# *Daphnia*-associated bacterial communities are influenced by infection status, environmental conditions and diet quantity across natural epidemics

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## Keywords

*Daphnia*, microbiome, wild, infection, long-term

# Abstract

Microbiomes associated with zooplankton play an important role for host health and contribute to ecosystem functions, such as nutrient cycling. Yet, few studies have assessed how environmental gradients and biotic interactions, such as parasitism and diet, shape the microbiome composition of wild zooplankton. Here, we used 16S rRNA gene sequencing and a long-term field dataset to investigate the microbiomes of water fleas belonging to the *Daphnia longispina* species complex, across six sampling events spanning 13 years. Sampling coincided with epidemics of the virulent eukaryotic gut parasite *Caullerya mesnili*. We further surveyed phytoplankton and zooplankton communities in the lake to explore associations between *Daphnia* microbiome composition and diet (phytoplankton density), as well as zooplankton density and community composition. We observed temporal variation and infection-dependent shifts in *Daphnia* microbiome composition. Importantly, host microbiome compositions co-varied with infection prevalence, indicating a correlation between host microbiome composition and epidemic size. Furthermore, environmental factors including temperature, dissolved oxygen and cyanobacterial abundance, previously found to drive *Caullerya* epidemics, were associated with distinct host microbiome compositions. Dominant bacterial taxa in the *Daphnia* microbiome correlated with *Daphnia* density, whereas the phylogenetic composition of rare bacterial taxa correlated with total zooplankton density. Lastly, *Daphnia* microbiome composition correlated with the density of green algae, protists and cyanobacteria in lake water, but not with their community composition. Our findings show the dynamic nature of *Daphnia*-associated microbiomes and suggest potential mechanisms through which they may mediate host disease dynamics, including associations with temperature, host density and diet quantity.

# Introduction

Zooplankton constitute essential component of aquatic ecosystems, serving as both consumers and prey. Their interactions with predators, phytoplankton and parasites can shape aquatic ecosystem functioning (Bakhtiyar et al. 2020). Among these interactions, parasites represent an often overlooked but critical functional group that can directly influence ecosystem level processes; either by the generation of biomass for consumption or indirectly by altering the population density and traits of their hosts (Preston et al. 2016). For instance, zooplankton-parasite associations have been shown to influence nutrient cycling within aquatic ecosystems (Cáceres et al. 2014; Frost et al. 2008).

Zooplankton harbour symbiotic microbes, which are orders of magnitude more abundant than their free-living counterparts (Tang 2005). For instance, copepods which are abundant species of zooplankton, selectively enrich specific bacterial taxa from their environment, which then disperse back into the water, shaping bacterioplankton community composition (Shoemaker et al. 2019). Bacterial epibionts associated with the water flea *Daphnia* contribute to nutrient cycling and the transfer of dissolved organic carbon in the pelagic food web (Eckert & Pernthaler 2014). Zooplankton-associated bacteria also aid in the digestion of toxic cyanobacteria by their host (Gorokhova et al. 2021), serving as a mechanism of adaptation to toxic diets (Macke et al. 2017). Thus, the symbiotic relationships between zooplankton and bacteria can exert cascading effects on ecosystem health and functioning, underscoring the importance of characterizing these relationships and assessing which abiotic conditions or biotic interactions, such as diet and parasitism, shape the diversity and community composition of symbionts.

Host-associated microbes shape the health and performance of various aquatic hosts (Infante-Vallamil et al. 2022) and are thus proposed to constitute the fourth vertex of a disease pyramid (Bernardo-Cravo et al. 2020). However, unravelling the forces influencing host-associated microbiomes can be challenging in natural settings, where they show temporal variability and are influenced by a complex interplay of deterministic (e.g. abiotic environmental gradients, biotic interactions of host species) and stochastic (e.g. direct horizontal transmission) processes (Sze et al. 2020). First, the composition of wild host microbiomes can vary across multiple temporal scales, spanning from hours to days within a single host generation (Aivelo, Laakkonen et al. 2016, Parris, Morgan et al. 2019, Caughman, Pratte et al. 2021) to longer

timescales of months (Krajacich, Huestis et al. 2018, Pierce and Ward 2019). The relatively long-term temporal variation in host microbiomes may correlate with seasonal environmental changes (Sun, Wang et al. 2016, Sharp, Pratte et al. 2017, Zhu 2022) or occur independently of seasonal patterns (Epstein, Smith et al. 2019). Second, environmental gradients such as temperature and dissolved oxygen shape the composition, diversity and function of aquatic host-associated microbiomes (reviewed in Sehnal et al. 2021). While diet composition may predict the composition of host-associated microbiota in a wide range of fishes (Escalas et al. 2021), this does not hold true for other aquatic species such as fire salamanders (Wang et al. 2021) or marine zooplankton (Xu et al. 2024). Lastly, horizontal transmission of host-associated microbes is a particularly prevalent process shaping host-microbe associations in aquatic habitats (Russell 2019). This mode of transmission is predicted to vary by the population density of hosts, as suggested by the metacommunity model of microbiome transmission (Miller et al. 2018).

