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Feeding ecology of a mysid species, *Neomysis awatschensis* in the Lake Kasumigaura: combining approach with microscopy, stable isotope analysis and DNA metabarcoding

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Abstract: *Neomysis awatschensis* is an important prey item for various fishes in Lake Kasumigaura, Japan. There is, however, a contradiction concerning the major food sources of this mysid species: whether "bottom mud", "particulate organic matter (POM) including phytoplankton", or "mesozooplankton" is the main diet, and this uncertainty may be due to differences in the methodology used to determine the prey items in previous studies. This study examined the main food sources of *N. awatschensis* by combining three methods to eliminate methodological biases: DNA metabarcoding, microscopy and stable isotope analysis. Planktonic diatoms and green algae sequences were the main taxa detected by DNA metabarcoding and microscopy on the fecal pellets. The δ^{15}N values of the mysids were similar to those of phytoplankton feeders rather than carnivorous planktonic crustaceans. These results suggest that diatoms and green algae were the major food sources for *N. awatschensis* in Lake Kasumigaura during the investigation period, and that its trophic level is as low as that of herbivores. However, the partial contribution of other "POM" (e.g., benthic diatoms) is also implied, considering the dispersion in δ^{13}C values. Mesozooplankton prey, such as copepods, were detected both using DNA metabarcoding and microscopy, but they could not be a major food source due to the low nitrogen stable isotopic signature of the mysids. The opportunistic feeding habits of *N. awatschensis* possibly explain the consistent dominance of this species in the lake, where the ecosystem structure was substantially changed because of successive desalination.

Key words: desalination, fecal pellets, Mysida, Mysidacea, zooplankton

Introduction

Lake Kasumigaura, located in the middle part of Japan (Fig. 1), was originally a brackish inland-sea lake. The lake has, however, been desalinated after the construction of sluice gates from 1963 to 1975, becoming a freshwater lake (Table 1) (Takamura 2012). *Neomysis awatschensis* (Brandt, 1851) (Fig. 2), a mysid species generally distributed in brackish waters, survived after the desalination, and this species maintains a large population in today's Lake Kasumigaura (Takamura et al. 2017). *Neomysis intermedia* (Czerniavsky, 1882) was also described in the same genus, but this species is now treated as a synonym of *N. awatschensis* (Price 2004). The present mysid species is generally distributed throughout the lake, and they show diel vertical and horizontal migration (Toda et al. 1982). This mysid species is an important food source for various fishes (e.g., *Hypomesus nipponensis* McAllister, 1963) and, consequently, is thought to be a key species in the ecosys-

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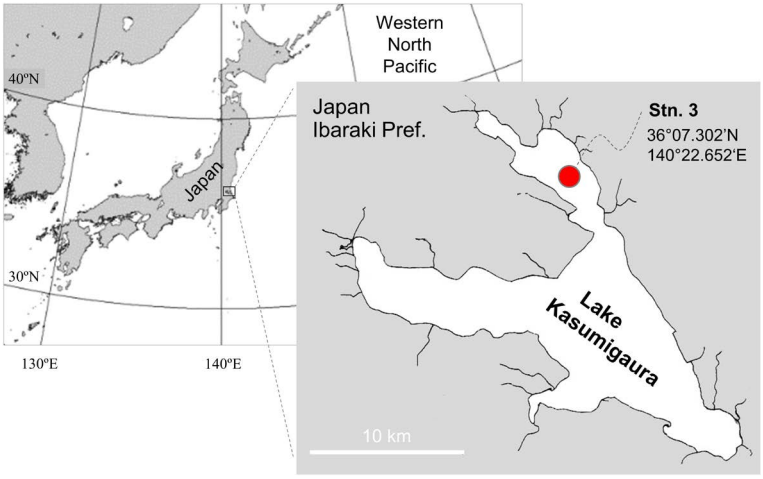

Fig. 1. Location of Lake Kasumigaura, where *N. awatschensis* was sampled in this study.

Fig. 2. Microscopic image of *Neomysis awatschensis* (Brandt, 1851). Adult male collected at the sampling site, Lake Kasumigaura.

Table 1. Studies on the feeding ecology of *N. awatschensis* in Lake Kasumigaura.

Investigation period	Method	Conclusion about the possible food sources	Reference
1959	microscopy of gut contents and feeding experiment	bottom mud (composed of organic matter including protists, rotifers and benthic algae)	Murano 1963
1963–1975	**construction of sluice gates causing the desalination of Lake Kasumigaura**		
1977–1979	*	zooplankton (cladocerans and copepods)	Kasuga 1981
1978–1993	*	zooplankton	Matsubara 1994
1979	feeding experiment	rotifers, bottom mud (including protists and bacteria)	Kasuga 1982
1980–1989	*	cladocerans, rotifers, copepods and bottom mud	Hanazato & Yasuno 1988, Hanazato 1998
1983–1985	*	cladocerans	Hanazato & Yasuno 1987
1984	stable isotope analysis	POM** (mostly phytoplankton)	Toda & Wada 1990
1986–1989	*	cladocerans	Hanazato & Aizaki 1991
2016, 2017	DNA metabarcoding, microscopy and stable isotope analysis	phytoplankton, benthic algae	this study

* Indirectly inferred from the seasonal fluctuation pattern in the abundance of both mysid and potential prey items. ** Particulate Organic Matter

tem of the lake (Suzuki & Ita 1977, Toda et al. 1982, Toda & Wada 1990).

Mysids exhibit high biomasses in the brackish and coastal waters of the world, and they are important food sources for many aquatic organisms (Mauchline 1980, Takahashi 2004). This crustacean group generally feeds on various materials (e.g., zooplankton and phytoplankton, bottom mud including microalgae and protists, land plants, detritus etc.) (Lasenby & Langford 1973, Mauchline 1980, Murtaugh 1981, Viitasalo & Rautio 1998), and because of this wide range of prey, it is difficult to precisely quantify the composition of their diet and to clarify their trophic level (Takahashi 2004). This is true for *N. awatschensis* living in Lake Kasumigaura, and there is a difference in opinion concerning the food sources. Murano (1963), who conducted the first intensive study of the feeding ecology of this mysid, reported that bottom mud including protists, rotifers and benthic algae is the main diet for this species, and this conclusion was supported by subsequent research with feeding experiments (Kasuga 1982). However, numerous studies conducted during 1980s–1990s reported that the main diet of *N. awatschensis* in the lake is zooplankton such as cladocerans, rotifers and copepods (Table 1), based on the typical fluctuation pattern of top down control in zooplankton abundance by this mysid species (Hanazato 1988). However, stable isotope analysis of nitrogen performed in the same period suggested that the major food source of *N. awatschensis* was particulate organic matter (POM), which is mostly composed of phytoplankton (Toda & Wada 1990). Such discrepancy of opinion is possibly due to the differences in analytical methods, which have different resolutions for determining the main food sources and trophic levels. Moreover, the timing of sampling could have affected the conclusions since the abundance and species composition of the planktonic community changed dramatically during the desalination of the lake (Fig. S1) (Takamura 2012, Takamura & Nakagawa 2017). It is consequently possible that this drastic change of environment affected the feeding habits of *N. awatschensis*.

