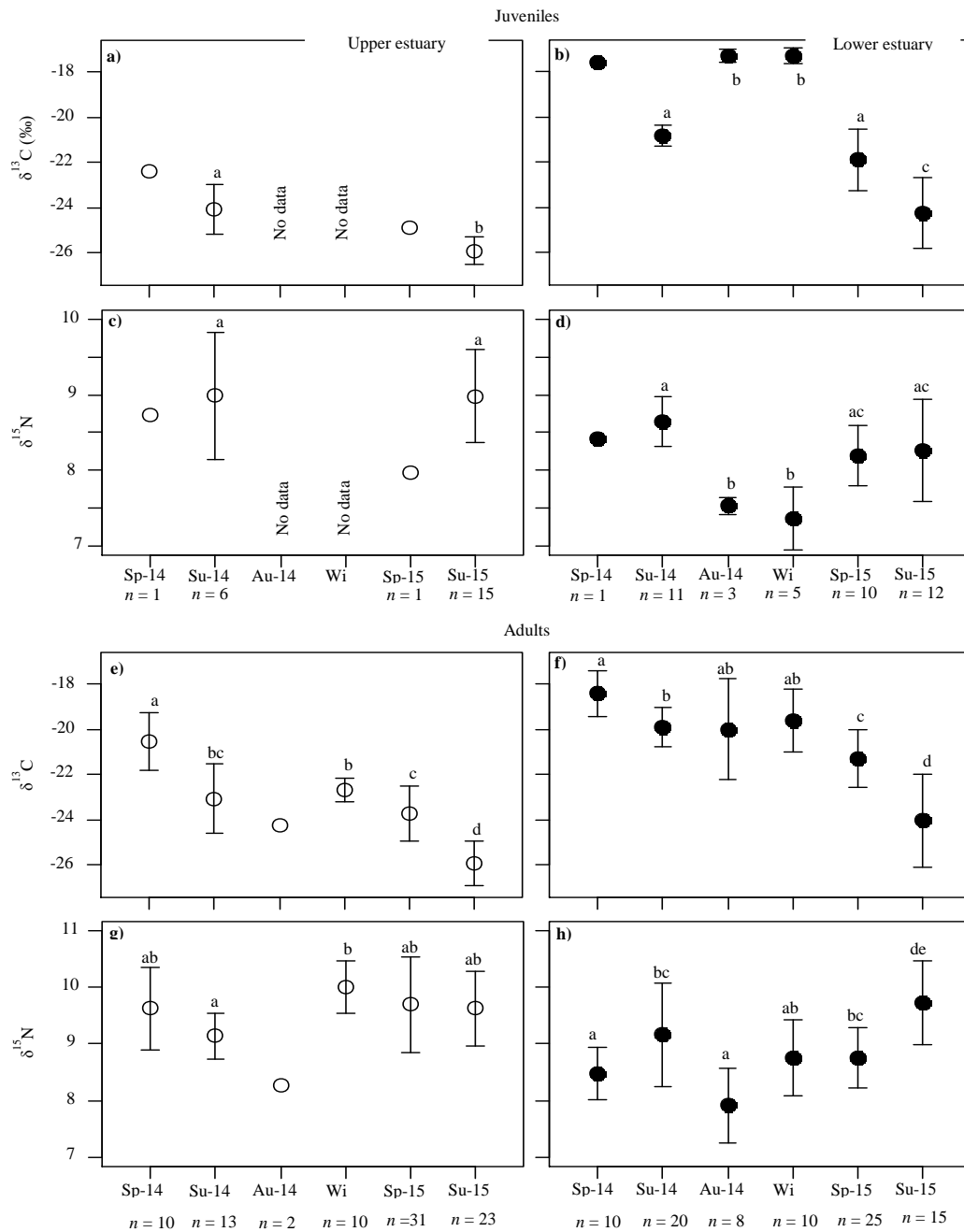


Fig. 3-5 Seasonal changes of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  (Mean  $\pm$  SD) in *Neomysis awatschensis*. Juveniles (a–d) and adults (e–h) sampled in the Yura River estuary. Open and closed circles indicate the upper and the lower parts of the estuary respectively. Significant differences are indicated by different letters above each error bar (Kruskal-Wallis Test and Conover *post-hoc* Test,  $p < 0.05$ ). Sp-14 is spring 2014; Su-14 is summer 2014; Au is autumn 2014; Wi is winter 2014; Sp-15 is spring 2015 and Su-15 is summer 2015.  $n$  is sample size.