Few studies have investigated changes in wild host microbiomes over time while simultaneously correlating these changes with infection by a natural parasite or pathogen. For instance, the microbiome of reef-building coral *Montastraea faveolata* undergoes strong abundance shifts in response to seasonal variation and the onset of Yellow Band Disease (Kimes, Johnson et al. 2013). In the fish host *Scomber japonicus*, both the microbiome composition and abundance of a pathogen *Photobacterium damselae* correlated with the summer season (Minich, Petrus et al. 2020). Moreover, worldwide amphibian declines attributed to the disease chytridiomycosis are linked with seasonally-induced changes in amphibian skin microbiota (Bernardo-Cravo, Schmeller et al. 2020, Schmeller, Cheng et al. 2022). Infection by the fungal parasite *Batrachochytrium dendrobatidis* in the frog *Philoria loveridgei* correlated with temporal fluctuations in the host's skin microbiome and specific bacterial taxa co-vary with the intensity of pathogen infection (Familiar Lopez, Rebollar et al. 2017). Thus, temporal variation in host microbiomes may influence host-parasite dynamics in natural populations and vice versa (Stencel 2021).

The water flea *Daphnia* is a globally prevalent zooplankton species known for its influence on phytoplankton communities (Hebert et al. 2016) and bacterioplankton abundance (De Corte et al. 2023) as well as community composition (Degans et al. 2002). *Daphnia* require their associated bacteria for survival and reproduction (Sison-Mangus et al. 2015), and their abundance in the aquatic environments is shaped by the presence of specific bacteria such as *Limnohabitans sp* in their microbiome (Peerakietkhajorn et al. 2015).

The microbiomes of *Daphnia* exhibit variability across a range of timescales: including diurnal feeding rates (Pfenning-Butterworth, Cooper et al. 2022) and host age in laboratory conditions (Callens, Watanabe et al. 2018), as well as seasonal variations in wild populations (Hegg, Radersma et al. 2021). Moreover, *Daphnia* populations are subjected to significant selective pressures from various microparasites, which can regulate their population densities (Ebert 2005), and subsequently, their symbiotic associations. For instance, *Daphnia* infected with the virulent eukaryotic gut parasite *Caullerya mesnili* (hereafter referred to as *Caullerya*) have experienced reduced diversity and altered community composition of their associated bacteria (Rajarajan et al. 2022b).

Despite advancements in understanding the factors shaping host-symbiont as well as host-parasite associations, few studies characterize the relative strength of these various effects on wild zooplankton communities, or assessed their potential role in long-term host disease dynamics. Here, we investigate variation in bacterial communities within a *Daphnia* population in Lake Greifensee, across six natural epidemics of *Daphnia* population with the *Caullerya* parasite, spanning a period of 13 years. We further explore correlations between *Daphnia* bacterial community composition and *Caullerya* infection prevalence, host density and physicochemical conditions (temperature, dissolved oxygen and abundance of cyanobacteria in lake water), which have been previously reported as drivers of *Caullerya* epidemics in this lake (Tellenbach, Tardent et al. 2016). Additionally, we test if the community composition of *Daphnia*-associated bacteria correlates with the community composition and density of dominant pelagic phytoplankton (cyanobacteria, green algae, protists, gold algae and diatoms), and zooplankton (copepods, cladocerans, rotifers). We hypothesize that (a) beta diversity (bacterial community composition) and dominant bacterial orders in *Daphnia* would vary both across years and by *Daphnia* infection status; (b) environmental gradients and biotic factors (specifically temperature, dissolved oxygen and cyanobacterial abundance), known to drive *Caullerya* epidemics, would also correlate with *Daphnia* bacterial communities and (c) diet (i.e. phytoplankton community composition in the lake) would predict *Daphnia* microbiome composition.