The aim of this study was, therefore, to examine the major food sources of *N. awatschensis* in Lake Kasumigaura at present by combining conventional microscopy with complementary modern methods. DNA metabarcoding using a massively parallel sequencer (or next-generation sequencer) is an effective method to obtain the taxonomic composition of prey: almost all the organisms in the predator's gut contents or fecal pellets can be detected more precisely than with microscopy (Cleary et al. 2015). The stable isotope analysis is widely used for clarifying trophic level based on average diet (Minagawa & Wada 1984). This study aimed to solve the confusion about the major food sources of *N. awatschensis* in Lake Kasumigaura, one of the most studied mysid populations in Japan.

Materials and Methods

Field sampling and treatment

All field samplings were conducted in June (in 2016 and 2017) since the target species is most abundant in this season (Takamura et al. 2017). In 2016, zooplankton were collected by a plankton net (mesh size: 200 μm) during the daytime (10:00–12:00 in local time) at Stn. 3 (36°07.302′N, 140°22.652′E, depth: ca. 4 m) (Fig. 1), where biological surveys have been conducted monthly by the National Institution for Environmental Studies (NIES) since 1978 (Takamura et al. 2017). The water temperature was 21.8°C, the pH was 7.8 and the dissolved oxygen was 7.4 mg L^{-1} in the surface water. Individuals of *N. awatschensis* (Fig. 2) were isolated with a large pipette immediately after the sampling on a research boat (n=13) (Fig. 3, Table S1). Adult mysids of ca. 10–11 mm in body length were chosen, and they were carefully rinsed with filtered lake water in order to remove any particles (e.g., other small plankton) attached to the body surface. The rinsed mysids were individually put into 15 mL tubes filled with filtered lake water and incubated 3–4 hours in the laboratory to let them excrete gut contents (fecal pellets). The fecal pellets excreted by each individual were picked up with DNA-free pipettes and preserved in 99% ethanol at −80°C.

Additional plankton sampling for the stable isotope analysis was conducted in June 2017 during the daytime at the same station. The water temperature was 23.0°C, the pH was 7.7 and the dissolved oxygen was 7.9 mg L^{-1} in the surface water. Large zooplankton that were abundant in the investigation period (mysids, cladocerans and copepods) were picked up from bulk samples (Fig. 3). A total of 22 mysids (adults of ca. 10–11 mm in body length) were individually incubated for the excretion of fecal pellets (Table S1). The mysid individuals were then rinsed with high-purity water and put into the wells of cell culture plates. Liquid around the body of each individual was removed as much as possible, and the cell culture plates were stored at −20°C. The same treatment was performed for cladocerans and copepods (*Diaphanosoma dubium* Manuilova, 1964 and "copepods", respectively). The sample "copepods" was mainly composed of various species (e.g., *Eodiaptomus*, *Pseudodiaptomus*), the body lengths of which were larger than ca. 1,000 μm. Approximately 5.0 L of surface water was also sampled and filtered through a GF/F filter (Whatman, U.S.A.) (mesh size: 0.7 μm) to obtain smaller zooplankton and POM. Two size fractions were prepared: 50–400 μm ("bulk zooplankton", including carnivorous species) and 0.7–50 μm (POM, phytoplankton dominant). The sample "bulk zooplankton" was chiefly composed of copepods and cladocerans, while POM mainly contained microalgae (e.g., centric diatoms). The samples on the filters were rinsed with high-purity water and stored at -20°C.

