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Symbiotic microbiome with three dominant crustacean zooplankton in Lake Biwa, Japan

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Keywords

Bacterial communities, Lake Biwa, Symbiotic bacteria, Ambient bacteria, Gut, Crustacean zooplankton, *Daphnia pulicaria*, *Eodiaptomus japonicus*, Cyclopoida spp., Flagellate, Ciliate, Epibionts.

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GENERAL INTRODUCTION

1. General Introduction

1.1 Zooplankton in Lake Biwa

Lake Biwa (35.1°N, 136.1°E) is the largest and the oldest freshwater lake in Japan, with a surface area of 670.3 km^2, a water volume of 27.5 km^3, and a maximum depth of 104 m (Tabata et al. 2016). It is the most species-rich aquatic habitat in Japan (Rossiter 2000), providing water for about 14.5 million people living in surrounding provinces for their daily lives and playing an important role in fisheries and agricultural irrigation (Kawanabe et al. 2012). Lake Biwa is monomictic; the water column becomes stratified from May to September, starts mixing from October, being mixed wholly in February every year (Kagami et al. 2006), and phytoplankton biomass in the epilimnion (0–20 m) generally increased from spring but low in winter (Hsieh et al. 2010, Liu et al. 2020).

In Lake Biwa, seven crustacean taxa, *Eodiaptomus japonicus*, Cyclopoida spp. including mainly *Mesocyclops dissimilis* and *Cyclops vicinus*, *Daphnia galeata*, *Daphnia pulicaria*, *Bosmina longirostris*, and *Diaphanosoma orientalis*, dominated and accounted for > 90% of the total crustacean biomass in the lake (Kawabata 1989, Yoshida et al. 2001, Urabe et al. 2003, Liu et al. 2020, 2022). Among the zooplankton taxa, calanoid copepod *E. japonicus* is the most dominant, followed by cladoceran *Daphnia* spp. and Cyclopoida spp. (Yoshida et al. 2001, Liu et al. 2020). *D. pulicaria* is the largest taxa among *Daphnia* spp. in the lake, having distinct morphological features including a short head without any helmet or projection, with five stout teeth in the middle pectin in the post-abdominal claw, compared to *D. galeata* (Urabe et al. 2003). *Daphnia* spp. show different seasonal succession in abundance among species (Liu et al. 2020). *D. galeata* usually increases after May, reaching its maximum in June. On the other hand, *D. pulicaria* shows large inter-annual variability during the main occurrence period, that is, mainly from January to June, reaching their maximum in April to May but declining after June toward winter (Liu et al. 2020). Cyclopoids usually increase in

the early summer and are stable until October, and a decline during the winter months (Kawabata 1989, Liu et al. 2020). The abundance of *E. japonicus* becomes low during winter and early spring, starts to increase after May, reaching a maximum in June to September, and decreases until December (Kawabata 1987, Liu et al. 2020).

The abundance of these zooplankton species serves as a major component of the biological condition by affecting water quality, phytoplankton densities, fish production, and nutrient cycling. They occupy the center of the open water food web, ingest bacteria and algae that form the base of the food web, and in turn, they are heavily preyed upon by fish, insects, and others (Yoshida et al. 2001, Kawabata et al. 2006, Liu et al. 2020). However, the effects of environmental disturbances can be detected through changes in zooplankton species composition and abundance (Liu et al. 2020).

In aquatic ecosystems, metazoan zooplankton are essential elements of the trophic food web and significant contributors to pelagic biodiversity and biochemical processes with its symbiotic microbiome (Tang et al. 2010). Recently, microbial symbionts have been known to be an integral part of zooplankton. Because cladoceran *Daphnia* and copepods depend on their bacterial symbionts for their development and growth (Sison-Mangus et al. 2014, Peerakietkhajorn et al. 2015, Callens et al. 2018, Akbar et al. 2020), nutrition (Macke et al. 2017, Cooper and Cressler 2020, Gorokhova et al. 2021), and pathogenic protection (Wahl et al. 2012). Interactions between the zooplankton and its symbiotic microbiome can be obligatory or facultative, and mutualistic, commensalistic, or parasitic (Wells and Varel 2011, Martin and Schwab 2012)**.**

Zooplankton ecologists generally tend to focus on interactions between zooplankton and their prey or predators, abiotic factors of surrounding environments that affect life history traits (Kawabata 2006, Boonmak et al. 2018, Liu et al. 2014, Gao et al. 2022) while symbiotic

microbiomes have been overlooked by aquatic microbial ecologists. During the last two decades, researchers have focused on host–microbiome interactions (Lim and Bordenstein 2020). However, studies on interactions between zooplankton and their symbionts are limited, especially in natural habitats, because the two are usually considered to represent different functional units with indirect connections in nutrient cycling and food webs in aquatic ecosystems (Azam and Malfatti 2007). It is necessary to study the interaction between these host-symbiont systems and their mode of interaction for a better understanding of the element fluxes, ecosystem responses, food web architecture, carbon metabolism, and unexplored trophic links in aquatic ecosystems (Eckert and Pernthaler 2014, Corno et al. 2013, Thébault and Fontaine 2010, Mougi and Kondoh 2012).

In Lake Biwa, zooplankton and microbial communities in the lake have been well-studied separately over various time scales (Kawabata 1989, Liu et al. 2014, Okazaki and Nakano 2016, Okazaki et al. 2019). However, interactions between zooplankton and their microbial symbionts have not been studied yet. In this study, I determined the microbial communities, such as bacteria and protists, associated with the three dominant zooplankton taxa in Lake Biwa.

1.2 Microbiome of host zooplankton

In aquatic habitats, crustacean zooplankton, including copepods and cladocerans, is a host of a wide range of microorganisms, such as viruses, bacteria, fungi, algae, and protozoans (Ho and Perkins 1985, Carman and Dobbs 1997, Bickel et al. 2012, Bass et al. 2021). These prokaryotic and eukaryotic microorganisms live in close physical contact with a host called its microbiome (Macke et al. 2017), and those inhabiting the host's body surface, including swimming appendages and filtering apparatus, are called ectosymbionts, whereas those in the internal organs, such as digestive tracts, hemocoel, and tissue called endosymbionts (Ho and Perkins 1985). The endosymbiotic microbiota may use organic matter available in the host's

gut and tissue, and ectosymbiotic ones can utilize organic matter excreted by the host (Wahl et al. 2012, Bickel and Tang 2014). By attaching to the host body surface, epibiotic microorganisms may evade zooplankton predators (Henebry and Ridgeway 1979) and travel to areas with available foods (Kankaala and Eloranta 1987, Wahl 1989) or use the host as a substrate for their growth and survival (Tang 2005, Tang et al. 2010, Corre et al. 2020, Lu et al. 2020)

Previous studies reported that symbiotic fungi have parasitic effects on their calanoid copepod hosts. Redfield and Vincent (1979) reported that a pathogenic ectosymbiotic fungus reduced the population size of *Diaptomus novamexicanus* by 48.8% in an alpine lake in California. An endosymbiotic fungus, *Leptolegnia baltica,* was responsible for the mortality of *Eureytemora hirundoides* in the Baltic Sea (Vallin 1951, Höhnk and Vallin 1953). Endosymbiotic parasitic fungi, *Thelohania* sp., affected the life history traits of *Daphnia pulex* in a vernal pond in Michigan (Brambilla 1983).

1.3 Bacterial symbionts

Several previous studies reported on the bacterial symbiont of daphnids and copepods. Bacterial components associated with their host bodies have been almost reported from cultured daphnids (Qi et al. 2009, Sison-Mangus et al. 2014, Callens et al. 2016, Akbar et al. 2020), and calanoid and cyclopoid copepods from marine habitats (Gerdts et al. 2013, De Corte et al. 2014, 2018, Sadaiappon et al. 2021, Velasquez et al. 2022). The information on symbiotic bacterial microbiota associated with copepods and cladocerans from natural freshwater habitats is still limited (Grossart et al. 2009, Samad et al. 2020, Wang et al. 2021, Eckert et al. 2021). However, all these studies described host-associated symbiotic bacteria using the whole body without dissecting the gut, thus containing limited information on the specific location of symbionts.

Furthermore, Chae et al. (2021) reported the gut bacterial microbiota of *Acratia tonsa*, *Sinocalanus tenellus*, and *Pseudodiaptomus inopinus* from a brackish habitat without separating the intestine from the host's body. Some other studies described host-associated surface-attached bacteria using microscopic images (Nagasawa and Nemoto 1985, Nagasawa and Terazaki 1987, Huq et al. 1983, Skovgaard et al. 2015). Harris (1993) and Carman and Dobbs (1996) described ectosymbiotic bacteria of calanoid hosts using culturing techniques. Moreover, Hansen and Bech (1996) described bacteria in the gut, body surface, and feces of *Acratia tonsa* using culture-dependent methods and without separating the gut from the host body. Such studies provided limited information about the species composition and diversity of bacterial microbiome attached to the host's surface and internal organs. To elucidate this matter, metagenomic analysis of bacterial DNA extracted separately from the gut and body parts without the gut allows the phylogenetic investigation of these host-associated uncultured bacteria in a broader range. And such a study will uncover knowledge on the complex interaction between host zooplankton and associated bacterial microbiome in the specific microhabitats.

1.4 Flagellated and Ciliated symbionts

Epibiosis involving a relationship between epizoic protists such as flagellates and ciliates and zooplankton is a widespread phenomenon in aquatic environments (Chiavelli 2003, Jones et al. 2016). Ciliates have free-living and symbiotic life (Mayén-Estrada et al. 2021), and about 2600 species are symbionts to metazoan zooplankton (Corliss 2000). The peritrich ciliates order Sessilida contains about 105 to 140 genera and more than 800 species (Foissner et al. 2010). The genus *Epistylis* under the Epistylididae family belonging to Sessilida contains over 260 nominal taxa that may adapt to diverse environmental conditions (Lu et al. 2020, Nitta 2022). Nitta (2022) reported the peritrich ciliate *Epistylis wuhanensis* attached to cyclopoid copepod *Lernaea cyprinacea* from the Arida River in Wakayama prefecture. Several previous

studies reported that heterotrophic peritrich ciliates attach to copepods and cladocerans (Foissner et al. 1999, Utz and Coats 2005, Clamp et al. 2016, Lu et al. 2020).

The protist *Colacium* and species of dinoflagellates colonize on zooplankton hosts (Ho and Perkins 1985, Rosowski and Kugrens 1973). The free-living stage of *Colacium* contains flagellum but leaves when it enters a sessile life attached to a substrate (Hubber-Pestalozzi 1955). Species of *Colacium* are autotrophs and can be heterotrophic in a light-limiting environment (Salmaso and Tolotti 2009). Several studies described *Colacium* as ectosymbionts of copepod and cladoceran hosts (Mohlenberg and Kaas 1990, Threlkeld et al. 1993, Chiavelli et al. 1993, Zalocar et al. 2011).

However, previous studies dealing with epibiotic ciliates and flagellates mainly focused on describing new records using morphological and molecular data, possible effects on hosts, and infection prevalence. But, limited information exists on the ecological aspect of the epibionts.

1.5 Methodology for identifying symbiotic bacteria

In the past, physical characteristics were the only basis for identifying and categorizing the bacteria, which proved to be challenging due to their wide range of variations. However, the emergence of DNA sequencing has transformed the field of biology, enabling more precise analysis of microorganisms. In 1990, Carl Woese and his team first began analyzing and sequencing the 16S rDNA genes of various bacteria using state-of-the-art DNA sequencing technology and used these sequences for phylogenetic studies. With the advent of PCR and automated DNA sequencing in the past two decades, as well as subsequent investigations on the 16S rDNA sequencing of bacteria and 18S rDNA sequencing of eukaryotes, vast amounts of sequence data on the rDNA genes of the smaller subunit of ribosomes in numerous living organisms were collected. Comparing these sequences showed that rDNA gene sequences are

highly conserved within living organisms of the same genus and species but differ between organisms of other genera and species (Woo et al. 2008).

Recently, researchers have conducted extensive research on zooplankton-associated bacteria, specifically focusing on sequencing the 16S rDNA gene (Shoemaker and Moisander 2015, 2017, Eckert et al. 2014, 2021). By using 16S rDNA sequences, researchers have been able to rename and reclassify bacterial genera and species, as well as identify and categorize new ones with possible functions (Qi et al. 2009, Sadaiappon et al. 2021). Moreover, using 16S rDNA sequences has greatly aided in classifying bacteria and their relationships, even for those that cannot be cultivated (Staley et al. 2013, Lentini et al. 2014). In this study, I utilized this advanced 16S rDNA gene sequencing technique and the Illumina Mi-seq platform, which proved to be highly effective in identifying different bacterial species.

1.6 The scientific approach: Thesis contents

In this research, I aimed to identify symbiotic bacteria associated with the bodies of the three most dominant crustacean zooplankton taxa, *E. japonicus*, Cyclopoida spp., and *Daphnia pulicaria* having different feeding habits from Lake Biwa, Japan. Secondly, I established a novel technique to separate the whole intact gut from these three crustacean zooplankton, which allowed me to identify resident symbiotic bacteria in the gut and body parts without the gut of these three zooplankton taxa for the first time.

The thesis, after a general introduction, is structured as follows:

In Chapter 1: Symbiotic bacterial communities of zooplankters from Lake Biwa, Japan

Chapter 2: Comparative analysis of bacterial compositions in the gut, body other than gut, and feces of the three dominant crustacean zooplankton in Lake Biwa, Japan.

Chapter 3: Community structures of symbiotic bacteria associated with the three dominant crustacean zooplankton in different depth habitats of Lake Biwa during the stagnation period.

Chapter 4: Taxonomic and ecological aspects on epibiotic ciliates and flagellates attached to cyclopoid copepods in Lake Biwa.

CHAPTER 1:

"Symbiotic bacterial communities of zooplankters from Lake Biwa, Japan.

This chapter is mainly based on the manuscript:

"Symbiotic bacterial communities of zooplankters from Lake Biwa, Japan" by Taiabur Rahman Tuhin, M.F.A. Anny, Xin Liu, Shoko Hosoi-Tanabe, and Syuhei Ban, accepted in **Plankton and Benthos Research.**

1. Introduction

Ecto- and endosymbiotic bacteria on zooplankton can form functional assemblages that, through diverse interactions, significantly impact surrounding environments (Green 1974, Ebert 2005). Interactions between the host and its symbiotic bacteria can be obligatory or facultative, and mutualistic, commensalistic, or parasitic (Wells and Varel 2011, Martin and Schwab 2012). Generally, the symbiotic bacteria promote host fitness by contributing to nutrition (Mangus et al. 2015, Cooper and Cressler 2020), detoxification (Macke et al. 2017a, Manakul et al. 2017), pathogen protection (Verschuere et al. 2000, Cooper and Cressler 2020), development (Mangus et al. 2015), and reproduction and growth (Peerakietkhajorn et al. 2015, 2016, Callens et al. 2018). However, studies on interactions between zooplankton and their bacterial symbionts are few, especially in natural habitats, because the two are usually considered to represent different functional units with indirect connections in nutrient cycling and food webs in aquatic ecosystems (Azam and Malfatti 2007).

Zooplankton ecologists generally stress interactions between zooplankton and their prey or abiotic factors of surrounding environments that affect life history traits (Boonmak et al. 2018, Liu et al. 2014, Gao et al. 2022) and overlook interactions with symbiotic bacteria. Study of interactions in host–symbiont systems will improve understanding of element flow, ecosystem dynamics, food web structure, and unknown trophic links between them in aquatic ecosystems (Eckert and Pernthaler 2014, Corno et al. 2013, Thébault and Fontaine 2010, Mougi and Kondoh 2012).

Although previous studies have reported microbial symbionts associated with the bodies of crustacean zooplankton from freshwater lakes (Grossart et al. 2009, Wang et al. 2021, Eckert et al. 2021), information on bacterial compositions in the feces released from the zooplankton is scarce. This is also important to understand carbon flux throughout the

water column because fecal material is an essential component of vertical flux throughout the water column (Poulsen and Kiørboe 2006). Previous studies suggested that zooplankton feces may contain bacteria that either originate from their digestive system (Gowing and Silver 1983, Tang 2005) or are acquired from the surrounding environment through the consumption of foods (Hansen and Bech 1996). Callens et al. (2020) found that both the host genotype and environmental microbes influenced the gut bacterial composition of *Daphnia magna* Straus, 1820. Similarly, Grossart et al. (2009) observed that the bacterial composition of a freshwater cyclopoid varies with the environmental bacterial assemblages and differs from that of its cladoceran counterpart. There are several other explanations for how these symbiotic bacterial communities could be constructed; bacterial compositions associated with zooplankton body rely on the feeding habits and behavior of their hosts (Datta et al. 2018, Chae et al. 2021, Velasquez et al. 2022), host specificity of microbial symbionts (Wang et al. 2021), or ambient environmental factors (Huq et al. 1984, Eckert et al. 2021, Chae et al. 2021).

It has been shown that major symbiotic bacterial classes in cultured daphnids, *Daphnia pulicaria* Forbes 1893, *Daphnia pulex* Leydig, 1860, and *D. magna*, include Betaproteobacteria and Gammaproteobacteria, and to a lesser degree Bacteroidetes and Actinobacteria (Qi et al. 2009). Callens et al. (2016) reported Burkholderiales, Aeromonadales, Cytophagales, and Flavobacteriales to be the most abundant order in cultured *D. magna* and *Aeromonas* sp. to be abundant in its gut. Species of *Limnohabitans*, ubiquitous in freshwater lakes, were dominant bacteria in the intestine of laboratory-reared *D. magna* (Freese and Schink 2011). Although this intensive knowledge of key bacterial components associated with their host bodies has been almost reported from cultured daphnids, the information from natural habitats is still limited for both copepods and cladocerans (Samad et al. 2020, Wang et al. 2021, Eckert et al. 2021).

Lake Biwa is the largest lake in Japan, and zooplankton and microbial communities in the lake have been well-studied separately over various time scales (Kawabata 1989, Liu et al. 2014, Okazaki and Nakano 2016, Okazaki et al. 2019). However, interactions between zooplankton and their bacterial symbionts have not been studied yet. In this study, we reported the bacterial communities associated with bodies and feces of three different zooplankton taxa with different feeding habits in Lake Biwa using a metagenomic analysis of 16S rDNA. We can enhance the information on the relationship between microbial symbionts and their zooplankton hosts with different feeding habits *in situ*.

2. Materials and Methods

2.1 Sample collection and preparation

Zooplankton were collected by a NORPAC net (mesh size, 200 μm; mouth diameter, 45 cm) hauled vertically from 20 m to the surface at a pelagic site in the north basin of Lake Biwa, Japan (35°18′32.6″N, 136°8′38.9″E, 70 m deep) on 25 September 2020, which enable to compare the bacterial community among multiple taxa because the biomass of dominant crustacean zooplankton increase from July to September above 20 m in the north basin of Lake Biwa (Liu et al. 2020). Live zooplankton were transferred in an insulated container to the laboratory within 1 h.

Three dominant zooplankton taxa were selected: *Daphnia pulicaria* Forbes, 1893, a suspension feeder that non-selectively ingests suspended materials, including phytoplankton (Ebert 2005, Rothhaupt 1997); *Eodiaptomus japonicus* Kiefer, 1932, a suspension feeder that selectively ingests phytoplankton and microzooplankton (Liu et al. 2021); and Cyclopoida spp., especially late copepodites and adults, which are raptorial feeders that ingest smaller zooplankton such as larval *E. japonicus* (Kawabata 1991). Two species in the cyclopoid copepods, i.e., *Mesocyclops dissimilis* Defaye and Kawabata, 1993 and *Cyclops vicinus* Uljanin,

1875, occurred throughout the study period. We did not discriminate the species because of the similar morphology and combined the two species as one taxon for the following analysis. Both species are at the same trophic level, i.e., raptorial feeders.

The number of animals, i.e., 100 and 120 adults of each taxon, were sorted from live samples under a dissecting microscope (Olympus, SZX12, Japan), washed twice with aged tap water filtered through a glass-fiber filter (Whatman, GF/F) (ATW), autoclaved at 120 °C for 20 min, and incubated into 1-L glass jars filled with ATW. Animals were incubated in a growth chamber (Sanyo, MLR-350, Japan) at ambient temperature (20 °C) for 24 h to evacuate gut contents, then removed from the jar and washed twice with Milli-Q water (better for DNA extraction than ATW to avoid contamination). Feces in jars were filtered through and collected upon a 0.2-μm pore membrane filter (Advantec, K020A047A, Japan) after extraneous materials such as broken body parts, carcasses, or newly hatched individuals had been removed from the sample (beneath a microscope).

Separate samples of 15–25 specimens (without eggs or embryos for females) from each taxon (15 individuals of *D. pulicaria*, 20 individuals of *E. japonicus*, and 25 individuals of Cyclopoida spp.) were transferred into sterile 1.5-mL micro-tubes (Eppendorf, Germany) filled with sodium phosphate (978 μL) and MT buffer (122 μL) in a Fast DNA® Spin Kit for Soil (MP Biomedicals, LLC, USA). In preliminary experiments, we confirmed that 12–15 individuals of *D. pulicaria*, 18–20 individuals of *E. japonicus*, and 22–25 individuals of Cyclopoida spp. were required for extracting a sufficient amount of DNA for metabarcoding analysis. The filters with feces were transferred into a 5-mL tube (Labcon, U.S.A.) and similarly treated. All samples were frozen at −80 °C until the following analysis.

2.2 Metabarcoding analysis using Illumina MiSeq

Samples were thawed and homogenized using a sterile disposable plastic pellet pestle. DNA was directly extracted from each sample using a Fast DNA® Spin Kit for Soil.

Metabarcoding targeted the V4 region of the 16S rRNA gene with primers 515F (5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNGTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNGGACTACHVGGGTWTCTAAT-3′) (Glenn et al. 2019). All PCR reactions were performed for each sample in a total of 10 μL reaction mixture, mixes with Ex TaqHS mix (TaKaRa Bio Inc., Japan), 10 μM of each forward and reverse primer, and about 5 ng of extracted DNA. Initial denaturation proceeded at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, elongation at 72 °C for 30 s, followed by a final extension at 72 °C for 5 min. Before the second PCR was performed, the first PCR product was purified by Beckman Coulter AMPure XP (Beckman Coulter, Inc., USA). To attach the index sequence to the products of the first PCR, products of the second PCR were processed with the primer set F: AATGATACGGCGACCACCGAGATCTACAC-Index-2(8bp)-ACACTCTTTCCCTACACGACGC and R: CAAGCAGAAGACGGCATACGAGAT-Index1(8bp)-GTGACTGGAGTTCAGACGTGTG (Tanaka et al. 2020). Initial denaturation was at 94 °C for 2 min, followed by 10 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, elongation at 72 °C for 30 s, and a final extension at 72 °C for 5 min. After purification using the Beckman Coulter AMPure XP, library quality was assessed using an Agilent fragment analyzer system and dsDNA 915 reagent kit (Advanced Analytical Technologies). Libraries were sequenced on an Illumina MiSeq platform, where 2 × 300 bp paired-end reads were generated. A total of 318,657 raw reads were obtained.

2.3 Data analysis

Raw data collected from the Illumina MiSeq sequencing platform were considered raw tags. Basic tags were prepared after removing the barcode and PCR primer sequences using the fastq_barcode_splitter from the Fastx Toolkit software (ver. 0.0.14). After removing the

primer sequence (50 bp at the 3′ end), chimera sequence, and noise sequence using the dada2 plugin of Qiime2 (ver. 2020.8), effective tags with 97% similarity were classified into one operational taxonomic unit (OTU). OTUs containing "unidentified" taxonomic annotations and singleton (only a single sequence across the entire dataset) were removed from data analysis. All OTUs with best Blast matches to non-bacterial organisms were also discarded from the analysis. OTU sequences belonging to bacteria were deposited to the DNA data bank of Japan (DDBJ) under the accession numbers DRR493548–493553.

Before analyzing OTUs, a rarefied OTU table was generated using the QIIME rarefaction analysis script with the rarefied depth in the minimum sequence number. The Shannon diversity index (*H*) was calculated for the bacterial community of each sample. We discarded nonbacterial OTUs such as chloroplasts (Eckert et al. 2021, Wang et al. 2021) and archaea (Wäge et al. 2019) from all samples because we used bacterial 16S rRNA gene primers. DNA sequences belonging to cyanobacteria had a closest match as a "partial chloroplast containing genome" of the eukaryotic euglenoid alga *Colacium* Ehrenberg, 1834 when compared against the NCBInt database using BLAST search. We, therefore, discarded the cyanobacterial OTUs as well.

We calculated *H* to examine the diversity of OTUs in each sample and performed a non-metric multidimensional scaling (NMDS) analysis to visualize dissimilarities in bacterial composition among samples using all OTUs. Heatmap analysis was performed using the most abundant bacterial families (≥ 100 reads for an OTU) in all samples. Analyses were performed using the R software ver. 4.0.3 (2020) 'vegan' package.

3. Results

A total of 293,018 sequences comprising 669 bacterial OTUs were generated, including three zooplankton bodies, in which 54 (*D. pulicaria*), 88 (*E. japonicus*), and 84 (Cyclopoida spp.) OTUs were identified, and in their feces, 132, 156, and 155 OTUs, respectively. Among

the identified OTUs, 7 (*D. pulicaria*), 48 (*E. japonicus*), and 51 (Cyclopoida spp.) OTUs were unique in the host bodies, and 13, 36, and 31 OTUs were in their feces, respectively (Table 1-1). *H* was lower in bodies (2.15–2.38) than in feces (2.44–2.83) and was highest for *E. japonicus*.

Table 1-1. Total numbers of sequences and identified OTUs, and Shannon's *H* index of bacteria associated with bodies and feces of *Daphnia pulicaria*, *Eodiaptomus japonicus*, and Cyclopoida spp. from Lake Biwa.

Taxa	Body or feces	Total no. of sequences	No. of OTUs	No. of unique OTUs	Shannon's *H*
D. pulicaria	Body	41529	54	7	2.157
E. japonicus	Body	46603	88	48	2.379
Cyclopoida spp.	Body	36561	84	51	2.153
D. pulicaria	Feces	56441	132	13	2.442
E. japonicus	Feces	50781	156	36	2.827
Cyclopoida spp.	Feces	61103	155	31	2.645

Bacteria were attributed to 15 identified phyla and 6 unknown phyla (termed as unidentified), with Proteobacteria, Bacteroidetes, Actinobacteria, and Firmicutes predominant in host bodies and feces (Fig. 1-1). Proteobacteria accounted for > 80% of all bacterial sequences for *D. pulicaria* and *E. japonicus* bodies, and Bacteroidetes ~9%. In Cyclopoida spp., Proteobacteria accounted for 55% and Bacteroidetes 35% of all bacterial sequences. Proteobacteria (52%–66%) were also abundant in the feces of all zooplankter taxa, followed by Actinobacteria (11.6%–34%), which was also more in feces. Proteobacteria and Bacteroidetes were less abundant in feces.

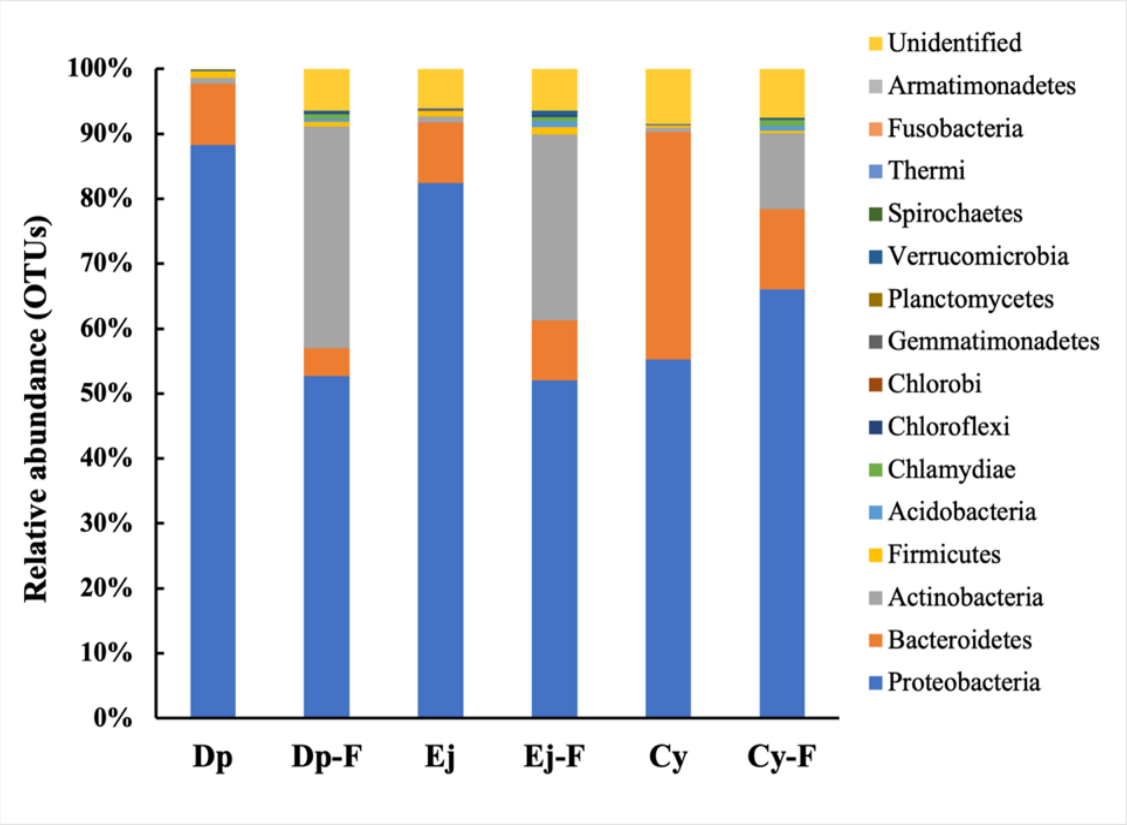

Fig. 1-1 Bacterial community (all OTUs) phyla associated with bodies and feces of *Daphnia pulicaria* (Dp), *Eodiaptomus japonicus* (Ej), and Cyclopoida spp. (Cy), from Lake Biwa.

At the order level, dominant bacterial OTUs in host bodies (based on numbers of sequences) were Aeromonadales (37%) and Burkholderiales (25%), while in feces, these were Actinomycetales (20%) and Rickettsiales (41%) (Fig. 1-2). Rhodobacterales was dominant only in cyclopoid bodies (4%). For *D. pulicaria* and *E. japonicus* bodies, Flavobacteriales (9.3% and 3.4%, respectively) and Pseudomonadales (2% and 7.5%, respectively), were relatively abundant, but not so (<0.2%) in Cyclopoida spp. Both Holophagales and Methylophilales occurred in bodies of *E. japonicus* but not *D. pulicaria* or Cyclopoida spp. Saprospirales occurred in bodies of *E. japonicus* and Cyclopoida spp., but not *D. pulicaria.*

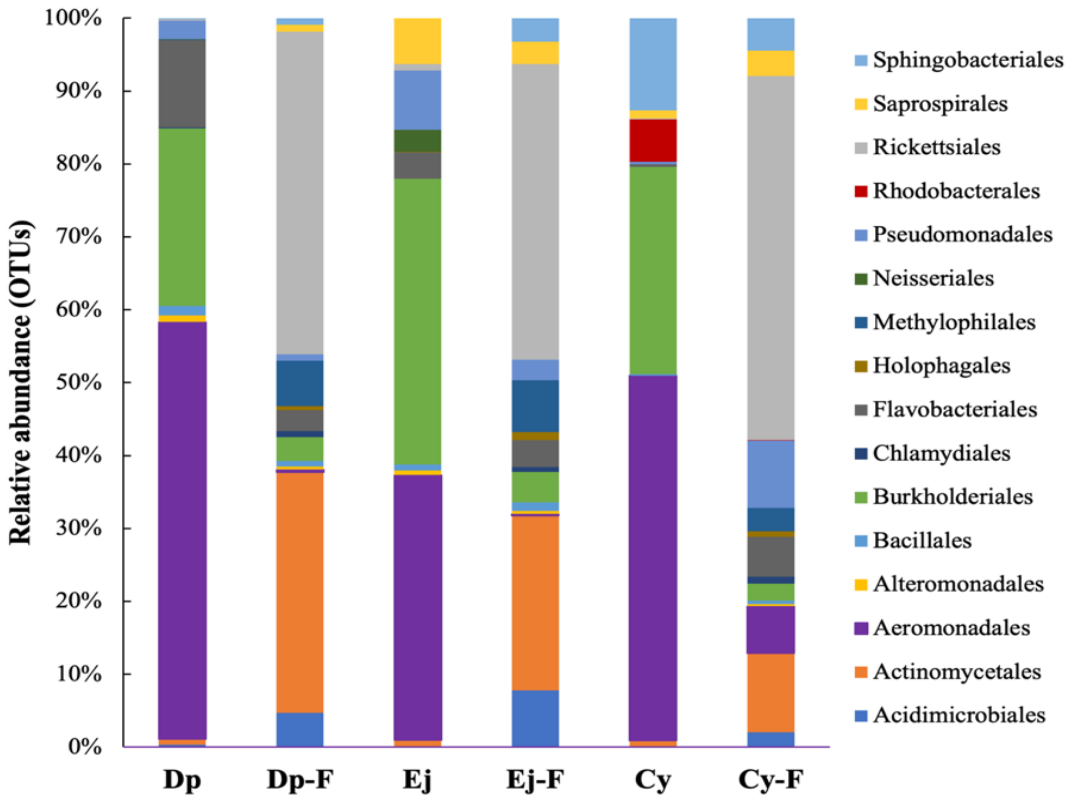

Fig. 1-2 Bacterial community (≥ 100 OTU reads) orders associated with bodies and feces of *Daphnia pulicaria* (Dp), *Eodiaptomus japonicus* (Ej), and Cyclopoida spp. (Cy), from Lake Biwa.