# Material & Methods

***Daphnia* sampling and processing:** *Daphnia* were collected from 0-30m depths of Lake Greifensee in Switzerland (N 47°20′41″, E 8°40′21″) using vertical towing with a 150µm zooplankton net at three sampling stations on the lake. Zooplankton samples were pooled into a single 10L canister half-filled with surface lake water. Samples were collected during the peaks of natural *Caullerya* epidemics on six occasions, spanning a period of 13 years (10/10/2007, 11/08/2011, 29/08/2013, 04/09/2014, 24/08/2017, 10/11/2020), as a part of a long-term *Daphnia* infection study (Turko, Tellenbach et al. 2018). These sampling occasions are hereafter referred to as "years", given that each zooplankton sample was taken in a different calendar year. Zooplankton samples were collected around the peak of each natural epidemic (Fig. S1). Upon collection, zooplankton samples were sieved through a ~250µm mesh and adult *Daphnia* specimens (>1mm in size, independent of infection status), belonging to the *Daphnia longispina* species complex, were gently lifted by the antennae and screened for the presence of *Caullerya* spores in the gut under 50× magnification (Lohr, Laforsch et al. 2010). Individuals containing visible *Caullerya* spores were classified as 'infected', whereas those without visible spore clusters were categorized as 'uninfected'. *Daphnia* specimens with resting eggs (ephippia), other infections or rare co-infections involving *Caullerya* and another parasite were excluded from the study. Individual *Daphnia* were then transferred to wells containing 20µL autoclaved MilliQ water and subsequently stored at -80°C until further processing.

**Bacterial community profiling of *Daphnia*:** DNA was extracted from individual *Daphnia* specimens (whole body) using the HotSHOT method (Montero-Pau, Gomez et al. 2008). Briefly, all samples were incubated in an alkaline lysis solution at 95°C for 30 min, transferred to ice for 3-4 min and eluted in a neutralizing solution. DNA extraction was carried out in three blocks (Table S1). Three DNA extraction negative controls containing only extraction reagents were added in the third DNA extraction block. Samples were then placed at -20°C until further processing. Subsequent PCR steps were carried out simultaneously for all samples. Bacterial DNA (V4-V5 region of the 16S gene) was amplified using barcoded universal 16S primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') (Caporaso, Lauber et al. 2011) and m806R (5'-GGACTACNVGGGTWTCTAAT-3') (Apprill, McNally et al. 2015) under the following cycling conditions: initial denaturation at 95°C – 3 min; (98°C – 20 s, 52°C – 15 s, 72°C – 15 s) 33 cycles; followed by a final extension at 72°C for 5 min. This PCR step was done with three technical replicates for each sample, and 10

PCR no-template negative controls were added to the workflow. Technical replicates were pooled before the next PCR step. For the second PCR, indexes from the Illumina Nextera XT V2 Library Prep Kit were then added to the samples under the conditions: initial denaturation at 95°C – 3 min; (95°C – 30 s, 55°C – 30 s, 72°C – 30 s) 10 cycles; followed by a final extension at 72°C for 5 min. The location of DNA samples (including a total of 13 negative controls, i.e. 3 "blank" DNA extraction and 10 PCR no template samples) in the 384-well plate was randomized during the entire workflow, though technical replicates in the first PCR step were in adjacent wells. Indexed PCR products were then purified using magnetic Agencourt AMPure XP beads (Cat #A63881). Samples were quantified using a Qubit HS Assay, normalized and pooled into a 1.2nM library for amplicon sequencing. Paired-end sequencing was carried out using MiSeq – 600 cycles (PE300) v3 run kit with 10% PhiX. See "Pre-processing of reads" and Table S1 for the final sample size of *Daphnia* analysed for each year and infection status in the study.

**Pre-processing of reads:** Sequencing resulted in a total of 7.6M paired-end reads, after exclusion of one infected sample from 2014 that had an unusually high number of reads (~720,000 as opposed to the average of 46229.93 reads in biological samples). Initial pre-processing steps were performed on the Euler computing cluster at ETH Zürich, as previously described (Rajarajan, Wolinska et al. 2022). Raw reads were 5'-end trimmed, merged, and quality filtered. Amplicons were clustered using UPARSE2 (Edgar 2013), denoised into Zero-radius Operational Taxonomic Units (ZOTUs) using UNOISE3 (Edgar 2016), further clustered based on 97% sequence similarity and annotated using non-Bayesian SINTAX classifier v.138 (Edgar 2016) and the Silva (v138) database (Quast, Pruesse et al. 2013). Biological samples had 320 to 237,358 reads and negative controls had 412 to 2188 reads, with one PCR no-template control unexpectedly showing 24,356 reads. Based on this distribution of reads in biological samples versus negative controls, biological samples with <3000 reads were excluded from downstream analyses. This resulted in a final sample size of 9-16 *Daphnia* individuals for each year and infection status and 71 uninfected and 90 infected *Daphnia* across all years (Table S1).