Table 3-3. Wilcoxon Signed-Rank Test for seasonal differences between the upper and the lower part of the estuary in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  for *Neomysis awatschensis* collected in the Yura River estuary, Sample sizes are shown in brackets.

Season	Variable (‰)	Estuary		<i>p</i> -value
		Upper	Lower	
<b>Juveniles</b>				
Summer 2014	$\delta^{13}\text{C}$	- 24.0 (06)	- 20.8 (11)	<0.05
Summer 2015	$\delta^{13}\text{C}$	- 25.9 (15)	- 24.2 (12)	<0.05
Summer 2014	$\delta^{15}\text{N}$	8.9 (06)	8.6 (11)	>0.05
Summer 2015	$\delta^{15}\text{N}$	8.9 (15)	8.2 (12)	<0.05
<b>Adults</b>				
Spring 2014	$\delta^{13}\text{C}$	- 20.5 (10)	- 18.4 (10)	<0.05
Summer 2014	$\delta^{13}\text{C}$	- 23.0 (13)	- 19.8 (20)	<0.05
Winter 2014	$\delta^{13}\text{C}$	- 22.6 (10)	- 19.6 (10)	<0.05
Spring 2015	$\delta^{13}\text{C}$	- 23.7 (31)	- 21.2 (25)	<0.05
Summer 2015	$\delta^{13}\text{C}$	- 25.9 (23)	- 24.0 (15)	<0.05
Spring 2014	$\delta^{15}\text{N}$	9.6 (10)	8.5 (10)	<0.05
Summer 2014	$\delta^{15}\text{N}$	9.1 (13)	9.1 (20)	>0.05
Winter 2014	$\delta^{15}\text{N}$	10.0 (10)	8.7 (10)	<0.05
Spring 2015	$\delta^{15}\text{N}$	9.7 (31)	8.7 (25)	<0.05
Summer 2015	$\delta^{15}\text{N}$	9.6 (23)	9.7 (15)	>0.05

In the upper estuary the  $\delta^{15}\text{N}$  mean values for juveniles were not significantly different between seasons in the upper estuary, whereas, in the lower estuary there were significant differences among the seasons. However, in the lower estuary, they were significantly different e.g summer 2014 (8.7‰) was different from winter 2014 (7.3‰) (Kruskal-Wallis test and Conover *post-hoc* test,  $p < 0.05$ , Fig. 3-5d). In the upper estuary, the  $\delta^{15}\text{N}$  mean values for adults were significantly different between summer 2014 (9.1‰) and winter 2014 (10.0‰). In the lower estuary in autumn 2014 (7.9‰)  $\delta^{15}\text{N}$  mean values were lower than in summer 2015 (9.7‰). For juveniles the  $\delta^{15}\text{N}$  mean values were significantly different between the upper estuary and the lower estuary in summer 2014 (Wilcoxon Signed-Rank Test, Table 3-3), but no differences were recorded in summer 2015. For adults, the  $\delta^{15}\text{N}$  mean values were significantly different between upper estuary and the lower estuary in winter 2014 (Wilcoxon Signed-Rank Test, Table 3-3) as well as in spring 2014 and 2015.

### 3.3.6. Contribution of potential food sources to the diet of *N. awatschensis*

The two sensitivity analysis scenarios produced almost identical results in the estimation of the contribution of potential food sources to the diet of *N. awatschensis* (Figs 3-6 and 3-7). In general, POMm and POMr were the main contributors in the lower and upper parts of the estuary. However, in 2015, the contribution of POMm decreased, when the contribution of POMr increased.

In juveniles, POMm contributed more than the three other potential food sources in summer and winter 2014 (Fig. 3-6). No difference in contribution was observed among the four potential food sources in spring 2014 and 2015 (upper estuary), autumn and winter 2014 (lower estuary).