The orders Spirobacillales, Legionellales, Chlamydiales, and Cerasicoccales occurred only in bodies of *D. pulicaria* (Fig. 1-3); Procabacteriales, Deinococcales, Gemmatales, and Holophagales occurred only in bodies of *E. japonicus* (Fig. 1-4); and Rhizobiales, Caulobacterales, Rhodospirillales, Bdellovibrionales, Xanthomonadales, Pasteurellales, Solirubrobacterales, Lactobacillales, Fimbriimonadales, Armatimonadales, Fusobacteriales, and Gemmatimonadales occurred only in cyclopoid bodies (Fig. 1-5).

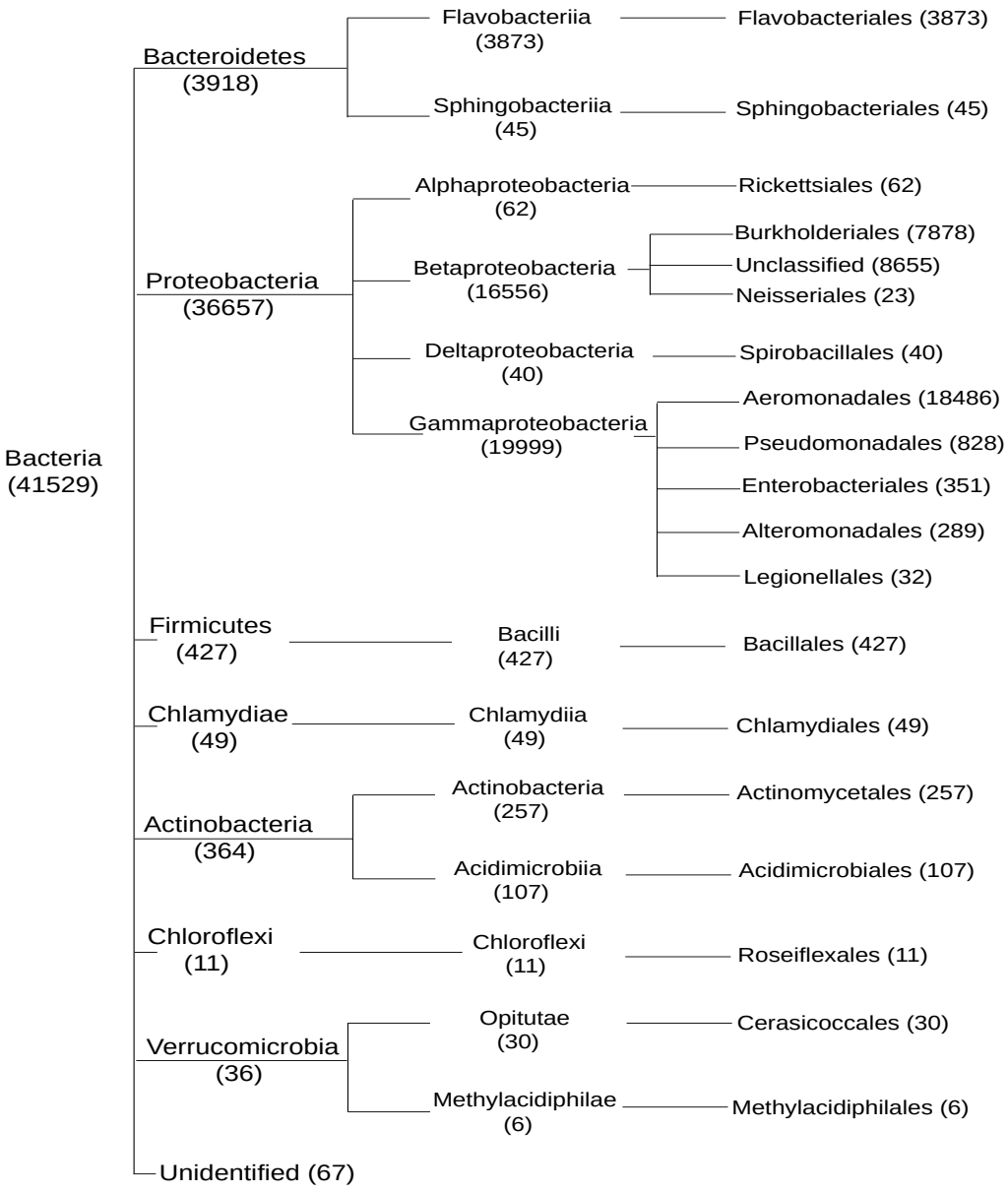

Fig. 1-3 Taxonomic tree of the bacteria associated with *Daphnia pulicaria*, collected from a pelagic site in the north basin of Lake Biwa on 25 September 2020 from phylum to order level taxonomic assignment using all OTUs. Figures in parentheses represent the cumulative number of OTUs assigned.

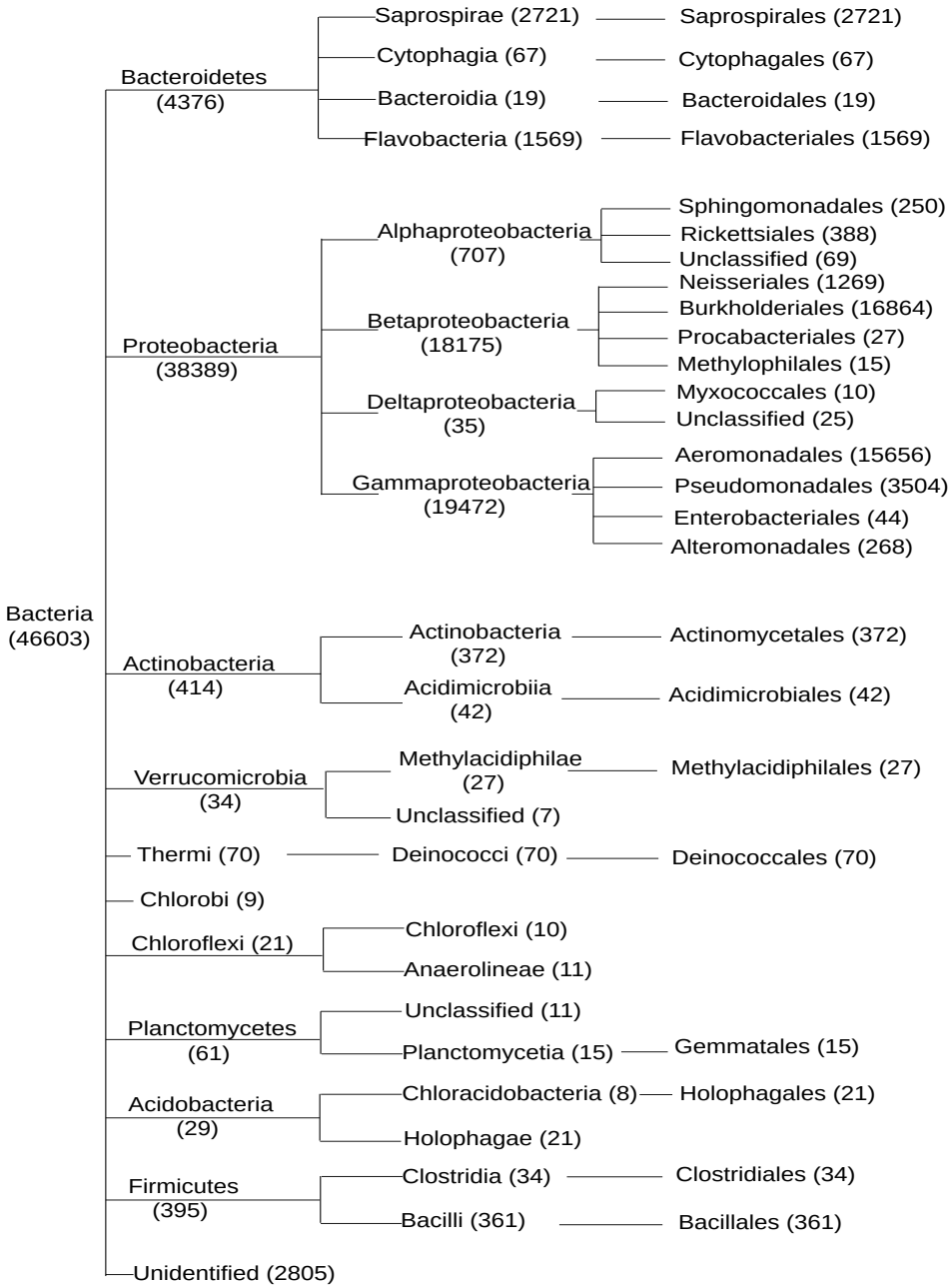

Fig. 1-4 Taxonomic tree of the bacteria associated with *Eodiaptomus japonicus*, collected from a pelagic site in the north basin of Lake Biwa on 25 September 2020 from phylum to order level taxonomic assignment using all OTUs. Figures in parentheses represent the cumulative number of OTUs assigned.

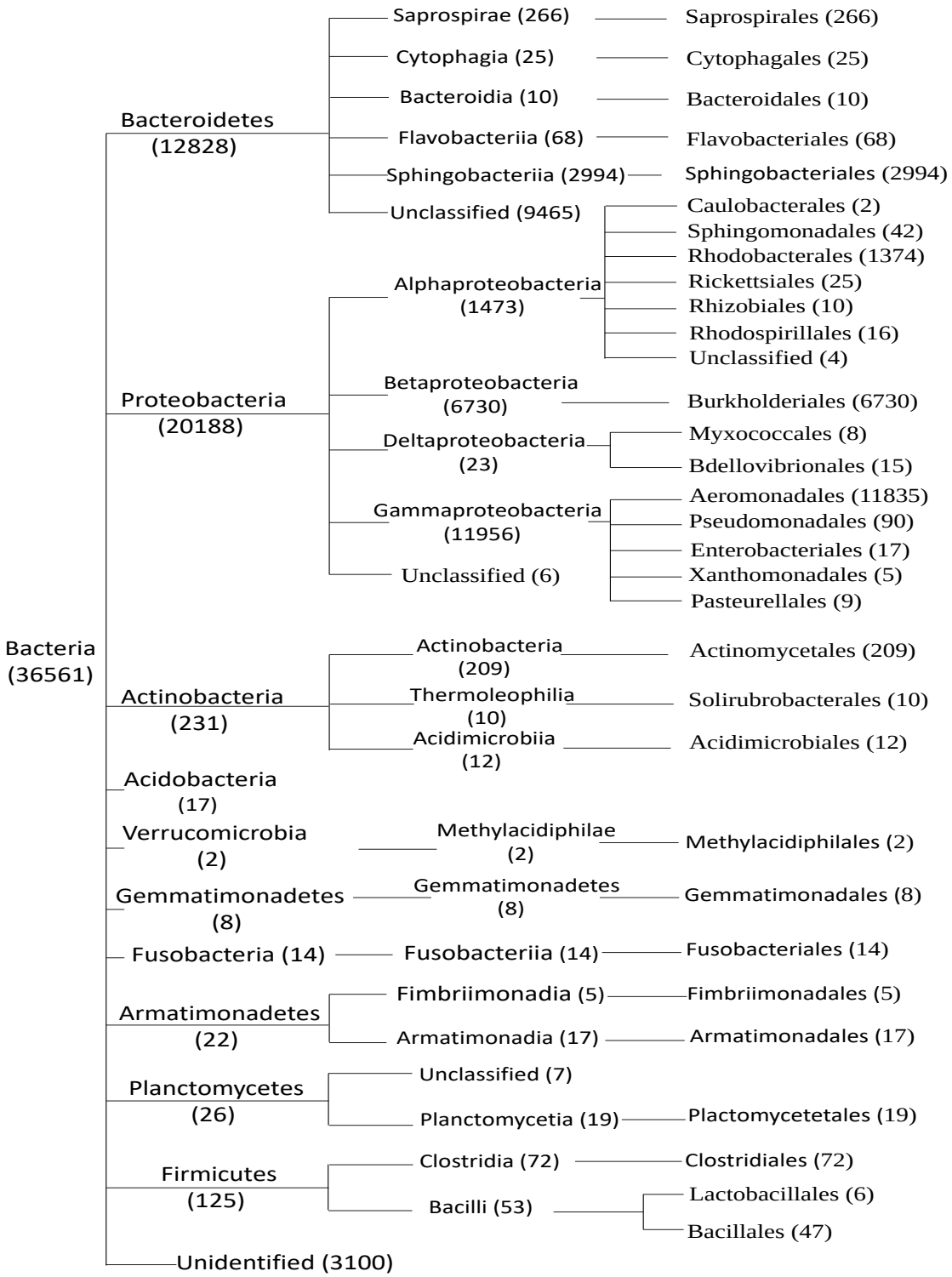

Fig. 1-5 Taxonomic tree of the bacteria associated with Cyclopoida spp., collected from a pelagic site in the north basin of Lake Biwa on 25 September 2020 from phylum to order level taxonomic assignment using all OTUs. Figures in parentheses represent the cumulative number of OTUs assigned.

Excluding the dominant Actinomycetales and Rickettsiales, zooplankton feces of all three taxa contained Methylophilales, Acidimicrobiales, Burkholderiales, Flavobacteriales and Pseudomonadales, and the bacterial composition differed from that of their bodies. Proportions of Methylophilales and Acidimicrobiales OTUs in feces of *D. pulicaria* (5.6% and 4%, respectively) and *E. japonicus* (6% and 7%, respectively) were slightly higher, while those of Pseudomonadales (8.3%), Aeromonadales (5.8%), and Flavobacteriales (5%) were relatively more abundant in feces of Cyclopoida spp. NMDS using all OTU sequences revealed that bacteria in feces differed from those in host bodies and that variation in the body was greater (Fig. 1-6).

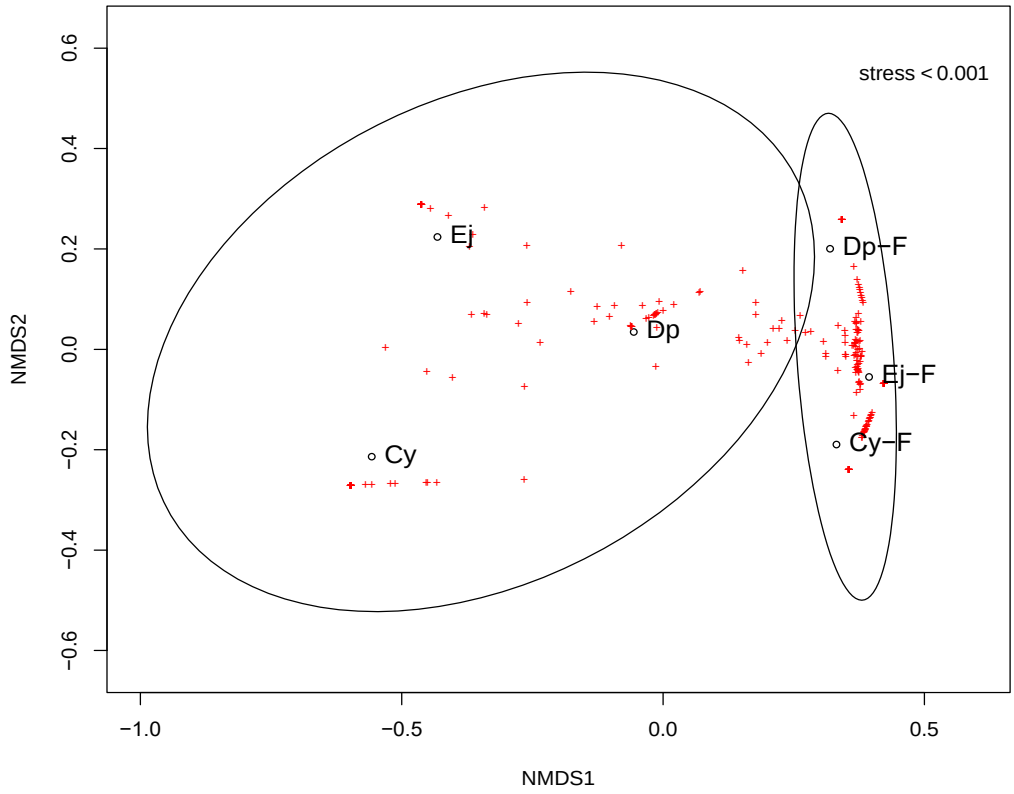

Fig. 1-6 Non-metric multidimensional scaling (NMDS) analysis (all OTUs) of dissimilarities among zooplankter bodies and feces. *Eodiaptomus japonicus* (Ej), *Daphnia pulicaria* (Dp), and Cyclopoida spp. (Cy); Ej-F, Dp-F, and Cy-F denote feces of each taxon. Encircled areas indicate confidence 95% intervals. Open circles represent zooplankter taxa; red crosses represent bacterial OTUs.

Heatmap analysis reveals a large difference in dominant bacterial families between zooplankter bodies and feces (Fig. 1-7). Bacterial families associated with *D. pulicaria* and Cyclopoida spp. bodies were most similar, whereas those of feces of *D. pulicaria* and *E. japonicus* were more similar.

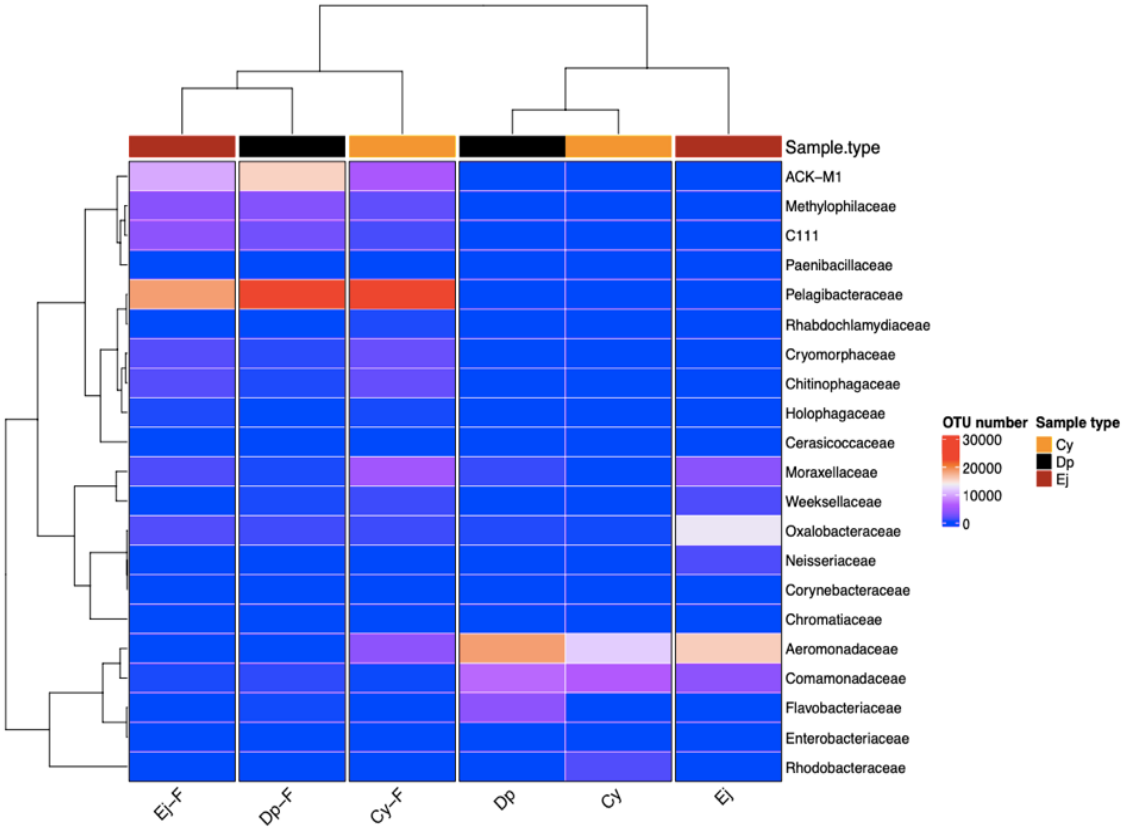

Fig. 1-7 Heatmap of abundant bacterial families (≥ 100 OTU reads) associated with zooplankter bodies and feces. *Eodiaptomus japonicus* (Ej), *Daphnia pulicaria* (Dp), and Cyclopoida spp. (Cy); Ej-F, Dp-F, and Cy-F denote feces of each taxon. Individual lines correspond to bacterial families; each column corresponds to a different sample. The upper dendrogram represents similarities in bacterial composition among zooplankter bodies and feces; left dendrogram represents similarities in patterns of occurrence of a bacterial family in a zooplankter body and feces. ACK-M1 and C111 are candidate Actinobacteria families.

For each zooplankton taxon, Aeromonadaceae was the dominant family in bodies, then for *D. pulicaria* and Cyclopoida spp., Comamonadaceae (17% of all bacterial OTUs), and for *E. japonicus*, Oxalobacteraceae (28%). In feces, the proportional contributions of Comamonadaceae and Oxalobacteraceae are lower than in zooplankton bodies, Pelagibacteraceae (40% of all sequences) was dominant, followed by ACK-M1 belonging to

Actinobacteria, Methylophilaceae, and C111 for *D. pulicaria* (5.6%) and *E. japonicus* (6%), and Moraxellaceae for Cyclopoida spp. (8%, below the percentage in bodies). Proportions of Chitinophagaceae, Cryomorphaceae, and Rhabdochlamydiaceae were higher in feces than in bodies of each zooplankton. Neither the Legionellaceae nor Rhabdochlamydiaceae occurred in either copepod body; Peptostreptococcaceae, Intrasporangiaceae, and Sphingomonadaceae occurred only in the cyclopoid body; and Trueperaceae occurred only in the *E. japonicus* body. Other notable families associated with the bodies of *D. pulicaria* included the Flavobacteriaceae (8.6%) and Moraxellaceae (2%), *E. japonicus* the Moraxellaceae (7%), Weeksellaceae (3%), and Neisseriaceae (3%), and Cyclopoida spp., the Rhodobacteraceae (4%) (Table 1-2).

Bacterial genera *Aeromonas*, *Ralstonia*, and *Acinetobacter* were commonly detected in the bodies of each zooplankton taxon. *Limnohabitans*, *Rhodoferax*, *Perlucidibaka*, and *Brevibacillus*, occurred only in the body of *D. pulicaria*. *Polaromonas*, *Hydrogenophaga*, *Comamonas*, *Corynebacterium*, *Truepera*, *Spirosoma*, *Staphylococcus*, and *Sphingomonas* occurred only in the body of *E. japonicus*. *Delftia*, *Rhodobacter*, *Segetibacter*, *Rodocytophaga*, and *Pontibacter* occurred only in the cyclopoid body (Table 1-2). *Flavisolibacter* and *Candidatus* Rhabdochlamydia occurred only in zooplankton feces, while *Flavobacterium*, *Polynucleobacter*, *Fluviicola*, and *Chryseobacterium* occurred in both bodies and feces (Table 1-3). *Flavobacterium*, which can digest polysaccharides (Gavriilidou et al. 2020), was relatively abundant in the body of *D. pulicaria*, less abundant in that of *E. japonicus*, and relatively scarce in the body of cyclopoids.

Table 1-2. Bacterial families for host taxa. Hyphens: family not detected, family < 0.1% of all OTU sequences, or unknown genus.

Order	Family	*Daphnia pulicaria*		*Eodiaptomus japonicus*		Cyclopoida spp.	
		Relative abundance (%)	Genus	Relative abundace (%)	Genus	Relative abundace (%)	Genus
Aeromonadales	Aeromonadaceae	45	*Aeromonas*	34	*Aeromonas*	32	*Aeromonas*
Burkholderiales	Comamonadaceae	17	*Rhodoferax, Limnohabitans*	8	*Polaromonas,Comamonas, Hydrogenophaga*	17	*Comamonas, Delftia*
	Oxalobacteraceae	1	*Ralstonia, Polynucleobacter*	28	*Ralstonia, Polynucleobacter*	1	*Ralstonia*
Pseudomonadales	Moraxellaceae	2	*Acinetobacter, Perlucidibaca*	7	*Acinetobacter*	0.2	*Acinetobacter*
Flavobacteriales	Flavobacteriaceae	8.6	*Flavobacterium*	0.7	*Flavobacterium*	–	–
	Cryomorphaceae	0.8	*Fluviicola*	–	–	0.1	*Fluviicola*
Aeromonadales	Chromatiaceae	0.7	*Rheinheimera*	0.6	*Rheinheimera*	–	–
Enterobacteriales	Enterobacteriaceae	0.8	–	–	–	–	–
Bacillales	Paenibacillaceae	0.2	*Brevibacillus*	–	–	–	–
Rhodobacterales	Rhodobacteraceae	–	–	–	–	4	*Rhodobacter*
Saprospirales	Chitinophagaceae	–	–	–	–	0.6	*Segetibacter*
Clostridiales	Peptostreptoccaceae	–	–	–	–	0.2	–
Actinomycetales	Intrasporangiaceae	–	–	–	–	0.2	–
Neisseriales	Neisseriaceae	0.1	*Chitinibacter*	3	*Chitinibacter*	–	–
Flavobacteriales	Weeksellaceae	–	–	3	*Chryseobacterium*	0.1	*Chryseobacterium*
Cytophagales	Cytophagaceae	–	–	0.1	*Spirosoma*	0.1	*Rodocytophaga, Pontibacter*
Pseudomonadales	Pseudomonadaceae	–	–	0.1	*Pseudomonas*	–	–
Actinomycetales	Corynebacteriaceae	–	–	0.2	*Corynebacterium*	–	–
Burkholderiales	Burkholderiaceae	–	–	0.2	*Burkholderia*	0.1	*Burkholderia*
Deinococcales	Trueperaceae	–	–	0.2	*Truepera*	–	–
Bacillales	Staphyllococcaceae	–	–	0.1	*Staphyllococcus*	–	–
Legionellales	Legionellaceae	0.1	–	–	–	–	–
Actinomycetales	Micrococcaceae	–	–	0.1	*Rothia*	–	–
Sphingobacteriales	Sphingomonadaceae	–	–	–	–	0.1	*Sphingomonas*

Table 1-3. Major bacterial OTUs from phylum to genus level taxonomic assignments and their relative abundance (%) found in the feces of the three different zooplankton species. Hyphen represents no detection in a family or unknown genus detected.

Zooplankton species (No. of sequences)	Phylum	Class	Order	Family	Genus
D. pulicaria (56441)	Proteobacteria (53%)	Alphaproteobacteria (40%)	Rickettsiales (40%)	Pelagibacteraceae (40%)	–
		Betaproteobacteria (11%)	Methylophilales (5.6%)	Methylophilaceae (5.6%)	–
			Burkholderiales (3%)	Oxalobacteraceae (1.7%)	*Polynucleobacter* (1.5%)
				Comamonadaceae (1%)	–
	Bacteroidetes (4%)	Flavobacteria (2.6%)	Flavobacteriales (2.6%)	Cryomorphaceae (1%)	*Fluviicola* (1%)
				Flavobacteriaceae (0.7%)	*Flavobacterium* (0.7%)
		Saprospirae (1%)	Saprospirales (1%)	Chitinophagaceae (1%)	*Flavisolibacter* (0.5%)
	Actinobacteria (34%)	Actinobacteria (30%)	Actinomycetales (30%)	–	–
		Acidimicrobiia (4%)	Acidimicrobiales (4%)	–	–
	Chlamydiae (0.8%)	Chlamydiia (0.8%)	Chlamydiales (0.8%)	Rhabdochlamydiaceae (0.6%)	*Candidatus* Rhabdochlamydia (0.6%)
	Firmicutes (0.7%)	Bacilli (0.7%)	Bacillales (0.7%)	–	–
E. japonicus (50781)	Proteobacteria (52%)	Alphaproteobacteria (37%)	Rickettsiales (36.6%)	Pelagibacteraceae (36%)	–
		Betaproteobacteria (10.6%)	Methylophilales (6%)	Methylophilaceae (6%)	–
			Burkholderiales (3.7%)	Oxalobacteraceae (2.7%)	*Polynucleobacer* (2.4%)
				Comamonadaceae (1%)	–
		Gammaproteobacteria (3%)	Pseudomonadales (2.5%)	Moraxellaceae (2.5%)	*Acinetobacter* (2.5%)
	Bacteroidetes (9%)	Flavobacteria (3%)	Flavobacteriales (3%)	Cryomorphaceae (2.8%)	*Fluviicola* (2.8%)
		Sphingobacteriia (2.8%)	Sphingobacteriales (2.8%)	–	–
		Saprospirae (2.8%)	Saprospirales (2.8%)	Chitinophagaceae (2.8%)	*Flavisolibacter* (2%)
	Actinobacteria (29%)	Actinobacteria (21.6%)	Actinomycetales (21.6%)	–	–
		Acidimicrobiia (7%)	Acidimicrobiales (7%)	–	–
	Firmicutes (1%)	Bacilli (1%)	Bacillales (1%)	–	–
	Acidobacteria (1%)	Holophagae (1%)	Holophagales (1%)	Holophagaceae (1%)	–
Cyclopoida spp. (61103)	Proteobacteria (66%)	Alphaproteobacteria (45.69%)	Rickettsiales (45%)	Pelagibacteraceae (44.7%)	–
		Gammaproteobacteria (14.5%)	Pseudomonadales (8.3%)	Moraxellaceae (8%)	*Acinetobacter* (8%)
			Aeromonadales (5.8%)	Aeromonadaceae (5.8%)	*Aeromonas* (5.8%)
		Betaproteobacteria (5%)	Methylophilales (2.9%)	Methylophilaceae (2.9%)	–
			Burkholderiales (2%)	Oxalobacteraceae (1.5%)	*Polynucleobacter* (1%)
	Bacteroidetes (12%)	Flavobacteria (5%)	Flavobacteriales (5%)	Cryomorphaceae (3%)	*Fluviicola* (3%)
				Weeksellaceae (1.5%)	*Chryseobacterium* (1.5%)
		Sphingobacteriia (4%)	Sphingobacteriales (4%)	–	–
		Saprospirae (3%)	Saprospirales (3%)	Chitinophagaceae (3%)	*Flavisolibacter* (2%)
	Actinobacteria (11.6%)	Actinobacteria (9.8%)	Actinomycetales (9.8%)	–	–
		Acidimicrobiia (1.8%)	Acidimicrobiales (1.8%)	–	–
	Chlamydiae (1%)	Chlamydiia (1%)	Chlamydiales (1%)	–	–
	Acidobacteria (0.7%)	Holophagae (0.7%)	Holophagales (0.7%)	Holophagaceae (0.7%)	–

4. Discussion

The heatmap analysis revealed that the bacterial communities of zooplankton bodies mostly differ from those of their feces. Bacteria from zooplankton bodies can be directly attached to the body surface (Carman and Dobbs 1997, Qi et al. 2009, Skovgaard et al. 2015) and/or colonize the gut (Tang et al. 2010). Some free-living environmental bacteria may also attach to potential zooplankter foods (Simon et al. 1992) and enter the gut through ingestion. Bacteria attached to the food ingested may be both intestinal residents, persisting in the gut (Gowing and Silver 1983), and transients, passing through it (Olafsen 1984, King et al. 1991, Nagasawa 1992). Transient bacteria might only remain in the gut for a short period of time (Harris 1993, Tang et al. 2010), and because some ingested material may remain undigested at egestion (Parsons et al. 1977), some may remain intact following egestion (Hansen and Bech 1996).

In the present study, the most abundant bacterial family in the feces of all three zooplankton hosts was Pelagibacteraceae, but it was quite low in abundance in the bodies. Actinobacterial ACK-M1 and C111 were also abundant in the feces but quite scarce in the bodies of the three hosts. In our other campaigns conducted in 2020 and 2021, Pelagibacteraceae, ACK-M1, and C111 were dominant in the epilimnetic waters of Lake Biwa in September (authors' unpublished data). This implies that the dominant bacterial families in the feces were similar to those in the water column. Okazaki and Nakano (2016) showed that Actinomycetales was predominant order in the epilimnion of Lake Biwa from April to December 2010, when strict stratification caused major nutrient concentrations to be reduced (Kim et al. 2006) and dissolved organic carbon (DOC) to be generally abundant (Maki et al. 2010). Pelagibacteraceae (Rickettsiales) can grow in nutrient-limited conditions (Ortmann and Santos 2016) using DOC (Giovannoni et al. 2005). This implies that the bacteria found in the

feces may be mostly transient and therefore differ from the residential bacteria associated with the host bodies.

Since our three zooplankton taxa were collected from the same depth and had different bacterial compositions in the feces, some fecal bacteria may be related to host feeding habits. The fecal bacteria of *D. pulicaria* and *E. japonicus* are similar and differ slightly from those of Cyclopoida spp. Both *D. pulicaria* and *E. japonicus* are suspension feeders that ingest particulate organic matter (POM), including bacterial aggregates, while Cyclopoida spp., especially adults, are fundamentally carnivorous and mainly ingest other small zooplankton. Adult *M. dissimilis*, a typical dominant cyclopoid in Lake Biwa, prey on the late nauplii and early copepodites of *E. japonicus* (Kawabata 1991).