Subsequent filtering steps were performed in R v4.0.2 using the package phyloseq (McMurdie and Holmes 2013). 62 ZOTUs (21 of unidentified phyla, 18 chloroplasts, 6 mitochondria, 16 singletons and 1 eukaryote) were filtered from the dataset. A further 42 ZOTUs were removed when samples were rarefied to an even depth of 3017 reads using vegan::*rarefy_even_depth*, resulting in 423 ZOTUs in the final dataset. There was

no consistent increase or decrease in ZOTU richness from older to newer samples, suggesting that long-term freezing of samples may not impact observed microbiomes in this study (Fig. S2, see also (Tap et al. 2019)).

**Environmental data for Lake Greifensee:** Sampling dates for physicochemical, zooplankton and phytoplankton data are listed in Table S1. Depth-resolved physico-chemical data on Lake Greifensee were obtained from the Office of Waste, Water, Energy and Air of the Canton of Zürich (AWEL, http://www.awel.zh.ch/). Temperature and dissolved oxygen measurements were averaged for the top 15m of the lake. Phytoplankton were sampled from 0-20m depth in the lake with a Schröder bottle. Phytoplankton (including cyanobacterial) cell counts in lake water were measured using the Utermöhl method (Utermöhl 1958) using sedimentation chambers and inverted light microscopy. Zooplankton samples were collected with three vertical tows of a 95µm plankton mesh from the same locations as *Daphnia* samples and fixed in 100% ethanol. *Caullerya* infection rates were assessed by visually screening 200 *Daphnia* adults for the presence of *Caullerya* spores in their gut (see above).

**Statistical analyses:** Statistical analyses were done in R v4.0.2 using the phyloseq package (McMurdie & Holmes 2013). Variation in beta diversity (Unifrac distance and Bray-Curtis Dissimilarity) by year and infection status was tested with two-way PERMANOVAs (9999 perm) using vegan::*adonis.* Then, the amount of variation explained by year and infection status was measured with a db-RDA using the function vegan::*capscale.* Given the substantial variation in bacterial communities uncovered between years (see Results), subsequent tests for variation by infection status were carried out separately for each year. Next, relative abundances of dominant bacterial orders were compared by infection status. Dominant orders were defined as those with an abundance of >1% of the dataset or present in >50% of all samples. Orders that did not meet these criteria were lumped into the category "Other". Differential abundance of orders was tested using the DESeq2 package; *p*-values were corrected for multiple comparisons using the "fdr" method (Love, Huber et al. 2014). Next, specific ZOTUs indicative of infection status were identified using Indicspecies::*signassoc* (De Caceres and Legendre 2009), 9999 perm and corrected for multiple comparisons using the Sidak method. Beta diversity in *Daphnia* bacterial communities were compared with population infection rates as well as the environmental variables temperature, dissolved oxygen and cyanobacterial cell counts using vegan::*envfit* and *p*-values were adjusted for multiple comparisons with the Bonferroni method. To test if phytoplankton or zooplankton community composition in lake water associates with *Daphnia*

bacterial communities, Jaccard dissimilarity matrix of *Daphnia* bacterial communities were modelled as a function of phytoplankton communities and correlated in a Mantel test.

# Results

**Bacterial community composition (beta diversity) differs by year and *Caullerya* infection status**

We compared variation in bacterial community composition (Bray-Curtis Dissimilarity and Unifrac distance), considering year and infection status as factors. In general, most variation in beta diversity was explained by the year (about 30%, Table 1), and comparatively little by infection status (about 1%, Table 1). The phylogenetic composition of *Daphnia*-associated bacterial communities, as assessed by Unifrac distance that includes phylogenetic relatedness between ZOTUs, changed significantly both with year and infection status (Fig. 1A, Table 1). Bray-Curtis Dissimilarity which weighs bacterial taxa by their relative abundances, varied significantly by year, and marginally by infection status. Neither beta diversity metric varied by the interaction of year and infection status.

**Relative abundances of dominant bacterial orders differ between *Caullerya*-infected and uninfected *Daphnia***

Bacterial communities of individual *Daphnia* across years comprised of 16 dominant orders; there was substantial variation among individual *Daphnia* sampled within the same year (Fig. 2). Overall, Burkholderiales was the most abundant bacterial order across all *Daphnia* individuals (51.29 ± 24.94%, mean ± SD), followed by Rickettsiales (15.1 ± 26.22%) and Flavobacteriales (14.58 ± 12.73%). Differential relative abundances of dominant bacterial orders between *Caullerya*-infected and uninfected *Daphnia* were observed primarily in epidemic years 2007, 2013 and 2017. Specifically, in 2007, Caulobacterales was more abundant in uninfected *Daphnia* (2.94 ± 2.36%) compared to infected ones (0.96 ± 0.7%). Chitinophagales was more abundant in infected *Daphnia* in both 2007 (1.34 ± 1.22%) and 2013 (2.51 ± 3.97%), compared to uninfected *Daphnia* in the respective years (0.45 ± 0.46% and 1.5 ± 2.9%). Rickettsiales was more abundant in infected *Daphnia* (5.18 ± 10.28%) compared to uninfected *Daphnia* (2.12 ± 4.7%), in 2013. In contrast, Aeromonadales was more abundant in uninfected *Daphnia* (4.64 ± 13.25%) than infected ones (1.66 ± 3.22%), while Pirellulales was more abundant in infected *Daphnia* (0.38 ± 0.59%) compared to uninfected ones (0.07 ± 0.12%), in 2017 (Table 2).