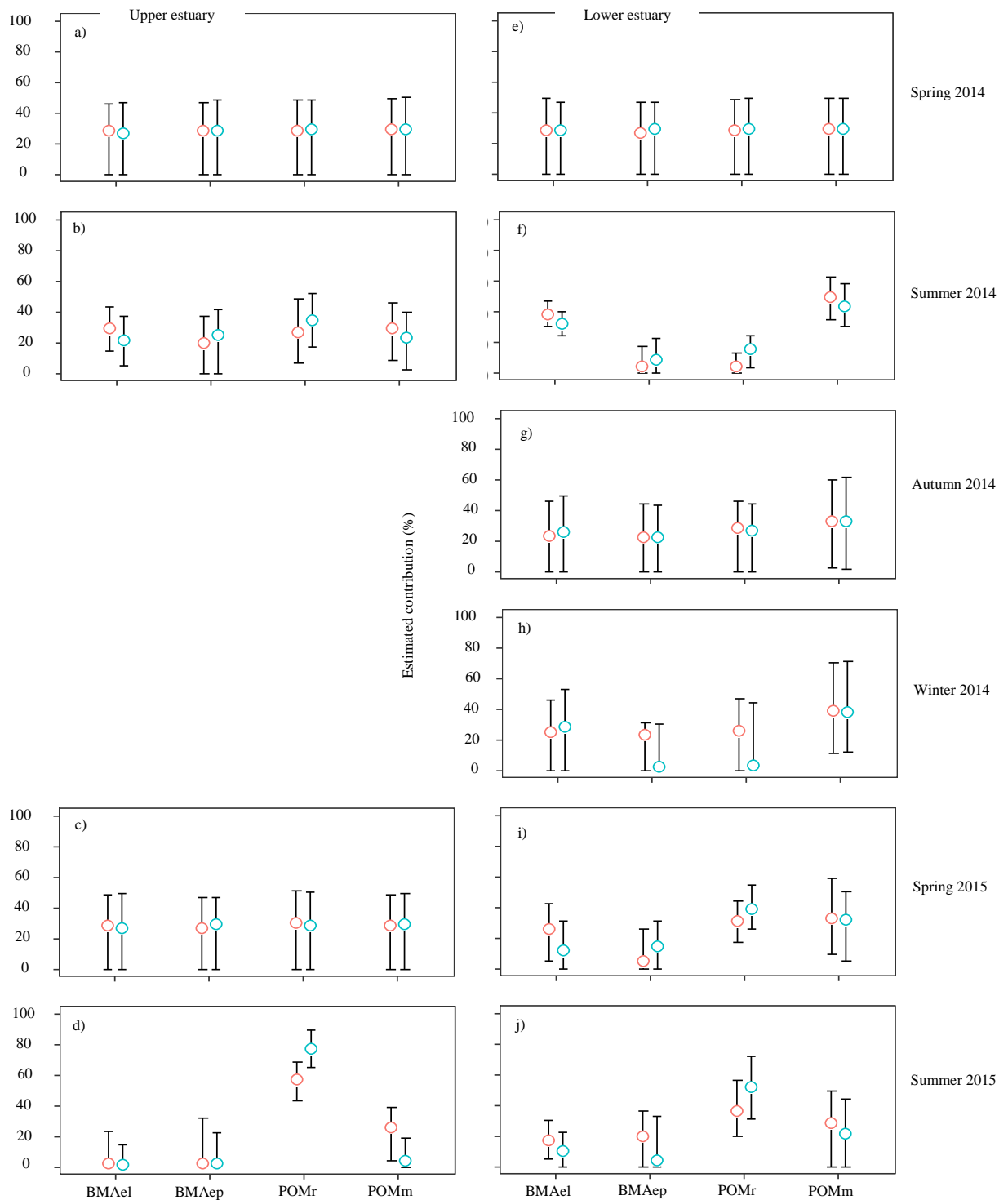


Fig. 3-6 Contribution of potential food source to the diet of juveniles. Seasonal changes in contribution of the potential food sources to the diet of *Neomysis awatschensis* juveniles estimated from stable isotope analysis. Red circles represent modes of scenario 1 and blue circles represent modes of scenario 2 using  $\delta^{13}\text{C}$  trophic enrichment factors of  $-0.73$  and  $0.5$ , respectively. Error bars represent the 95% credibility interval.