Of the three zooplankton taxa, *H* indices revealed the fecal bacterial community of *E. japonicus* to be the most diverse. Potential foods for this species are more variable than either of the other zooplankton taxon, including phytoplankton and microzooplankton (Yoshida et al. 2001, Wang et al. 2021). This omnivorous habit may lead to the bacterial community of its feces being more diverse than that of the herbivorous *D. pulicaria* and carnivorous Cyclopoida spp.

Flavobacterium, capable of degrading polysaccharides (Gavriilidou et al. 2020, Inoue et al. 2016), was more abundant in the body of herbivorous *D. pulicaria* than in the body of omnivorous *E. japonicus*, and it was only present at low abundances in carnivorous adult cyclopoids. Phytoplankton cell walls are mainly composed of polysaccharides (Qi et al. 2009) such as cellulose, hemicellulose, and pectins, which are preferred substrates for *Flavobacterium* bacteria. The association between *Flavobacterium* spp. and the bodies of *D. pulicaria* and *E. japonicus* might indicate that this bacterium is an intestinal symbiont that contributes to phytoplankton digestion. Furthermore, species of Aeromonadaceae show high chitinase activity (Tran et al. 2018). A plausible explanation for the prevalence of

Aeromonadaceae in the bodies of the three hosts could be the plentiful suspended chitin-based organic particulate aggregates within their ambient environments, which serve as an available food source for zooplankton. These particles would be derived from the remains of crustacean zooplankton, such as carcasses and molts, in freshwater systems (Grossart and Simon 1998, Grossart et al. 1997). In Lake Biwa, the absence of small phytoplankton during summer to autumn (Yoshida et al. 2001) may imply that herbivorous suspension-feeding hosts, like *D. pulicaria* and *E. japonicus*, consume more of these organic particles, which contain a high amount of chitin (Tang et al. 2010). Contrastingly, the carnivorous cyclopoid primarily feeds on other zooplankton that have chitin-based exoskeletons.

Bacterial communities associated with host bodies differed among the three zooplankton taxa. Bacterial OTUs referable to Trueperaceae occurred only with *E. japonicus*; those of Peptostreptococcaceae, Intrasporangiaceae, Rhodobacteraceae, and Sphingomonadaceae occurred only with Cyclopoida spp.; and Legionellaceae and Rhabdochlamydiaceae occurred only with *D. pulicaria*. Additionally, Oxalobacteraceae, Moraxellaceae, Neisseriaceae, and Weeksellaceae were abundant with *E. japonicus* but scarce with Cyclopoida spp.; and the family Rhodobacteraceae was abundant in Cyclopoida spp., but was not detected in *E. japonicus*. Some bacteria associated with aquatic organisms are host-specific and play protective roles (Wahl et al. 2012), protecting a host from biological and physical stresses (Erken et al. 2015). Attached benign bacteria may also contribute to the probiotic effect of the host *Artemia* body against pathogenic bacteria (Verschuere et al. 2000). Therefore, the specific bacterial components of host zooplankton may play important roles in maintaining host health.

Heatmap analysis reveals bacterial families associated with host cyclopoid and *D. pulicaria* bodies to be similar but differ from those of *E. japonicus*. During the stagnation period in Lake Biwa, *E. japonicus* usually occurs shallower than 20 m depth within the

epilimnion (Kawabata 1987). Because *Mesocyclops thermocyclopoides* Harada, 1931 (close relative of *M. dissimilis*) and *D. pulicaria* can occur in deeper layers during the day and migrate into shallow waters at night (Liu and Hu 2001, Stich and Lampert 1981), their environments may be more similar to each other than to that of *E. japonicus*. Habitat and behavioral differences may affect bacterial assemblages in the bodies of our three zooplankton taxa. Migrating *D. magna* can transport bacteria across depth layers and release them during migration (Grossart et al. 2010). In Lake Biwa's summer months, *E. japonicus* is the most abundant species and does not exhibit diel vertical migration (Kawabata 1987, Yoshida et al. 2001). A higher prevalence of Oxalobacteraceae associated with this host during September could be attributed to the mesophilic characteristics of this bacteria, thriving at temperatures of 20 °C or higher (Baldani et al. 2014).

The dominant families of symbiotic bacteria associated with *D. pulicaria* bodies in the present study were very similar to those found in the bodies of *Daphnia obtusa* Kurz, 1874 and *Daphnia galeata* Sars, 1864 from freshwater lake/pond in Italy (Eckert et al. 2021), and those found from the whole-bodies of the cultured daphnids, *D. magna, D. pulicaria and Daphnia pulex* (Leydig, 1860) (Freeze and Schink 2011, Qi et al. 2009, Peerakietkhajorn et al. 2015, Callens et al. 2016) (see Appendix 1)). This implies that this symbiotic bacterial family associated with daphnid species may follow a pattern termed phylosymbiosis, that is, a group of taxonomically similar zooplankton species shows similarity in their microbial symbionts (Lim and Bordenstein 2020). Symbiotic *Aeromonas* sp., which was dominant in the present study and a lot of previous studies (see Appendix 1), has been shown to support the growth of its daphnid host in laboratory experiments (Mangus et al. 2015).

In the previous studies, the dominant bacterial families reported in the bodies of freshwater calanoids, *Sinocalanus doerrii* (Brehm, 1909) and *Sinodiaptomus sarsi* (Rylov, 1923), were Pseudomonadaceae, Bacillaceae, and Moraxellaceae (Wang et al. 2021), while

those in *Eudiaptomus padanus* (Burckhardt, 1900) and Calanoida spp. were Comamonadaceae, Pseudomonadaceae, Flavobacteriaceae, Neisseriaceae, and Oxalobacteraceae (Eckert et al. 2021). These families were different from those found in *E. japonicus* in the present study (see Appendix 1). The prevalent bacterial families associated with the body of Cyclopoida spp. in the present study also differed from those found in other cyclopoid copepods, e.g., *Thermocyclops oithonoides* (Sars, 1863) and *Mesocyclops leuckarti* (Claus, 1857) from freshwater lakes in Europe (see Appendix 1). These facts suggest that copepod-associated bacteria may be host-specific and/or depend on the ambient environmental conditions, including available food types and bacterial communities in the surrounding waters (Grossart et al. 2009, Velasquez et al. 2022).

Bacterial communities associated with the bodies of *E. japonicus* and Cyclopoida spp. were more diverse than in *D. pulicaria*. The cephalothorax of copepods has many sensory organs and excretory pores (Koomen and von Vaupel Klein 1998), whereas daphniids do not, and they also have reduced carapace segmentation (e.g., Sars 1993). This implies that the surface of copepods is structurally more complex than the outer shield of daphniids. Bacteria on zooplankter body surfaces may attach to and/or colonize the fine pits and holes of sensory organs.

In this study, since the sampling was conducted during the period when the biomass of the zooplankton hosts increased in Lake Biwa, a comparative analysis of bacterial communities among the three hosts could be done. Moreover, analyzing the bacterial compositions associated with not only whole bodies of the hosts but also feces, which has not been previously done in zooplankton (see Appendix 1), has allowed us to discuss the relationship between the symbiotic bacterial communities and feeding habits of the hosts. On the other hand, seasonal succession and generality of the bacterial community is still an open question. An additional analysis, e.g., in the period of lower zooplankton biomass (e.g., winter), is necessary.

CHAPTER 2:

“Comparative analysis of bacterial compositions in the gut, body other than gut, and feces of the three dominant crustacean zooplankton in Lake Biwa, Japan.”

1. Introduction

Symbiotic bacteria can be associated with their hosts' external surfaces and internal organs through various adherence and penetration mechanisms (De Corte et al. 2014, 2018). Zooplankton may acquire bacterial symbionts from their mother during embryonic development (Shoemaker and Moisander 2017) and from their surrounding environments (Tang et al. 2010. Wahl et al. 2012). The digestive tracts, especially the intestine of zooplankton, harbors a diverse symbiotic microbiota (Nagasawa and Nemoto 1988, Harris 1993, Peter and Sommaruga 2008) that may digest the food materials ingested by their hosts and convert them into essential metabolites accessible for their hosts (Flint et al. 2008, Wu et al. 2012). Resident bacteria in the intestine may also take part in the degradation of egested fecal materials and play a profound role in nutrient cycling in an aquatic ecosystem (Gowing and Silver 1983, Tang et al. 2011, Yeh et al. 2020). In addition, the intestinal environment may contain an acidic and hypoxic to anoxic condition, which may influence the colonization of anaerobic bacteria (Tang et al. 2011, Shoemaker and Moisander 2015, 2017).

The microbial composition in the digestive tract of zooplankton can be changed by the diet of the hosts (Tang 2005, Tang et al. 2009, Sadaiappon et al. 2021), species-specific feeding behaviors (Chae et al. 2021, Velasquez et al. 2022) and surrounding environmental factors including bacterial community structures (Grossart et al., 2009, Callens et al. 2020). The remaining parts of the zooplankton body, excluding the digestive tract, also provide suitable environments for colonizing diverse bacterial microflora (Tang et al. 2010, Wang et al. 2021). Bacteria may directly attach to the surface of the chitinous exoskeleton (Hansen and Bech 1996, Carman and Dobbs 1997, Qi et al. 2009), filtering apparatus of a filter feeder (Qi et al. 2009), oral area and the feeding appendages (Huq et al. 1983, Nagasawa 1988, Skovgaard et al. 2015) and anal region (Bickel and Tang 2014), which

may attract bacteria as microhabitats enriched with organic matters. Bacteria attached to the exoskeleton of zooplankton may protect their hosts from pollutants in the surrounding environment and smoothen mobility (Manakul et al. 2017, Nagasawa 1989). However, knowledge of the community composition and functions of the bacterial community in/on the exterior of the zooplankton body is scarce to date.

In most studies dealing with the bacterial community associated with crustacean zooplankton, whole body of the hosts without dissecting their body parts were used for analyzing the microbial symbionts (Grossart et al. 2009, Chae et al. 2021, Eckert et al. 2021, Valesquez et al. 2022). Therefore, the information on the locations of residential bacteria, e.g., in the gut and body surface of the hosts, was limited. Previously, symbiotic bacteria on the external surface of the hosts' body were detected by culturing them (Harris 1993, Carman and Dobbs 1997, Huq et al. 1983) or using microscopic analysis (Nagasawa and Nemoto 1985, Qi et al. 2009, Skovgaard et al. 2015). However, such methods may not provide sufficient information about species composition and diversity of the microbiome attached to the hosts' exterior. Metagenomic analysis allows us to know sufficient information on the diversity and phylogeny of the symbiotic microbiota, including uncultured ones.

It has been shown that zooplankton suffer from reduced fitness and negatively impact host life-history traits without symbiotic microbiota (Mangus et al. 2015, Akbar et al. 2020). However, there is limited information on community structure and diversity of host-associated bacteria in the specific sites of the exterior and interior of the host zooplankton collected from freshwater lakes. In addition, because of the difficulty of separating a gut from a small-sized zooplankton body (<1mm of body length), the studies dealing with bacterial communities associated with the body surface and gut separately were limited

(Tang et al. 2010). There were no studies dealing with host-associated bacterial community compositions separately in the gut and body parts other than the gut of zooplankton from both culture and natural habitats. In this chapter, I focused on identifying and characterizing the bacteria in the gut and body parts excluding the gut (BP) of the three dominant zooplankton with different feeding habits in Lake Biwa, Japan, and compared with bacterial compositions in their feces and surrounding environment.

2. Materials and methods

2.1 Sample collection and preparation

Zooplankton were collected with a vertical net haul from 20 m to the surface using a NORPAC net (mesh size, 200 μm; mouth diameter, 45 cm) at a pelagic site (35°18′.793″N, 136°8′.854″E, 70 m deep) in the north basin of Lake Biwa, Japan on 4 March 2021. A vertical profile of water temperature was obtained with a conductivity-temperature-depth (CTD) profiler (AAQ-Rinko, JFE advantec). The lake waters for analyzing ambient bacterial compositions were also collected from 0, 10, and 20 m with a Van-Dorn bottle (volume, 6 L) and then combined with each other at the same volume. The collected live zooplankton and lake water were transferred in an insulated container to the laboratory within 1 h.

I selected three dominant zooplankton taxa, i.e., *Daphnia pulicaria* which is a non-selective suspension feeder and mainly ingests phytoplankton (Ebert 2005, Rothhaupt 1997), *Eodiaptomus japonicus*, which is an omnivorous suspension feeder and ingests both phytoplankton and microzooplankton (Yoshida et al. 2001), and Cyclopoida spp. which are raptorial feeders and ingest smaller zooplankton such as larval *E. japonicus* (Kawabata 1991).

A sufficient number, i.e., 100 –120 adult individuals, of each taxon were sorted from live samples under a dissecting microscope (Olympus, SZX12, Japan), washed twice with aged tap

water filtered through a glass-fiber filter (Whatman, GF/F) (ATW), autoclaved at 120 °C for 20 min, and incubated separately into 1-L glass jars filled with ATW. The animals were incubated in a growth chamber (Sanyo, MLR-350, Japan) at ambient temperature (8 °C) for 24 h to evacuate gut contents, then removed from the jar and washed twice with Milli-Q water. Feces in the jars were filtered through and collected upon a 0.2-μm pore membrane filter (Advantec, K020A047A, Japan) after extraneous materials such as broken body parts, carcasses, or newly hatched individuals had been removed from the jar under a microscope.

For the following DNA extraction experiments, 15 individuals of > 2 mm in *D. pulicaria*, 30 individuals of adult males and females in *E. japonicus*, and 35 individuals of adult males and females in cyclopoids were sorted from the zooplankton evacuated the gut contents. In each individual, the gut was removed from the body with a sterile needle under a dissecting microscope (Olympus, SZX9) at a magnification of 40x–50x (Fig. 2-1). Each of the removed gut and all other body parts excluding the gut in each taxon was separately transferred into a sterile 1.5-mL micro-tube (Eppendorf, Germany) filled with sodium phosphate (978 μL) and MT buffer (122 μL) in a Fast DNA® Spin Kit for Soil (MP Biomedicals, LLC, USA) for following DNA extraction. A batch of the feces collected on a filter in each taxon was transferred into a 5-mL tube (Labcon, U.S.A.) filled with the same reagents as treating the body.

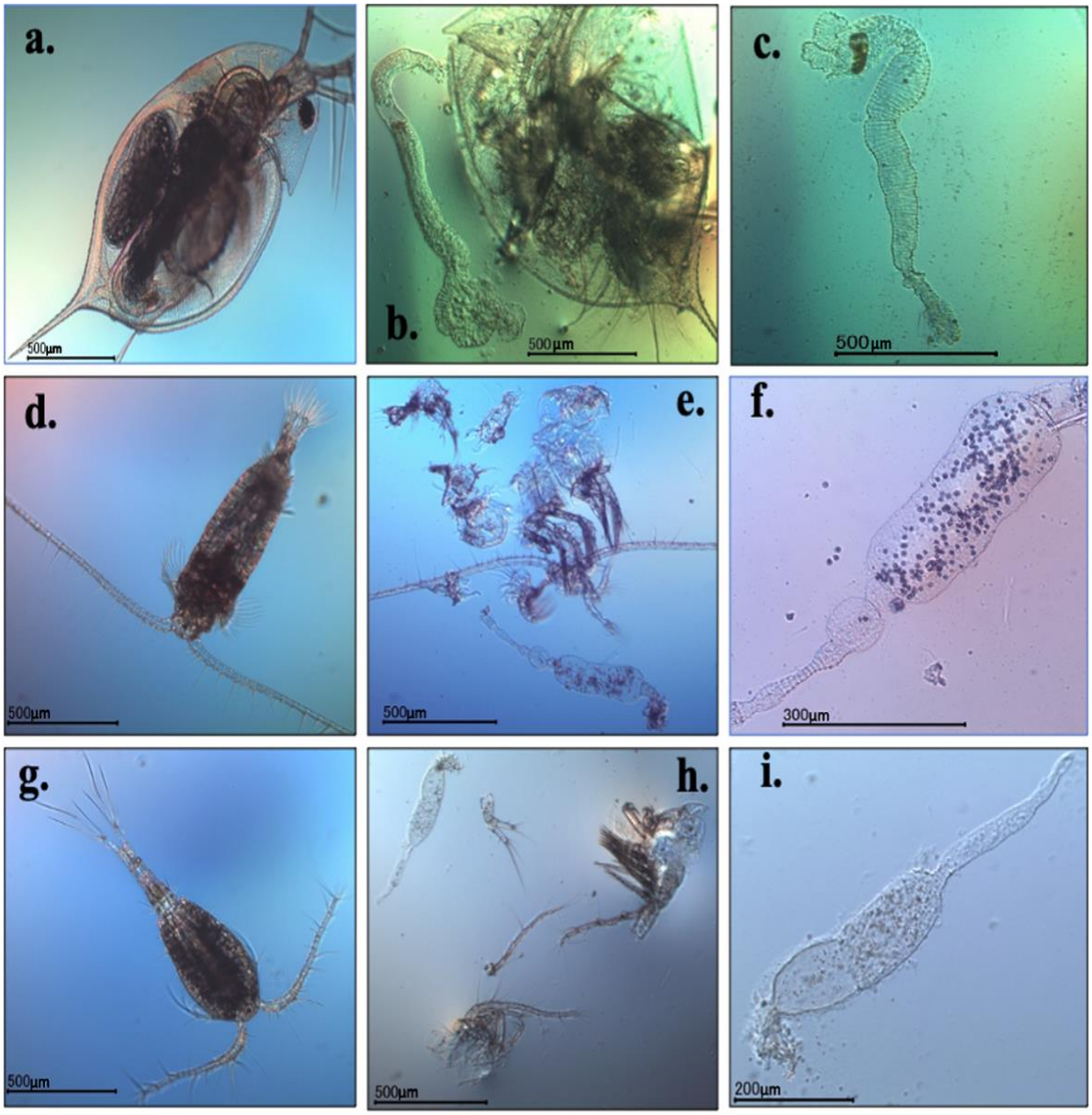

Fig. 2-1 Microscopic images in outer shapes of three zooplankton taxa tested (a, d, g), their body parts (b, e, h), and digestive tract (c, f, i) dissected from the body of *Daphnia pulicaria* (a-c), *Eodiaptomus japonicus* (d-f) and Cyclopoida spp. (g-i) collected at a pelagic site in the north basin of Lake Biwa on 4 March 2021.

To analyze bacterial compositions in the ambient lake waters, 1 L of the lake waters combined from the three depths was firstly sieved with a 20-μm mesh netting. A 300 ml of the pre-sieved water was filtered with a 0.2-μm pore polycarbonate membrane filter (Advantec, K020A047A, Japan). The filter was transferred into a 5-mL tube (Labcon, U.S.A.) filled with sodium phosphate (978 μL) and MT buffer (122 μL) in a Fast DNA® Spin Kit for Soil (MP Biomedicals, LLC, USA) for DNA extraction.

I prepared another zooplankton, i.e., laboratory-reared *D. magna* fed on axenic *Chlamydomonas reinhardtii* (IMA C–9). Healthy 1–2 days old animals were randomly selected from the stock culture and reared in a 1 L glass jar filled with autoclaved ATW for 21 days with excess food of axenic *C. reinhardtii* (>10^5 cells mL^{-1}). When the animals grew up adult, ten animals were sorted, washed twice with ATW to remove loosely attached materials on a carapace surface, and transferred to a new jar filled with ATW to evacuate gut contents for 24 hours at 20˚C. Then, 8 individuals were sorted from the jar, washed twice with MilliQ water, and dissected gut from the body in an aseptic condition. The guts and all other body parts excluding the gut, were separately transferred to a sterile 1.5-mL micro-tube (Eppendorf, Germany) for DNA extraction with the same method for the field samples. All samples were frozen at −80 °C until analysis.

2.2 Metabarcoding analysis using Illumina MiSeq

The frozen samples were thawed and homogenized using a sterile disposable plastic pellet pestle. DNA was directly extracted from each sample using a Fast DNA® Spin Kit for Soil. Metabarcoding targeted the V4 region of the 16S rRNA gene with primers 515F (5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNGTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNGGACTACHVGGGTWTCTAAT-3′) (Glenn et al. 2019). All PCR reactions were performed in each sample with a 10

μL mixture of an Ex TaqHS-mix (TaKaRa Bio Inc., Japan), 10 μM of each forward and reverse primer, and about 5 ng of extracted DNA. Initial denaturation proceeded at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, elongation at 72°C for 30 s, followed by a final extension at 72°C for 5 min. Before the second PCR was performed, the first PCR product was purified by Beckman Coulter AMPure XP (Beckman Coulter, Inc., USA). To attach the index sequence to the products of the first PCR, products of the second PCR were processed with the primer set F: AATGATACGGCGACCACCGAGATCTACAC-Index-2(8bp)-ACACTCTTTCCCTACACGACGC and R: CAAGCAGAAGACGGCATACGAGAT-Index1(8bp)-GTGACTGGAGTTCAGACGTGTG (Tanaka et al. 2020). Initial denaturation was at 94 °C for 2 min, followed by 10 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, elongation at 72 °C for 30 s, and a final extension at 72 °C for 5 min. After purification using the Beckman Coulter AMPure XP, library quality was assessed using an Agilent fragment analyzer system and dsDNA 915 reagent kit (Advanced Analytical Technologies). Libraries were sequenced on an Illumina MiSeq platform, generating 2×300 bp paired end reads. A total of 501,356 raw reads were obtained.

2.3 Data analysis

Raw data collected from the Illumina MiSeq sequencing platform were considered raw tags. Basic tags were prepared after removing the barcode and PCR primer sequences using the fastq_barcode_splitter from the Fastx Toolkit software (ver. 0.0.14). After removing the primer sequence (50 bp at the 3′ end), chimera sequence, and noise sequence using the dada2 plugin of Qiime2 (ver. 2020.8), effective tags with 97% similarity were classified into one operational taxonomic unit (OTU). OTUs containing “unidentified” taxonomic annotations and singleton (only a single sequence across the entire dataset) were removed from data

analysis. All OTUs with the best BLAST matches to non-bacterial organisms were also discarded from the analysis.

Before analyzing OTUs, a rarefied OTU table was generated using the QIIME rarefaction analysis script with the rarefied depth in the minimum sequence number. We discarded OTUs belonging to chloroplast, mitochondria, cyanobacteria, and archaea from all samples (Chapter 1). The Shannon diversity index (*H*) was calculated for the bacterial community of each sample.

We calculated *H* to examine the diversity of OTUs in each sample and performed a non-metric multidimensional scaling (NMDS) analysis to visualize dissimilarities in bacterial composition among zooplankton's BP, gut, feces, and ambient water samples using all OTUs. Heatmap analysis was performed using the dominant bacterial families (≥ 200 reads for OTUs) in all samples. Analyses were performed using the R software ver. 4.0.3 (2020) 'vegan' package.

3. Results

A total of 472,383 sequences comprising 1,125 bacterial OTUs were generated, including the gut, body parts other than gut (BP), and feces of the three field-caught zooplankton, in which 117, 119, and 91 OTUs in *D. pulicaria*, 49, 39, and 120 OTUs in *E. japonicus*, and 63, 19, and 173 OTUs in Cyclopoida spp., respectively. The numbers of OTUs were not so different between the gut and BP in any taxa, while those in feces were more than double than those in the gut and BP for two copepod taxa. In laboratory-rearing *D. magna*, the numbers of OTUs were relatively much lower than those in wild animals, 28 and 29 from the gut and BP, respectively. From the ambient waters, the highest bacterial OTUs, 278, were found. Shannon *H* diversity indices in feces were higher than those in the gut of the two copepod taxa while lower than those in the gut of *D. pulicaria* (Table 2-1). The lowest *H* was

found in BP (1.09) of laboratory-rearing *D. magna*, while the highest one was found (4.16) in ambient waters.

Table 2-1. Total numbers of sequences and OTUs, and Shannon's *H* indices of bacterial communities associated with body parts other than gut (BP), gut and feces of *Daphnia pulicaria*, *Eodiaptomus japonicus*, and Cyclopoida spp., and Ambient bacteria (AB) collected at a pelagic site in the north basin of Lake Biwa on 4 March 2021, and laboratory rearing *Daphnia magna*.

Host taxa/environment	Sample type	Total no. of sequences	No. of OTU's	Shannon's *H*
D. magna	BP	45288	29	1.09
D. magna	Gut	47316	28	1.85
D. pulicaria	BP	39838	119	2.47
D. pulicaria	Gut	51746	117	2.96
D. pulicaria	Feces	49132	91	2.60
E. japonicus	BP	31032	39	2.21
E. japonicus	Gut	45915	49	1.41
E. japonicus	Feces	43454	120	2.74
Cyclopoida spp.	BP	5373	19	1.94
Cyclopoida spp.	Gut	22608	63	2.09
Cyclopoida spp.	Feces	47217	173	2.81
Ambient water	AB	43464	278	4.16

Bacterial OTU sequences were mostly assigned to 14 identified phyla (99.6%), with the predominance of Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria. These four phyla accounted for 99% of the total sequences from all host gut, BP, and feces, while 0.44 % of the total sequences were assigned to unknown phyla (termed as unidentified) (Fig. 2-2). Among the total sequences obtained from BP and the gut of four zooplankton taxa, Proteobacteria accounted for 68% of all bacterial sequences on average, following Bacteroidetes, Firmicutes, and Actinobacteria. Among the total sequences obtained from the feces of the field-collected zooplankton, both Proteobacteria and Bacteroidetes accounted for 21% on average, following Firmicutes and Actinobacteria. In the ambient bacterial assemblage, Actinobacteria were the most dominant at 27%, while quite low percentages of <6% in gut, BP, and feces of the field-collected zooplankton. Compositions of Firmicutes were higher in the

feces (31%–64%) than those in the gut (~2%–19%) of the three field-collected zooplankton, which was not found in the ambient bacterial assemblage.

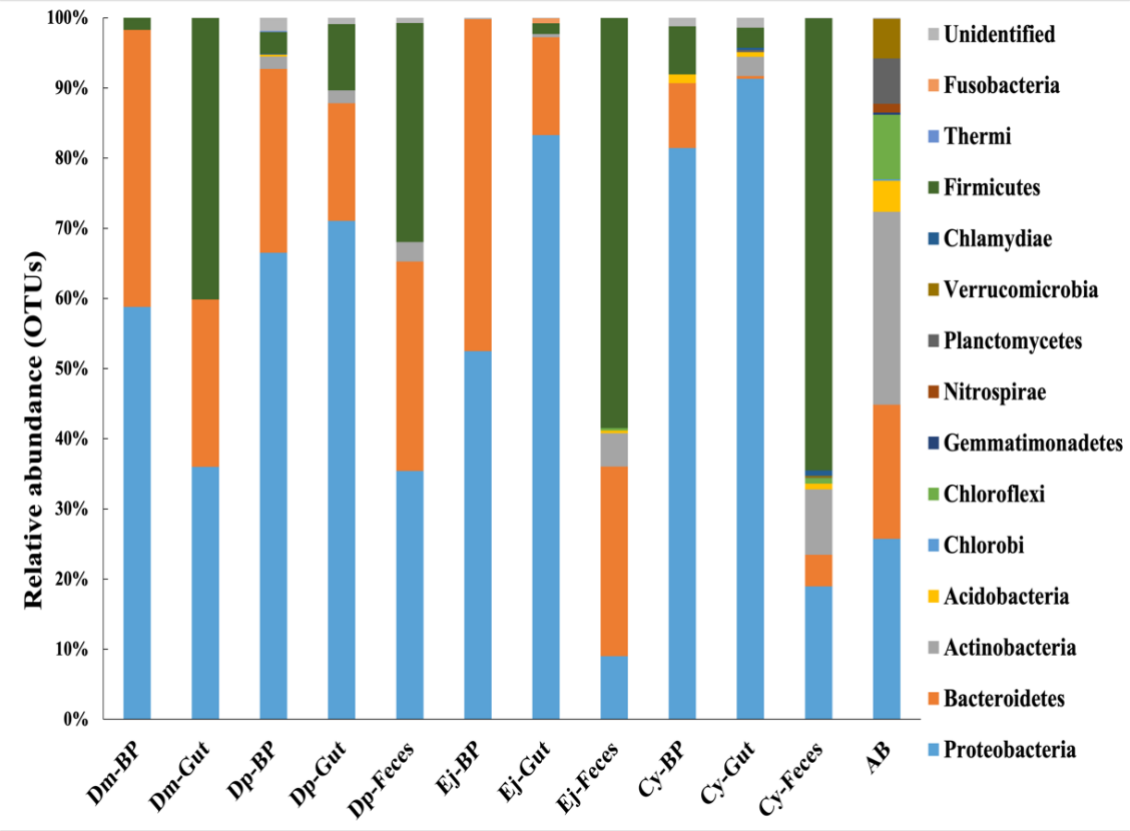

Fig. 2-2 Phylum compositions of bacterial OTUs associated with body parts without gut (BP), gut, and feces in *Daphnia pulicaria* (Dp), *Eodiaptomus japonicus* (Ej), Cyclopoida spp. (Cy) and ambient bacteria (AB) collected at a pelagic site in the north basin of Lake Biwa on 4 March 2021, and laboratory rearing *Daphnia magna* (Dm).

The most prevalent bacterial family in all the BP, gut, and feces of *D. pulicaria* were Comamonadaceae (mostly including genus *Limnohabitans*) at 57%, 23%, and 25% in BP, gut, and feces, respectively (Fig. 2-3). It was mostly followed by Flavobacteriaceae (including *Flavobacterium* alone) in the BP, by Flavobacteriaceae, Moraxellaceae, and Pseudomonadaceae in the gut, and by Flavobacteriaceae and Paenibacillaceae in the feces. Interestingly, the dominant composition of bacteria associated with laboratory-rearing *D. magna* was quite similar to those in *D. pulicaria*.

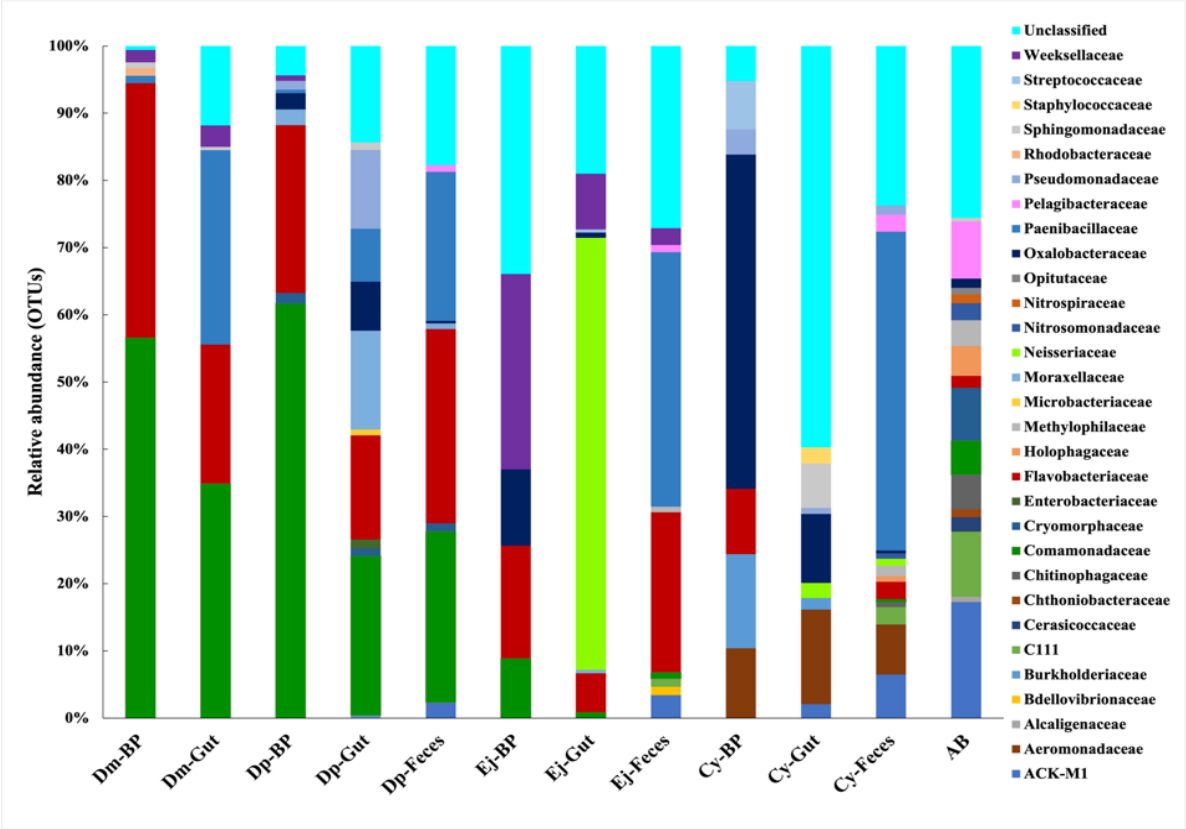

Fig. 2-3 Family compositions of bacterial OTUs (≥ 200 reads) associated with body parts without gut (BP), gut, and feces in *Daphnia pulicaria* (Dp), *Eodiaptomus japonicus* (Ej), Cyclopoida spp. (Cy) and ambient bacteria (AB) collected at a pelagic site in the north basin of Lake Biwa on 4 March 2021, and laboratory rearing *Daphnia magna* (Dm).