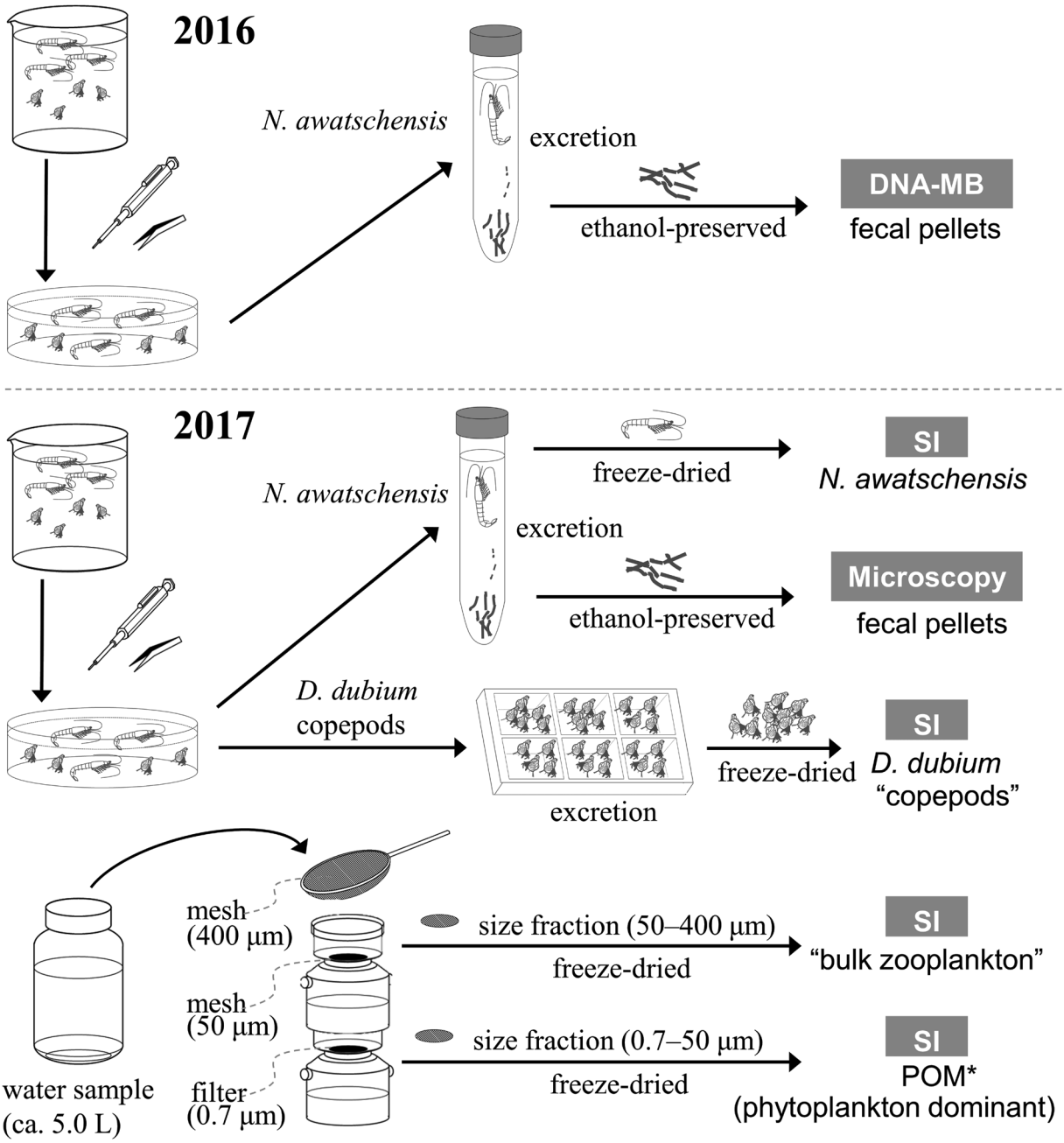

Fig. 3. Sample treatment procedures for the DNA metabarcoding (DNA-MB), microscopy and stable isotope analysis (SI) in this study.
* Particulate Organic Matter

DNA metabarcoding

Library preparation

Fecal pellets were chosen to analyze the food sources because the dissection of small plankton to obtain gut contents involves a high risk of contamination. The ethanol-fixed fecal pellets were centrifuged at 15,000 rpm for 10 min. After removing the supernatant (mainly ethanol), 100 uL of guanidine-containing extraction buffer (GITC buffer, Decelle et al. 2012) was added, and the DNA was extracted according to the method described by Nakamura et al. (2015).

The V4 hypervariable region (ca. 380 bp) in *18S* ribosomal DNA was amplified following the procedure of Toju (2016). The Peptide Nucleic Acid (PNA) specified for the mysid's sequence (*N. awatschensis*) was newly designed referring to Cleary et al. (2015) (Table 2), and the initial Polymerase Chain Reaction (PCR) was conducted with universal primers for eukaryotes. The first fusion primers were designed by combining P5 or P7 adapters, a series of "N" and V4-specific sequences for eukaryotes (Table 2). The reaction volume was 25.0 μL, containing 1.0 μL of extracted DNA, 12.5 μL of Q5 High-Fidelity 2x Master Mix (New England Biolabs, U.S.A), 2.5 μL of mysid spe-

Table 2. PCR primers and the Peptide Nucleic Acid (PNA) used in the first PCR for the DNA metabarcoding. Note that the V4-specific sequences were partially modified from Bråte et al. (2010).

	Name	Direction	Sequence
Primer	3NDf1	forward	GGCAAGTCTGGTGCCAG
	V4_euk_R2B	reverse	RYGGTATCTRATCRYCTTCG
PNA	Block-Neomysis	—	TTCTTGAGATGCTCT

cific PNA, 1.25 μL of 10 μM first fusion primers (containing sequences corresponding to the sequencing primers) (forward and reverse) and 6.5 μL of RNase-free water. An initial denaturation at 98℃ for 1 min was followed by 35 cycles of denaturation at 98℃ for 5 s, annealing at 65℃ for 20 s and at 59℃ for 10 s, and extension at 67℃ for 30 s. A final extension was conducted at 67℃ for 2 min. The second PCR amplification was performed with 25 μL reaction mixture: 2.0 μL of the initial PCR product, 12.5 μL of Q5 High-Fidelity 2x Master Mix, 1.25 μL of 10 μM second fusion primers (including indices of 8 mers and P5 or P7 adapters) (forward and reverse) and 8.0 μL of RNase-free water. The thermocycling conditions of the second PCR were as follows: initial denaturation at 98℃ for 30 s; 15 cycles of denaturation at 98℃ for 10 s and annealing at 72℃ for 30 s; and final extension at 72℃ for 2 min.

The PCR products were purified using AMPure XP (Beckman Coulter, U.S.A.), and the length of the products was confirmed using Tape Station 4200 (Agilent Technologies, U.S.A.). In order to measure DNA concentration and amplification efficiency, quantitative Polymerase Chain Reaction (qPCR) was performed using a Thermal Cycler Dice Real Time System (TaKaRa Bio, Japan) with Library Quantification Kit (TaKaRa Bio, Japan).

Amplicon sequencing and Data analysis

The pooled samples were adjusted to 4 pM before amplicon sequencing. A run of sequencing was conducted using MiSeq (Illumina, U.S.A.) with MiSeq Reagent kit v3 (600 cycles) (Illumina, U.S.A.), following the recommended protocol and default settings. The data was analyzed with the computer software package Claident ver. 0.2.2016.07.05 (Tanabe & Toju 2013) according to the Claident manual (Tanabe 2018). The procedure is briefly outlined below. The forward and reverse sequences were first concatenated, and low quality sequences, whose quality scores were less than 30 in average, were removed. Chimera sequences were also excluded, and the rest of the sequences were clustered into operational taxonomic units (OTUs) (the minimum identification score was 0.97). The OTU composition of each sample (fecal pellets from each mysid) was summarized in a table, where the minimum length of a sequence was 200 mer and the minimum number of OTUs was 200 reads. The OTUs were then taxonomically identified by NCBI BLASTN (https://www.ncbi.nlm.nih.gov/), according to the procedures in the Claident manual. Raw sequence data were deposited in the DDBJ database with the accession number DRA009589.

Additional PCRs were conducted with the same primers and conditions as the first PCR, in order to identify several OTUs (calanoid copepods) detected from the fecal pellets in this study. The sequences of the V4 region were obtained for two copepod species distributed in the lake: *Eodiaptomus japonicus* (Burckhardt, 1913) and *Pseudodiaptomus japonicus* Kikuchi, 1928 (previously referred to *P. inopinus* in Lake Kasumigaura) (Orui & Ueda 2018). These sequences were registered in the DDBJ database: LC413686 and LC413687, respectively.