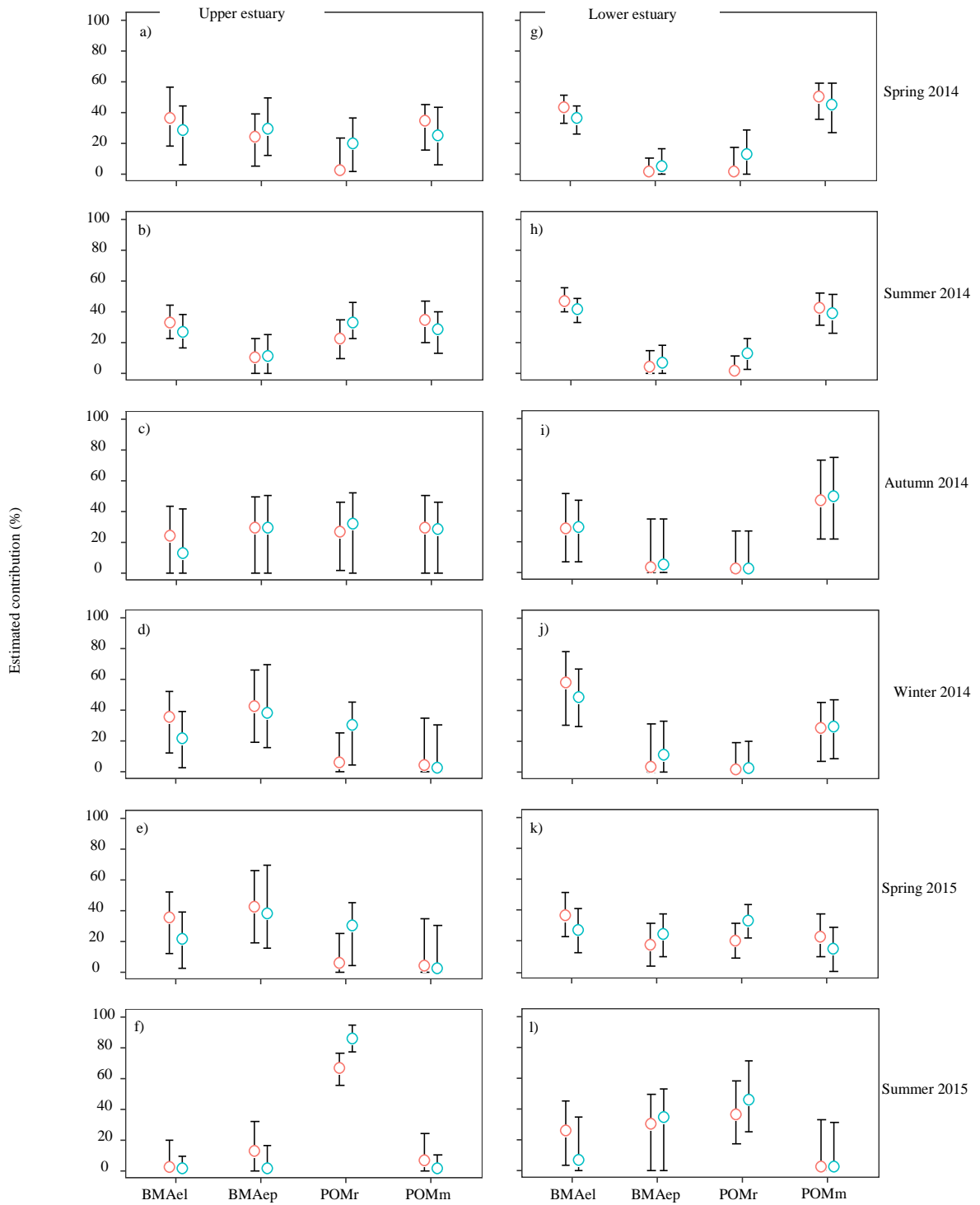


Fig. 3-7 Contribution of potential food sources to the diet of adults. Seasonal changes in contribution of the potential food sources to the diet of *Neomysis awatschensis* adults estimated from stable isotope analysis. Red circles represent modes of scenario 1 and blue circles represent modes of scenario 2 using  $\delta^{13}\text{C}$  trophic enrichment factors of  $-0.73$  and  $0.5$ , respectively. Error bars represent the 95% credibility interval.