In the two copepod taxa, the dominant bacterial compositions were quite different among BP, gut, and feces. Those in the BP of *E. japonicus* were Weeksellaceae at 28%, followed by Flavobacteriaceae at 16% and Oxalobacteraceae at 11%, while those in the gut were Neisseriaceae at 63%, followed by Weeksellaceae at 8%. Those in the feces were Paenibacillaceae at 37%, followed by Flavobacteriaceae at 23% and ACK-M1 at 3%. Those in the BP of Cyclopoida spp. were Oxalobacteraceae at 47%, followed by Burkholderiaceae at 13% and Aeromonadaceae at 10%, while those in the gut were Aeromonadaceae at 13%, followed by Oxalobacteraceae at 10%. Those in the feces were Paenibacillaceae at 46%, followed by Aeromonadaceae (7%). On the other hand, the abundant family in the ambient bacteria was quite different from zooplankton-associated bacterial compositions, being ACK-M1 at 17% and C111 at 9% (Fig. 2-3, Table 2-2).

Table 2-2. List of families with which occurred at least 0.1% within one of the body parts without gut (BP), gut, and feces of the three zooplankton taxa, and ambient bacteria (AB) from ambient water collected at a pelagic site in the north basin of Lake Biwa on 4 March 2021, Japan. Hyphens: family was not detected, or < 0.1% of all OTU sequences.

Family	*Daphnia pulicaria*			*Eodiaptomus japonicus*			Cyclopoida spp.			Ambient water
	BP (%)	Gut (%)	Feces (%)	BP (%)	Gut (%)	Feces (%)	BP (%)	Gut (%)	Feces (%)	AB (%)
Comamonadaceae	57	23	25	8.6	1	1	–	0.7	0.5	5
Flavobacteriaceae	2	15	28	16	6	23	9	0.4	2.4	2
Oxalobacteraceae	2	7	0.4	11	0.8	0.4	47	10	0.4	1
Paenibacillaceae	0.2	8	22	–	0.3	37	–	–	46	–
Neisseriaceae	–	0.1	0.2	0.2	63	–	–	2.1	1	–
Pseudomonadaceae	1	11	0.2	0.4	–	0.2	4	1	1.3	–
Weeksellaceae	1	–	–	28	8	2.4	–	–	–	–
Streptococcaceae	0.1	0.2	–	–	0.5	–	7	0.6	–	–
Staphylococcaceae	–	0.2	–	–	–	–	–	2	–	–
Aeromonadaceae	10	0.1	–	–	–	–	10	13	7	–
Burkholderiaceae	0.2	0.4	–	0.4	–	–	13	2	–	–
Bradyrhizobiaceae	–	–	–	–	–	–	5	–	0.1	–
ACK-M1	–	0.1	2	–	0.1	3	–	2	6	17
C111	–	–	0.1	–	–	1	–	–	2.5	9.3
Alcanivoracaceae	–	0.1	–	–	–	–	–	–	–	–
Bacillaceae	1	–	–	–	–	–	–	–	–	–
Bdellovibrionaceae	–	–	–	0.2	0.1	1	–	–	–	–
Caulobacteraceae	–	–	–	0.1	–	–	–	–	–	–
Cerasicoccaceae	–	–	–	–	–	–	–	–	0.1	2
Chitinophagaceae	0.4	–	–	–	–	0.3	–	–	0.7	5
Chromatiaceae	–	–	0.3	–	–	–	–	–	–	–
Clostridiaceae	1	–	–	–	–	–	–	–	–	–
Corynebacteriaceae	–	0.2	–	–	0.2	–	–	0.5	–	–
Cryomorphaceae	1	1	1	–	–	0.1	–	–	0.3	7.5
Enterobacteriaceae	–	1	0.3	0.2	–	–	–	0.3	–	0.1
Geobacteraceae	–	–	–	–	–	–	–	–	0.6	–
Holophagaceae	–	–	–	–	–	0.4	–	–	1	4.2
Lachnospiraceae	0.5	–	–	–	–	–	–	–	–	–
Leptotrichiaceae	–	–	–	–	0.7	–	–	–	–	–
Methylobacteriaceae	0.1	–	–	–	–	–	–	–	–	–
Methylophilaceae	–	–	0.1	0.2	–	1	–	–	1.5	3.7
Microbacteriaceae	–	1	–	–	–	–	–	–	–	–
Micrococcaceae	0.6	0.3	–	–	–	–	–	–	–	–
Moraxellaceae	2	14	1	0.4	0.5	–	–	–	–	–
Mycobacteriaceae	–	0.2	–	–	–	–	–	–	–	–
Nitrosomonadaceae	–	–	–	–	–	0.4	–	–	1	2.5
Nocardioidaceae	0.6	–	–	–	–	–	–	–	–	–
Pasteurellaceae	–	0.4	–	–	0.3	0.1	–	–	–	–
Pelagibacteraceae	0.1	0.3	1	–	–	1	–	–	2	8
Porphyromonadaceae	0.2	–	–	–	–	–	–	–	–	–
Procabacteriaceae	–	–	–	–	–	–	–	–	0.1	0.1
Rhabdochlamydiaceae	–	–	–	–	–	–	–	–	0.3	–
Rhodobacteraceae	0.3	–	–	–	–	–	–	–	–	–
Rhodospirillaceae	–	–	–	–	–	–	–	0.5	–	0.1
Saprospiraceae	0.1	–	–	–	–	–	–	–	–	–
Sphingomonadaceae	0.3	1	–	–	0.2	–	–	6	0.4	–
Thermicanaceae	0.2	–	–	–	–	–	–	–	–	–
Xanthomonadaceae	0.1	–	–	–	–	–	–	0.5	–	–
Trueperaceae	0.2	–	–	–	–	–	–	–	–	–
Acetobacteraceae	–	–	0.1	–	–	–	–	–	–	0.1
Opitutaceae	–	–	–	–	–	–	–	–	–	1
Chthoniobacteraceae	–	–	–	–	–	–	–	–	–	1
Nitrospiraceae	–	–	–	–	–	–	–	–	–	1.2

Dominant bacterial genera identified were *Limnohabitans*, *Flavobacterium*, *Brevibacillus*, *Acinetobacter*, and *Pseudomonas* in both *D. pulicaria* and *D. magna* (Table 2-3). Whereas, those in *E. japonicus* were *Andreprevotia*, *Chryseobacterium*, and *Polaromonas*, and those in Cyclopoida spp. were *Ralstonia*, *Burkholderia*, *Aeromonas*, *Sphingomonas*, *Streptococcus*, and *Staphylococcus*.

Table 2-3. List of dominant genera occurred in the body parts without gut (BP), gut, and feces of the three zooplankton taxa collected at a pelagic site in the north basin of Lake Biwa, Japan, on 4 March 2021, including laboratory rearing *D. magna*. Hyphens: Genus was not detected, or < 1% of all OUT sequences.

Host taxa	Bacterial genus	Body parts (%)	Gut (%)	Feces (%)
D. pulicaria	*Limnohabitans*	56	23	25
	Flavobacterium	23	15	28
	Acinetobacter	2	14	–
	Ralstonia	2	6	–
	Pseudomonas	–	11	–
	Fluviicola	–	–	1
	Brevibacillus	–	8	22
	Perlucidibaka	–	–	1
	ACK-M1	–	0.1	2
	C111	–	–	0.1
E. japonicus	*Chryseobacterium*	28	8	2
	Ralstonia	11	–	–
	Polaromonas	8	–	–
	Andreprevotia	–	63	–
	Flavobacterium	–	6	23
	Brevibacillus	–	0.3	37
	ACK-M1	–	0.1	2
	C111	–	–	1
Cyclopoida spp.	*Ralstonia*	47	10	–
	Burkholderia	13	–	–
	Aeromonas	10	13	7
	Flavobacterium	9	0.4	2
	Streptococcus	7	0.6	–
	Sphingomonas	–	6	–
	Deefgea	–	2	–
	Staphylococcus	–	2	–
	Brevibacillus	–	–	46
	ACK-M1	–	2	6
	C111	–	–	3
D. magna	*Limnohabitans*	55	34	–
	Flavobacterium	37	20	
	Chryseobacterium	2	3	
	Rhodobacter	1	–	
	Brevibacillus	–	29	

Non-metric multidimensional scaling (NMDS) analysis clearly separated the ambient bacteria from those associated with the gut and BP of the three field-collected zooplankton (Fig. 2-4). Bacterial compositions among the three zooplankton were more similar in feces than those in BP and gut. Although those in BP and gut were quite different among the taxa, those in BP between the two copepod taxa were similar.

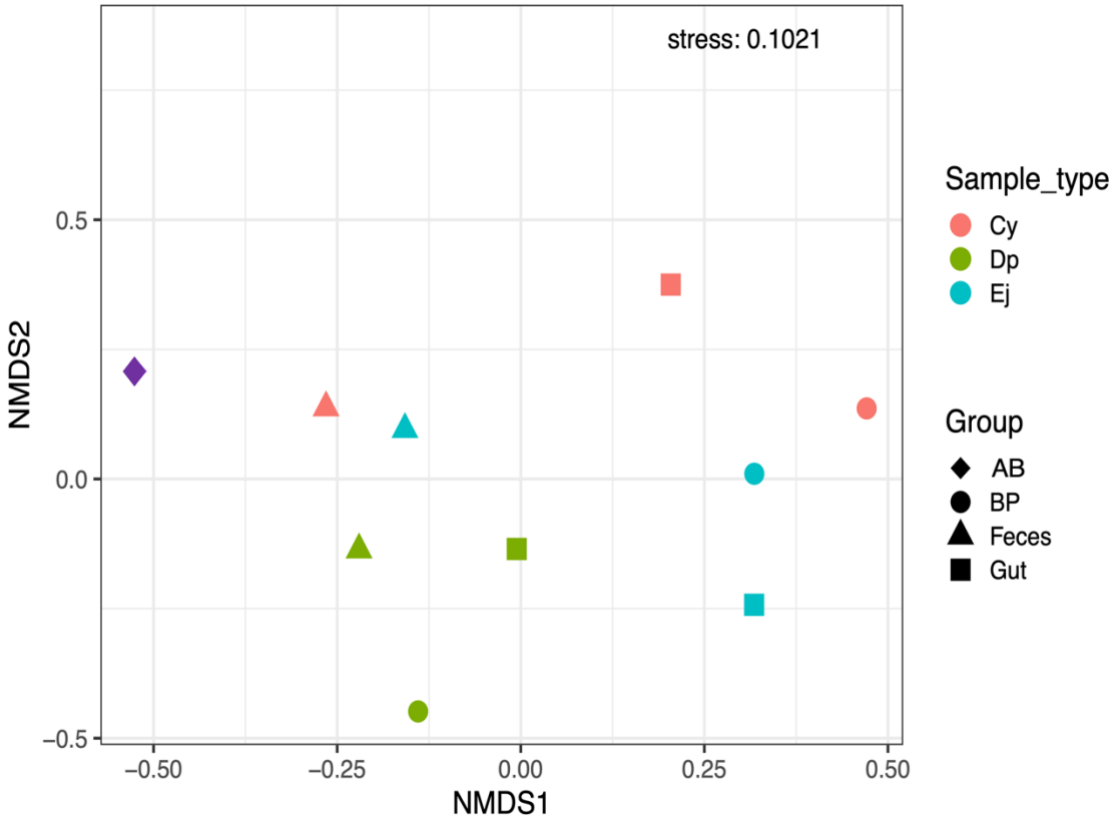

Fig. 2-4 Non-metric multidimensional scaling (NMDS) analysis using all OTUs demonstrating dissimilarities in bacterial composition between ambient bacteria(AB) from ambient water and all zooplankton gut, BP, and feces samples. Ej, Dp, and Cy represent *Eodiaptomus japonicus*, *Daphnia pulicaria*, and Cyclopoida spp., respectively; BP denotes body parts other than the gut of each taxon.

Heatmap analysis showed that the dominant family composition of the bacteria associated with BP and gut was quite similar between the two daphnid species (Fig. 2-6). Those associated with feces of *D. pulicaria* were also similar. Those in the feces of the two copepod taxa and those in the gut and BP of Cyclopoida spp. were both similar, while those in the gut of *E. japonicus* were far from the others.

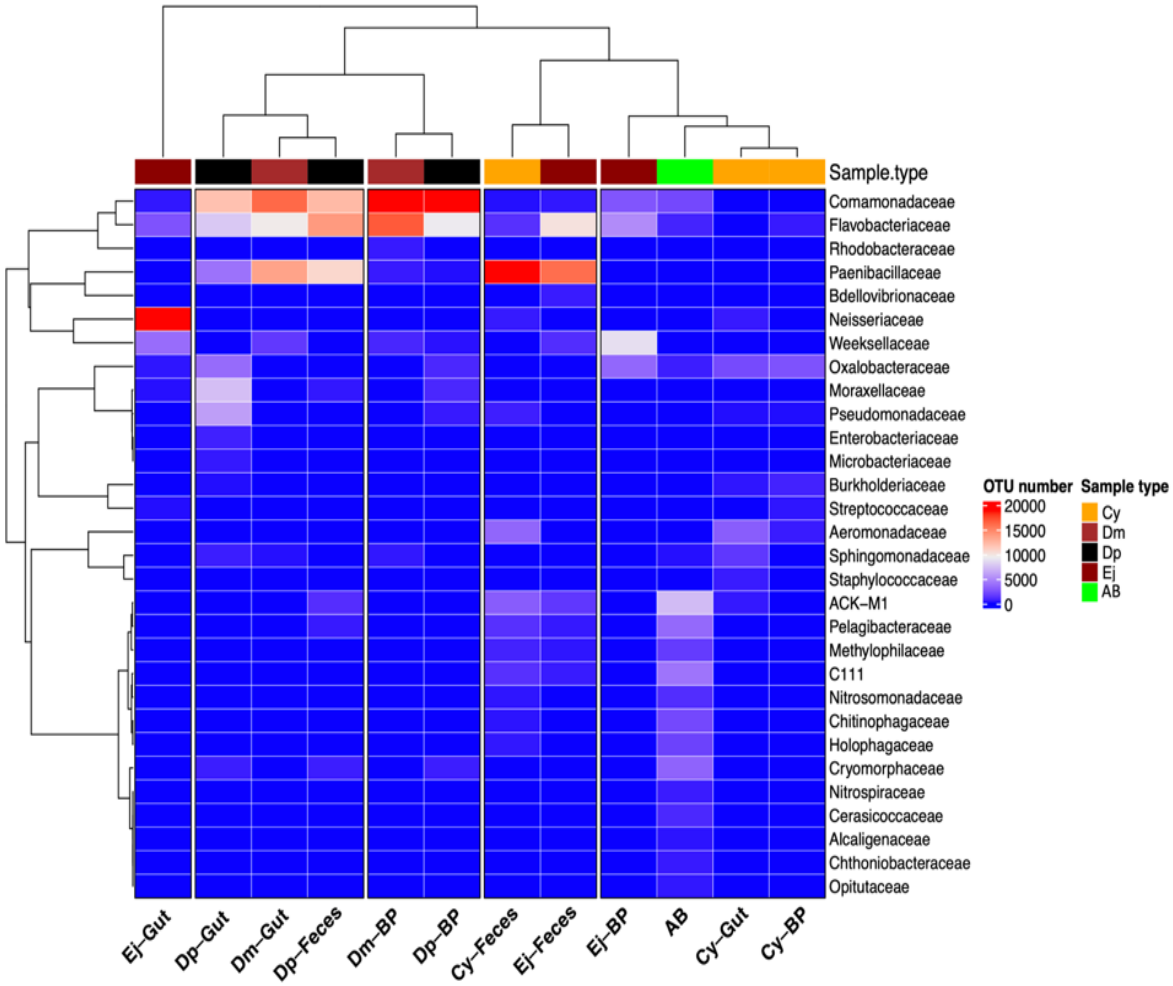

Fig. 2-6 Heatmap of major bacterial families (≥ 200 OUT reads) associated with zooplankton body parts without gut (BP), gut, and feces in *Eodiaptomus japonicus* (Ej), *Daphnia pulicaria* (Dp), Cyclopoida spp. (Cy), *Daphnia magna* (Dm) and ambient bacteria (AB) were collected at a pelagic site in the north basin of Lake Biwa on 4 March 2021. Individual lines correspond to bacterial families; each column corresponds to a different sample. The upper dendrogram represents similarities in bacterial composition among zooplankter gut, BP, and feces; the left dendrogram represents similarities in patterns of occurrence of a bacterial family in a zooplankter sample and SS. ACK-M1 and C111 are candidate Actinobacteria families.

4. Discussion

According to the bacterial composition at the phylum and family level and the NMDS analysis, the bacterial community associated with BP, gut, and feces in the three field-collected zooplankton taxa were quite different from those in the ambient waters. Actinobacteria is the most dominant bacterial phyla in the water column in Lake Biwa (Okazaki and Nakano 2016), while it was less found in the zooplankton bodies and feces in this study. The other abundant

phylum in the ambient waters, including Chloroflexi, Planctomycetes, Verrucomicrobia, Gemmatimonadetes, and Chlorobi, were also not found in both BP and gut of the field-collected three zooplankton, suggesting that some specific bacteria might be selected from the ambient waters by the zooplankton hosts (Macke et al., 2020). On the other hand, a part of bacterial phyla in the ambient waters, e.g., Verrucomicrobia and Chloroflexi, were also found in the feces-associated bacteria. As mentioned above, Actinobacteria were found quite less amount from the host bodies but more abundant from the feces, implying that bacteria in the feces may include the bacteria from outside of the hosts' bodies, that is, from the ambient waters passing through the gut, as described in Chapter 1.

In this study, bacterial compositions in the gut and BP of laboratory-rearing *D. magna* were quite similar to those in *D. pulicaria* collected from Lake Biwa; the dominant families were Comamonadaceae, Flavobacteriaceae, Moraxellaceae, Paenibacillaceae, and Pseudomonadaceae. These families were also found to be abundant in the whole-body microbiota of laboratory-rearing *D. magna* and wild animals of *D. galeata* and *D. obtusa* collected from freshwater lakes and ponds in Italy (Eckert et al. 2021). Freese and Schink (2011) also showed that Comamonadaceae, Moraxellaceae, and Pseudomonadaceae were the dominant bacterial families from the gut of laboratory-rearing *D. magna*. It has been also shown that Comamonadaceae, Flavobacteriaceae, and Pseudomonadaceae are the most abundant families in the host-associated bacteria in laboratory-rearing *D. magna*, *D. pulex*, and *D. pulicaria* originated from different laboratories (Qi et al. 2009). This similarity among several daphnid species from different regions and laboratories suggest a strict relationship between the daphnid species and the bacterial families, implying that these bacterial families might construct a stable symbiotic relationship with the daphnids.

Limnohabitans was the most dominant genus in Comamonadaceae from both wild *D. pulicaria* and laboratory-reared *D. magna* in this study and has been proven to be a beneficial

symbiont of its daphnid host in several laboratory experiments (Peerakiethajorn 2015, 2016, Akbar et al. 2020). *Limnohabitans* spp. possesses various metabolic abilities (Zeng et al. 2012) that may benefit *Daphnia* nutrition (Cooper and Cressler 2020).

Acinetobacter and *Pseudomonas* were the dominant genera in Moraxellaceae and Pseudomonadaceae, respectively, from the gut of *D. pulicaria* in this study. *Acinetobacter* can accumulate large amounts of phosphorus as phosphate in its cell and release it under anaerobic conditions (Deinema et al. 1985). A strain of *Pseudomonas* symbiont aided survival and maintained the fecundity of its host *D. magna* under mercury stress conditions in a laboratory experiment (Fong et al. 2019).

The gut of *E. japonicus* was constituted by a unique microbiota mostly dominated by Neisseriaceae, followed by Weeksellaceae, Flavobacteriaceae, Comamonadaceae, and Oxalobacteriaceae. Interestingly, all these families were also detected in BP, but the relative abundance was different. These bacterial families were also found in Chapter 1 on the bacterial composition of whole-body *E. japonicus*. *Andreprevotia* in Neisseriaceae possesses a higher chitinolytic potential to biodegrade chitin from the exoskeleton of crustaceans (Tran et al. 2018). Chitin-rich crustacean carcasses, exoskeletons from molting, and other chitinous organisms like fungi may produce an enormous amount of chitinous organic substances aggregate in the water column (Yu et al. 1991, Grossart and Simon 1998). In addition, based on the vertical temperature profile of the water column during this sampling time, it was a water mixing period in Lake Biwa, which may facilitate more chitinous particulate organic matter in the epilimnion from that previously deposited in the hypolimnion during summer stagnation (Yoshida et al. 2001). *E. japonicus* are suspension feeders which ingest particulate organic matter (POM), including chitinous aggregates, and microzooplankton like fungal zoospores available in the ambient water column, thus leading to a chitin-rich diet which might

facilitate an increased abundance of *Andreprevotia* in *E. japonicus* gut (Tang et al. 2001, Tang 2005, Yeh et al. 2020).

On the contrary, chitin digestion may increase carbon and nitrogen in the intestine, thus could enhance bacterial proliferation (Pruzzo et al. 2008), consequently, higher prevalence. In Chapter 1, the reason behind the *Flavobacterium* association in this host's gut and feces has also been described. The genus *Chryseobacterium* in Weeksellaceae, abundant in BP, has keratolytic potential and can utilize keratin and biosynthesis of microviridin, a protease inhibitor (Kang et al. 2021). Most strains of *Chryseobacterium* are resistant to a broad range of antimicrobial agents (Bernardet et al. 2015) and thus can modulate the host's interaction with pathogens and other foulers as an epibiotic bacteria (Wahl et al. 2012). However, there is limited information about the functions of zooplankton's surface-attached bacteria and their impact on the host's physiology.

Oxalobacteraceae, Sphingomonadaceae, Staphylococcaceae, Neisseriaceae, and Burkholderiaceae dominated the gut and BP microbial composition in host cyclopoids. But the abundance and occurrence of each taxon differed greatly between gut and BP bacterial compositions. Oxalobacteraceae and Burkholderiaceae were highly abundant in the BP at 47%, and 13%, respectively, while those in the gut were very low in abundance, at 10% and 1.6 %, respectively. Sphingomonadaceae, Staphylococcaceae, and Neisseriaceae were abundant in the gut while absent in the BP. This difference can be due to the bacteria in the gut being exposed to higher amounts of different kinds of substrates ingested by the hosts as food (Flint et al. 2008, Wu et al. 2012) than those exposed by the surface-attached bacteria. On the other hand, the host's surface-attached bacteria may use organic matter excreted by the hosts themselves (Tang et al. 2010).

Furthermore, *Burkholderia*, in the cyclopoids' body parts without gut, may produce antibiotics, such as bacteriocin and bioactive secondary metabolites, and can function as a

biocontrol agent against pathogenic fungi, bacteria, protozoa, and nematodes (Elshafie and Camele 2021). These crustacean hosts do not have adaptive immunity (Vazquez et al. 2009). Therefore, their association with bacteria that can produce antimicrobial agents may offer protection against pathogens (Wahl et al. 2012, Elshafie and Camele 2021).

CHAPTER 3:

“Community structures of symbiotic bacteria associated with the three dominant crustacean zooplankton in different depth habitats of Lake Biwa during the stagnation period.”

1. Introduction

The bacteria related to these zooplankters' external and internal body structures are referred to as bacterial microbiota and are in close physical contact with the host organisms (Macke et al. 2017b). The zooplankton body provides a lucrative microhabitat for its microbiota as its exterior, while the interior surfaces are rich in organic matter, supporting bacteria to proliferate rapidly (Bickel and Tang 2014). In turn, abundant microbiota may benefit its host physiological processes (Tang et al. 2010, Akbar et al. 2020). Such bacterial microbiota has been found from both gut and body surfaces from marine and freshwater zooplankton (Sochard et al. 1979, Hansen and Bech 1996, Tang et al. 2010), and also from dominant crustaceans in Lake Biwa, as already shown in the previous chapters. However, there is still limited information on the relationship between these bacterial compositions and host habitats in different seasons and depths yet.

The availability of foods for host zooplankton and bacterial communities in the surrounding waters may influence the microbial compositions associated with the hosts (Grossart et al. 2009). The biotic and abiotic environmental factors including temperature, pH, and food availability greatly vary with season and habitats of the zooplankton hosts (Bickel and Tang 2014, Velasquez et al. 2022). Such differences can also impact the life history traits of zooplankton hosts (Liu et al. 2014, Boonmak et al. 2018, Gao et al. 2022) and influence the community structure of both zooplankton-associated and free-living bacteria (Huq et al.1984, Bickel and Tang 2014). Whereas knowledge of the effects of these factors on zooplankton-associated microbiota and their consequential influences on physiology and ecology of the hosts still needs to be improved (Samad et al. 2020). Moisander et al. (2015) showed that temporal changes in food types available for zooplankton were fundamental in controlling host-associated bacterial composition in a temperate marine coastal habitat. In an aquatic

environment, temperature and amounts of organic substrates are essential factors for bacterial proliferation and abundance (Shish and Ducklow 1994, Autio 1998).

On the other hand, zooplankton-associated microbiota may adapt to spatio-temporally varying environmental conditions (Tang et al. 2010). Gerdts et al. (2013) found no seasonality in the bacterial communities related to the copepod hosts during two years study period, though the ambient bacterial community showed clear seasonality. Whereas Qi et al. (2009) showed that *Daphnia* spp. from different geographic regions harbored similar bacterial composition. Symbiotic bacteria are found in every body parts including the gut and should be regarded as distinct functional units due to their distribution patterns, functions and contributions to their host's physiology (Tang et al. 2010, Wahl et al. 2012). The bacterial microbiota in a specific site of the host body can be an unambiguous source for understanding microenvironmental variation in a host body and the effect of microbiota on the host's biology (Macke et al. 2017b, Mangus et al. 2015).

In Lake Biwa, *Eodiaptomus japonicus*, Cyclopoida spp., and *Daphnia pulicaria* are dominant crustacean zooplankton throughout the year but show different seasonality regarding their abundance and vertical habitat selection (Kawabata 1989, Yoshida et al. 2001). It is important to clarify how the host habitat selection influences the microbiota in the different parts of the host body, especially during the stagnation period, when the environmental factors, such as temperature, dissolved oxygen, phytoplankton, and microbiota in the surrounding waters, vary with depth (Tezuka 1982, Okazaki and Nakano 2016, Gurung et al. 2001) and affect the physiology of the host zooplankton.

Population dynamics, spatio-temporal distributions, and the food availability of these three zooplankton have been well-studied in Lake Biwa (Kawabata, 1987, 1989, Yoshida et al. 2001, Liu et al. 2020). I've already described bacterial community structure associated with the zooplankton hosts in the epi-pelagic zone of the lake in specific seasons in previous chapters.

In this chapter, the zooplankton-associated bacterial compositions were compared between two different depth layers, i.e., epilimnion (0–20 m) and hypolimnion (20–50 m), in June and September 2021, to clarify the depth-mediated effects on the composition of the zooplankton-associated bacteria during the vertically stagnated water column in Lake Biwa.

2. Materials and methods

2.1 Sample collection and preparation

Zooplankton were collected from the two different depths of the water column, from 20 m to the surface and from just above the bottom to 20 m, with a vertical net haul using a NORPAC net (mesh size, 200 μm; mouth diameter, 45 cm), and with oblique tow of a closing net (mesh size, 100 μm; mouth diameter, 30 cm), respectively, at a pelagic site (35°18′.793″N, 136°8′.854″E, 70 m deep) in the north basin of Lake Biwa, Japan on 17 June and 21 September 2021. Another zooplankton samples for enumerating individual numbers were simultaneously collected from the same two depth layers with vertical discrete haul using the closing net. The zooplankton were immediately killed with Lugol's solution and preserved with sugar-formalin at a final concentration of 4%. Samples were collected in the morning between 9:30 and 11:30. Vertical profiles of water temperature, dissolved oxygen (DO), and chlorophyll *a* concentration were obtained with a conductivity-temperature-depth profiler (AAQ-Rinko, JFE advantec). Lake waters for determining ambient bacteria were collected from 0, 10, 20, 30, 40, and 50 m with a Van-Dorn bottle (volume, 6 L), and the lake waters from 0, 10, and 20 m and those from 30, 40 and 50 m were combined with the same volume of each depth water for following metagenomic analysis on the ambient bacteria. The live zooplankton and lake waters collected were transferred in an insulated container to the laboratory within 1 h.

Approximately 100 adult individuals of each zooplankton taxon, i.e., *Daphnia pulicaria* (>2 mm), *Eodiaptomus japonicus,* and Cyclopoida spp., were sorted from the live zooplankton sample under a dissecting microscope (Olympus, SZX12, Japan), washed twice with aged tap water filtered through a glass-fiber filter (Whatman, GF/F), autoclaved at 120 °C for 20 min (ATW), and incubated separately into 1-L glass jars filled with ATW for evacuating the gut contents. The zooplankton sorted from each depth of 0–20 m and 20–50 m were incubated separately in a growth chamber (Sanyo, MLR-350, Japan) at ambient water temperatures, i.e., 17 °C in June and 22 °C in September above 20 m, and 10 °C below 20 m in both months, for 24 h, picked up from the jar and washed twice with Milli-Q water, and then used for following DNA extraction.

For enumerating zooplankton abundance, each of the three dominant zooplankton taxa, *E. japonicus* adult males and females, adult cyclopoids, and large *D. pulicaria* (>2 mm) was counted under a dissecting microscope (Olympus, SZH10, Japan) at a magnification of 40x.

For the following DNA extraction, 30 adult *E. japonicus*, 35 adult cyclopoids, and 15 adult *D. pulicaria* (>2 mm) were sorted from the zooplankton specimens evacuated the gut contents, and removed the gut from the body with a sterile tungsten needle under a dissecting microscope (Olympus, SZX9) at a magnification of 40–50x,. Each of the gut removed and all other body parts excluding the gut was transferred into a sterile 1.5-mL micro-tube (Eppendorf, Germany) filled with sodium phosphate (978 µL) and MT buffer (122 µL) in a Fast DNA® Spin Kit for Soil (MP Biomedicals, LLC, USA), and then frozen at -80˚C for the following analysis.

To analyze bacterial compositions in the ambient waters, each 1 L of the combined waters from the two different depth strata (0–20 m and 30–50 m) was filtered with a 20-µm-mesh sieve. A 300 ml of the pre-sieved water was filtered with a 0.2-µm pore polycarbonate membrane filter (Advantec, K020A047A, Japan). The filter was transferred into a 5-mL tube

(Labcon, U.S.A.) filled with sodium phosphate (978 µL) and MT buffer (122 µL) in a Fast DNA® Spin Kit for Soil (MP Biomedicals, LLC, USA) for DNA extraction. All samples were frozen at −80 °C until the following analysis.

2.2 Metabarcoding analysis using Illumina MiSeq

Samples were thawed and homogenized using a sterile disposable plastic pellet pestle. DNA was directly extracted from each sample using a Fast DNA® Spin Kit for Soil. Metabarcoding targeted the V4 region of the 16S rRNA gene with primers 515F (5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNGTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNGGACTACHVGGGTWTCTAAT-3′) (Glenn et al. 2019). All PCR reactions were performed in each sample with a 10 µL mixture of an Ex TaqHS-mix (TaKaRa Bio Inc., Japan), 10 µM of each forward and reverse primer, and about 5 ng of extracted DNA. Initial denaturation proceeded at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, elongation at 72°C for 30 s, followed by a final extension at 72°C for 5 min. Before the second PCR was performed, the first PCR product was purified by Beckman Coulter AMPure XP (Beckman Coulter, Inc., USA). To attach the index sequence to the products of the first PCR, products of the second PCR were processed with the primer set F: AATGATACGGCGACCACCGAGATCTACAC-Index-2(8bp)-ACACTCTTTCCCTACACGACGC and R: CAAGCAGAAGACGGCATACGAGAT-Index1(8bp)-GTGACTGGAGTTCAGACGTGTG (Tanaka et al. 2020). Initial denaturation was at 94 °C for 2 min, followed by 12 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, elongation at 72 °C for 30 s, and a final extension at 72 °C for 5 min. After purification using the Beckman Coulter AMPure XP, library quality was assessed using an

Agilent fragment analyzer system and dsDNA 915 reagent kit (Advanced Analytical Technologies). Libraries were sequenced on an Illumina MiSeq platform, generating 2 × 300 bp paired-end reads. A total of 12,22,427 raw reads were obtained.

2.3 Data analysis

Raw data collected from the Illumina MiSeq sequencing platform were considered raw tags. Basic tags were prepared after removing the barcode and PCR primer sequences using the fastq_barcode_splitter from the Fastx Toolkit software (ver. 0.0.14). After removing the primer sequence (50 bp at the 3′ end), chimera sequence, and noise sequence using the dada2 plugin of Qiime2 (ver. 2021.11), effective tags with 97% similarity were classified into one amplicon sequence variant (ASV), hereinafter operational taxonomic unit (OTU) (Frankel-Bricker et al. 2020). OTUs containing "unidentified" taxonomic annotations and singleton (only a single sequence across the entire dataset) were removed from data analysis. All OTUs with the best Blast matches to non-bacterial organisms were also discarded from the analysis.

Before analyzing OTUs, a rarefied OTU table was generated using the QIIME rarefaction analysis script with the rarefied depth in the minimum sequence number. We discarded the OTUs belonging to cyanobacteria, chloroplast, mitochondria, and archaea from all samples (Chapters 1, 2).