### ***Daphnia* bacterial community composition correlates with environmental conditions and plankton density**

Both Bray-Curtis dissimilarity and Unifrac distance of *Daphnia* bacterial communities correlated significantly with temperature, dissolved oxygen content and *Caullerya* infection prevalence. Moreover, both Bray-Curtis dissimilarity and Unifrac distance of *Daphnia* bacterial communities correlated significantly with *Daphnia* density, as well as the density of several phytoplankton and microbial groups in the lake water, including cyanobacteria, green algae and protists (Fig 1, Table 3). In contrast, only Unifrac distance of *Daphnia* bacterial communities correlated with total zooplankton density and gold algal density in the lake water.

### ***Daphnia* bacterial community composition does not correlate with diet (phytoplankton) or zooplankton community composition**

Jaccard dissimilarity matrices of *Daphnia* bacterial communities did not correlate with those of phytoplankton, which included green algae, gold algae, protists, cyanobacteria or diatoms ($p > 0.05$, Mantel test). Similarly, *Daphnia* bacterial community composition did not correlate with the composition of pelagic zooplankton included copepods, rotifers and *Daphnia* (Table 4).

# Discussion

We compared the bacterial community composition of wild *Caullerya*-infected and uninfected *Daphnia* individuals collected across six natural epidemics spanning a remarkable 13-year period. To our knowledge, this is the first survey of aquatic wild animal microbiomes over such an extensive timeframe. We found that bacterial community composition of wild *Daphnia* varies by year, both in terms of relative abundances of dominant taxa and their phylogenetic composition. Furthermore, we observed differences in the phylogenetic composition of rare taxa (Unifrac distance) between infected and uninfected *Daphnia*, whereas variations in dominant taxa were only marginal. Certain dominant bacterial orders, such as Chitinophagales, varied in their average relative abundances with infection status. However, this variation displayed temporal inconsistency. Specifically, changes in any dominant bacterial order associated with infection status were evident in only three of the six surveyed epidemics (2007, 2013 and 2017), with the specific bacterial orders and ZOTUs influenced by infection status being different each year. The specific environmental conditions

known to drive *Caullerya* disease epidemics i.e. cyanobacterial abundance in lake water and water temperature in the preceding month, as well as *Caullerya* infection prevalence, correlated significantly with *Daphnia* microbiome compositions. Thus, we report the first correlation between aquatic host microbiomes and epidemic size in a long-term, natural setting. Our results align with previous studies conducted in other animal species, such as the frog *Philoria loveridgei* (Familiar Lopez, Rebollar et al. 2017) and the sponge *Porites astreoides* (Meyer, Paul et al. 2014), which also showed a generally strong temporal variation in host microbiomes, with the specific taxa associated with infected hosts varying from year to year. Lastly, contrary to our hypothesis, diet (phytoplankton community) composition did not predict microbiome compositions in *Daphnia*.

*Caullerya* epidemics are associated with temporal changes in the population genetic structure of *Daphnia* (Schoebel, Wolinska et al. 2010, Turko, Tellenbach et al. 2018). Additionally, *Daphnia* microbiomes are known to be influenced by a host genetic component (Frankel-Bricker, Song et al. 2020, Macke, Callens et al. 2020, Rajarajan, Wolinska et al. 2022). In the present study, we show the dynamic nature of host microbiome alterations associated with infection status. For instance, the order Rickettsiales was enriched in infected *Daphnia* in 2013, but this was not true across the other five years surveyed, including 2020. In contrast, we previously noted a putative increase in abundance of Rickettsiales in uninfected compared to *Caullerya*-infected *Daphnia* among animals collected during a *Caullerya* epidemic in 2020 (Rajarajan, Wolinska et al. 2022). Specifically, we observed an enrichment of the orders Enterobacteriales and Pseudomonadales in infected *Daphnia* collected from December 2020. However, no differences in dominant bacterial orders by infection status were detected in the present study among *Daphnia* sampled in November 2020 (see Fig. S1). Thus, our findings suggest that *Daphnia* microbiome compositions may shift over the course of single epidemic. We speculate that these shifts in individual microbiome compositions over time may occur partially due to host clonal turnover during an epidemic (Turko et al. 2018).