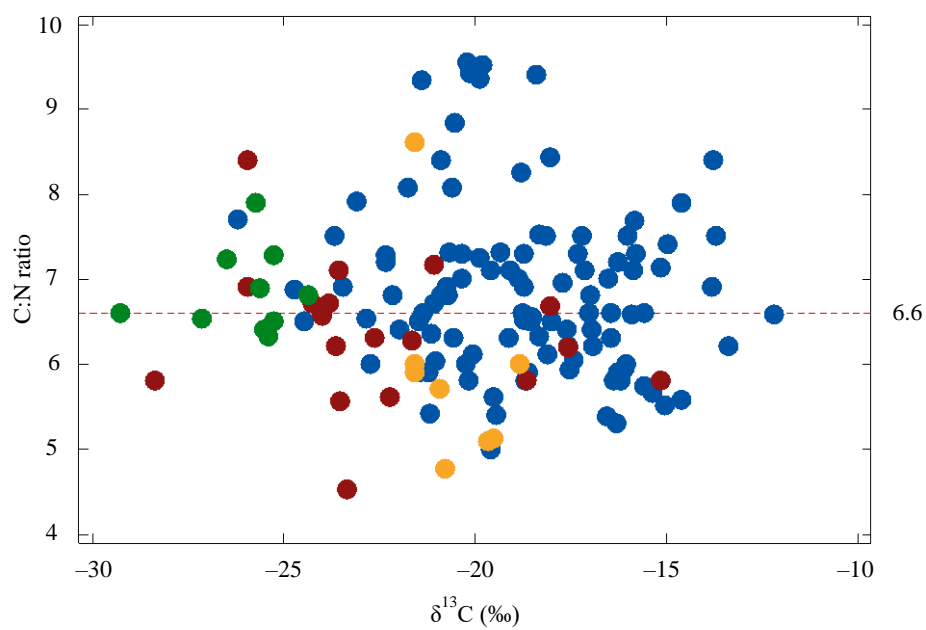


Fig. 3-8 The C:N ratios.

The C:N ratios compared with  $\delta^{13}\text{C}$  of potential food sources. Blues circles represent BMAel, red circles BMAep, green circles POMr and orange circles POMm. The red dotted line is the Redfield C: N ratio average (Redfield, 1963).

The greatest contribution of POMm (mode: 50%) was found in the lower estuary in summer 2014 (Fig. 3-6f). In contrast, the contribution of POMr exceeded that of POMm, with its highest contribution (mode: 60%) in the upper estuary in summer 2015 (Fig. 3-6d).

In adults, POMm and BMAel usually contributed more than the other two potential food sources in both the upper and lower parts of the estuary in 2014 (mode: 30–50%, Fig. 3-7). In 2015, POMr replaced POMm as the main contributor, with its highest value (mode: 80%) in the upper estuary.

The C:N ratios of BMAel  $7.9 \pm 1.1$  (mean  $\pm$  SD), BMAep  $7.4 \pm 0.9$ , POMr  $7.9 \pm 0.5$  and POMm  $6.8 \pm 1.3$  are distributed around the Redfield ratio of 6.6 (Fig. 3-8).