Microscopy

Fecal pellets were collected from selected individual mysids used for the stable isotope analysis (nine specimens, Table S1). Each sample of fecal pellets was partly decomposed with a dissecting needle on a microscope slide. After some drops of high-purity water were added, the decomposed sample was sealed with a cover glass and observed with an inverted microscope (TMS, Nikon, Japan). An area of ca. 900 μm in diameter was randomly selected under the microscope (×200), and all the identifiable particles in the area were counted (the cell number was counted for phytoplankton). The selection of area and the counting of particles were repeated three times for each specimen, and the proportion (%) was calculated based on the number of particles. Contents of some fecal pellets (including particles) were photographed with a digital camera (Nikon 1 V1, Nikon, Japan).

Stable isotope analysis

Frozen samples (zooplankton individuals, "bulk zooplankton" and POM) were freeze-dried and ground with a micro-spatula. The lipids within the samples were removed using a solution comprising chloroform and methanol at a ratio of 2 : 1 (Sugisaki et al. 2013), and ca. 0.5 mg of each sample and standard was then wrapped in a tin capsule. Stable isotope analyses were performed with a continuous flow interface (ConFlo IV, Thermo Fisher Scientific, Germany) and an isotopic ratio mass spectrometer (Delta V Advantage, Thermo Fisher Scientific, Germany). All stable isotope values are registered in the δ notation: $\delta^{15}N=([(^{15}N_{sample}/^{14}N_{sample})/(^{15}N_{standard}/^{14}N_{standard})]-1)\times 1{,}000$, where the standard is atmospheric nitrogen, and $\delta^{13}C=([(^{13}C_{sample}/^{12}C_{sample})/(^{13}C_{standard}/^{12}C_{standard})]-1)\times 1{,}000$, where the standard is PeeDee Belemnite (Peterson & Fry 1987). The standard deviation for each working standard

Table 3. Major organisms detected by DNA metabarcoding on the fecal pellets of each mysid specimen. Note that the proportion (%) was calculated based on the selected OTUs, where the read numbers were more than 200 and sequence length was more than 200 mer. The first, second and third highest values for each specimen are shown in red, orange and cream, respectively. Others were composed of protists (mainly cercozoans).

	Higher group	*Genus*	Specimen of *Neomysis awatschensis*													Total read
			KU1	KU2	KU3	KU4	KU5	KU6	KU7	KU8	KU9	KU10	KU11	KU12	KU13	
Phyto-plankton	Chlorophyta	*Coelastrum*	11.5	0.0	25.1	94.2	0.0	0.0	0.0	0.0	0.0	0.0	39.5	0.0	7.3	9146
	Bacillariophyceae	*Cyclostephanos / Stephanodiscus*	18.9	1.2	2.7	0.0	30.5	0.2	0.3	0.6	7.0	53.6	8.6	15.9	2.3	3830
	Bacillariophyceae	*Cyclotella*	22.4	18.6	8.4	0.0	4.9	0.2	75.4	75.4	92.7	45.6	51.4	12.1	0.0	3495
	Chlorophyta	*Chlorella / Micractinium*	1.6	0.0	26.1	0.0	45.5	0.0	11.9	0.0	0.0	0.0	0.0	0.0	1.2	2737
	Charophyta	*Cosmarium / Euastrum*	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	76.8	909
	Chlorophyta	*Follicularia / Planktosphaeria*	28.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	51.0	0.0	637
	Chlorophyta	*Desmodesmus*	0.0	63.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.3	0.0	521
	Chlorophyta	*Dictyosphaerium*	16.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	11.2	462
	Chlorophyta	Chlorophyceae	0.0	0.0	0.0	0.0	9.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	450
	Bacillariophyceae	*Aulacoseira*	0.4	7.2	1.3	3.0	0.3	0.0	2.4	13.1	0.0	0.5	0.4	10.2	0.6	437
	Chlorophyta	*Tetrastrum*	0.0	0.0	0.0	0.0	0.0	3.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	235
Zoo-plankton	Copepoda	*Pseudodiaptomus japonicus**	0.0	0.0	9.0	0.0	0.0	54.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4316
	Copepoda	*Eodiaptomus japonicus**	0.0	0.0	26.0	0.0	0.0	42.0	8.2	0.0	0.0	0.0	0.0	0.0	0.0	3692
		Others	0.2	9.6	1.3	2.8	9.5	0.0	1.8	10.9	0.3	0.3	0.2	9.6	0.7	858
		Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	—

* These OTUs were identified at species-level because the sequences corresponded with those of the copepod species dwelling in the investigation area (Lake Kasumigaura).

was less than 0.15‰.

Results

DNA metabarcoding (samples obtained in 2016)

Sequences from *Neomysis awatschensis* were the most frequently detected, but their read numbers were ca. 30–50% compared with results obtained without the blocking of the mysid's DNA with PNA (data not shown), indicating that the blocking PCR partly succeeded in the initial PCR. Other sequences (expected to be from the food organisms of *N. awatschensis*) were also detected, and the OTUs with more than 200 reads are listed in Table 3. Zooplankton sequences (*Pseudodiaptomus japonicus* and *Eodiaptomus japonicus*) were detected from two specimens (KU3 and KU6). However, most of the sequences were from phytoplankton belonging to diatoms (Bacillariophyta) and green algae (Chlorophyta and Charophyta), and only a few sequences of benthic algae were detected (Table 3).

Microscopy (samples obtained in 2017)

Particles of phytoplankton-origin were the most frequently observed in all the fecal pellets (99.5% on average) (Table 4). Centric diatom-like particles, (e.g., *Aulacoseira* and cyclostephanoid diatoms, Fig. 4a, e–g) and pennate diatom-like particles (e.g., *Fragilaria,* Fig. 4d) were the most frequently observed (98.3%). Green algae (e.g., *Desmodesmus* and *Pediastrum*, Fig. 4b, h) less often appeared (1.2%). Crustacea-origin particles (fragments of crustaceans, Fig. 4c) were less frequently detected (0.5%). Unidentifiable substances were also seen in the fecal pellets. A large part of these substances was apparently composed of organic materials, and inorganic materials (e.g., mud, sand particles) were seldom observed.

Stable isotope analysis (samples obtained in 2017)

The nitrogen stable isotope ratio (δ^{15}N) was highest in "copepods" (18.2‰), followed by "bulk zooplankton" (16.7‰), *Diaphanosoma dubium* (15.7‰ and 13.9‰), *Neomysis awatschensis* (14.8‰±0.7) and POM (11.8‰ and 11.4‰) (Fig. 5). The same tendency was seen in the carbon stable isotope ratio (δ^{13}C), where "copepods" showed the highest value (−22.1‰), followed by "bulk zooplankton" (−22.4‰), *D. dubium* (−23.3‰ and −23.5‰), *N. awatschensis* (−23.0‰±1.1) and POM (−26.4‰ and −26.0‰). The δ^{15}N values of *N. awatschensis* were similar to those of the phytoplankton feeder (*D. dubium*), i.e. between POM and "bulk zooplankton" (including carnivorous zooplankton). For the mysids, the variation in δ^{13}C values was relatively large (ca. 4‰), compared with that in the δ^{15}N values (ca. 2.5‰) (Fig. 5). There was appar-

Table 4. Major organisms detected by microscopy on the fecal pellets of each mysid specimen. Note that the proportion (%) was calculated based on the number of identifiable particles (the cell number for phytoplankton). The first, second and third highest values for each specimen are shown in red, orange and cream, respectively.