*Caullerya* disease incidence is driven by temperature and cyanobacterial abundance in lake water (Tellenbach et al. 2016). Specifically, low temperatures have been associated with higher infection prevalence in experimental settings (Schoebel et al. 2010). Natural epidemics are driven by cyanobacterial biomass in lake water, along with high temperatures in the preceding month (Tellenbach et al. 2016). Moreover, *Daphnia* are more susceptible to *Caullerya* infection when fed a diet consisting of cyanobacteria

(regardless of toxicity), as confirmed with a laboratory experiment (Tellenbach et al. 2016). *Daphnia* microbiome compositions are also shaped by temperature (Sullam, Pichon et al. 2018, Frankel-Bricker, Song et al. 2020) as well as by cyanobacterial diets (Akbar et al. 2021; Macke et al. 2017). In the present study we found significant correlations between these environmental factors and *Daphnia* microbiome compositions, across six natural epidemics. We also found that *Daphnia* microbiomes correlate with *Caullerya* infection prevalence; individuals sampled from small epidemics differ in microbiome composition compared to those from larger epidemics. Thus, host microbiomes are distinctly shaped by environmental conditions that drive disease epidemic size.

In the present study, we found that the order Burkholderiales dominated *Daphnia* microbiomes across all surveyed years. Additionally, we observed a general correlation between *Daphnia* microbiome composition and host population density, however, the directionality of this correlation requires further investigation. For instance, *Limnohabitans* sp. (Order: Burkholderiales) was previously demonstrated to enhance *Daphnia* fitness (Peerakietkhajorn et al. 2015). Thus, *Daphnia* microbiome composition in natural environments may play a role in enhancing *Daphnia* fitness, thus driving high host abundances. Alternatively, it is also possible that high host population densities influence the microbiomes of individual hosts via horizontal transmission of microbiota (Miller et al. 2018). Both freshwater (Eckert et al. 2021; Rajarajan et al. 2022a) and marine (Boscaro et al. 2022) zooplankton microbiomes are known to be shaped by their environment with no evidence of phylosymbiosis i.e. host species identity or relatedness do not determine microbiome compositions. Therefore, horizontal transmission of associated microbiota may be an important factor shaping the microbiome composition of wild zooplankton (Russell 2019), including *Daphnia* (Cooper et al. 2022). The high abundance of Burkholderiales in the *Daphnia* microbiome in the present study could therefore also be attributed to efficient horizontal transmission of the strain driven by high host population density. We also found that the composition of rare, but not dominant taxa, in wild *Daphnia* microbiomes varied phylogenetically with total zooplankton density in the lake water. This suggests that the presence and abundance of other species in a host community may also contribute to the structure of wild host microbiomes, albeit to a lesser extent.

In a natural setting, *Daphnia*-associated bacterial communities vary independently of the composition of bacterioplankton communities over a season (Hegg et al. 2021). Here, we tested if the community

composition of larger-sized microbes (protists, phytoplankton), which are grazed on by *Daphnia*, associate with distinct host microbiomes. Previous research has shown that *Daphnia* microbiomes are influenced by diet quality (Akbar et al. 2022) and can contribute to the digestion and tolerance of toxic cyanobacteria (Macke et al. 2017). However, contrary to our expectations, our data did not support the idea that similarity in diet composition (phytoplankton community in lake water) predicts similar host microbiome composition, consistent with observations for marine zooplankton (Xu et al. 2024). Nevertheless, we observed that the density of green algae and protists (in addition to cyanobacteria) showed weak but significant correlations with *Daphnia* bacterial community composition. In general, the quantity of available food can influence microbiome community composition and its beneficial function for the host (Callens et al. 2016; Freese & Schink 2011). Our results posit that the abundance of available food, rather than the specific composition of the diet, may drive host-associated bacterial community composition in the wild.

Overall, we found that *Daphnia* bacterial community composition variation over time, was influenced by infection status (i.e. differences between infected and uninfected individuals) and infection prevalence in the population. Further, environmental conditions, specifically temperature and dissolved oxygen levels, along with the diet quantity (cyanobacteria, green algae, protists) in lake water significantly impacted the composition of host microbiomes. Thus, host microbiomes may contribute to disease dynamics in the wild by influencing host health.