### **3.4. Discussion**

#### *3.4.1. Stomach contents*

Diatoms were the most dominant food items in the stomach contents of *Neomysis awatschensis* occurring in the Yura River estuary, irrespective of the season, location or life stage. Diatoms mainly comprised of benthic species (e.g. *Bacillaria paxillifer* and *Navicula* spp.) and to a lesser extend planktonic species (i.e. Thalassiosiraceae and *Melosira varian*). These observations are consistent with previous works on other mysid species such as *Neomysis mercedis* which ingests mainly benthic species (*Bacillaria* spp.) among other algae species in the San Francisco Bay-Delta estuary (Siegfried and Kopache, 1980) and *Neomysis americana* which consumes benthic diatoms species (*Navicula* sp.) in the tidal creeks North Inlet, South Carolina (Zagursky and Feller, 1985). Considering that the observation of stomach content is a “snapshot” analysis, providing information on the ingested food over a short time frame (Vander Zanden *et al.*, 1999; Galván *et*

*al.*, 2012), the results suggested that *N. awatschensis* ingests diatoms from both the substratum and to a lesser extent the water column, along the Yura River estuary. This finding is consistent with the results of Katayama *et al.* (2011) on the near-bottom distribution of *N. awatschensis* during day-time feeding on benthic diatoms. Whereas some individuals moved to the surface and middle layers at night-time, where they might feed on planktonic phytoplankton. The relative occurrence of planktonic diatom species compared to benthic species in the stomach of mysid caught during the day can be attributed to the fact that only few planktonic diatoms might sink to the sediment surface (Kasim and Mukai, 2006), which might be consumed by *N. awatschensis*. In addition, considering that sampling occurred at different times during the day and all stomachs were full, *N. awatschensis* is thought to feed continuously throughout the day. This result concurs with the finding of Katayama *et al.* (2011).

#### 3.4.2. Stable isotope analyses

The two stringent scenarios of the sensitivity analysis were used and generated almost identical results, therefore the results of this study using the stable isotope analysis is thought to be reliable. Stable isotope results indicated that both BMA and POM contributed to the diet of *N. awatschensis*. The C:N ratios of BMA and POM suggested that this potential food sources might mainly be composed of phytoplankton. Indeed their C:N ratios are in the order of 6.6 (i.e Redfield ratio) the average phytoplankton C:N ratio in molar base (Redfield, 1963; Thomas *et al.*, 1999). The results of the stable isotope analysis appear to be in contradiction with the previous results of the stomach content analysis, in which benthic diatoms dominated. However, the integration time of the information provided by both the stomach content and the stable isotope analysis is different: the first reports food source contribution at the time of sampling (i.e snapshot),



while the latter reports the food source contribution over a couple of months (Vander Zanden *et al.*, 1999; Galván *et al.*, 2012; Phillips *et al.*, 2014). The higher contribution of phytoplankton to the diet detected by stable isotope analysis might be because of *N. awatschensis* migration to water column and the surface where it can feed on phytoplankton. Phytoplankton might be digested fast enough not to be observed in the mysid collected during the day and feeding mainly on benthic diatoms. This hypothesis is supported by several observations of diel vertical migration for food in different mysid species e.g. *Neomysis mercedis*, *N. awatschensis* and *Orientomysis mitsukurii* (Murtaugh, 1989; Katayama *et al.*, 2011; Takahashi *et al.*, 2017).

Most of the year, *N. awatschensis* depended on BMA for nutrition but would shift its diet when other food sources are available. Indeed, the origin of POM changes in response to the prevailing environmental conditions in the Yura River estuary. Marine phytoplankton were accessible to *N. awatschensis* when salt-wedge condition appeared in the estuary (Kasai *et al.*, 2010; Funahashi *et al.*, 2013) and densities were always higher in the lower estuary than in the upper estuary. In contrast, riverine phytoplankton were available when there was almost no saline water intrusion even in the lower estuary due to the clogging of the river mouth (Omweri *et al.*, 2017). As a result, *N. awatschensis* depended on marine phytoplankton in summer 2014, whereas in summer 2015 it had to settle for riverine phytoplankton (Figs 3-6f and i) In winter 2014, juveniles of *N. awatschensis* depended mainly on marine phytoplankton, while adults depended mainly on both BMA and marine phytoplankton. When the water is cold in the Yura River estuary, the density of phytoplankton decreases (Watanabe *et al.*, 2014). Seasonal diet shifts according to food availability have been recorded in other mysid species (Winkler *et al.*, 2007; Vilas *et al.*, 2009). Although, the stable isotope results may not always be accurate in reconstructing multiple potential food source diets and possible overlapping of food source isotopic signature (Franco-Trecu *et al.*, 2013; Phillips *et al.*, 2014).