The DNA samples of this study were included in three separate sequencing runs, i.e., *E. japonicus*, Cyclopoida spp., and *D. pulicaria* and ambient water samples (AB). The Shannon diversity index (*H*) was calculated for the bacterial community of each sample. Non-metric multidimensional scaling (NMDS) analysis was performed to compare bacterial composition between sample types for each zooplankton taxa and water samples using all OTUs. To examine statistical differences in bacterial communities between sampling months and between habitats, I performed analysis of similarities (ANOSIM). Heatmap analysis was also

performed in family level using ≥ 200 reads of OTUs. All statistical analyses were performed using the R software package 'vegan' ver. 4.0.3 (2020).

3. Results

3.1 Vertical structure of the water column

In both June and September 2021, the temperature and dissolved oxygen (DO) profiles in the water column showed that lake water was stratified, with a shallower mixing layer in June than that in September (Fig 3-1). Thermoclines were developed at 4–15 m in June, while at 13–15 m in September. The lake waters below 15 m were considered as hypolimnion in both months. I separated the water column to two layers, i.e., above 20 m and below 20 m, and therefore former layer included epilimnion and metalimnion while later one just hypolimnion. Average water temperatures above 20 m were 17.1 °C on 17 June while 22.0 °C on 21 September, being 5 °C higher in September than that in June. The average water temperature below 20 m was around 10 °C in both months. Chlorophyll *a* concentration above 20 m were higher in June (2.86 μg/L) than that in September (0.98 μg/L). Although percentages of DO saturation were higher throughout the water column in June, ca. 26% higher above the thermocline than in September. More than 100% above 10 m in June might probably be attributed to photosynthesis of phytoplankton. The three zooplankton taxa tested were mainly distributed above 20 m during the sampling period (Table 3-1). *E. japonicus* was mostly colonized in epilimnion, >10 fold than that in the hypolimnion, while not so large difference between the depth layers in Cyclopoida spp., >3 fold. *D. pulicaria* was relatively low in number in both layers compared to those two copepods.

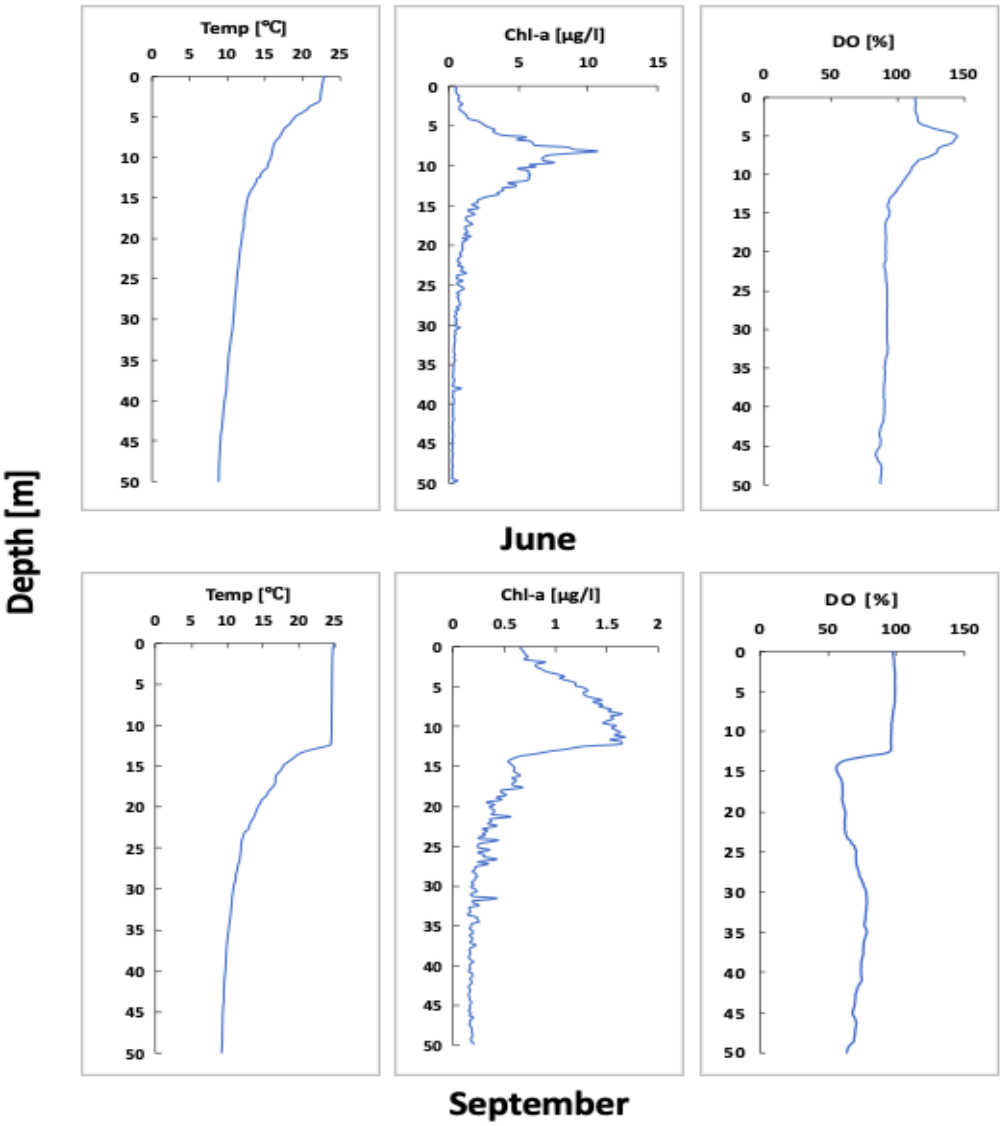

Figure 3-1. Vertical profiles of temperature, chlorophyll *a* concentration, and dissolved oxygen content throughout the water column at a pelagic site in the north basin of Lake Biwa (35°18′.793″N, 136°8′.854″E) on 17 June and 21 September 2021.

Table 3-1. Average water temperature, chlorophyll *a* concentration (Chl-a), % of dissolved oxygen, and individual (adults) densities of three zooplankton hosts at a pelagic site (35°18′.793″N, 136°8′.854″E) of the north basin of Lake Biwa on 17 June and 21 September 2021.

Environmental parameters and densities of zooplankton hosts	June		September	
	0–20 m	20–50 m	0–20 m	20–50 m
Water temperature (° C)	17.09	10.34	22.02	10.49
Chl-a (μg/L)	2.86	0.46	0.98	0.22
% saturation of dissolved oxygen	109.3	90.59	85.18	71.81
Eodiaptomus japonicus (indiv. / m^3)	3950	450	8650	850
Cyclopoida spp. (indiv. / m^3)	1500	350	1800	500
Daphnia pulicaria (indiv. / m^3)	450	100	100	50

3.2 Bacterial compositions in zooplankton and ambient waters

A total of 1,091,297 sequences, comprising 2,850 bacterial OTUs, were generated from 24 samples of the gut (Gut) and body parts other than gut (BP) of the three zooplankton species and ambient bacteria (AB) from two different depth layers in June and September (Table 3-2). A total of 876,673 sequences and 1,396 OTUs were found from Gut and BP, while 214,624 sequences and 1,454 OTUs from AB. The bacterial communities in AB showed extremely higher diversity than those associated with any zooplankton taxa and higher in the hypolimnion than those in the epilimnion regarding both numbers of OTU and Shannon's *H* (Table 3-2). The diversities of the bacteria associated with zooplankton were higher above 20 m than those below 20 m except for the two copepod taxa in June, where Shannon's *H*s were almost similar between the depths though the number of OTUs was relatively higher in the upper layer. Shannon's *H*s of the BP-associated bacteria were mostly higher than those of the Gut-associated bacteria except for *D. pulicaria* collected below 20 m in June when the diversity was higher in the Gut-associated bacteria.

Table 3-2. Total numbers of sequences and identified OTUs, and Shannon's *H* index of bacteria associated with body parts without gut (BP), and gut of *Daphnia pulicaria*, *Eodiaptomus japonicus*, and Cyclopoida spp., and ambient bacteria (AB), collected from Lake Biwa on 17 June, and 21 September 2021.

Host taxa/ environment	Month	Depth	Site	Total no. of sequences	No. of OTU's	Shannon's *H*
D. pulicaria	Jun	0–20m	BP	45,788	102	3.20
	Jun	0–20m	Gut	49,801	77	2.79
	Jun	20–50m	BP	41,266	55	2.00
	Jun	20–50m	Gut	42,539	55	2.5
	Sep	0–20m	BP	39,216	75	2.50
	Sep	0–20m	Gut	38,823	78	2.15
	Sep	20–50m	BP	38,309	42	1.64
	Sep	20–50m	Gut	29,477	63	1.50
E. japonicus	Jun	0–20m	BP	36,899	39	2.41
	Jun	0–20m	Gut	42,420	68	2.04
	Jun	20–50m	BP	17,092	51	2.79
	Jun	20–50m	Gut	28,028	49	2.50
	Sep	0–20m	BP	31,810	59	2.61
	Sep	0–20m	Gut	20,903	66	2.52
	Sep	20–50m	BP	26,963	45	1.97
	Sep	20–50m	Gut	30,801	18	1.27
Cyclopoida spp.	Jun	0–20m	BP	36,356	45	2.22
	Jun	0–20m	Gut	48,196	84	1.73
	Jun	20–50m	BP	37,997	34	2.25
	Jun	20–50m	Gut	39,283	43	1.87
	Sep	0–20m	BP	31,344	76	2.80
	Sep	0–20m	Gut	41,587	83	2.46
	Sep	20–50m	BP	38,962	43	2.30
	Sep	20–50m	Gut	42,813	46	1.77
Ambient waters	Jun	0–20m	--	52,968	317	3.98
	Jun	30–50m	--	57,774	380	4.46
	Sep	0–20m	--	48,981	357	3.77
	Sep	30–50m	--	54,901	400	4.10

More than 99.6% of bacterial OTU sequences generated were assigned to 16 identified phyla. A few sequences with an unknown taxonomic assignment at the phylum level (0.36% of the total sequences) were designated as unidentified. The dominant bacterial phyla in BP and Gut of all three hosts were Proteobacteria and Bacteroidetes (82% and 14% of the total sequences, respectively, on average), while those in AB were Proteobacteria, Actinobacteria and Bacteroidetes (35%, 22%, and 14% of the total sequences, respectively) (Fig. 3-2).

Chloroflexi and Planctomycetes additionally dominated in AB from hypolimnion in both June and September. No Firmicutes and Tenericutes were found from AB, while no Chloroflexi, Planctomycetes, Nitrospirae, Chlorobi, Armatimonadetes, and Gemmatimonadetes were found from zooplankton hosts.

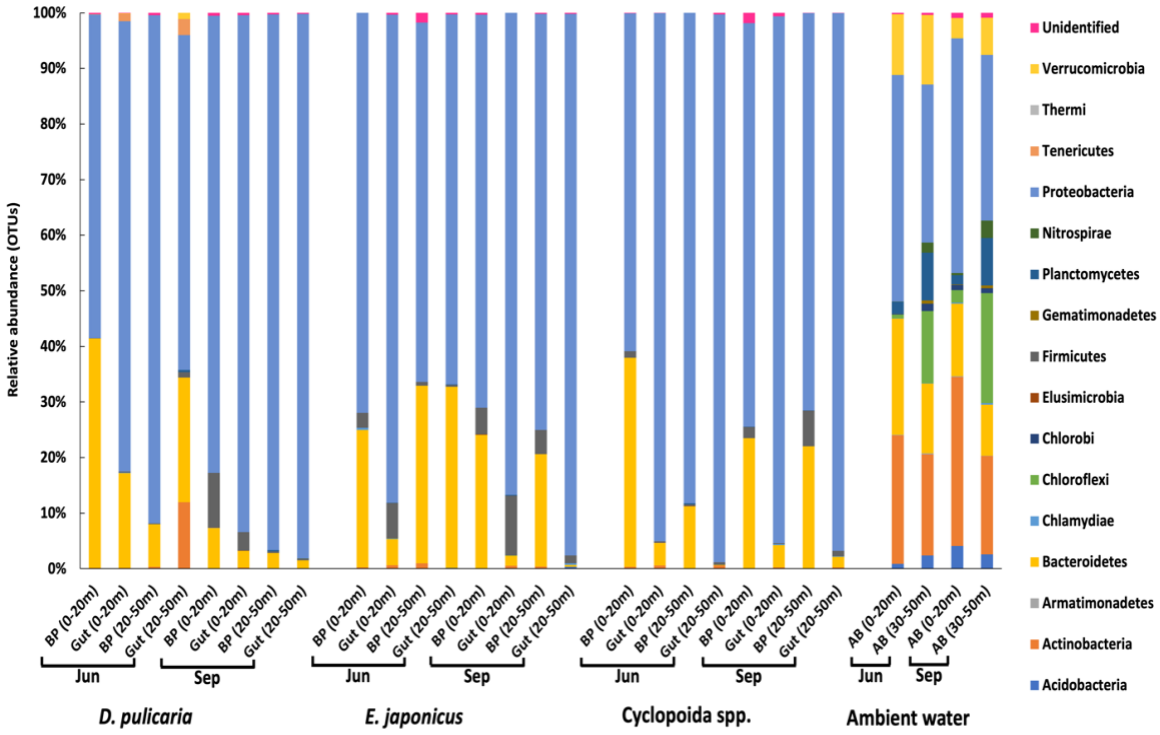

Fig. 3-2 Phylum composition of bacterial OTUs associated with body parts without gut (BP) and gut (Gut) in *Daphnia pulicaria*, *Eodiaptomus japonicus*, Cyclopoida spp., and ambient bacteria (AB) in ambient water, collected from a pelagic site in the north basin of Lake Biwa on 17 June and 21 September 2021.

The most abundant bacterial family associated with *D. pulicaria* from epilimnion in June were Aeromonadaceae (41%) and Flavobacteriaceae (15%) in the Gut, and Flavobacteriaceae (24%) and Comamonadaceae (22%) in the BP. Whereas, in hypolimnion, those of the Gut was dominated by Neisseriaceae (30%) and Flavobacteriaceae (22%), and those in BP were Neisseriaceae (41%) and Comamonadaceae (9%), being quite different from those of both Gut and BP in epilimnion. In September, dominant bacterial families in epilimnion were Aeromonadaceae (51%) and Oxalobacteraceae (25%) in the Gut, Oxalobacteraceae (40%) and Neisseriaceae (12%) in the BP, being different from those in June.

In hypolimnion, Oxalobacteraceae and Aeromonadaceae were dominated in both the Gut (71% and 13%, respectively) and BP (60% and 13%, respectively), being also quite different from those in June. The Major abundant bacterial components in the Gut and BP from both epi- and hypolimnion in September were similar, but different between the depths. Flavobacteriaceae was abundant from both Gut and BP in June at 17% on average, while that in September was <1% (Fig. 3-3). Oxalobacteraceae, Aeromonadaceae, Comamonadaceae, Flavobacteriaceae and Neisseriaceae were also found in Gut and BP at 3-28% and 7-28%, respectively, on average from epi- and hypolimnion in both June and September. Moraxellaceae was commonly found from the BP in both months.

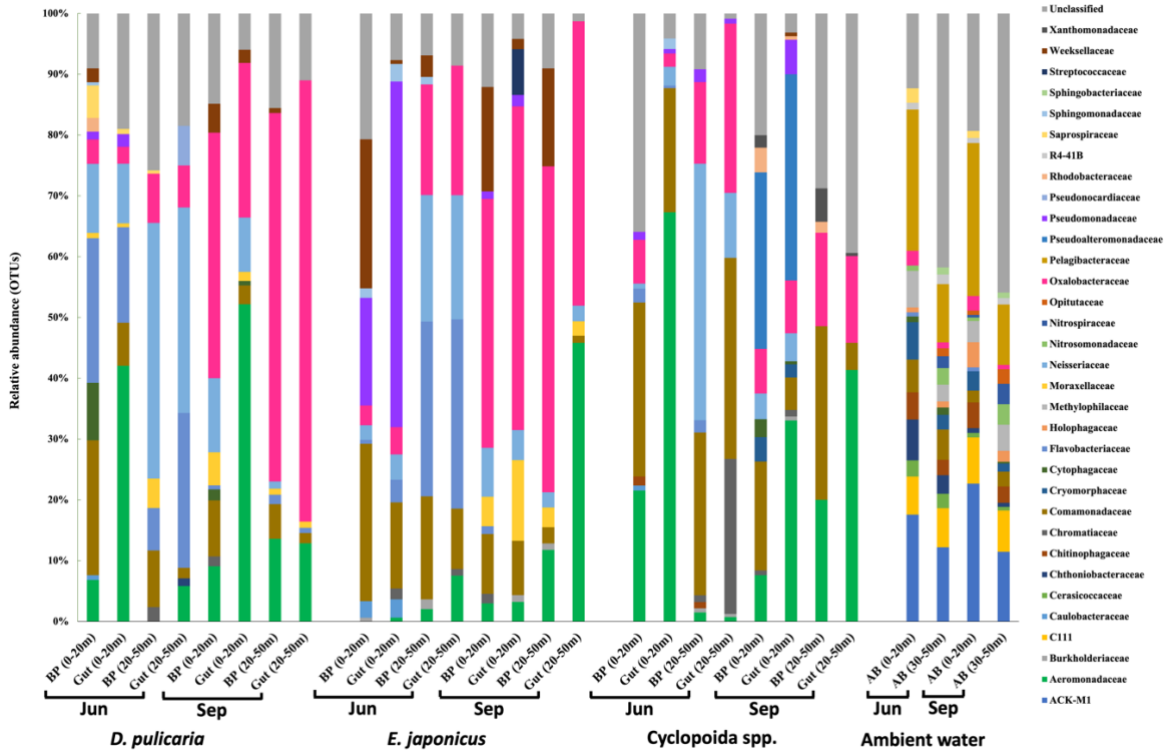

Fig. 3-3 Family composition of bacterial OTUs (≥ 200 reads) associated with body parts without gut (BP) and gut (Gut) in *Daphnia pulicaria*, *Eodiaptomus japonicus*, Cyclopoida spp. and ambient bacteria (AB) in ambient water, collected from a pelagic site in the north basin of Lake Biwa on 17 June and 21 September 2021. ACK-M1 and C111 are candidate Actinobacteria families.

Dominant bacterial families in *E. japonicus* from epilimnion in June were Pseudomonadaceae (56%) and Comamonadaceae (14%) in the Gut, Comamonadaceae (25%)

and Weeksellaceae (24%) in the BP, while those from hypolimnion were Flavobacteriaceae and Neisseriaceae in the Gut (30% and 20%, respectively) and the BP (27% and 20%, respectively), being quite different from those from epilimnion. On the contrary, in September, Oxalobacteraceae was the most dominant family in the Gut and BP from both epi- and hypolimnion; 51% and 40%, respectively, in epilimnion, while 46% and 53%, respectively, in hypolimnion. Next dominant bacterial family in the Gut were Moraxellaceae (13%) and facultative anaerobic Streptococcaceae (7%), in epilimnion, while Aeromonadaceae (45%) from hypolimnion, greatly contrasting with those in June. Flavobacteriaceae was found from both the Gut and BP in June but absent in September. Oxalobacteraceae, Comamonadaceae, Neisseriaceae, Weeksellaceae and Aeromonadaceae were found from the Gut and BP in both June and September.

In Cyclopoida spp., the most dominant bacterial family in epilimnion of June were Aeromonadaceae and Comamonadaceae from the Gut (66% and 20%, respectively) and BP (21% and 28%, respectively). Whereas, those in hypolimnion were Comamonadaceae (32%), Oxalobacteraceae (27%) and Chromatiaceae (25%) from the Gut, Neisseriaceae (42%), Comamonadaceae (27%) and Oxalobacteraceae (13%) from the BP. On the contrary, those in epilimnion of September was dominated by Pseudoalteromonadaceae in both of Gut and BP at 33% and 28%, respectively, followed by Aeromonadaceae at 33% in the Gut and Comamonadaceae at 17% in BP. Those in hypolimnion were Aeromonadaceae (41%) and Oxalobacteraceae (14%) from the Gut and Comamonadaceae (28%) and Aeromonadaceae (20%) from the BP.

The dominant bacterial families in AB were very different from those associated with the three hosts, but almost similar between the months and depths, being Pelagibacteraceae, ACK-M1, C111, Comamonadaceae, Methylophilaceae, Chthoniobacteraceae and Chitinophagaceae.

Non-metric multidimensional scaling (NMDS) analysis using all bacterial OTUs for each zooplankton taxa in June and September showed different dissimilarity patterns of the bacterial compositions associated with the host among the taxa (Fig. 3-4). In *D. pulicaria*, bacterial compositions were similar between the Gut and BP but different between the two months and depths. In *E. japonicus*, those in June were also similar between the Gut and BP but different between the depths, while those from the Gut were quite different from others in September. In Cyclopoida spp., bacterial compositions were different between two months, two depths, and body parts, i.e., Gut and BP. Those in AB also differed between months and depths.

Table 3-3. ANOSIM results comparing dissimilarities between June and September and between epi- (0–20m) and hypolimnion (20–50m) for zooplankton-associated bacterial community compositions, collected from Lake Biwa on 17 June and 21 September 2021. R-value (1, -1), R-value close to 1 indicates significant differences between groups, -1 indicates within the group in more different than between groups.

Zooplankton taxa	**Treatments**		**Replicates in each group**	**Permutations**	**ANOSIM R statistic**	***P*-value**
	Group 1	**Group 2**				
D. pulicaria	June	September	4	999	0.74	0.03
D. pulicaria	0–20m	20–50m	4	999	0.36	0.05
E. japonicus	June	September	4	999	0.74	0.03
E. japonicus	0–20m	20–50m	4	999	-0.04	0.57
Cyclopoida spp.	June	September	4	999	0.44	0.03
Cyclopoida spp.	0–20m	20–50m	4	999	0.14	0.29

ANOSIM showed that dissimilarities of bacterial composition associated with *D. pulicaria* (Gut and BP) were significantly different between the two months and two depths, while those with two copepod taxa were also significantly different between the two months but not different between the two depths (Table 3-3).

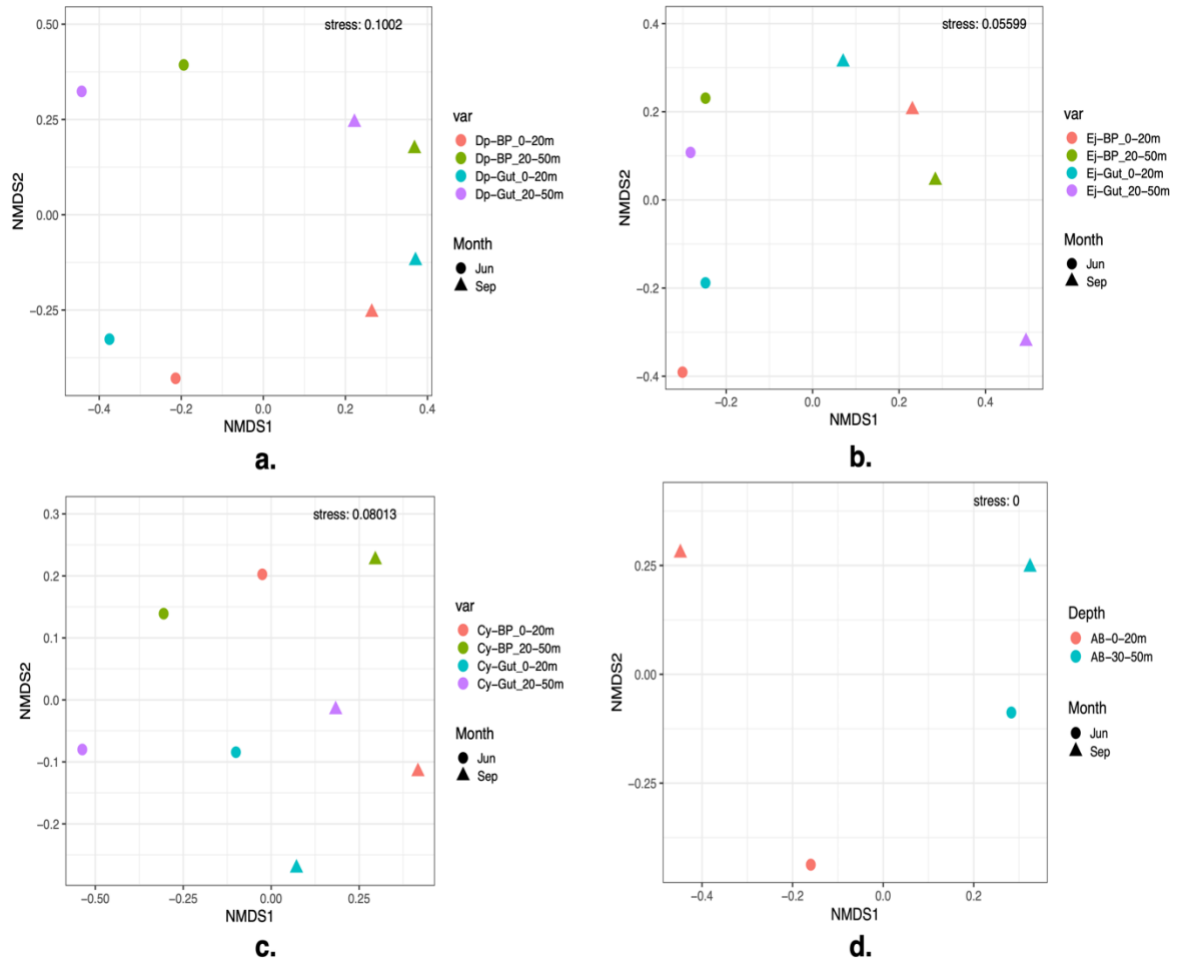

Fig. 3-4 Non-metric multidimensional scaling (NMDS) analysis using all OTUs demonstrating dissimilarities among samples from June and September for each zooplankton taxa; *D. pulicaria* (a), *E. japonicus* (b), Cyclopoida spp. (c), and ambient bacteria (AB) (d) from ambient water (d). Dp, Ej, and Cy represent *Daphnia pulicaria*, *Eodiaptomus japonicus*, and Cyclopoida spp., respectively, BP denotes body parts other than the gut of each taxon.

Heatmap analysis showed that the dominant family composition of bacteria associated with zooplankton hosts were similar between the two depths in each month, and therefore, five large clusters were constructed (Fig. 3-6). In each cluster, those in the same month were mostly constituted. Cluster 1 constitutes dominant family compositions with *E. japonicus* and *D. pulicaria* in both depths of September; those associated with the Gut and BP of *E. japonicus* in the epilimnion and with the Gut in the hypolimnion, those with the Gut and BP of *D. pulicaria* in the hypolimnion and with the BP in epilimnion were relatively similar. The dominant families were Oxalobacteraceae, Aeromonadaceae, and Comamonadaceae. Cluster

2 included those with *E. japonicus* and *D. pulicaria* in both epi- and hypolimnion of June; those associated with the Gut and BP of *E. japonicus* and *D. pulicaria* and BP of Cyclopoida spp. from hypolimnion were relatively similar. The dominant families were Neisseriaceae, Flavobacteriaceae, Comamonadaceae, and Oxalobacteraceae. In cluster 3, those with *E. japonicus* and Cyclopoida spp. in both depths of both months were constituted with dominant families of Comamonadaceae and Oxalobacteraceae. In cluster 4, those with the Gut of *E. japonicus* and Cyclopoida spp. in the hypolimnion and the Gut of *D. pulicaria* and Cyclopoida spp. in the epilimnion of September, dominating Aeromonadaceae and Oxalobacteraceae. In cluster 5, those with the Gut of *D. pulicaria* and Cyclopoida spp. in the epilimnion of June were constituted with the dominance of Aeromonadaceae, Comamonadaceae, and Neisseriaceae.

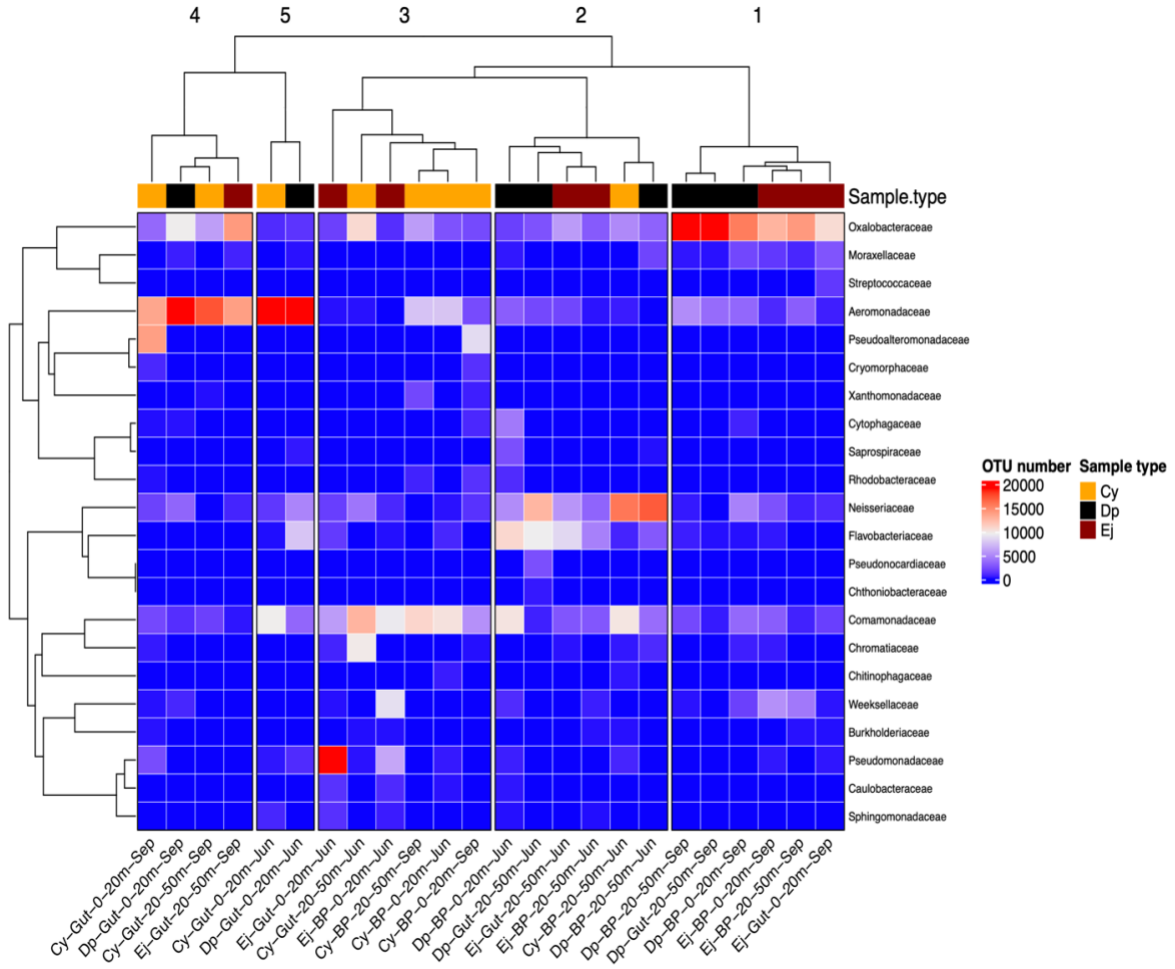

Fig. 3-6 Heatmap of abundant bacterial families (≥ 200 OTU reads) associated with zooplankter BP and gut of *Daphnia pulicaria* (Dp), *Eodiaptomus japonicus* (Ej), Cyclopoida spp. (Cy); BP denotes body parts other than the gut. Individual lines correspond to bacterial families; each column corresponds to a different sample. The upper dendrogram represents similarities in bacterial composition among zooplankter BP and gut; the left dendrogram represents similarities in patterns of occurrence of a bacterial family in zooplankter samples. The number at the top indicates each cluster.

4. Discussion

In this chapter, I examined how the bacterial compositions associated with the Gut and BP of the three zooplankton hosts differed between the two months and the depth habitats at a pelagic site in the north basin of Lake Biwa. The lake water was tightly stratified during the study period, and June was start of the stagnation period while September was the end of the period. Therefore, abiotic and biotic conditions of the environment, including temperature, dissolved oxygen, phytoplankton, and zooplankton community, and environmental bacterial

compositions differed between the months and the depths (Tezuka 1984, Kawabata 1989, Hsieh et al. 2010, Okazaki and Nakano 2016). Ambient bacterial compositions observed were remarkably different between two different water layers, 0–20 m and 30–50 m. Dominat phylum of ambient bacteria were Chloroflexi and Planctomycetes in hypolimnion in both June and September, and it is similar to those previously reported by Okazaki et al. (2013) and Okazaki and Nakano (2016). The Gut and BP of *D. pulicaria*, *E. japonicus*, and Cyclopoida spp. in epilimnion contained higher microbial diversity (*H*) than those in hypolimnion. It might be associated with consistently higher bacterial abundance in epilimnion with higher temperature and DO than those in hypolimnion during the summer stagnation period in Lake Biwa (Gurung et al. 2001). Moreover, during the stagnation period, dissolved organic carbon (Maki et al. 2010) and phytoplankton (Tezuka 1984) become more abundant in the epilimnion compared to the hypolimnion. These conditions might be considered to facilitate a favorable condition for diverse bacterial attachment and proliferation in the gut and body surface of the three zooplankton hosts in epilimnion (Huq et al. 1984, Bickel and Tang 2014). Whereas symbiotic bacteria with the zooplankton may utilize organic matter imported and produced by the hosts themselves through ingestion and egestion (Møller 2005, Møller et al. 2007), being independent of the availability of organic substrates in their surrounding environment (Bickel and Tang 2014).