	Higher group	*Genus*	Specimen of *Neomysis awatschensis*												Average
			KU16	KU17	KU18	KU19	KU20	KU21	KU23	KU24	KU25	KU26	KU27	KU30	KU34	
Phyto-plankton	Bacillariophyceae	*Aulacoseira*	81.5	85.4	84.4	87.1	100.0	61.4	69.0	82.1	47.1	79.4	72.5	73.7	90.6	78.0
		cyclostephanoid diatom	7.6	9.8	11.1	8.6	0.0	15.9	10.3	10.3	11.8	6.3	10.0	18.4	6.3	9.7
		Other centric diatom	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.6	0.0	0.2
		Fragilaria	7.6	4.9	4.4	0.0	0.0	13.6	17.2	5.1	41.2	7.9	7.5	2.6	0.0	8.6
		Other pennate diatom	3.4	0.0	0.0	1.4	0.0	6.8	3.4	0.0	0.0	0.0	5.0	2.6	0.0	1.7
	Chlorophyta-like fragment		0.0	0.0	0.0	1.4	0.0	0.0	0.0	2.6	0.0	6.3	5.0	0.0	0.0	1.2
Zoo-plankton	Crustacea-like fragment		0.0	0.0	0.0	1.4	0.0	2.3	0.0	0.0	0.0	0.0	0.0	0.0	3.1	0.5
	Total		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	—

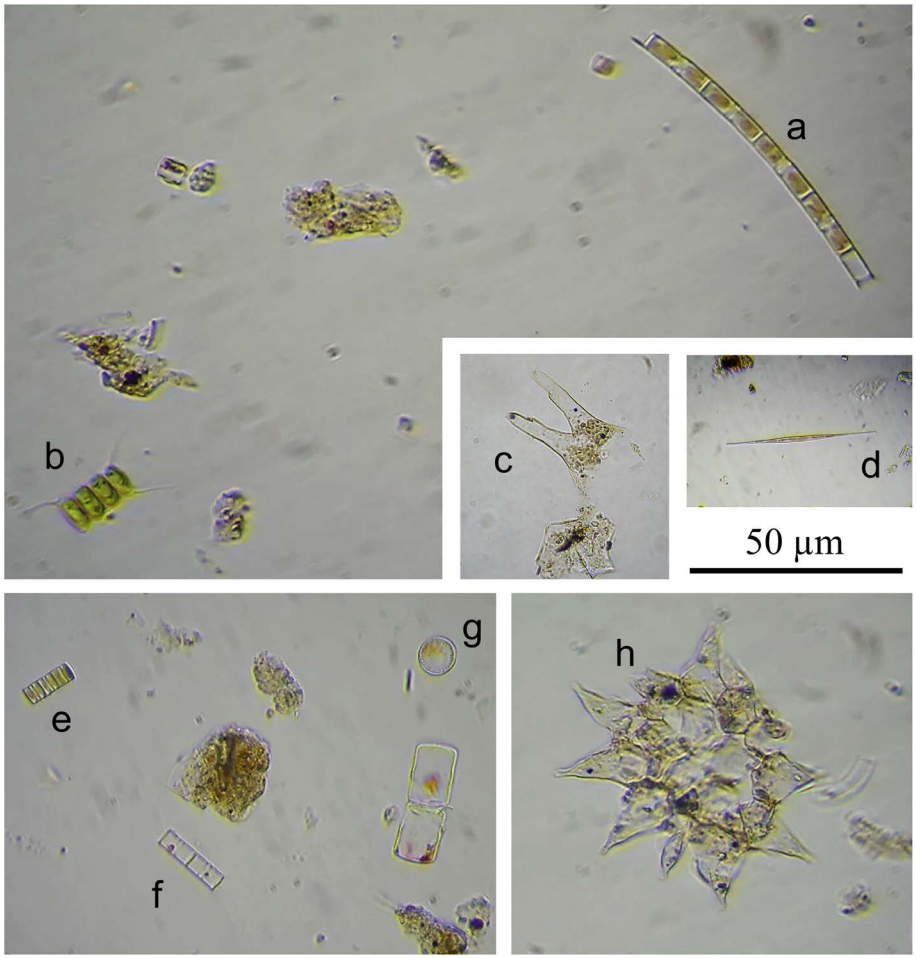

Fig. 4. Light microscopic images of items found in the fecal pellets of *N. awatschensis*. *Aulacoseira* sp. (a, e and f), *Desmodesmus* sp. (b), Crustacea-like particle (c), *Fragilaria* sp. (d), cyclostephanoid diatom (g) and *Pediastrum* sp. (h).

ently no correlation between the result of the stable isotope analysis and feeding habit revealed by microscopy, though some specimens were used for both analyses. For example, the δ^{15}N values of the specimen KU30 is relatively high (suggesting a relatively carnivorous diet), but no zooplankton-like particles were detected in its fecal pellets (Fig. S2).

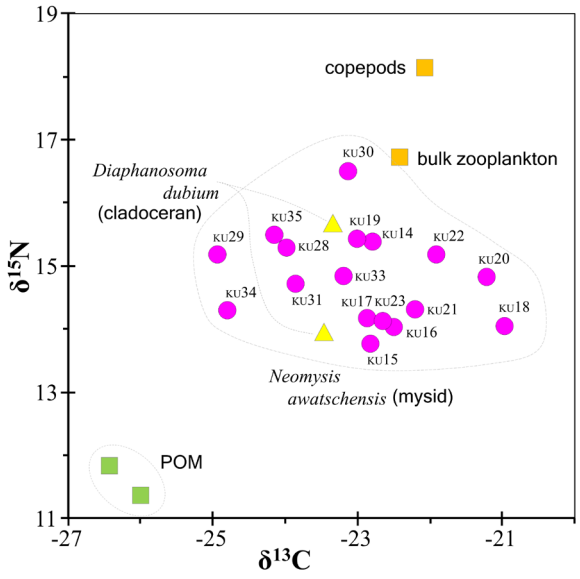

Fig. 5. δ^{15}N–δ^{13}C map of the plankton community in Lake Kasumigaura. Note that the two size fractions (400–50 μm and 50–0.7 μm) are shown as "bulk zooplankton" and POM, respectively.