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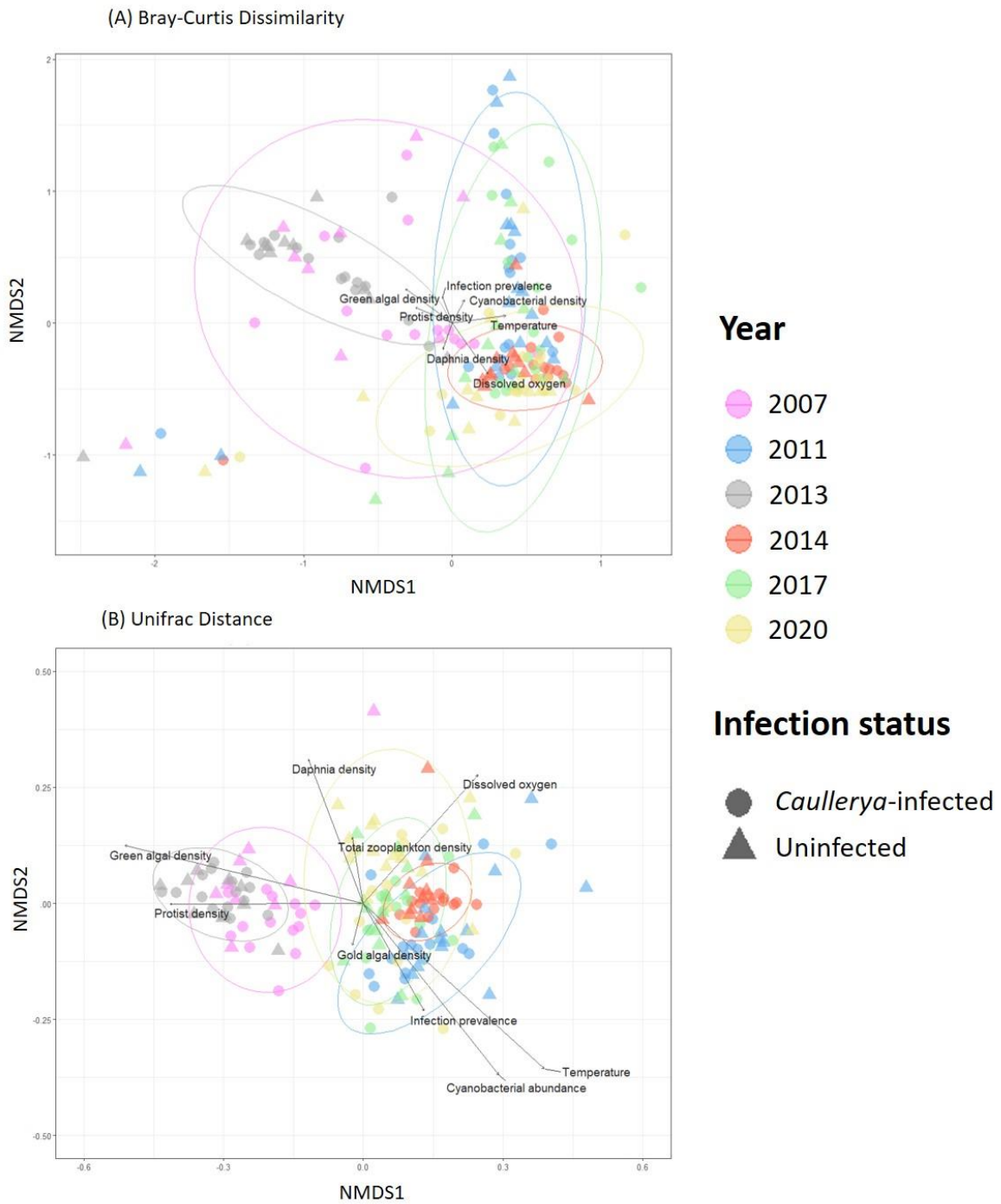


**Figure 1**. Nonmetric Multi-Dimensional Scaling (NMDS) of beta diversity indices (A) Bray-Curtis Dissimilarity and (B) Unifrac distance in individual *Daphnia* by year and infection status. Data point shapes indicate infection status and colors indicate year of sampling. Arrows indicate abiotic (temperature, dissolved oxygen) and biotic variables (infection prevalence, zooplankton and phytoplankton densities in lake water) that correlate significantly with beta diversity, length of arrows indicate strength of correlation. Ellipses show 95% confidence intervals for each year.