Chl-*a* concentrations were mainly distributed at 0~20 m depth in Lake Biwa and abundant in June while low in September (Fig. 3-1). NMDS and ANOSIM showed that the bacterial communities in the BP and Gut of the three zooplankton hosts differed significantly between June and September. Flavobacteriaceae, which is a type of bacteria specialized in digesting phytoplankton-derived substrates (Qi et al. 2009), in the Gut and BP of *D. pulicaria* and *E. japonicus*, had a lower contribution in September than in June. This may be associated with the phytoplankton diet available for *D. pulicaria* and *E. japonicus* in June but not in

September. Commamonadaceae, which has been shown to be a widely occurring bacterial family in *Daphnia* as also shown in Chapter 2, and ubiquitous during phytoplankton bloom in various freshwater bodies (Eckert and Pernthaler 2014), was lower in September than in June.

In September, Oxalobacteraceae was detected in the Gut, and BP of *E. japonicus* and *D. pulicaria* in the hypolimnion, where water temperatures were lower than 10˚C in both months, may be attributed to the availability of nutrients in the host microhabitat (Tang et al. 2010) that fosters the growth of this mesophilic bacterial group disregarding temperature limitation (Pomeroy and Wiebe 2001). Bickel and Tang (2014) reported that temperature had a limited impact on *Acartia tonsa* and *Balanus* sp.-associated bacterial abundance in Chesapeake Bay. During the stratification period, the availability of organic substrate for heterotrophic microbial processes in the hypolimnion may be rather more important than temperature (Bickel and Tang 2014). The members of Oxalobacteraceae are heterotrophic and can be aerobic or microaerobic to anaerobic (Baldani et al. 2014). Thus, abundant Oxalobacteraceae detected in the Gut and BP of *E. japonicus* and *D. pulicaria* inhabiting in hypolimnion may be attributed to the availability of nutrients in the host microhabitat (Tang et al. 2010, Bickel and Tang 2014) that may foster the growth of this bacterial group disregarding temperature and oxygen limitation (Pomeroy and Wiebe 2001).

In the Gut and BP of Cyclopoida spp., Pseudoalteromonadaceae including *Vibrio cholerae* was dominant just from the epilimnion in September. Water temperature might be one of the key parameters affecting the spatio-temporal distribution of *Vibrio cholerae* associated with Cyclopoida spp. in Lake Biwa. *Vibrio* spp. are predominant symbiotic bacterium in the gut and body surface of marine copepods (Sochard et al. 1979, Huq et al. 1983, Tang et al. 2010) and are prevalent in warm water conditions (Huq et al. 1984). The presence of *Vibrio* sp. in the gut of cyclopoids from the epilimnion may be attributed to their consumption of chitin-rich diets. *Vibrio* spp. are specialized to digest chitin (Cottrell et al.

2000). The adult *Mesocyclops* is a carnivore and preys on nauplii and juvenile *E. japonicus* (Kawabata 1991), having a chitinous body mainly distributed in epilimnion during the stagnation period (Kawabata 1987). Consequently, Cyclopoida spp. inhabiting in hypolimnion might face a lack of such food and may feed on other microzooplankton available (Kawabata 1987).

The microbial difference in Cyclopoida spp. between two habitats also could correlate with a species-specific difference and their habitat selection. In Lake Biwa, Cyclopoida spp. mainly include two species, *Mesocyclops dissimilis* and *Cyclops vicinus* (Kawabata 1989). The adult *M. dissimilis* is abundant during the stagnation period and restricted to epilimnion, whereas *C. vicinus* is mainly distributed to the deeper layer (Kawabata 1989). Therefore, interspecies variability and their different habitat selection in the study period can be important reasons for harboring diverse microbiota among individuals from different habitats (Datta et al. 2018, Sadaiappon et al. 2021).

CHAPTER 4:

“Taxonomic and ecological aspects on epibiotic ciliates and flagellates attached to cyclopoid copepods in Lake Biwa.”

1. Introduction

Epibiosis is a type of symbiotic relationship where one organism spends its sessile life stage attached to the other organism's surface. The organism that attaches to another living organism, which is referred to as the basibiont or host, is called epibiont (Wahl, 1989). Such episymbiotic relationships can have both positive and negative effects for both epibiont and host (Wahl 1989, Wahl et al.1997, Cabral et al. 2017), or it could be impartial, depending on the ecological aspect (Wahl 2008). Occasionally, this relationship could be detrimental to hosts (Herman and Mihursky 1964, Turner et al. 1979, Visse 2007), decreasing their life span (Gilbert and Schroder 2003) and fecundity (Henebry and Ridgeway 1979, Threlkeld and Willey 1993), increasing burden and interfering host's motility (Willey et al. 1990, Jones et al. 2016); altering their physiology and behavior (Barea-Arco et al. 2001, Willey and Threlkeld 1993); competing for the food, if size range of ingestible food particles overlaps (Kankaala and Eloranta 1987). On the contrary, epibionts may protect the host (basibiont) against predators (Wahl 1989) and sometimes provide a food source for the host when usual planktonic feed is at low levels (Barea-Arco et al. 2001). By attaching to the host bodies, epibionts may evade zooplankton predators (Henebry and Ridgeway 1979), travel to areas with available foods (Kankaala and Eloranta 1987, Wahl 1989), and have optimal conditions for growth (Regali-Seleghim and Godinho 2004).

Epibiosis involving a relationship between epizoic protists and zooplankton is a widespread phenomenon in aquatic environments (Chiavelli 2003, Jones et al. 2016). The flagellated green alga *Colacium* attaches and colonizes on the body surface of crustacean zooplankton, including *Daphnia* spp., cyclopoid and calanoid copepods (Willey et al. 1990, Threlkeld et al. 1993, Zalocar et al. 2011) from both fresh- (Huber-Pestalozzi 1955) and brackish waters (Møhlenberg and Kaas 1990). Previous studies showed that *Colacium vesiculosum* was mutualistic to its calanoid hosts (Møhlenberg and Kaas 1990, Zalocar et al.

2011). Epizoic *Colacium* spp. was prevalent when their zooplankton hosts were abundant (Møhlenberg and Kaas 1990, Chiavelli et al. 1993), and the infestation rate was positively correlated with ambient temperature (Møhlenberg and Kaas 1990). However, the ecological context and significance of the relationship between these zooplankton hosts and *Colacium* spp. is poorly understood. The epizoic flagellate *Colacium* may prefer zooplankton taxa as a single host for colonization over other taxa (Chiavelli et al. 1993, Møhlenberg and Kaas 1993).

Many species of peritrich ciliates have been shown to attach as epibionts on the body of various crustacean zooplankton species (Khal 1935; Sprague and Couch 1971; Fernandez-Leborans and Tato-Porto 2000; Bickel et al. 2012). There are many reports on colonial peritrich ciliate *Epistylis* spp. attached to the exoskeletons of cyclopoid and calanoid copepods and cladocerans (Foissner et al. 1999, Bickel et al. 2012, Clamp et al. 2016, De Souza Santos et al. 2020). However, knowledge on the effect of *Epistylis* spp. on its crustacean hosts is very limited, though high infestation by *Epistylis* sp. has been shown to reduce the survival of its host *Pseudodiaptomus stuhlmanni* in a laboratory experiment (Jones et al. 2016). De Souza Santos et al. (2020) showed that the prevalence of epibiont *Epistylis* sp. on calanoid and cyclopoid hosts was related to its host size and abundance but not to any ambient abiotic variables. On the contrary, Utz and Coats (2005) didn't find any relationship between host abundance and the prevalence of *Epistylis* infestation. Cabral et al. (2017) reported that both physico-chemically and biologically (food availability and host abundance) environmental parameters regulated the populations of epibiotic ciliate *Epistylis* spp. in the floodplain. *Epistylis* sp. also shows host-specificity (Utz and Coats 2005, Clamp et al. 2016, Lu et al. 2020) as shown in *Colacium*, and tends to attach to just a couple of host species even when other zooplankton species coexist (Xie et al. 2001, Cabral et al. 2017).

In this chapter, I focused on the following steps, 1) species of epibionts occurred were identified based on their morphological features, 2) seasonal changes and vertical distribution

of the epibiont and infestation rate were determined with environmental variables, and 3) factors affecting these spatio-temporal variation in infestation of the epibionts were discussed.

2. Materials and Methods

Zooplankton samples were collected monthly with a vertical net haul using a conical plankton net (mouth diameter, 30 cm: mesh size, 0.1 mm) with a flowmeter from just above the bottom to the surface at two fixed pelagic sites, K4 (35°18′56.6″N, 136°11′27.0″E; depth ∼50 m) from 15 January 2018 to 19 December 2019, and a station near Japan water agency (JWA) observatory buoy (35°18.672′ N, 136°08.593′ E; depth ∼70 m) from 17 March 2020 to 16 December 2021 and from 20 June to 21 December 2022 in north basin of Lake Biwa. To determine vertical distribution of zooplankton and epibionts, zooplankton samples were also collected with discrete vertical net hauls from 8 depth layers, 0–5, 5–10, 10–15, 15–20, 20–25, 25–30, 30–50 m and 50 m – just above the bottom, using a closing plankton net (mouth diameter, 30 cm; mesh size, 0.1 mm) in 24 August, 14 September, 17 October, and 15 November at the same time with the monthly sampling in 2022. The zooplankton collected were immediately killed with Lugol's Iodine solution and preserved with 5% sugar formalin. Vertical profiles of water temperature, dissolved oxygen (DO), and chlorophyll *a* concentration throughout the water column were determined with a CTD profiler (AAQ-Rinko, JFE advantec) on each occasion.

Each crustacean zooplankton species and individuals attached by epibionts were identified and enumerated under a dissecting microscope (OLYMPUS SZH10) at the magnification of 30–70x. Infestation prevalence as total infestation rate (total number of infested individuals), co-infestation rate of ciliates and flagellates, and infection rate of each epibiont was calculated as the number of zooplankton infested by epibionts, i.e., ciliates and flagellates, divided by the total number of the host zooplankton (Bush et al. 1997). The

epibionts attached to just cyclopoid copepods (see Results in detail), and then the epibionts attached to the body parts of the host cyclopoids, i.e., antennule, cephalothorax, thorax segments, swimming legs, caudal rami, and setae were also examined.

Another zooplankton sample was collected with a vertical net haul from 30 m to the surface using a Norpac net (mouth diameter, 45 cm; mesh size, 0.2 mm) to examine the morphological features of the epibionts. Live zooplankton collected were immediately transferred to the laboratory with an insulated container. In the laboratory, the body size and shape of the epibionts and types of colonies were examined with a phase contrast microscope (OLYMPUS IX70, Japan) at a magnification of 100–400x, equipped with a digital camera (WRAYCAM-NF1000) connected to a computer. Measurements were performed using the software WraySpect ver. 2.1. and identified the species of the flagellate and ciliate epibionts. Silver-nitrate staining was performed to identify some specific characteristics of the zooid, including pellicular striation, telotroch band, infundibular ciliature, and shape of macronucleus of the ciliate epibionts (Waggoner et al. 2016). Identification of epibionts was made based on mainly Foissner et al. (1999) and Lu et al. (2020) for ciliates, and Huber-Pestalozzi (1955), Rosowski and Kugrens (1973) and Zalocar et al. (2011) for flagellates.

3. Results

Two types of protistan epibionts, i.e., stalked colonial peritrich ciliate *Epistylis* sp. and epizoic flagellate *Colacium* sp., were exclusively found on adults and copepodites of Cyclopoida spp. There were three types of infestation observed; cyclopoids were infested by the ciliate alone, the flagellate alone, or both (hereinafter co-infestation). *Colacium* sp. was commonly attached to the cephalosome, metasome, urosome, antennule, caudal rami and setae, and swimming legs of the cyclopoid hosts (Fig. 4-1), while *Epistylis* sp. was mainly attached to metasome and urosome of the adult and late copepodite (Fig. 4-2).

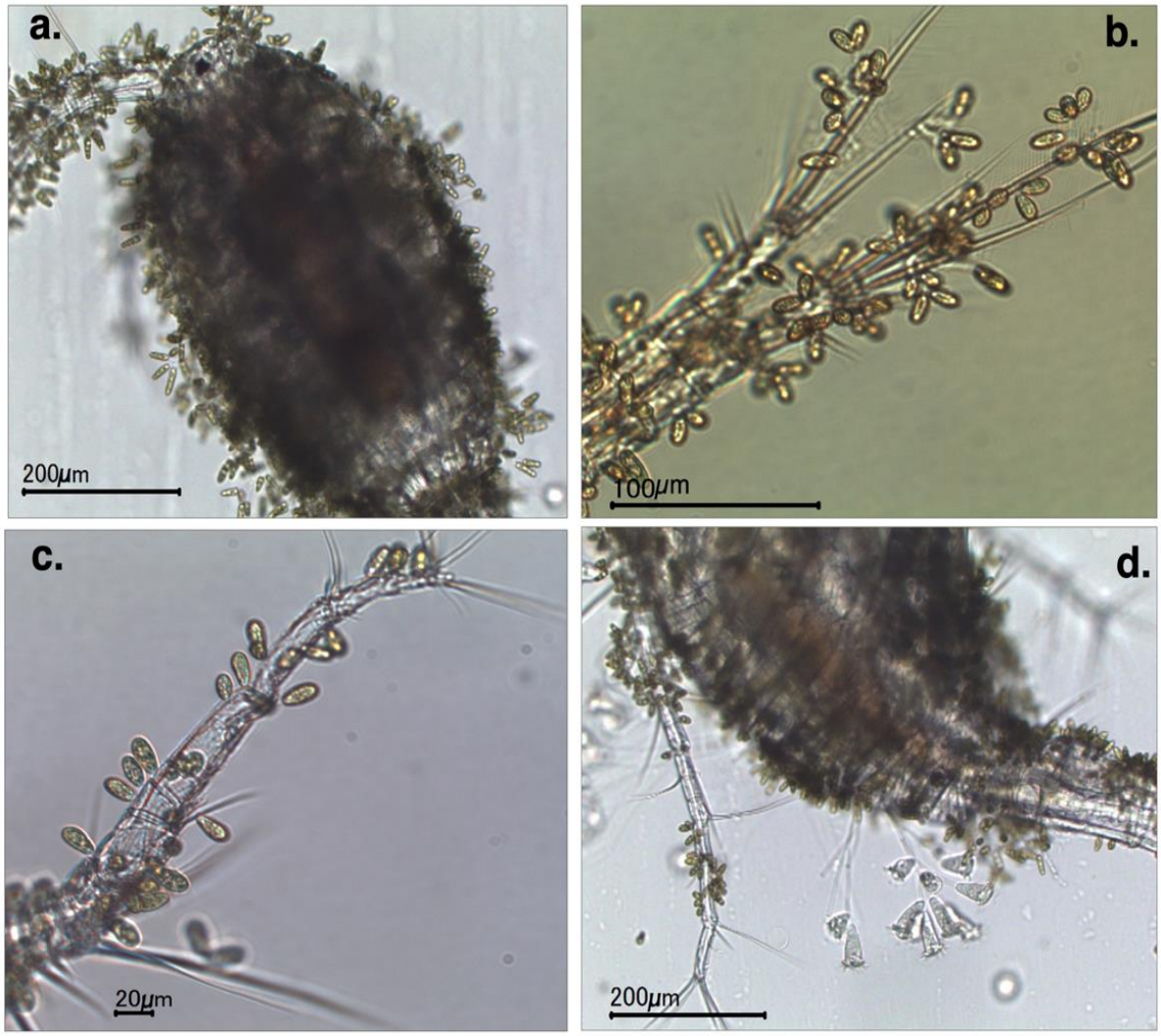

Fig. 4-1 Photographs of cyclopoid copepods infested by flagellate epibiont, *Colacium vesiculosum*, in Lake Biwa; different sites of the body, cephalosome and antennae (a), urosome and caudal rami (b) and right antennae of adult male (c), and a co-infestation (both flagellates and ciliates infected a cyclopoid) (d).

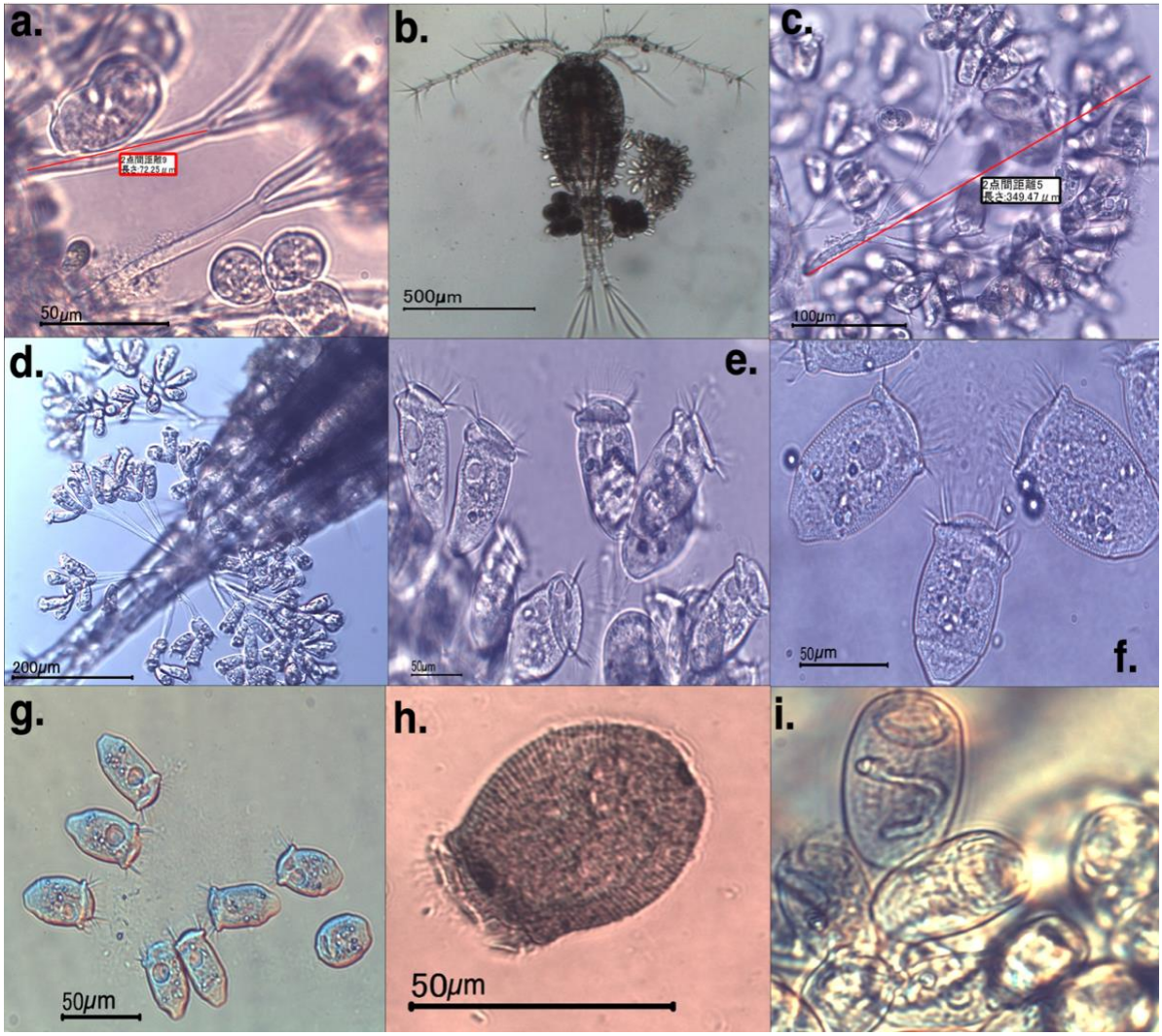

Fig. 4-2 Photographs of colonies and zooids of ciliate epibionts, *Epistylis anastatica*, attached to Cyclopoida spp. in Lake Biwa; a–g, and i (live specimen), h (silver nitrate staining specimen). A contractile dichotomously branched stalk with a smooth surface (a), shape of colonies attached to the metasome and urosome of cyclopoid copepods (b–d), zooids with characteristic features, i.e., peristomial lip, peristomial collar, shape of peristomial disc, contractile vacuole, and its position (e–g), transversely striated pellicle (h), and c-shaped macronucleus (i).

3.1 Morphological features of the epibionts

3.1.1 Flagellates

Solitary or colonial non-motile green vegetative cells without flagellum attached to the surface of the outer shell of the cyclopoid body by a mucilaginous dichotomously branched stalk. Cells often grouped together, forming amorphous colonies. Vegetative cells were ovoid or spindle to somewhat pyriform, having paramylon bodies, and 13.86–23.28 μm long and

6.00–9.98 μm width on average. Cell apices obtuse-shaped. It was difficult to observe chloroplast and pyrenoid in the cell body due to excess food reserved (Rosowski and Kugrens 1973) (Table 4-1, Fig. 4-1). According to the morphological features of this flagellate with descriptions by Huber-Pestalozzi (1955), Rosowski and Kugrens (1973), and Zalocar et al. (2011), this epizoic flagellate should be identified *Colacium vesiculosum* (Ehrenberg 1833).

Table 4-1. Morphological features of flagellate epibiont infested Cyclopoida spp. in Lake Biwa.

Identifying features	Morphological features of *Colacium* sp. in this study	*Colacium vesciculosum* described by Ehrenberg (1833)
Vegetative cell	Solitary or colonial	Solitary or colonial
Shape of cell body	Ovoid to spindle, cell apices obtuse	Ovoid to spindle
Length of cell	13.86–23.28 μm long (mean = 18.61±2.50, n=34) × 6–9.98 μm wide (mean = 8.07±1.01, n=34)	Sessile cell 15-21 and 11-14 wide
Stalk	Stalk formed and dichotomously branched	Gelatinous stalk forming dichotomously branching colony
Paramylon bodies	Present	Present
Chloroplast and pyrenoid	Not observed	Chloroplast saucer-shaped with a pyrenoid, 5 to 10 in number
Flagella	Absent	Sessile cell without flagella

3.1.2 Ciliates

Acontractile stalk was dichotomously branched with a smooth surface, and the branching pattern of the colony is umbellate. The height of the colony ranged from 241.6–482.0 μm, and the stalk were 41.1–113.1 μm long and 6.5–13.0 μm wide. Extended zooids were elongated and bell-shaped, 45.8–79.2 μm long and 18.8–39.2 μm wide. Pellicle was striated transversely with a well-defined peristomial lip and peristomial collar as wide as the zooid's body Peristomial disc was slightly convex, and a single contractile vacuole was located at the ventral wall of the infundibulum underneath the peristomial collar. C-shaped macronucleus was longitudinally oriented. Infundibular polykinety, including oral ciliature, was not studied

well in this study (Table 4-2, Fig. 4-2). According to the morphological features of this ciliate with descriptions by Foissner et al. (1999) and Lu et al. 2020, this peritrich ciliate might be identified as *Epistylis anastatica* (Linnaeus 1767, Ehrenberg 1830).

Table 4-2. Morphological features of ciliate epibiont infested Cyclopoida spp. in Lake Biwa.

Characteristics	**Morphological features of ciliate in this study**	***Epistilis anastatica* Ehrenburg (1830)**	**References other than Ehrenburg (1830)**
Size of zooids (in vivo)	45.79–79.19 µm long (mean = 56.93±7.40, n=40) ×18.75–39.3 µm wide (mean = 24.20±4.99, n=40)	60–100 µm × 20–35 µm	Foissner et al. 1999, Lu et al. 2020
Shape of zooids	Conical or elongate bell-shaped, pellicle transversely striated	Slightly conical, elongate bell-shaped, pellicle with transverse striae	Foissner et al. 1999, Lu et al. 2020
Colony shape/height	Umbellate, 241.62–482 µm (mean = 347.91±65.43, n=25)	Umbellate, usually <500 µm	Foissner et al. 1999, Lu et al. 2020
Stalk	Dichotomously branched, acontractile, and smooth	Dichotomously branched, acontractile, and smooth	Foissner et al. 1999
Macronucleus	C-shaped and longitudinally oriented	longitudinally oriented, slightly curved to C-shaped, upper part lying under peristome and lower part curved	Foissner et al. 1999
Contractile vacuole	Slightly underneath the peristomal collar	Slightly underneath the peristomal collar, at the ventral wall of the vestibulum	Foissner et al. 1999
Peristomal collar	Narrow and about as wide as body	Narrow and about as wide as body	Foissner et al. 1999
Peristomal disc	Slightly convex	Slightly convex	Foissner et al. 1999

3.2 Seasonal changes in temperature, dissolved oxygen, and chlorophyll *a* concentration

Lake water temperature increased from late April at the surface, and the thermocline developed at 10–20 m from June to October every year during the study period (Fig. 4-3). DO saturations were >100% due to photosynthesis above 10 m throughout the study period but

decreased to <80% from June to September, probably because of decomposition of sinking organic matters originated by phytoplankton bloom in June. Chl-*a* concentration increased above 20 m from June to July and October to November in 2018 to 2020. Another increase throughout the water column in March and a weak increase in October in 2021. No such large seasonality was found in 2022.

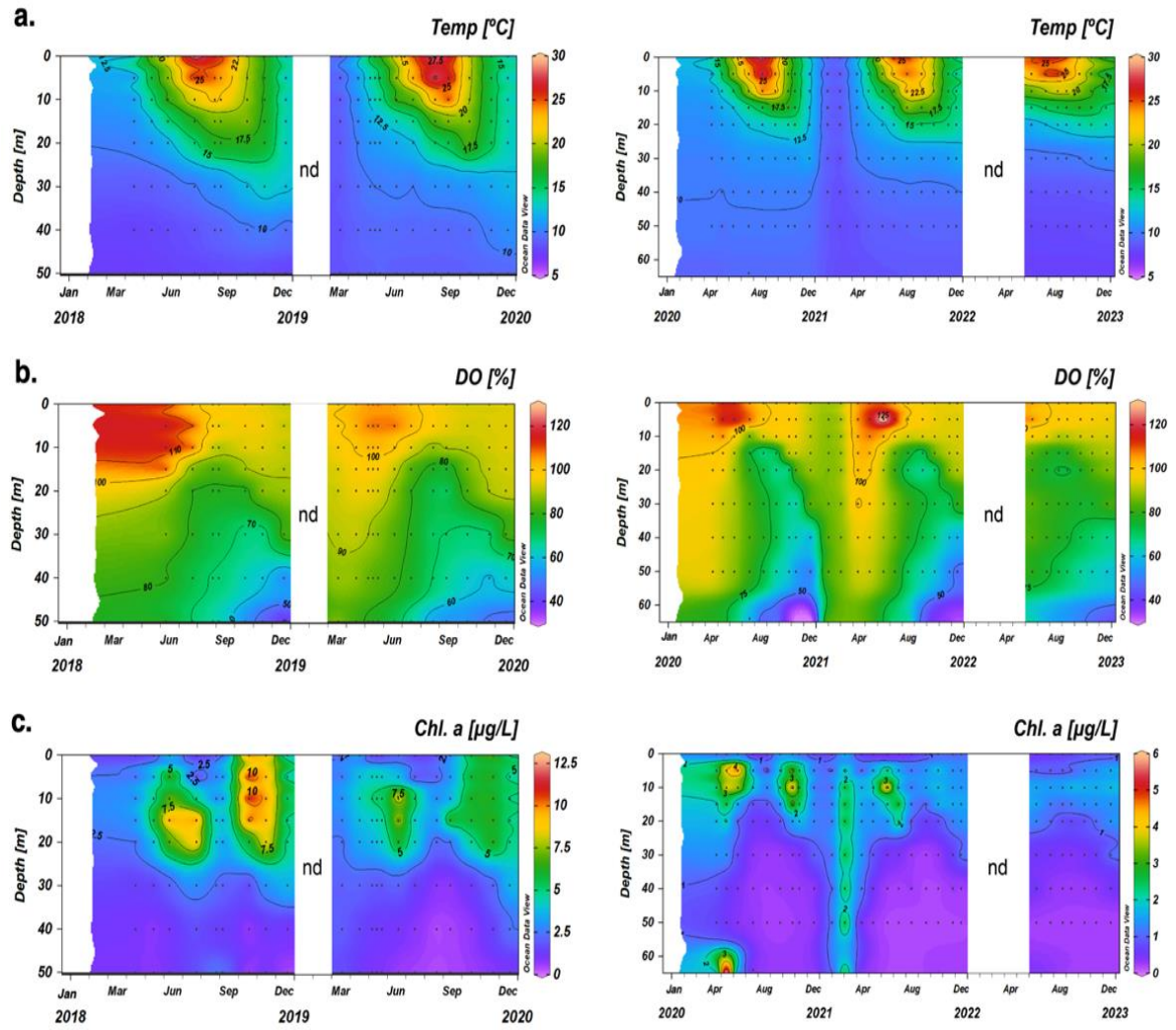

Fig. 4-3 Vertical profiles of water temperatures (a) dissolved oxygen (DO) saturation (b), and Chlorophyll *a* concentration (c) from January 2018 to December 2019 at site K4 and from March 2020 to December 2022 at JWA bouy in the North basin of Lake Biwa.

Average water temperature above 30 m increased from May onwards, peaked in August and remained almost the same until September, and then gradually decreased toward the next March (Fig. 4-4). On the contrary, average DO saturation above 30 m gradually decreased

during the stratification period to September and then increased up to 100% by the next May every year. Average chlorophyll-*a* concentration above 30 m increased in June and October in 2018–2021, while no such increase was found in 2022.

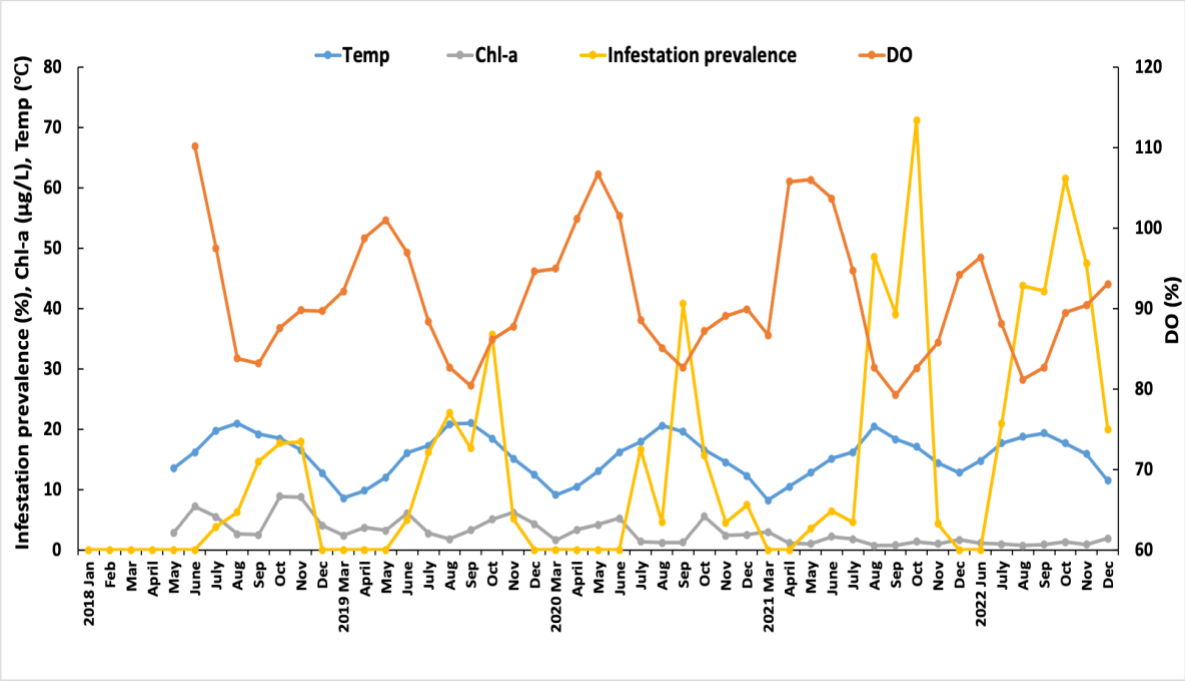

Fig. 4-4 Seasonal changes in total infestation rate (infestation prevalence) of epibionts and average water temperature (Temp), dissolved oxygen (DO) saturation, and chlorophyll *a* (Chl-*a*) concentrations above 30 m at a pelagic site in the north basin of Lake Biwa from 15 January 2018 to 21 December 2022.

3.3 Seasonal changes of host abundance and the prevalence of the epibionts

The epibionts occurred just in cyclopoid copepods from July to November in 2018 and 2019, May to November in 2021, and July to December in 2020 and 2022. The infestation was mostly found in the adult and copepodite stages but not in the naupliar stage, except for July 2022, when the nauplii infected by flagellates was found. Total infestation rates gradually increased from June or July every year (Fig. 4-5). The maximum rates were usually found in October or November and increased year to year from 18% in 2018 to 71% in 2022, then declined toward December. The total number of cyclopoid copepods varied from October/November to December with a mean value of 3.7 to 1.8 ind. L^{-1} with clear seasonality

(Fig. 4-5). The timing of the increase was different among the year; mostly from June except for 2020 and 2022, when it was September. Infested cyclopoids increased from July almost every year except for 2020 when they increased from September. During the study period, no such infection was detected in other crustacean zooplankton taxa, including the most dominant calanoid copepod *E. japonicus* and *Daphnia* spp.