Discussion

According to our preliminary observation, *Neomysis awatschensis* excretes fecal pellets several hours after feeding. The sampling for this study was conducted during daytime, and therefore, the species detected by the DNA metabarcoding and microscopy would reflect the prey on which the mysids fed after sunrise. The following phytoplankton taxa were most frequently detected by the DNA metabarcoding: Green algae, *Coelastrum*, diatoms *Cyclostephanos*/*Stephanodiscus*/*Cyclotella* and green algae, *Chlorella*/*Micractinium* (Table 3). However, centric and pennate diatoms were most frequently detected in the microscopy. Judging from the detection frequency of the two analyses, diatoms and green algae could be the most important food sources of *N. awatschensis* during the daytime for the sampling period. Most of the diatoms and green algae detected in the present DNA metabarcoding corresponded with the species that were most abundant *in situ* during the investigation period (May–July 2016) according to the phytoplankton monitoring data from Lake Kasumigaura Database (Takamura & Nakagawa 2012), suggesting that mysids opportunistically fed on abundant phytoplankton taxa.

Green algae were much more frequently detected by DNA metabarcoding than by microscopy (Tables 3–4). Although the samples for the microscopy and DNA metabarcoding were collected in different years (Table S1), the composition of phytoplankton in the investigation area did not differ substantially between 2016 and 2017 (Takamura et al. 2012). We, therefore, consider that the soft cells of green algae are difficult to detect with microscopy because their soft bodies are more easily digested, unlike diatoms with hard shells, which sometimes remain even after digestion (e.g., Peterson 1987). Another possibility for the discrepancy between the microscopy and DNA metabarcoding is a bias caused by the DNA gene copy number, because the read number generated from the massively parallel sequencer could be affected by DNA copy number of each organism (Cleary et al. 2012).

Several sequences of rotifers were detected in the OTUs, although the read numbers were less than 200. Cladocerans, which are occasionally reported as important prey for mysid species in other ecosystems (Lasenby & Langford 1973, Murtaugh 1981), were not detected (data not shown). Although *Diaphanosoma dubium*, a cladoceran species, was abundant during the investigation period (Takamura et al. 2017), this species may be too large to be preyed on by the mysid. The detection of copepod sequences from two mysid individuals (Table 3, KU3 and KU6) is possibly caused by the feeding on nauplius larvae of copepods. Nauplius larvae are major food sources for various mysids (Takahashi & Kawaguchi 1998, Takahashi 2004), and they were abundant at the sampling site during the investigation period (162.66–263.30 inds. L^{-1} in May–July 2016, Takamura et al. 2017).

Previous studies also reported that "unidentifiable substances" (or detritus) were often detected in the gut contents of mysids (e.g., Murano 1963), although the origin of these substances was undeterminable. Bottom mud containing various benthic organisms has been suggested as a source of the unidentifiable substances, but its ingestion has so far only been confirmed by feeding experiments in the laboratory (Murano 1963, Kasuga 1982). Moreover, DNA metabarcoding and microscopy performed in the present study found little evidence for the ingestion of benthic plants and animals by this species. Considering the result that sand particles (inorganic materials) were also seldom observed by microscopy, most of the unidentifiable substances are supposed to be digested organic materials originating from the organisms that were detected by our DNA metabarcoding.

The δ^{15}N of an organism reflects its trophic level through isotopic fractionation (Minagawa & Wada 1984, Sugisaki et al. 2013). The δ^{15}N of *N. awatschensis* was comparable with those of herbivorous cladocerans analyzed in this study, and this tendency was also reported in a previous study (Toda & Wada 1990). Hence, the trophic level of this mysid is similar to that of phytoplankton feeders, and this agreed with the results of DNA metabarcoding and microscopy. On the other hand, their δ^{13}C values were widely dispersed (Fig. 5), though the composition of detected food items were generally similar among the mysid individuals used in the analysis (Tables 3–4). Since the stable isotope ratio is influenced by long-term diet, the dispersed δ^{13}C value may suggest the partial contribution of alternative food sources, such as benthic microalgae and land plants (Matsuzaki et al. 2010, Okuda 2012, Sugisaki et al. 2013). It also needs to be considered that the

Table 5. Major food sources of *N. awatschensis* in Lake Kasumigaura revealed by the three different methods used in this study. The advantages of each method are also shown.

Method	DNA metabarcoding	Microscopy	Stable isotope analysis
Major food sources presumed by each method	planktonic diatoms and chlorophytes (cyclostephanoids, *Coelastrum* and *Chlorella*/*Micractinium*)	planktonic diatoms (*Aulacoseira*, cyclostephanoids and *Fragilaria*)	primary producers (microalgae)
Advantage Possible…	—to detect most of the prey organisms in the feces (including soft or fragile prey). —to determine the food sources of short period.	—to physically confirm the morphology of prey organisms in the feces. —to provide quantitative data (comparable to previous studies). —to determine the food sources of short period.	—to estimate the trophic level of the predator based on the assimilated prey organisms. —to estimate the food sources of long period.
Disadvantage Impossible… (or difficult…)	—to physically confirm the morphology of prey organisms. —to know whether the organisms were assimilated.	—to detect soft or fragile prey (e.g., soft algae and zooplankton). —to know whether the organisms were assimilated.	—to know the species composition of prey organisms. —to estimate the trophic level of the predator feeding on benthic algae (because of the irregular isotopic fractionation).

δ^{13}C value of phytoplankton can vary especially in early summer, due to the gradual increase of water temperature and highly fluctuating availability of dissolved carbon (Zohary et al. 1994). Mysids are reported to feed on terrestrial plants (Takahashi 2004), and some studies suggest that pollens could be a major food source of mysids including *N. awatschensis* (Murano 1963, Bonsdorff & Bonsdorff 2005). Further examination about the importance of food sources other than phytoplankton is required to reveal the long-term variation (season to year) of food sources in this species.

Overall, considering the results of the DNA metabarcoding (Table 3), microscopy (Table 4) and stable isotope analysis (Fig. 5), we conclude that diatoms and green algae were the major food sources for *Neomysis awatschensis* in Lake Kasumigaura during the investigation period, and its trophic level was as low as that of herbivores (Fig. 5). Mysids actively move into the water column and feed on mesozooplankton (e.g., cladocerans, copepod nauplii) during the night (Mauchline 1971, Lasenby & Langford 1973, Takahashi 2004, Katayama et al. 2011). Therefore, it is also possible that *N. awatschensis* in today's Lake Kasumigaura feeds on mesozooplankton during the night, though the stable isotope value indicates that they could not be a major food source of the present mysid species.