In addition, in the present research, some sample for juvenile was small (n<5) limited further estimation of the food contribution. But they provide good insight on the type of ingested food over a long-time frame compared to the observation of stomach contents.

Ontogenetic changes in the diet composition of mysid were not observed in *N. awatschensis*. Because diatoms are readily available in the sediments and can be accessed both by juveniles and adults. In contrast, ontogenetic changes in diet have been reported in *Mysis diluviana* (Whall and Lasenby, 2009). Hence, the feeding habit of different mysid species is most likely to be species-specific.

#### 3.4.3. Herbivorous feeding habit

The present study on the mysid of the Yura River estuary suggests that *N. awatschensis* mainly ingests benthic and planktonic microalgae. Hence, the mysid *N. awatschensis* can certainly be described as an herbivores feeder. A previous study also indicates that *N. awatschensis* in Kasumigaura lagoon primarily consumes phytoplankton (Toda and Wada, 1990). Other species of the genus *Neomysis* (Czerniavsky, 1882) are considered as omnivorous feeders, capable of utilizing a variety of food sources such as phytoplankton, microzooplankton and small invertebrates (Takahashi, 2004; Vilas *et al.*, 2009; Porter, 2016).

### 3.5. Conclusion

The combination of these two independent methods could provide meaningful insight as to understand the feeding habits of *N. awatschensis* distributed in the Yura River estuary. This species is an herbivorous feeder, primarily feeding on benthic and planktonic microalgae. Marine microalgae are available when salt-wedge conditions appear in the estuary, whereas riverine

particulate organic matter which is mainly composed of phytoplankton would be accessible when the river mouth was clogged.



## Chapter 4

### General discussion and summary

The present study aims to clarify ecology and production dynamics of the mysid *Neomysis awatschensis* occurring in the Yura River estuary. The specific objectives are (1) to investigate the population structures of *N. awatschensis* in relation to the fluctuating environmental conditions and (2) to determine the feeding habits of *N. awatschensis* in the Yura River estuary.

#### 4.1. Major findings

In chapter two, the research results revealed that *N. awatschensis* is dominant species in the mysid community in the estuary consisting of 98% of the total mysid catches and that its reproduction is partially suppressed by variable and oligotrophic conditions in the Yura River estuary. Combined with the flexibility to seasonal changes in temperature and food availability, the great tolerance to acute salinity fluctuations allows *N. awatschensis* to maintain its population in the estuary.

Chapter three describes feeding habits. Stomach content analysis indicated that benthic diatoms such as *Bacillaria paxillifer* and *Navicula* spp. are the main food items of *N. awatschensis* irrespective of the season, the locality and the life stage. On the contrary, the stable isotope analysis and subsequent Bayesian mixing model which allow for an approximate estimate of long-term feeding habit of *N. awatschensis* indicated that BMA and POM were the main contributors.

Considering that BMA and POM had C:N ratios close to the Redfield ratio, *N. awatschensis* would depend on both benthic and planktonic microalgae for nutrition. The apparent discrepancy between the stomach content and stable isotope analysis can be explained if *N. awatschensis* feeds on benthic microalgae during daytime and on planktonic microalgae at nighttime. This hypothesis is supported by previous studies that reported diel changes in distribution and feeding in *N. awatschensis* (Katayama *et al.*, 2011) and *Orientomysis mitsukrii* (Takahashi *et al.*, 2017). The contribution of marine phytoplankton to the diet of *N. awatschensis* increased under salt-wedge intrusion, whereas without salt-wedge intrusion, riverine phytoplankton replaced marine phytoplankton.