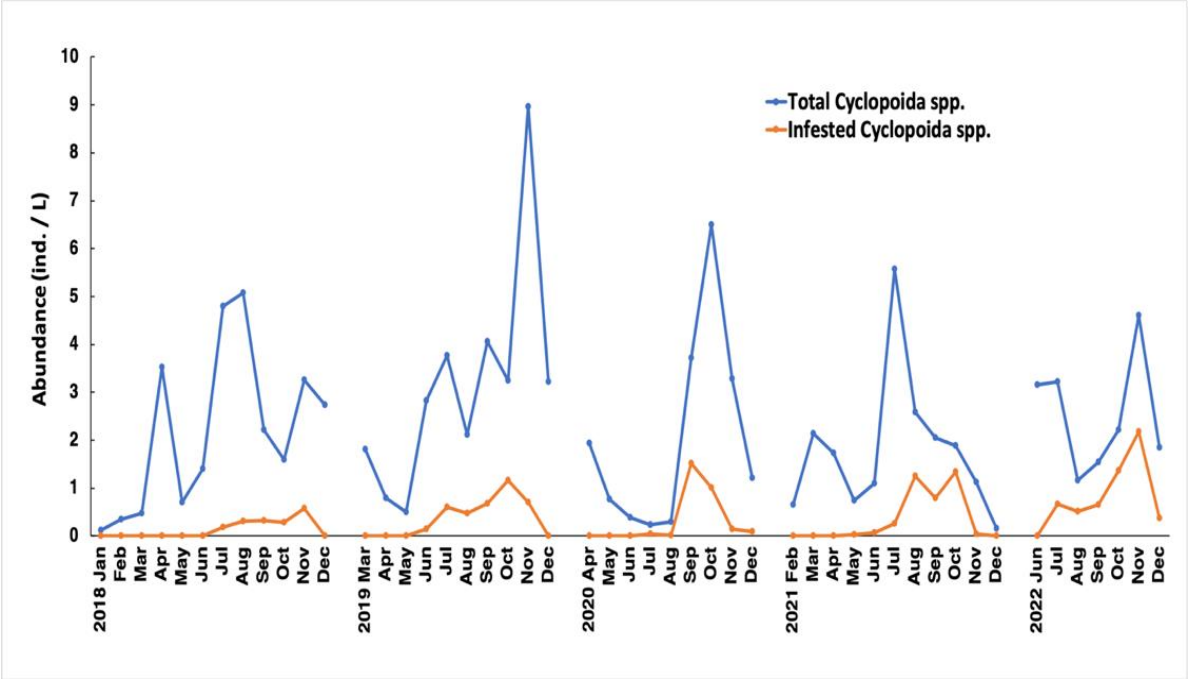

Fig. 4-5 Seasonal distributions in abundance of total Cyclopoida spp. (adult and copepodites) and Cyclopoida spp. infested by both ciliates and flagellates at a pelagic site in the north basin of Lake Biwa from 15 January 2018 to 21 December 2022.

Infestation rates of the flagellate *C. vesiculosum* alone increased year to year, from 20% in 2018 to 53% in 2022, while those of ciliate *E. anastatica* alone showed no such year-to-year variation. Seasonally, the highest infestation rate of the flagellates was found in July at 61% on average among the five years and the lowest at 2.5% in December, while the highest infestation rate of the ciliates alone was found in November at 46%, and the lowest in June. Co-infestation, i.e., infested by both flagellates and ciliates, was found throughout the study period with no seasonality and varied from 5.19 to 10.34 on the annual mean (Fig. 4-6).

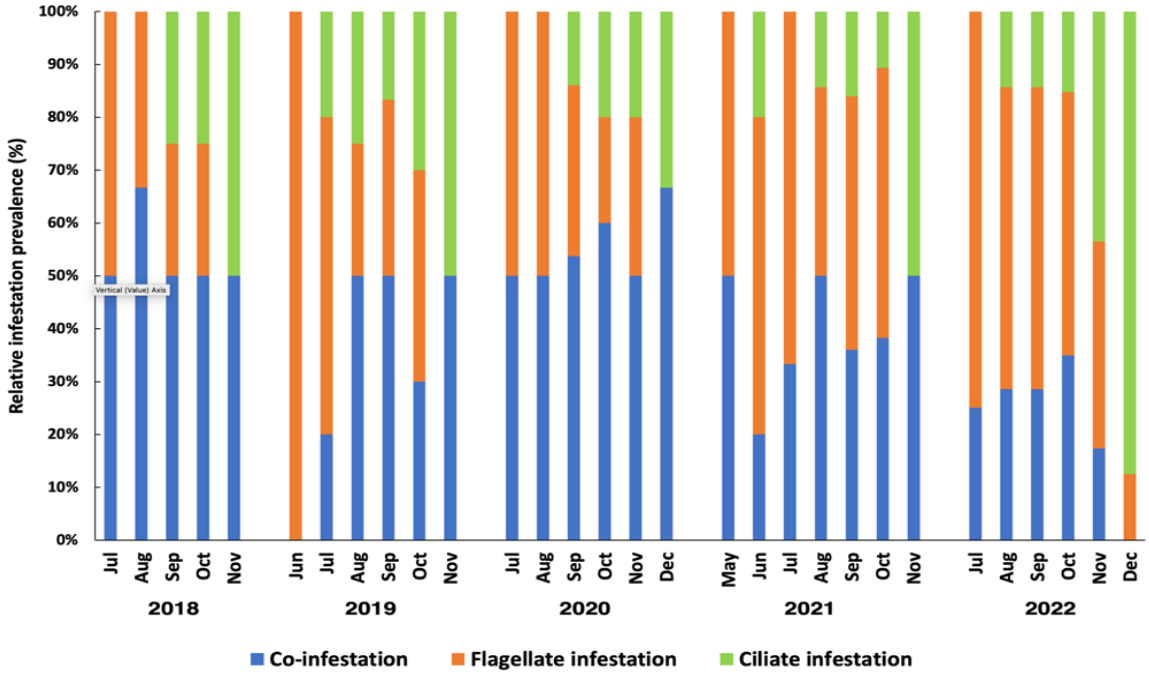

Fig. 4-6 Compositions in co-infestation (both flagellates and ciliates), flagellates alone, and ciliates alone infestation for Cyclopoid spp. at a pelagic site in the north basin of Lake Biwa from 15 January 2018 to 21 December 2022.

In 2022, vertical distributions of the dominant zooplankton species and epibionts-infested cyclopoids were investigated. All zooplankton species including infested cyclopoids were mostly distributed above 20 m in August and September, expanded to 25 m in October and 30 m in November, depending on the thermocline (Fig. 4-7).

Total infestation rate was positively correlated with cyclopoid abundance and average water temperature above 30 m while negatively correlated with average DO saturation (Fig. 4-8). There was no such relationship between the infestation rate and average chlorophyll *a* concentration above 30 m.

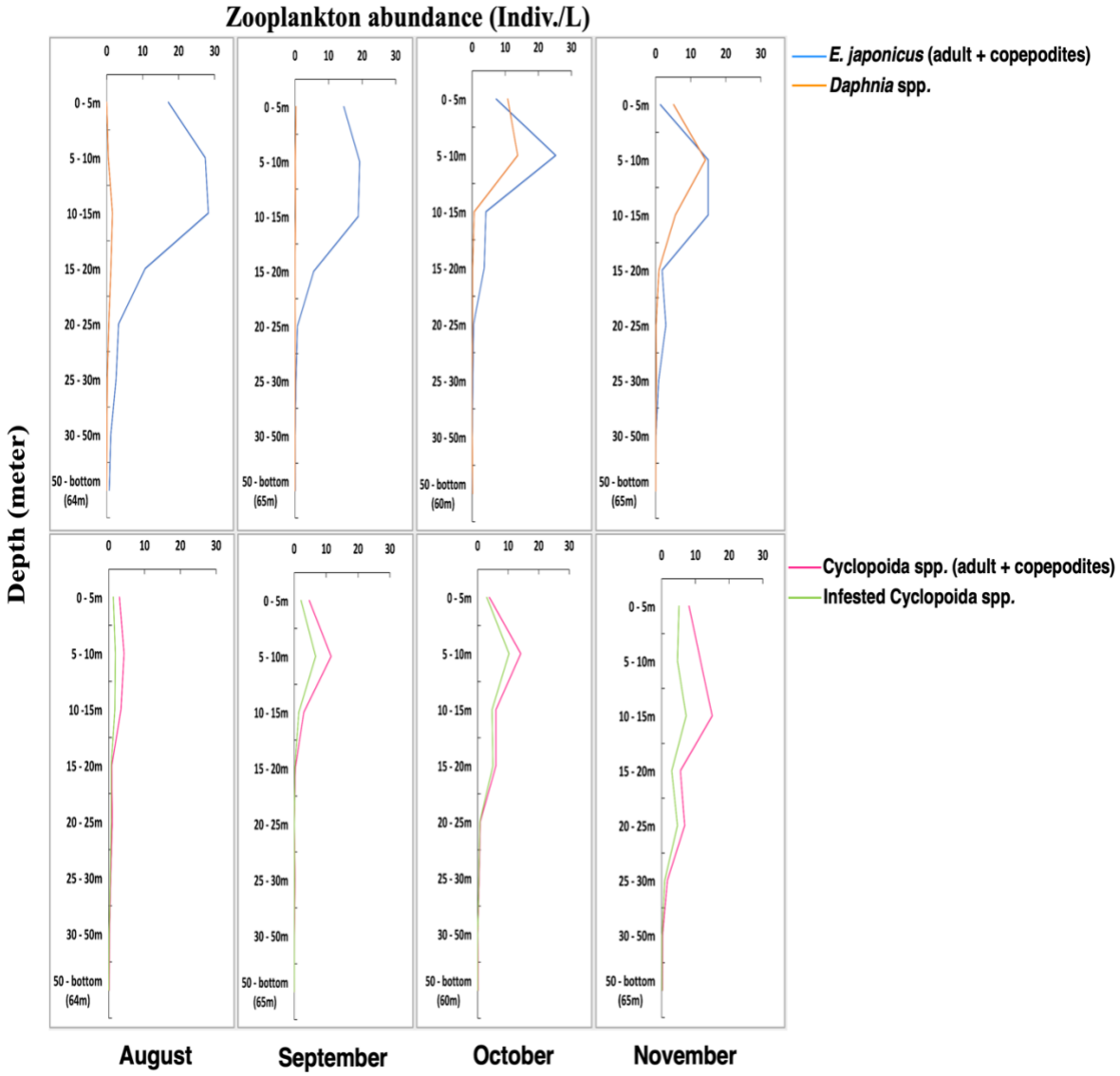

Fig. 4-7 Vertical distributions of dominant zooplankton, *Eodiaptomus japonicus*, *Daphnia* spp., Cyclopoida spp., and infected cyclopoids at a station (35°18.672′ N, 136°08.593′ E) near the JWA buoy in the north basin of Lake Biwa on 24 August, 14 September, 17 October and 15 November 2022.

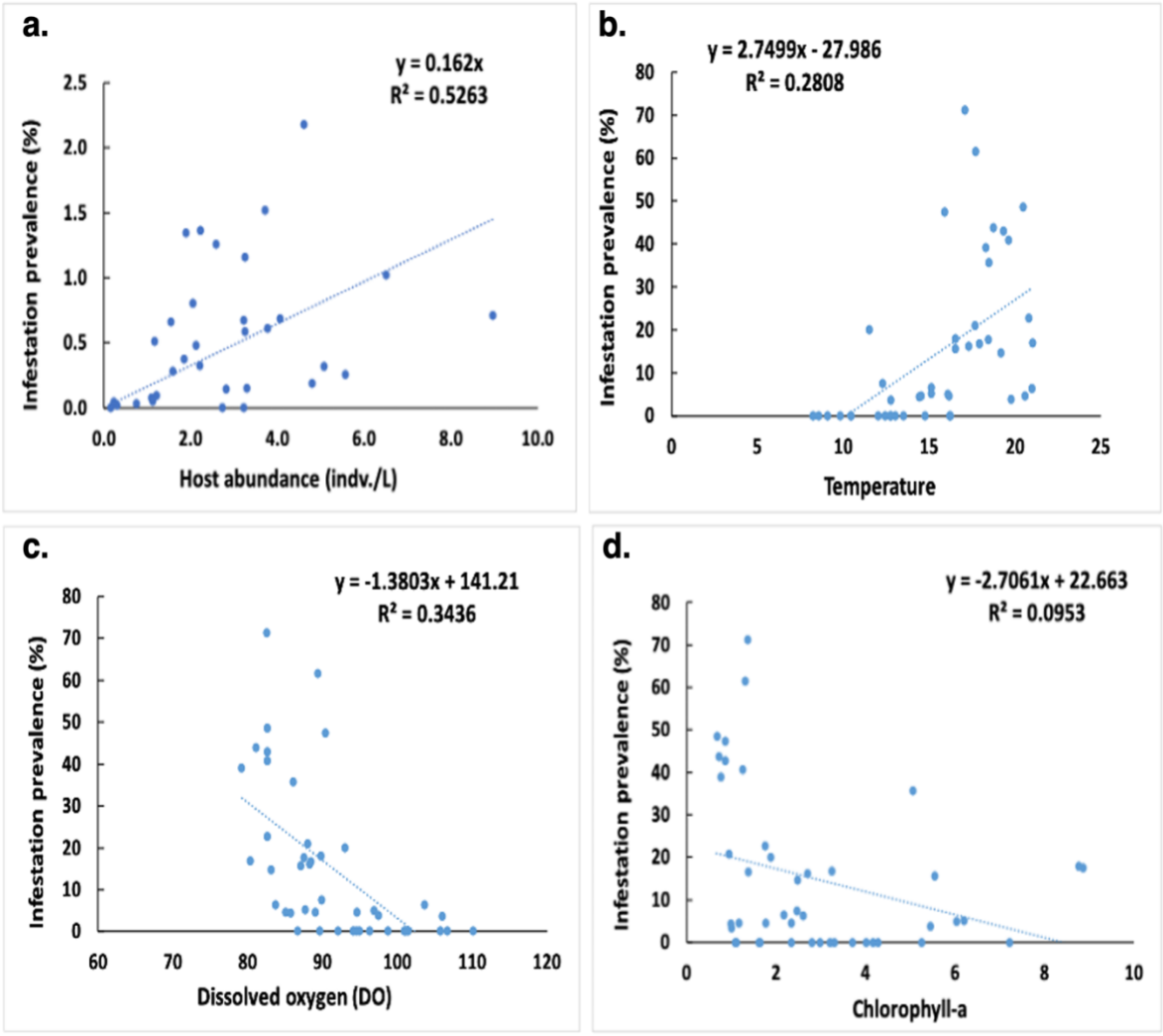

Fig. 4-8 Scatter plots showing the relationship between infestation prevalence (%) and host abundance (a), temperature (b), dissolved oxygen (%) (c), and chlorophyll-*a* (d). For temperature, dissolved oxygen, and chlorophyll-a, an average value (from 0 to 30 m depth) was used.

4. Discussion

This is the first report on epibiosis for crustacean zooplankton in Lake Biwa. Identification of the epibionts based on morphological features might play a crucial role in studying the interaction of the organisms and their ecological aspects. Since the oral ciliature of the identified species of *E. anastatica* and the number of chloroplasts with or without pyrenoid in the *C. vesiculosum* cell have not been appropriately studied, an integrated approach by combining these morphological features with DNA sequencing is recommended.

The epibiont flagellate *C. vesiculosum* and ciliate *E. anastatica* strictly attached to cyclopoid copepods even when other zooplankton species were co-existent throughout the water column. Several previous studies reported that species of *Epistylis* and *Colacium* showed specificity for some species of zooplankton hosts for colonization (De Souza Santos et al. 2020, Corre et al. 2020, Gilbert and Schroder 2003, Utz and Coats 2005, Xie et al. 2001). According to Clamp et al. (2016), only *Mesocyclops isabellae* was infested with *Epistylis anastatica* despite the coexistences of calanoid *Neodiaptomus lindbergi* and *Heliodiaptomus viduus* in an Indian pond. Lu et al. (2020) also reported that *E. anastatica* exclusively infested cyclopoid copepods in two freshwater ponds in Qingdao, China. Jones et al. (2016) observed that *Epistylis* sp. attached to *Pseudodiaptomus stuhlmanni* but not to *Acartiella natalensis* and *Oithona brevicornis* in St. Lucia Estuary, South Africa. Cabral et al. (2017) found that each of the two different species of *Epistylis* was specific to *Notodiaptomus henseni* and *Thermocyclops minutus* in a river floodplain in Brazil.

It can be assumed that the hydrodynamic disturbance created by continuous swimming or feeding currents of suspension feeder *E. japonicus* and *Daphnia* spp. may trigger an escaping response in *E. anastatica* and *C. vesiculosum* epibionts roaming in the ambient water (Threlkeld et al. 1993). Vavra (1963) mentioned that characteristics of swimming movements by hosts are related to the survival of *Epistylis* spp. The dominant zooplankton taxa in the present study, *E. japonicus*, *D. pulicaria,* and Cyclopoida spp., have distinct swimming characteristics compared to each other. *D. pulicaria* moves in a well-known characteristic "hop-and-sink" style (Uttieri et al. 2014, CO'Keefe et al. 1998). Hwang and Turner (1995) found that Cyclopoida spp. moved in a jerking pattern with frequent rest, while calanoid *Temora turbinata* swam continuously without rest. Additionally, Drenner and McComas (1980) demonstrated that diaptomid copepod moves in a zigzag or erratic motion. It is crucial to examine the distinct movement characteristics of these three zooplankters in Lake Biwa to

understand why cyclopoids are the only hosts preferred by both epibionts for adherence and colonization.

There are many previous reports on photosynthetic *C. vesiculosum* colonizing body surface of both copepods and daphnids (Møhlenberg and Kaas 1990, Chiavelli et al. 1993, Zalocar et al. 2011, Cabral et al. 2014). In this study, *C. vesiculosum* is also attached to cyclopoids. For the photosynthetic flagellate like *C. vesiculosum*, attachment to other organisms may benefit from its mobile hosts, which may allow the epibionts to access dissolved nutrients and the photic environment (Threlkeld et al. 1993). On the other hand, motile cells released from both sessile epibionts can be good food sources even for the host cyclopoids (Barea-Arco et al. 2001).

C. vesiculosum attached to cyclopoids dominated in the epilimnetic waters of Lake Biwa, implying that warm water with higher solar irradiation during summer to late autumn may provide a favorable environment for the attached alga (Salmaso and Tolotti 2009). A high abundance of cyclopoid hosts during the growing seasons for the alga might increase adhesion sites available for new colonization (Genc and Bozkurt 2009), consequently increasing infestation prevalence. In this study, though the cyclopoid hosts were distributed even below the euphotic zone, *C. vesiculosum* was also attached there. It can be related to the trophic state of the genus *Colacium*, which are facultative heterotroph and can grow in dark hypolimnion using dissolved organic carbon (DOC) (Salmaso and Tolotti 2009).

The ciliate epibiont *Epistylis* mainly feed on bacteria, pico- and nano-sized plankton, and particulate organic matter (Kankaala and Eloranta 1987, Bickel et al. 2012), while flagellates may take dissolved nutrient and DOC (Salmaso and Tolotti 2009). In this study, the low DO concentrations in the mid-depth layer during the stagnation period might be associated with increased microbial activities related to deposited organic materials from the phytoplankton

bloom. The stratified lake water in the early summer of every year may induce favorable conditions in metalimnion for this epizoic ciliate and flagellate.

Summary

Chapter 1

I report bacterial communities from bodies and feces of the three most abundant zooplankton taxa (*Eodiaptomus japonicus*, *Daphnia pulicaria*, Cyclopoida spp.) from Lake Biwa using meta-barcoding analysis targeting the prokaryote-specific V4 region of 16S rDNA. A total of 293,018 sequences comprising 669 bacterial OTUs were generated, including three zooplankton bodies, of which 54 (*D. pulicaria*), 88 (*E. japonicus*), and 84 (Cyclopoida spp.) OTUs were identified, and in their feces, 132, 156, and 155 OTUs, respectively. Among the identified OTUs, 7 (*D. pulicaria*), 48 (*E. japonicus*), and 51 (Cyclopoida spp.) OTUs were unique in the host bodies, and 13, 36, and 31 OTUs were in their feces, respectively. Bacteria associated with the body and feces of *E. japonicus* were most divergent. The bacterial compositions of the bodies and feces in all three host taxa differed significantly. Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria dominated the bacterial phyla in the zooplankton bodies and feces. At lower taxonomic levels, the bacteria of bodies (particularly) and feces differed among zooplankton taxa. Prevalent bacteria of *D. pulicaria* and Cyclopoida spp. bodies were Aeromonadales (Gammaproteobacteria), and those of E. japonicus were dominated by Burkholderiales (Betaproteobacteria). Aeromonadaceae was the most dominant bacterial family in zooplankton bodies, while Pelagibacteraceae was dominant in feces. The bacterial communities present in the feces were similar among the taxa but differed from those found in the bodies of the animals themselves, implying that the feces contained transient bacteria passing through the digestive system. The bacterial composition associated with the zooplankton bodies may be related to the host's feeding habits, habitat selection, and behavior.

Chapter 2

In this chapter, the resident bacteria in the gut and body parts without gut (BP) of the three dominant zooplankton with different feeding habits were identified, characterized, and compared with bacterial compositions in their feces and ambient waters. To determine dominant and stable symbiotic bacteria in the gut and other body parts, the gut was dissected from the animals' bodies. zooplankton and water samples were collected from the epilimnion (0–20 m) at a pelagic site in the north basin of Lake Biwa on 4 March 2021. Symbiotic bacterial communities in the gut, body other than the gut, and feces differed from those in the ambient waters. The gut, body other than the gut, and feces of the three zooplankton contained different bacterial communities, which also differed among the three species. The most dominant family in the gut and BP of *D. pulicaria* was Comamonadaceae for each site, at 23% and 57%, in *E. japonicus* Neisseriaceae at 63%, and Weeksellaceae at 28%, and in Cyclopoida spp. Aeromonadaceae at 13% and Oxalobacteraceae at 47%, respectively. These results suggested that the differences in the symbiotic bacterial communities in the gut and body may depend on feeding habits and the host's specific preferences and immune responses in the three taxa living in the same habitat.

Chapter 3

To analyze bacterial composition in zooplankton gut and body excluding gut, and ambient waters, samples were collected from the two different depths of the water column, from 20 m to the surface and from the 50 m to 20 m at the same pelagic site in the north basin of Lake Biwa, on 17 June, and 21 September 2021. Vertical profiles of water temperature, dissolved oxygen (DO), and chlorophyll *a* concentrations were recorded at the same time. The lake water was vertically stagnated in June and September. Water temperature, dissolved oxygen, and chlorophyll *a* concentration were very different

between the two depths and months. The gut and body parts without gut (BP) of *D. pulicaria, E. japonicus*, and Cyclopoida spp. inhabited 0–20 m contained higher microbial diversity than their 20–50 m inhabiting counterpart. The symbiotic bacterial community in the gut and body parts of each zooplankton taxa were significantly different between June and September, while different between habitats in *D. pulicaria* and not significantly different in the two copepods. These results suggested that changes in the abiotic and biotic factors of the ambient environment affect bacterial communities in the gut and body parts of the three zooplankton.

Chapter 4

This report presents findings on the presence of epibiotic infestation on cyclopoid copepods in Lake Biwa. From January 2018 to December 2022, zooplankton samples were collected monthly at two pelagic sites in the north basin of Lake Biwa using a vertical net haul. At the same time, temperature, dissolved oxygen (DO), and Chlorophyll-a were measured throughout the water column. The epibionts identified through its gross morphological features were the epizoic flagellate *Colacium vesiculosum* and Peritrich ciliate *Epistylis anastatica*, which infested both adult and copepodites of Cyclopoida spp. To confirm the morphological identification, a DNA base phylogenetic analysis is recommended. These two epibionts were specific only to cyclopoid copepods, despite the presence of *E. japonicus* and *Daphnia* spp. in the water layers. Three types of epibiotic infestation were observed: cyclopoids infested with only flagellate, ciliate, or both. The epibiotic infestation was generally found from May to December, with no infestation found from January to April. The infestation prevalence was highest from August to November of each year. A positive relationship was found between infestation prevalence and temperature and host abundance, while dissolved oxygen showed a negative association, indicating a stable interaction between abiotic and biotic ecological processes that regulate this episymbiotic relationship in Lake Biwa.

References

Akbar S, Gu L, Sun Y, Zhou Q, Zhang L, Lyu K, Huang Y, Yang, Z. 2020. Changes in the life history traits of *Daphnia magna* are associated with the gut microbiota composition shaped by diet and antibiotics. Science of the Total Environment. 705:135827.

Autio R. 1998. Response of seasonally cold-water bacterioplankton to temperature and substrate treatments. Estuarine, Coastal, and Shelf Science. 46(4):465–474.

Azam F, Malfatti F. 2007. Microbial structuring of marine ecosystems. Nature Reviews Microbiology. 5(10):782–791.

Baldani JI, Rouws L, Cruz LM, Olivares FL, Schmid M, Hartmann A. 2014. The family Oxalobacteraceae. InRosenberg E DeLong E Lory S Stackebrandt E Thompson F eds The Prokaryotes. Springer Berlin Heidelberg. 919–974.

Bass D, Rueckert S, Stern R, Cleary AC, Taylor JD, Ward GM, Huys R. 2021. Parasites, pathogens, and other symbionts of copepods. Trends in Parasitology. 37(10):875–889.

Barea-Arco J, Pérez-Martínez C, Morales-Baquero R. 2001. Evidence of a mutualistic relationship between an algal epibiont and its host, *Daphnia pulicaria*. Limnology and Oceanography, 46(4):871–881.

Bernardet JF, Hugo CJ, Bruun B. 2015. *Chryseobacterium*. Bergey's Manual of Systematics of Archaea and Bacteria. 1–35.

Bickel SL, Tang KW, Grossart HP. 2012. Ciliate epibionts associated with crustacean zooplankton in German lakes: distribution, motility, and bacterivory. Frontiers in Microbiology. 3:243.

Bickel SL, Tang KW. 2014. Zooplankton-associated and free-living bacteria in the York River, Chesapeake Bay: comparison of seasonal variations and controlling factors. Hydrobiologia. 722:305–318.

Boonmak P, Liu X, Ban S, Sanoamuang LO. 2018. Effects of different algal diets and carbon supplies on larval development growth and survival in the freshwater copepod *Mongolodiaptomus malaindosinensis* (Copepoda: Calanoida). Plankton and Benthos Research. 13(4):163–172.

Boyle PJ, Mitchell R. 1978. Absence of microorganisms in crustacean digestive tracts. Science. 200(4346):1157–1159.

Brambilla DJ. 1983. Microsporidiosis in a *Daphnia pulex* population. Hydrobiologia. 99:175–188.

Bush AO, Lafferty KD, Lotz JM, Shostak AW. 1997. Parasitology meets ecology on its own terms: Margolis et al. revisited. The Journal of parasitology. 575–583.

Cabral AF, Dunck B, Lansac-Toha FM, Rodrigues L, Pinto Utz LR, Machadovelho LF. 2014. First report of *Colacium vesiculosum* Ehrenberg 1853 (Euglenophyceae), as epibiont on planktonic Copepods (Crustacea, Copepoda), in a Brazilian floodplain lake. Acta Protozoologica. 53(4).

Cabral AF, Utz LRP, Velho LFM. 2017. Structure and distribution of ciliate epibiont communities in a tropical floodplain. Hydrobiologia. 787:167–180.

Callens M, Macke E, Muylaert K, Bossier P, Lievens B, Waud M, Decaestecker E. 2016. Food availability affects the strength of mutualistic host–microbiota interactions in Daphnia magna. The ISME journal. 10(4):911–920.

Callens M, Watanabe H, Kato Y, Miura J, Decaestecker E. 2018. Microbiota inoculum composition affects holobiont assembly and host growth in *Daphnia*. Microbiome. 6:1–12.

Callens M, De Meester L, Muylaert K, Mukherjee S, Decaestecker E. 2020. The bacterioplankton community composition and a host genotype-dependent occurrence of taxa shape the *Daphnia magna* gut bacterial community. FEMS Microbiology Ecology. 96:128.

Carman KR, Dobbs FC. 1997. Epibiotic microorganisms on copepods and other marine crustaceans. Microscopy research and technique. 37(2):116–135.

Chae YJ, Oh HJ, Chang KH, Kwak IS, Jo H. 2021. Application of Next-Generation Sequencing for the Determination of the Bacterial Community in the Gut Contents of Brackish Copepod Species (*Acartia hudsonica Sinocalanus tenellus* and *Pseudodiaptomus inopinus*). Animals. 11(2):542.

Chiavelli DA, Mills EL, Threlkeld ST. 1993. Host preference, seasonality, and community interactions of zooplankton epibionts. Limnology and Oceanography. 38(3):574–583.

Chiavelli DA. 2003. Host-epibiont population dynamics. Dartmouth College.

Clamp JC, Chatterjee T, Fernandez-Leborans G. 2016. Occurrence of *Epistylis anastatica* (Linnaeus, 1767)(Ciliophora: Peritrichia) on *Mesocyclops isabellae* Dussart and Fernando (Crustacea: Copepoda: Cyclopoida) in India, with an annotated checklist of species of *Epistylis* reported as Epibionts of Cyclopoid Copepods and resources for their identification. Zootaxa. 4168(3):525–540.

CO'Keefe T, C Brewer M, Dodson SI. 1998. Swimming behavior of *Daphnia*: its role in determining predation risk. Journal of Plankton Research. 20(5):973–984.

Cooper RO, Cressler CE. 2020. Characterization of key bacterial species in the *Daphnia magna* microbiota using shotgun metagenomics. Scientific reports. 10(1):652.

Corliss JO. 2000. Biodiversity, classification, and number of species of protists. In Nature and Human Society: The Quest for a Sustainable World. Proceedings of the 1997 Forum on Biodiversity, Board on Biology, National Research Counc.130–155 pages. National Academy Press.

Corno G, Villiger J, Pernthaler J. 2013. Coaggregation in a microbial predator-prey system affects competition and trophic transfer efficiency. Ecology. 94(4):870–881.

Corre PS, Abadilla MRCS, Arnaldo MNL. 2020. *Vorticella* and *Colacium* as epibionts of copepods in Pasig river, Philippines. Philippine Journal of Systematic Biology. 14(1):1-8.

Cottrell MT, Wood DN, Yu L, Kirchman DL. 2000. Selected chitinase genes in cultured and uncultured marine bacteria in the α-and γ-subclasses of the proteobacteria. Applied and environmental microbiology. 66(3):1195–1201.

Datta MS, Almada AA, Baumgartner MF, Mincer TJ, Tarrant AM, Polz MF. 2018. Inter-individual variability in copepod microbiomes reveals bacterial networks linked to host physiology. The ISME journal. 12(9):2103–2113.

De Corte D, Lekunberri I, Sintes E, Garcia JAL, Gonzales S, Herndl GJ. 2014. Linkage between copepods and bacteria in the North Atlantic Ocean. Aquatic Microbial Ecology. 72(3):215–225.

De Corte D, Srivastava A, Koski M, Garcia JAL, Takaki Y, Yokokawa T, Nunoura T, Elisabeth NH, Sintes E, Herndl GJ. 2018. Metagenomic insights into zooplankton-associated bacterial communities. Environmental microbiology. 20(2):492–505.

De Souza Santos G, Ibraim VRC, Silva EEC, Eskinazi-Sant'Anna EM. 2020. Interaction between *Epistylis* sp. and copepods in tropical lakes: responses of epibiont infestation to species host density. Limnologica. 84:125815.

Deinema MH, van Loosdrecht M, Scholten A. 1985. Some physiological characteristics of Acinetobacter spp. accumulating large amounts of phosphate. Water Science and Technology. 17(11-12):119–125.

Drenner, R.W. and McComas, S.R., 1980. The roles of zooplankter escape ability and fish size selectivity in the selective feeding and impact of planktivorous fish In: Kerfoot WC (ed.) Evolution and ecology of the zooplankton community.

Ebert D. 2005. Ecology epidemiology and evolution of parasitism in *Daphnia*. National Library of Medicine. 2–4.

Eckert EM, Pernthaler J. 2014. Bacterial epibionts of *Daphnia*: a potential route for the transfer of dissolved organic carbon in freshwater food webs. The ISME journal. 8:1808–1819.

Eckert EM, Anicic N, Fontaneto D. 2021. Freshwater zooplankton microbiome composition is highly flexible and strongly influenced by the environment. Molecular ecology. 30(6):1545–1558.

Ehrenberg C. 1834. Dritter Beitrag zur Erkenntniss grosser Organisation in der Richtung des kleinsten Raumes. Berlin: Konigl. Akad. d. Wiss. 1833:145–336.

Elshafie HS, Camele I. 2021. An overview of metabolic activity, beneficial and pathogenic aspects of *Burkholderia* spp. Metabolites. 11(5):321.

Erken M, Lutz C, McDougald D. 2015. Interactions of *Vibrio* spp. with zooplankton. Microbiology Spectrum. 3(10):10–1123.

Fernandez-Leborans G, Tato-Porto ML. 2000. A review of the species of protozoan epibionts on crustaceans. I. Peritrich ciliates. Crustaceana. 643–683.

Flint HJ, Bayer EA, Rincon MT, Lamed R, White BA. 2008. Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. Nature Reviews Microbiology. 6(2):121–131.

Foissner W, Berger H, Schaumburg J. 1999. Identification and ecology of limnetic plankton ciliates.