The results of this study mostly agree with those of Toda & Wada (1990), in which the herbivorous feeding habit of this mysid is reported. However, this species has also been regarded to be detritivorous (to feed on bottom mud including benthic organisms) or carnivorous (to eat mesozooplankton like cladocerans and rotifers) (Table 1). Such controversies may reflect the flexible opportunistic feeding habit of *N. awatschensis*, and the gradual desalination from 1963, which has had a substantial impact on the local ecosystem, and could have affected the feeding habits of this mysid species. Since the taxonomic composition of phytoplankton and mesozooplankton changed during the 1980s–2000s, after the construction of the sluice gates (Fig. S1), it is possible that *N. awatschensis* switched its major prey from zooplankton to phytoplankton (Viitasalo et al. 1998) during the drastic changes caused by desalination of the lake. The opportunistic feeding habit of *N. awatschensis* possibly explains the consistent dominance of this species in the lake, even though the ecosystem structure was changed substantially due to the successive desalination.

The methodology used would also affect the conclusions of previous studies, because most of them adopted a single approach in which the opportunistic feeding habits of *N. awatschensis* are difficult to be precisely evaluated (Table 1). The present study combined microscopy and DNA metabarcoding in order to detect organisms with soft bodies. Stable isotope analysis was also applied, focusing on δ^{15}N values and also on δ^{13}C values, which were not measured by previous studies. By combining these methods, each with different advantages (Table 5), the food sources of *N. awatschensis* in Lake Kasumigaura was able to be more precisely estimated. Future studies on its feeding habits, including diel, seasonal and year-to-year sampling, would further clarify the food sources of this mysid.

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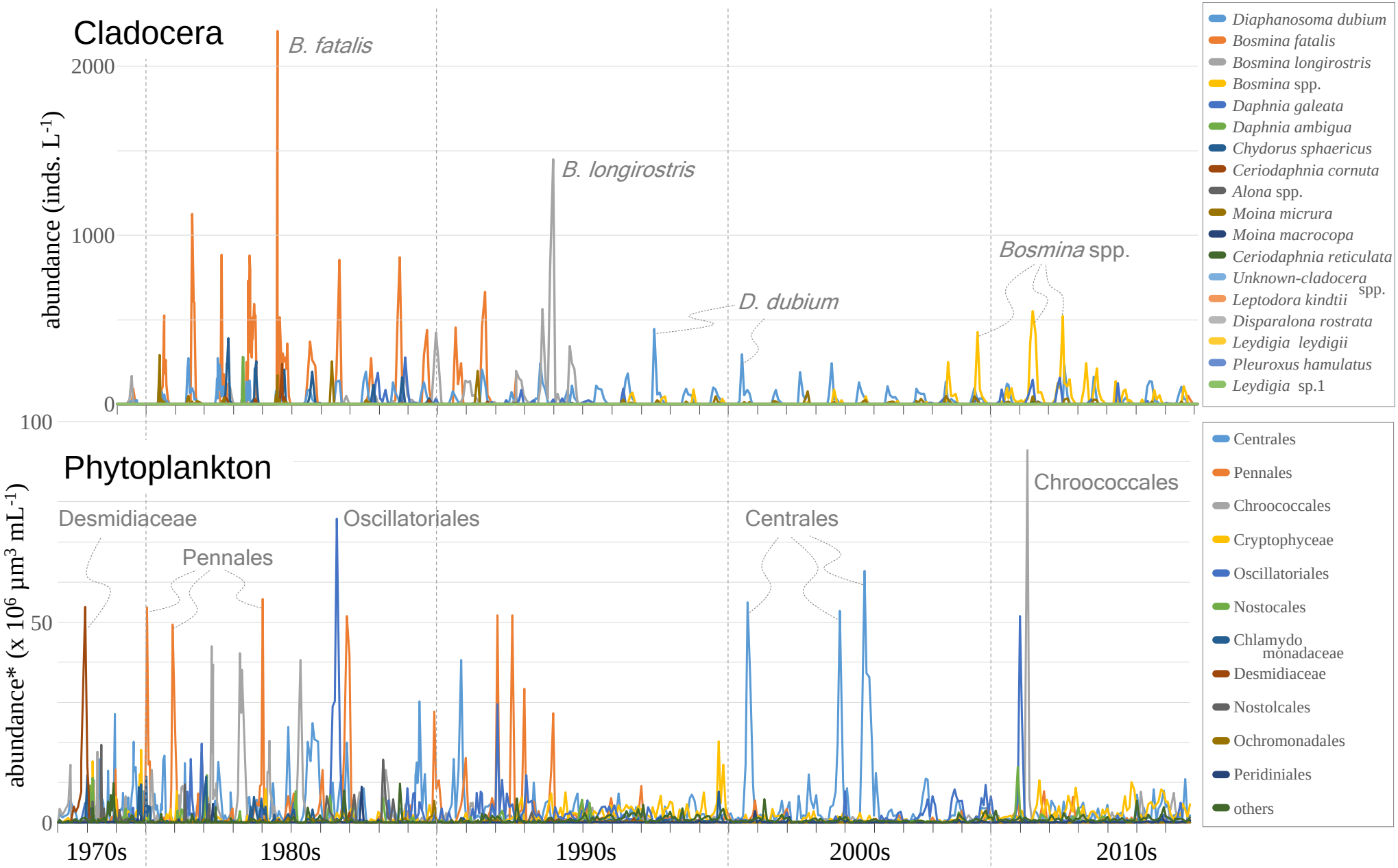

Fig. S1. Seasonal fluctuation of phytoplankton and cladoceran abundance at Sta. 3 in Lake Kasumigaura. The data were derived from the Lake Kasumigaura Database (Takamura & Nakagawa 2012, Takamura et al. 2017).

* The abundance of phytoplankton is expressed in units of volume (µm^3) per milliliter of lake water.