#### **4.2. Survival strategies of *N. awatschensis* in the Yura River estuary**

Despite variable and oligotrophic conditions in the Yura River estuary, the densities of the mysid *N. awatschensis* occurring in the Yura River estuary always peaked in the early summer. The current investigations revealed that the female *N. awatschensis* produced many juveniles into the population when water temperature conditions were favourably moderate and biological productivity was at its highest to maintain its population in the estuary. The population growth of *N. awatschensis* is thought to be triggered by the seasonal relaxation of harsh winter conditions, such as high river discharge low food availability and low temperatures. Seasonal changes in water temperature is a well-known condition that influences reproduction and growth of mysid in estuaries (Mauchline, 1980; David *et al.*, 2005; Yamada *et al.*, 2007; Bae *et al.*, 2016). The density peak of *N. awatschensis* in early summer is supported by the combination of high egg production rate of large-sized females and low mortality rate of juveniles. Low temperature could suppress reproduction in winter, whereas in summer high temperatures could increase the mortality of juveniles and subsequent low population density to next early spring. Even if temperature is

favorable in autumn 2014, decreasing temperature and low chlorophyll might not be suitable for reproduction. In addition, growth is more pronounced in warmer seasons.

The present research results have demonstrated that the mysid *N. awatschensis* occurring in the Yura River estuary is tolerant to a wide range of salinity conditions i.e. 0–32, the results could suggest that the mysid can survive in this estuary, irrespective of the sudden salinity changes characterized in the estuary due to the salt-wedge regime (Kasai *et al.*, 2010; Funahashi *et al.*, 2013). For example; previous studies revealed that generally mysids have species specific suitable salinity ranges (Mees *et al.*, 1994; Rappé *et al.*, 2011; Bae *et al.*, 2016). Therefore, the wide acceptable salinity range for *N. awatschensis* may allow this species to be the dominant mysid as the major primary consumer in river estuaries where physical conditions are greatly variable.

In the hyper-eutrophic Kasumigaura lagoon, *N. awatschensis* production was high mainly due to stable and favorable environmental conditions prevalent through the year (Toda *et al.*, 1982, 1983; Toda and Wada, 1990; Soma *et al.*, 1995; Yoshimura *et al.*, 2012). In comparison, the Yura River estuary is high variables and oligotrophic, the production of *N. awatschensis* relatively low due to unstable and low primary production (Watanabe *et al.*, 2014) in this estuary.

The variability in Yura River discharge contributed to the seasonality of *N. awatschensis* densities observed during the present study. Other studies revealed that sustained river flows contributed substantially to seasonal and/or spatial distribution patterns of zooplanktons in estuaries (Jerling and Wooldridge, 1995; Grange *et al.*, 2000).

Although *N. awatschensis* is primarily an herbivorous feeder, it depends on both benthic and planktonic algae for nutrition. In response to the prevailing environmental conditions in the estuary, it can interchangeably utilize riverine and marine phytoplankton. Such flexible feeding habits are advantageous characteristics to *N. awatschensis* inhabiting in the variable and oligotrophic Yura River estuary.

The Mysid *N. awatschensis* has been recorded in the gut contents of juvenile temperate seabass *Lateolabrax japonicus* (Fuji *et al.*, 2010, 2014, 2016) and other fishes (Antonio *et al.*, 2010, 2012; Aoki *et al.*, 2014) in the estuary. This suggests that *N. awatschensis* is important as a trophic link between primary producers and secondary consumers in the Yura River estuary, mainly because it is a single species of dominant zooplankton community withstanding the variable and oligotrophic conditions in the present research area.

### **4.3. Study limitations and future works**

The current study was conducted only during the daytime, information on the vertical migration of *N. awatschensis* is required to fully understand the diel vertical migration and diel change of feeding habit of this species in the estuary. Monthly sampling campaigns yielded substantial information in the present study. However, river discharges influenced mysid densities and recruitment of juveniles in a shorter timing. At least weekly sampling is thought to be ideal to analyze detailed mechanisms of population dynamics in mysid.

Gut content analyses provided snapshots of the food ingested by *N. awatschensis*, while stable isotope analysis provides information on feeding habit over a longer period, there is no evidence that predicted prey items are really consumed. Other methods to estimate feeding habit such as fatty acid analysis and stable isotope analysis of amino acids (Alfaro *et al.*, 2006; Ando and Nozaki, 2007) could give further supporting information on the types of foods ingested by this mysid.



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