Foissner W, Blake N, Klaus WOLF, Breiner HW, Stoeck T. 2010. Morphological and molecular characterization of some peritrichs (Ciliophora: Peritrichida) from tank bromeliads, including two new genera: Orborhabdostyla and Vorticellides. Acta Protozoologica. 48(4):291.

Fong JC, De Guzman BE, Lamborg CH, Sison-Mangus MP. 2019. The mercury-tolerant microbiota of the zooplankton *Daphnia* aids in host survival and maintains fecundity under mercury stress. Environmental science and technology. 53(24):14688–14699.

Frankel-Bricker J, Song MJ, Benner MJ, Schaack S. 2020. Variation in the microbiota associated with *Daphnia magna* across genotypes, populations, and temperature. Microbial ecology. 79(3):731–742.

Freese H, Schink B. 2011. Composition and stability of the microbial community inside the digestive tract of the aquatic crustacean *Daphnia magna*. Microbial Ecology. 62:882–894.

Gao H, Liu X, Ban S. 2022. Effect of acute acidic stress on survival and metabolic activity of zooplankton from Lake Biwa Japan. Inland Waters. 12: 488–498.

Gavriilidou A, Gutleben J, Versluis D, Forgiarini F, van Passel MW, Ingham CJ, Smidt H, Sipkema D. 2020. Comparative genomic analysis of Flavobacteriaceae: Insights into carbohydrate metabolism gliding motility and secondary metabolite biosynthesis. BMC genomics. 21(1):1–21.

Genc E, Bozkurt A. 2009. First record of the epibiont protozoan *Epistylis* sp. on zooplankton (Copepoda, Cladocera, and Rotifera) from the Asi River, Turkey. Crustaceana. 82(5):515–530.

Gerdts G, Brandt P, Kreisel K, Boersma M, Schoo KL, Wichels A. 2013. The microbiome of North Sea copepods. Helgoland Marine Research. 67(4):757–773.

Gilbert JJ, Schröder T. 2003. The ciliate epibiont *Epistylis pygmaeum*: selection for zooplankton hosts, reproduction and effect on two rotifers. Freshwater Biology. 48(5): 878–893.

Giovannoni SJ, Tripp HJ, Givan S, Podar M, Vergin KL, Baptista D, Bibbs L, Eads J, Richardson TH, Noordewier M, Rappé MS. 2005. Genome streamlining in a cosmopolitan oceanic bacterium. Science. 309(5738):1242–1245.

Glenn TC, Pierson TW, Bayona-Vásquez NJ, Kieran TJ, Hoffberg SL, Thomas Iv JC, Lefever DE, Finger JW, Gao B, Bian X, Louha S. 2019. Adapterama II: universal amplicon sequencing on Illumina platforms (TaggiMatrix). PeerJ. 7:7786.

Gorokhova E, El-Shehawy R, Lehtiniemi M, Garbaras A. 2021. How copepods can eat toxins without getting sick: Gut bacteria help zooplankton to feed in cyanobacteria blooms. Frontiers in Microbiology. 11:589816.

Gowing MM, Silver MW. 1983. Origins and microenvironments of bacteria mediating fecal pellet decomposition in the sea. Marine Biology 73(1):7–16

Green J. 1974. Parasites and epibionts of Cladocera. The Transactions of the Zoological Society of London. 32(6):417–515.

Grossart HP, Simon M, Logan BE. 1997. Formation of macroscopic organic aggregates (lake snow) in a large lake: The significance of transparent exopolymer particles plankton and zooplankton. Limnology and Oceanography. 42(8):1651–1659.

Grossart HP, Simon M. 1998. Bacterial colonization and microbial decomposition of limnetic organic aggregates (lake snow). Aquatic Microbial Ecology 15(2):127–140.

Grossart HP, Dziallas C, Tang KW. 2009. Bacterial diversity associated with freshwater zooplankton. Environmental Microbiology Reports. 1(1):50–55.

Grossart HP, Dziallas C, Leunert F, Tang KW. 2010. Bacteria dispersal by hitchhiking on zooplankton. Proceedings of the National Academy of Sciences. 107(26):11959–11964.

Gurung TB, Kagami M, Yoshida T, Urabe J. 2001. Relative importance of biotic and abiotic factors affecting bacterial abundance in Lake Biwa: an empirical analysis. Limnology. 2:19–28.

Hansen B, Bech G. 1996. Bacteria associated with a marine planktonic copepod in culture. I. Bacterial genera in seawater body surface intestines and fecal pellets and succession during fecal pellet degradation. Journal of Plankton Research. 18(2):257–273.

Harris JM. 1993. The presence nature and role of gut microflora in aquatic invertebrates: a synthesis. Microbial Ecology. 25(3):195–231.

Henebry MS, Ridgeway BT. 1979. Epizoic ciliated protozoa of planktonic copepods and cladocerans and their possible use as indicators of organic water pollution. Transactions of the American Microscopical Society. 495–508.

Herman SS, Mihursky JA. 1964. Infestation of the copepod Acartia tonsa with the stalked ciliate *Zoothamnium*. Science, 146(3643):543–544.

Höhnk W, Vallin S. Epidemisches Absterben von Eurytemora im Bottnischen Meerbusen, verursacht durch Leptolegnia baltica nov. spec. 1953.

Ho JS, Perkins PS. 1985. Symbionts of marine Copepoda: an overview. Bulletin of marine science. 37(2):586–598.

Hsieh CH, Ishikawa K, Sakai Y, Ishikawa T, Ichise S, Yamamoto Y, Kuo TC, Park HD, Yamamura N, Kumagai M. 2010. Phytoplankton community reorganization driven by eutrophication and warming in Lake Biwa. Aquatic Sciences. 72:467–483.

Huber-Pestalozzi G. 1955. Euglenophyceen. Huber-Pestalozzi's Das Phytoplankton des Susswassers, Die Binnengewasser XVI.

Huq A, Small EB, West PA, Huq MI, Rahman R, Colwell RR. 1983. Ecological relationships between *Vibrio cholerae* and planktonic crustacean copepods. Applied and environmental microbiology. 45(1):275–283.

Huq A, West PA, Small EB, Huq MI, Colwell RR. 1984. Influence of water temperature salinity and pH on survival and growth of toxigenic Vibrio cholerae serovar 01 associated with live copepods in laboratory microcosms. Applied and Environmental Microbiology. 48(2):420–424.

Hwang JS, Turner JT. 1995. Behaviour of cyclopoid, harpacticoid, and calanoid copepods from coastal waters of Taiwan. Marine Ecology. 16(3):207–216.

Inoue A, Nishiyama R, Ojima T. 2016. The alginate lyases FlAlyA, FlAlyB, FlAlyC, and FlAlex from *Flavobacterium* sp. UMI-01 have distinct roles in the complete degradation of alginate. Algal research. 19:355–362.

Jones S, Carrasco NK, Perissinotto R, Vosloo A. 2016. Association of the epibiont *Epistylis* sp. with a calanoid copepod in the St Lucia Estuary, South Africa. Journal of Plankton Research. 38(6):1404–1411.

Kagami M, Gurung TB, Yoshida T, Urabe J. 2006. To sink or to be lysed? Contrasting fate of two large phytoplankton species in Lake Biwa. Limnology and oceanography. 51(6):2775–2786

Kang D, Shoaie S, Jacquiod S, Sørensen SJ, Ledesma-Amaro R. 2021. Comparative genomics analysis of keratin degrading *Chryseobacterium* species reveals their keratinolytic potential for secondary metabolite production. Microorganisms. 9(5):1042.

Kankaala P, Eloranta P. 1987. Epizooic ciliates (*Vorticella* sp.) compete for food with their host *Daphnia longispina* in a small polyhumic lake. Oecologia. 73:203–206.

Kawabata K. 1987. Abundance and distribution of *Eodiaptomus japonicus* (Copepoda: Calanoida) in Lake Biwa. Bull Plankton Soc Japan. 34:173–183.

Kawabata K. 1989. Seasonal changes in abundance and vertical distribution of *Mesocyclops thermocyclopoides*, *Cyclops vicinus* and *Daphnia longispina* in Lake Biwa. Japanese Journal of Limnology. 50:9–13

Kawabata K. 1991. Ontogenetic changes in copepod behavior: an ambush cyclopoid predator and a calanoid prey. *Journal of Plankton Research*. 13(1):27–34.

Kawabata K, Narita T, Nishino M. 2006. Predator-prey relationship between the landlocked dwarf ayu and planktonic Crustacea in Lake Biwa, Japan. Limnology. 7:199–203.

Kawanabe H, Nishino M, Maehata M. 2020. Lake Biwa: interactions between nature and people. Springer Nature.

Khal A. 1935. Artiere order protozoa I: Wimpertiere odder ciliate (Infusoria) 4. Peritricha and Chonotrica. In Dahl F (ed.), Die Tierwelt Deutschlands. Jena (Germany): G. Fischer 30:651–805

Kim C, Nishimura Y, Nagata T. 2006. Role of dissolved organic matter in hypolimnetic mineralization of carbon and nitrogen in a large monomictic lake. Limnology and Oceanography. 51(1):70–78.

King CH, Sanders RW, Shot Jr EB, Porter KG. 1991. Differential survival of bacteria ingested by zooplankton from a stratified eutrophic lake. Limnology and Oceanography. 36(5):829–844.

Koomen P, JC von Vaupel Klein. 1998. The integumental perforation pattern of the *Undeuchaeta incisa* female (Copepoda Calanoida). Journal of Marine Systems. 15:401–412.

Lentini V, Gugliandolo C, Bunk B, Overmann J, Maugeri TL. 2014. Diversity of prokaryotic community at a shallow marine hydrothermal site elucidated by Illumina sequencing technology. Current Microbiology. 69:457–466.

Lim SJ, Bordenstein SR. 2020. An introduction to phylosymbiosis. Proceedings of the Royal Society B. 287(1922):20192900.

Liu X, Beyrend-Dur D, Dur G, Ban S. 2014. Effects of temperature on life history traits of *Eodiaptomus japonicus* (Copepoda: Calanoida) from Lake Biwa (Japan). Limnology. 15(1):85–97.

Liu X, Dur G, Ban S, Sakai Y, Ohmae S, Morita T. 2020. Planktivorous fish predation masks anthropogenic disturbances on decadal trends in zooplankton biomass and body size structure in Lake Biwa Japan. Limnology and Oceanography. 65(3):667–682.

Liu X, Dur G, Ban S, Sakai Y, Ohmae S, Morita T. 2021. Quasi-decadal periodicities in growth and production of the copepod *Eodiaptomus japonicus* in Lake Biwa Japan related to the Arctic Oscillation. Limnology and Oceanography. 66(10):3783–3795.

Liu Z, Hu Y. 2001. Vertical distribution of the dominant planktonic crustaceans in a mesotrophic reservoir xujiahe reservoir (central china). Limnologica. 31(3):171–178.

Lu B, Shen Z, Zhang Q, Hu X, Warren A, Song W. 2020. Morphology and molecular analyses of four epibiotic peritrichs on crustacean and polychaete hosts, including descriptions of two new species (Ciliophora, Peritrichia). European journal of protistology. 73:125–670.

Macke E, Callens M, De Meester L, Decaestecker E. 2017a. Host-genotype dependent gut microbiota drives zooplankton tolerance to toxic cyanobacteria. Nature Communications. 8(1):1608.

Macke E, Tasiemski A, Massol F, Callens M, Decaestecker E. 2017b. Life history and eco-evolutionary dynamics in light of the gut microbiota. Oikos. 126(4):508–531.

Macke E, Callens M, Massol F, Vanoverberghe I, De Meester L, Decaestecker E. 2020. Diet and Genotype of an Aquatic Invertebrate Affect the Composition of Free-Living Microbial Communities. Frontiers in microbiology. 11:380.

Maki K, Kim C, Yoshimizu C, Tayasu I, Miyajima T, Nagata T. 2010. Autochthonous origin of semi-labile dissolved organic carbon in a large monomictic lake (Lake Biwa): carbon stable isotopic evidence. Limnology. 11(2):143–153.

Manakul P, Peerakietkhajorn S, Matsuura T, Kato Y, Watanabe H. 2017. Effects of symbiotic bacteria on chemical sensitivity of *Daphnia magna*. Mar. Environ. Res. 128:70–75.

Martin BD, Schwab E. 2012. Current usage of symbiosis and associated terminology. International Journal of Biology. 5(1):32–45.

Mayén-Estrada R, Dias RJP, Ramírez-Ballesteros M, Rossi M, Reyes-Santos M, Durán-Ramírez CA, Cruz-Jiménez G. 2021. Ciliates as Symbionts. In Plankton Communities. IntechOpen.

Møhlenberg F, Kaas H. 1990. *Colacium vesiculosum* Ehrenberg (Euglenophyceae), infestation of planktonic copepods in the western Baltic. Ophelia. 31(2):125–132.

Moisander PH, Sexton AD, Daley MC. 2015. Stable associations masked by temporal variability in the marine copepod microbiome. PLoS one. 10(9):0138967.

Møller EF, Thor P, Nielsen TG. 2003. Production of DOC by *Calanus finmarchicus*, *C. glacialis* and *C. hyperboreus* through sloppy feeding and leakage from fecal pellets. Marine Ecology Progress Series. 262:185–191.

Møller EF. 2005. Sloppy feeding in marine copepods: prey-size-dependent production of dissolved organic carbon. Journal of Plankton Research. 27(1):27–35.

Møller EF, Riemann L, Søndergaard M. 2007. Bacteria associated with copepods: abundance, activity and community composition. Aquatic Microbial Ecology. 47(1):99–106.

Mougi A, Kondoh M. 2012. Diversity of interaction types and ecological community stability. Science. 337(6092):349–351.

Nagasawa S, Simidu U, Nemoto T. 1985. Scanning electron microscopy investigation of bacterial colonization of the marine copepod Acartia clausi. Marine Biology. 87:61–66.

Nagasawa S, Terazaki M. 1987. Bacterial epibionts of the deep-sea copepod calanus-cristatus kroyer. Oceanologica acta. 10(4):475–479.

Nagasawa S. 1988. Copepod-bacteria associations in Zielony lake, Poland. Journal of plankton research. 10(3):551–554.

Nagasawa S, Nemoto T. 1988. Presence of bacteria in guts of marine crustaceans and on their fecal pellets. Journal of Plankton Research, 10(3):559–564.

Nagasawa S. 1989. Bacterial epibionts of copepods. Science Progress (1933-):169–176.

Nagasawa S. 1992. Concurrent observations on gut interior and fecal pellets of marine crustaceans. Journal of plankton research. 14(11):1625–1630.

Narita T, Okamoto K. 1984. Zooplankton. 313-321. In S. Horie (ed.), Lake Biwa. Dr. W. Junk.

Okazaki Y, Hodoki Y, Nakano SI. 2013. Seasonal dominance of CL500-11 bacterioplankton (phylum Chloroflexi) in the oxygenated hypolimnion of Lake Biwa, Japan. FEMS microbiology ecology. 83(1):82–92.

Okazaki Y, Nakano SI. 2016. Vertical partitioning of freshwater bacterioplankton community in a deep mesotrophic lake with a fully oxygenated hypolimnion (Lake Biwa Japan). Environmental Microbiology Reports. 8(5):780–788.

Okazaki Y, Nishimura Y, Yoshida T, Ogata H, Nakano Si. 2019. Genome-resolved viral and cellular metagenomes revealed potential key virus-host interactions in a deep freshwater lake. Environmental Microbiology. 21:4740–4754.

Olafsen JA. 1984. Ingestion of bacteria by cod (*Gadus morhua* L.) larvae. In The Propagation of Cod *Gadus morhus* L. An International Symposium Arendal Norway 14–17 June 1983. Institute of Marine Research.

Ortmann AC, Santos TT. 2016. Spatial and temporal patterns in the Pelagibacteraceae across an estuarine gradient. FEMS microbiology ecology. 92(9):133.

Parsons TR, Takahashi M, Hargrave B. 1977. Biological oceanographic processes 2nd edition Pergamon press Oxford.

Peerakietkhajorn S, Tsukada K, Kato Y, Matsuura T, Watanabe H. 2015. Symbiotic bacteria contribute to increasing the population size of a freshwater crustacean *Daphnia magna*. Environ. Microbiol. Rep. 7:364–372.

Peerakietkhajorn S, Kato Y, Kasalický V, Matsuura T, Watanabe H. 2016. Betaproteobacteria *Limnohabitans* strains increase fecundity in the crustacean *Daphnia magna*: symbiotic relationship between major bacterioplankton and zooplankton in a freshwater ecosystem. Environ. Microbiol. 18:2366–2374.

Peter H, Sommaruga R. 2008. An evaluation of methods to study the gut bacterial community composition of freshwater zooplankton. Journal of plankton research. 30(9):997–1006.

Pomeroy LR, Wiebe WJ. 2001. Temperature and substrates as interactive limiting factors for marine heterotrophic bacteria. Aquatic Microbial Ecology. 23(2):187–204.

Poulsen LK, Kiørboe T. 2006. Vertical flux and degradation rates of copepod fecal pellets in a zooplankton community dominated by small copepods. Marine Ecology Progress Series. 323:195–204.

Pruzzo C, Vezzulli L, Colwell RR. 2008. Global impact of *Vibrio cholerae* interactions with chitin. Environmental microbiology. 10(6):400–1410.

Qi WH, Nong G, Preston JF, Ben-Ami F, Ebert D. 2009. Comparative metagenomics of Daphnia symbionts. BMC Genomics. 10:1–21.

Redfield GW, Vincent WF. 1979. Stages of infection and ecological effects of a fungal epidemic on the eggs of a limnetic copepod. Freshwater Biology. 9(6):503–510.

Regali-Seleghim MH, Godinho MJ. 2004. Peritrich epibiont protozoans in the zooplankton of a subtropical shallow aquatic ecosystem (Monjolinho Reservoir, São Carlos, Brazil). Journal of Plankton Research. 26(5):501–508.

Rosowski JR, Kugrens P. 1973. Observations on the euglenoid Colacium with special reference to the formation and morphology of attachment material. Journal of Phycology. 9(4):370–383.

Rossiter A. 2000. Lake Biwa as a topical ancient lake. Advances in Ecological Research. 31:571–598.

Rothhaupt KO. 1997. Grazing and nutrient influences of *Daphnia* and *Eudiaptomus* on phytoplankton in laboratory microcosms. Journal of plankton research. 19(1):125–139.

Sadaiappan B, PrasannaKumar C, Nambiar VU, Subramanian M, Gauns MU. 2021. Meta-analysis cum machine learning approaches address the structure and biogeochemical potential of marine copepod associated bacteriobiomes. Scientific Reports, 11(1):3312.

Salmaso Nico, Tolotti Monica. 2009. Other phytoflagellates and groups of lesser importance. In Encyclopedia of inland waters (No. 1, pp. 174–183). Elsevier.

Samad MS, Lee HJ, Cerbin S, Meima-Franke M, Bodelier PL. 2020. Niche differentiation of host-associated pelagic microbes and their potential contribution to biogeochemical cycling in artificially warmed lakes. Frontiers in Microbiology. 11:582.

Sars GO. 1993. On the freshwater crustaceans occurring in the vicinity of Christiania translated from Norwegian by A. Brettingen. University of Bergen.197.

Shish FK, Ducklow HW. 1994. Temperature regulation of heterotrophic bacterioplankton abundance, production, and specific growth rate in Chesapeake Bay. Limnology and Oceanography. 39(6):1243–1258.

Shoemaker, K.M. and Moisander, P.H., 2015. Microbial diversity associated with copepods in the North Atlantic subtropical gyre. FEMS Microbiology Ecology. 91(7):64.

Shoemaker KM, Moisander PH. 2017. Seasonal variation in the copepod gut microbiome in the subtropical North Atlantic Ocean. Environmental microbiology. 19(8):3087–3097.

Simon M, Welschmeyer NA, Kirchman DL. 1992. Bacterial production and the sinking flux of particulate organic matter in the subarctic Pacific. Deep Sea Research Part A. Oceanographic Research Papers. 39(11-12):1997–2008.

Sison-Mangus MP, Mushegian AA, Ebert D. 2015. Water fleas require microbiota for survival growth and reproduction. The ISME journal 9(1):59–67.

Skovgaard A, Castro-Mejia JL, Hansen LH, Nielsen DS. 2015. Host-specific and pH-dependent microbiomes of copepods in an extensive rearing system. PLoS One. 10(7):0132516.

Sochard MR, Wilson DF, Austin B, Colwell RR. 1979. Bacteria associated with the surface and gut of marine copepods. Applied and Environmental Microbiology. 37(4):750–759.

Sprague V, Couch J. 1971. An annotated list of protozoan parasites, hyperparasites, and commensals of decapod Crustacea. The Journal of Protozoology. 18(3):526–537.

Staley C, Unno T, Gould TJ, Jarvis B, Phillips J, Cotner JB, Sadowsky MJ. 2013. Application of Illumina next-generation sequencing to characterize the bacterial community of the Upper Mississippi River. Journal of Applied Microbiology. 115(5):1147–1158.

Stich HB, Lampert W. 1981. Predator evasion as an explanation of diurnal vertical migration by zooplankton. Nature. 293:396–398.

Tabata R, Kakioka R, Tominaga K, Komiya T, Watanabe K. 2016. Phylogeny and historical demography of endemic fishes in Lake Biwa: the ancient lake as a promoter of evolution and diversification of freshwater fishes in western Japan. Ecology and Evolution. 6(8):2601–2623.

Tanaka D, Fujiyoshi S, Maruyama F, Goto M, Koyama S, Kanatani JI, Isobe J, Watahiki M, Sakatoku A, Kagaya S, Nakamura S. 2020. Size resolved characteristics of urban and suburban bacterial bioaerosols in Japan as assessed by 16S rRNA amplicon sequencing. Scientific Reports. 10(1):12406.

Tang KW, Visscher PT, Dam HG. 2001. DMSP-consuming bacteria associated with the calanoid copepod *Acartia tonsa* (Dana). Journal of experimental marine biology and ecology. 256(2):185–198.

Tang KW. 2005. Copepods as microbial hotspots in the ocean: effects of host feeding activities on attached bacteria. Aquatic Microbial Ecology. 38(1):31–40.

Tang KW, Bickel SL, Dziallas C, Grossart HP. 2009. Microbial activities accompanying decomposition of cladoceran and copepod carcasses under different environmental conditions. Aquatic Microbial Ecology. 57(1):89–100.

Tang KW, Turk V, Grossart HP. 2010. Linkage between crustacean zooplankton and aquatic bacteria. Aquat Microb Ecol. 61:261–277.

Tang KW, Glud RN, Glud A, Rysgaard S, Nielsen TG. 2011. Copepod guts as biogeochemical hotspots in the sea: evidence from microelectrode profiling of *Calanus* spp. Limnology and Oceanography. 56(2):666–672.

Tezuka Y. 1982. Seasonal variation of dominant phytoplankton, chlorophyll a and nutrient levels in nearshore waters of the south basin of Lake Biwa. Japanese Journal of Limnology (Rikusuigaku Zasshi). 43(3):215–220.

Thébault E, Fontaine C. 2010. Stability of ecological communities and the architecture of mutualistic and trophic networks. Science. 329:853–856.

Threlkeld ST, Willey RL. 1993. Colonization, interaction, and organization of cladoceran epibiont communities1. Limnology and Oceanography. 38(3):584–591.

Threlkeld ST, Chiavelli DA, Willey RL. 1993. The organization of zooplankton epibiont communities. Trends in Ecology and Evolution. 8(9):317–321.

Tran DM, Sugimoto H, Nguyen DA, Watanabe T, Suzuki K. 2018. Identification and characterization of chitinolytic bacteria isolated from a freshwater lake. Bioscience biotechnology and biochemistry 82(2):343–355.

Turner JT, Postek MT, Collard SB. 1979. Infestation of the estuarine copepod Acartia tonsa with the ciliate Epistylis. Transactions of the American Microscopical Society. 136–138.

Uttieri M, Sandulli R, Spezie G, Zambianchi E. 2014. From small to large scale: a review of the swimming behaviour of *Daphnia*. *Daphnia:* Biology and mathematics perspectives. Nova Science Publishers Inc, New York. 309–322.

Urabe J, Ishida S, Nishimoto M, Weider LJ. 2003. *Daphnia pulicaria*, a zooplankton species that suddenly appeared in 1999 in the offshore zone of Lake Biwa. Limnology. 4:0035–0041.

Utz LR, Coats DW. 2005. Spatial and temporal patterns in the occurrence of peritrich ciliates as epibionts on calanoid copepods in the Chesapeake Bay, USA. Journal of Eukaryotic Microbiology. 52(3):236–244.

Vallin S. 1951. Plankton mortality in the northern Baltic caused by a parasitic water-mould. Rept. Inst. Freshwater Res., Drottningholm. 32:139–148.

Vavra J. 1963. Studies on symphorious Ciliata Peritrichida. I. *Epistylis anastatica* (L.). Acta Soc. zool. Bohemoslov. 4:243.

Vazquez L, Alpuche J, Maldonado G, Agundis C, Pereyra-Morales A, Zenteno E. 2009. Immunity mechanisms in crustaceans. Innate immunity. 15(3):179–188.

Velasquez X, Morov AR, Belkin N, Kurt TT, Rubin-Blum M, Tchernov D, Meron D, Guy-Haim T. 2022. Feeding strategy and dietary preference shape the microbiome of epipelagic copepods in a warm nutrient-impoverished ecosystem. Environmental DNA. 5(1):38–55.

Verschuere L, Heang H, Criel G, Sorgeloos P, Verstraete W. 2000. Selected bacterial strains protect *Artemia* spp. from the pathogenic effects of *Vibrio proteolyticus* CW8T2. Applied Environmental Microbiology. 66:1139–1146.

Visse M. 2007. Detrimental effect of peritrich ciliates (*Epistylis* sp.) as epibionts on the survival of the copepod *Acartia bifilosa*. Proc Estonian Acad Sci Biol Ecol. 56(3):173–178.

Wäge J, Strassert JF, Landsberger A, Loick-Wilde N, Schmale O, Stawiarski B, Kreikemeyer B, Michel G, Labrenz M. 2019. Microcapillary sampling of Baltic Sea copepod gut microbiomes indicates high variability among individuals and the potential for methane production. FEMS Microbiology Ecology 95(4): 024.

Waggoner B, Newman T, Tarbutton T. 2016. Quick, Safe, and Simple Silver Staining for Ciliates and Other Protists. Proceedings of the Association for Biology Laboratory Education. 37:63.

Wahl M. 1989. Marine epibiosis. I. Fouling and antifouling: some basic aspects. Marine ecology progress series. 175–189.

Wahl M, Hay ME, Enderlein P. 1997. Effects of epibiosis on consumer-prey interactions. In Interactions and Adaptation Strategies of Marine Organisms: Proceedings of the 31st European Marine Biology Symposium, held in St. Petersburg, Russia, 9–13 September 1996 (pp. 49–59). Springer Netherlands.

Wahl, M., 2008. Ecological lever and interface ecology: epibiosis modulates the interactions between host and environment. Biofouling. 24(6):427–438.

Wahl M, Goecke F, Labes A, Dobretsov S, Weinberger F. 2012. The second skin: ecological role of epibiotic biofilms on marine organisms. Front. Microbial. 3:292.

Wang Qianhong, Zheng Hao, Ruirui Ding, Huabing Li, Xiangming Tang, Feizhou Chen. 2021. Host Dependence of Zooplankton-Associated Microbes and Their Ecological Implications in Freshwater Lakes. *Water*. 13(21):2949.

Wells J, Varel V. 2011. Chapter 9: Symbiosis of plants animals and microbes. In: Pond W.G. Bazer F.W. and Rollin B.E. (ed). Animal Welfare in Animal Agriculture: Husbandry Stewardship and Sustainability. New York: CRC Press.185–203.

Willey RL, Cantrell PA, Threlkeld ST. 1990. Epibiotic euglenoid flagellates increase the susceptibility of some zooplankton to fish predation. Limnology and Oceanography. 35(4):952–959.

Willey RL, Threlkeld ST. 1993. Organization of crustacean epizoan communities in a chain of subalpine ponds. Limnology and oceanography. 38(3):623–627.

Woo PC, Lau SK, Teng JL, Tse H, Yuen KY. 2008. Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. Clinical Microbiology and Infection. 14(10):908–934.

Wu S, Wang G, Angert ER, Wang W, Li W, Zou H. 2012. Composition, diversity, and origin of the bacterial community in grass carp intestine. PloS one. 7(2):30440.

Xie P, Sanderson B, Frost T, Magnuson JJ. 2001. Manipulation of host density affects infestation of a peritrich ciliate (*Epistylis lacustris*) on a calanoid copepod (*Leptodiaptomus minutus*) in Crystal Lake, Wisconsin, USA. Journal of Freshwater Ecology. 16(4):557–564.

Yeh HD, Questel JM, Maas KR, Bucklin A. 2020. Metabarcoding analysis of regional variation in gut contents of the copepod *Calanus finmarchicus* in the North Atlantic Ocean. Deep Sea Research Part II: Topical Studies in Oceanography. 180:104738.

Yoshida T, Gurung T, Kagami M, Urabe J. 2001. Contrasting effects of a cladoceran (*Daphnia galeata*) and a calanoid copepod (*Eodiaptomus japonicus*) on algal and microbial plankton in a Japanese lake Lake Biwa. Oecologia. 129(4):602–610.

Yu C, Lee AM, Bassler BL, Roseman S. 1991. Chitin utilization by marine bacteria. A physiological function for bacterial adhesion to immobilized carbohydrates. Journal of Biological Chemistry. 266(36):24260–24267.

Zalocar Y, Frutos SM, Casco SL, Forastier ME, Vallejos SV. 2011. Prevalence of *Colacium vesiculosum* (Colaciales: Euglenophyceae) on planktonic crustaceans in a subtropical shallow lake of Argentina. Revista de Biología Tropical. 59(3):1295–1306.

Zeng Y, Kasalický V, Šimek K, Koblížek M. 2012. Genome sequences of two freshwater betaproteobacterial isolates, Limnohabitans species strains Rim28 and Rim47, indicate their capabilities as both photoautotrophs and ammonia oxidizers. Journal of Bacteriology. 194(22):6302–6303.

APPENDIX

Appendix 1 Dominant bacterial symbionts associated with zooplankton from freshwater environment and laboratory culture.

	Host-zooplankton taxa	Surrounding environment	Sample type	Dominant bacterial family	Methods/ platform	Reference
***Daphnia* spp.**	*D. pulicaria*	Freshwater lake	Whole body	Comamonadaceae, Flavobacteriaceae, Aeromonadaceae, Moraxellaceae	16S rRNA; Illumina MiSeq	Present study
	D. galeata	Freshwater lake / pond	Whole body	Comamonadaceae, Flavobacteriaceae, Aeromonadaceae, Moraxellaceae, Neisseriaceae	16S rRNA; Illumina MiSeq	Eckert et al. 2021
	D. obtusa			Comamonadaceae, Flavobacteriaceae, Aeromonadaceae, Moraxellaceae, Pseudomonadaceae, Burkholderiaceae		
	D. magna	Laboratory culture	Whole body	Comamonadaceae, Flavobacteriaceae, Rhodobacteraceae, Burkholderiaceae, Enterobacteriaceae	16S rRNA; 454 pyrosequencing (GS 20 and GS FLX)	Qi et al. 2009
	D. pulicaria			Comamonadaceae, Flavobacteriaceae, Aeromonadaceae, Pseudomonadaceae, Methylobacteriaceae, Burkholderiaceae		
	D. pulex			Comamonadaceae, Flavobacteriaceae, Pseudomonadaceae, Enterobacteriaceae, Methylobacteriaceae		
	D. magna	Laboratory culture	Whole body	Comamonadaceae, Flavobacteriaceae, Oxalobacteraceae	16S rRNA	Peerakiethajorn et al. 2015
	D. magna	Laboratory culture	Whole body	Comamonadaceae, Flavobacteriaceae, Aeromonadaceae, Spirosomaceae	16S rDNA; 454 pyrosequencing	Callens et al. 2016

Calanoid copepods	*E. japonicus*	Freshwater lake	Whole body	Aeromonadaceae, Oxalobacteraceae, Comamonadaceae, Moraxellaceae, Neisseriaceae, Weeksellaceae	16S rRNA; Illumina MiSeq	Present study
	Sinocalanus dorrii, Sinodiaptomus sarsi	Freshwater lake	Whole body	Pseudomonadaceae, Bacillaceae, Moraxellaceae	16S rRNA; Illumina Nova 6000	Wang et al. 2021
	Eudiaptomus padanus, Calanoida spp.	Freshwater lake	Whole body	Comamonadaceae, Pseudomonadaceae, Flavobacteriaceae, Neisseriaceae, Oxalobacteraceae	16S rRNA; Illumina MiSeq	Eckert et al. 2021
Cyclopoid copepods	Cyclopoida spp.	Freshwater lake	Whole body	Aeromonadaceae, Comamonadaceae, Rhodobacteraceae	16S rRNA; Illumina MiSeq	Present study
	Thermocyclops oithonoides	Freshwater lake	Whole body	Comamonadaceae, Flavobacteriaceae, Neisseriaceae, Oxalobacteraceae, Microbacteriaceae, Flexibacteriaceae	DGGE analysis & 16S rRNA	Grossert et al. 2009
	Mesocyclops leuckarti	Freshwater lake	Whole body	Comamonadaceae, Flavobacteriaceae, Pseudomonadaceae	16S rRNA; Illumina MiSeq	Eckert et al. 2021