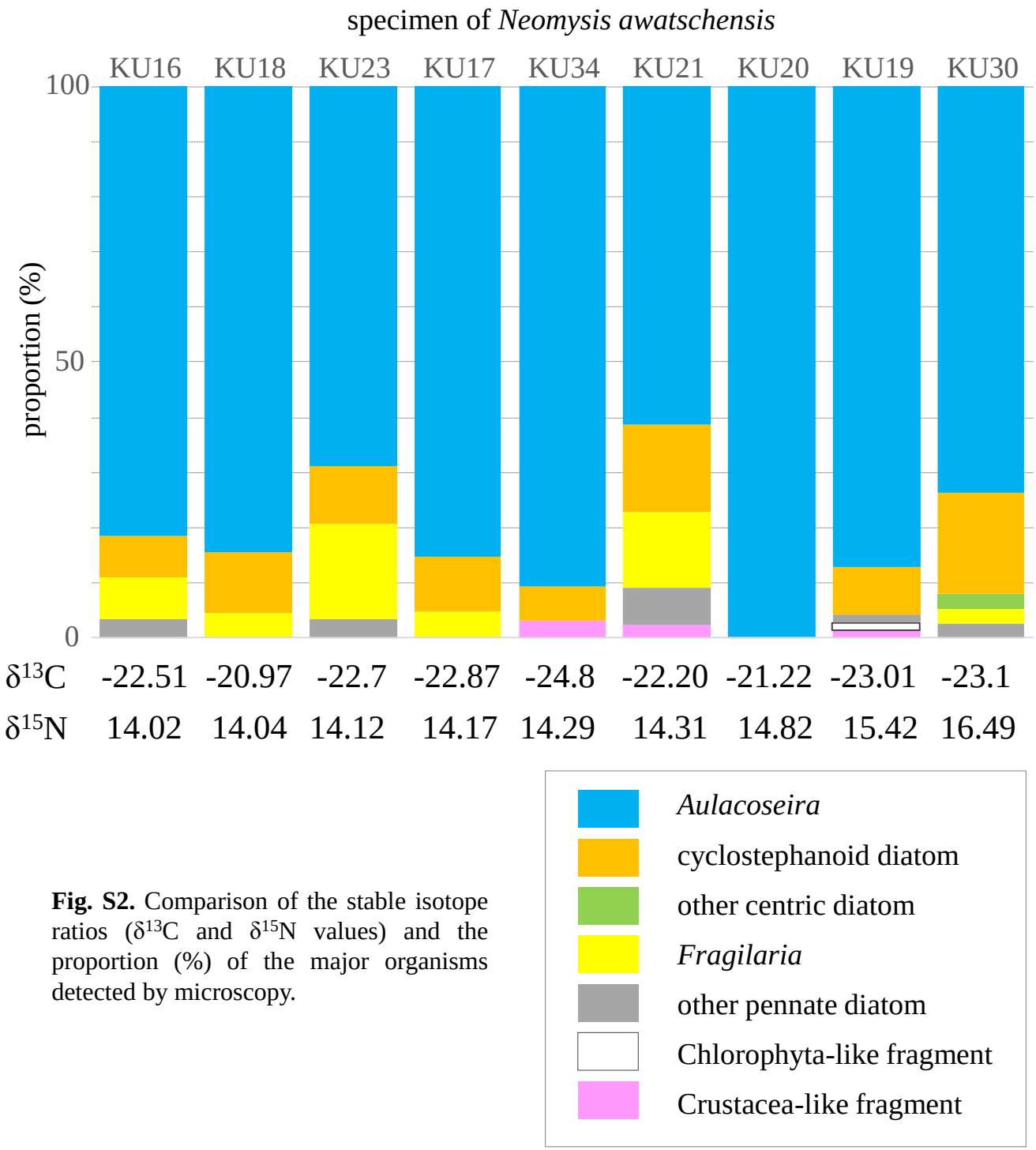

Fig. S2. Comparison of the stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$ values) and the proportion (%) of the major organisms detected by microscopy.

Table S1. Specimens of *N. awatschensis* analyzed in this study. Note that all specimens were collected at Stn. 3 (Fig. 1).

Analysis*	Specimen	Taxon	Sex	Sampling season
DNA-MB	KU1	*N. awatschensis*	male	June, 2016
DNA-MB	KU2	*N. awatschensis*	female	June, 2016
DNA-MB	KU3	*N. awatschensis*	male	June, 2016
DNA-MB	KU4	*N. awatschensis*	female	June, 2016
DNA-MB	KU5	*N. awatschensis*	male	June, 2016
DNA-MB	KU6	*N. awatschensis*	female	June, 2016
DNA-MB	KU7	*N. awatschensis*	male	June, 2016
DNA-MB	KU8	*N. awatschensis*	female	June, 2016
DNA-MB	KU9	*N. awatschensis*	male	June, 2016
DNA-MB	KU10	*N. awatschensis*	female	June, 2016
DNA-MB	KU11	*N. awatschensis*	male	June, 2016
DNA-MB	KU12	*N. awatschensis*	male	June, 2016
DNA-MB	KU13	*N. awatschensis*	male	June, 2016
SI	KU14	*N. awatschensis*	female	June, 2017
SI	KU15	*N. awatschensis*	male	June, 2017
SI / MS	KU16	*N. awatschensis*	male	June, 2017
SI / MS	KU17	*N. awatschensis*	female	June, 2017
SI / MS	KU18	*N. awatschensis*	male	June, 2017
SI / MS	KU19	*N. awatschensis*	male	June, 2017
SI / MS	KU20	*N. awatschensis*	female	June, 2017
SI / MS	KU21	*N. awatschensis*	male	June, 2017
SI	KU22	*N. awatschensis*	male	June, 2017
SI / MS	KU23	*N. awatschensis*	male	June, 2017
MS	KU24	*N. awatschensis*	male	June, 2017
MS	KU25	*N. awatschensis*	female	June, 2017
MS	KU26	*N. awatschensis*	male	June, 2017
MS	KU27	*N. awatschensis*	female	June, 2017
SI	KU28	*N. awatschensis*	male	June, 2017
SI	KU29	*N. awatschensis*	male	June, 2017
SI / MS	KU30	*N. awatschensis*	male	June, 2017
SI	KU31	*N. awatschensis*	female	June, 2017
SI	KU32	*N. awatschensis*	male	June, 2017
SI	KU33	*N. awatschensis*	female	June, 2017
SI / MS	KU34	*N. awatschensis*	female	June, 2017
SI	KU35	*N. awatschensis*	male	June, 2017
SI	-	*Diaphanosoma dubium*	-	June, 2017
SI	-	*Diaphanosoma dubium*	-	June, 2017
SI	-	copepods	-	June, 2017
SI	-	SS	-	June, 2017
SI	-	SS	-	June, 2017
SI	-	bulk zooplankton	-	June, 2017

* DNA-MB: DNA metabarcoding. MS: microscopy. SI: stable isotope analysis.