**Figure 2**. Relative abundances of dominant bacterial orders in individual *Daphnia* across years with *Caullerya*-infected *Daphnia* in the top row and uninfected *Daphnia* in the bottom row. Each vertical bar corresponds to the bacterial community of a single, whole *Daphnia* individual. "Other" was defined as bacterial orders comprising <1% of the dataset and present in <50% of samples. Missing bars correspond to samples that were excluded from analyses (see Methods).

| **Unifrac Distance** | | | | | | | **% Variation** |
|---|---|---|---|---|---|---|---|
| | Df | Sums Sq | Mean Sq | F value | R-squared | *p* value | |
| year | 5 | 11.1 | 2.219 | 13.7 | 0.303 | **0.0001*** | 30.28 |
| infection | 1 | 0.3 | 0.292 | 1.8 | 0.008 | **0.0337*** | 0.79 |
| Residuals | 156 | 25.3 | 0.162 | | 0.689 | | |
| Total | 162 | 36.7 | | | 1 | | |

| **Bray Curtis Dissimilarity** | | | | | | | **% Variation** |
|---|---|---|---|---|---|---|---|
| | Df | Sums Sq | Mean Sq | F value | R-squared | *p* value | |
| year | 5 | 15 | 2.99 | 15.36 | 0.327 | **0.0001*** | 33 |
| infection | 1 | 0.4 | 0.408 | 2.09 | 0.009 | 0.0581 | 0.92 |
| Residuals | 156 | 30.4 | 0.195 | | 0.664 | | |
| Total | 162 | 45.7 | | | 1 | | |

**Table 1**. Two-way PERMANOVAs on beta diversity indices Unifrac distance (top section) and Bray-Curtis Dissimilarity (lower section) by year and infection status (9999 permutations). Significant *$p < 0.05$ are indicated in bold. The percent variation column indicates percentage of variation explained by the explanatory variable with db-RDA. Significant variation in beta diversity by year (but not infection status) may be due differential dispersion of bacterial communities among years, as indicated by vegan::*betadisper*

| | 2007 | | 2011 | | 2013 | | 2014 | | 2017 | | 2020 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Bacterial Orders** | log2Change | *p* value | log2Change | *p* value | log2Change | *p* value | log2Change | *p* value | log2Change | *p* value | log2Change | *p* value |
| Absconditabacteriales | -1.96 | 0.12 | -0.96 | 0.48 | 1.48 | 0.97 | 0.28 | 0.80 | -1.55 | 0.17 | -0.94 | 0.71 |
| Aeromonadales | -1.11 | 0.38 | -1.31 | 0.37 | -0.53 | 0.97 | 1.84 | 0.25 | **3.54** | **0.02** | -1.20 | 0.75 |
| Bacillales | 0.03 | 0.99 | 0.38 | 0.84 | -0.01 | 1.00 | 0.88 | 0.25 | 0.93 | 0.10 | 0.32 | 0.75 |
| Burkholderiales | -1.02 | 0.08 | 0.76 | 0.37 | 0.00 | 1.00 | -0.57 | 0.25 | 0.06 | 0.92 | -0.08 | 0.94 |
| Caulobacterales | **1.03** | **0.04** | 0.52 | 0.96 | 0.03 | 1.00 | 0.06 | 0.98 | 0.27 | 0.68 | -0.48 | 0.71 |
| Chitinophagales | **-2.04** | **0.04** | -0.09 | 0.97 | **-3.09** | **0.00** | -0.42 | 0.44 | -0.20 | 0.68 | 0.64 | 0.57 |
| Enterobacterales | 0.08 | 0.94 | -1.67 | 0.37 | 0.47 | 0.97 | 1.45 | 0.25 | 0.97 | 0.17 | 1.04 | 0.57 |
| Flavobacteriales | -1.07 | 0.38 | 0.30 | 0.84 | -0.14 | 1.00 | -0.40 | 0.33 | -0.44 | 0.62 | -0.61 | 0.57 |
| Other | 0.28 | 0.51 | -0.60 | 0.37 | 0.11 | 0.97 | 0.52 | 0.42 | -0.47 | 0.55 | -0.49 | 0.57 |
| Pirellulales | -0.02 | 0.99 | -0.54 | 0.81 | 1.74 | 0.63 | 0.64 | 0.75 | **-2.91** | **0.02** | -1.95 | 0.57 |
| Pseudomonadales | 0.29 | 0.38 | -0.52 | 0.51 | -0.02 | 1.00 | 0.89 | 0.19 | 1.20 | 0.09 | -0.60 | 0.57 |
| Rhizobiales | 0.32 | 0.55 | 0.13 | 0.97 | 0.07 | 0.98 | 0.96 | 0.75 | 0.46 | 0.62 | -0.05 | 0.96 |
| Rhodobacterales | 0.48 | 0.61 | 0.18 | 0.97 | -0.25 | 0.98 | 2.05 | 0.49 | -1.50 | 0.34 | -0.68 | 0.71 |
| Rickettsiales | 1.98 | 0.12 | 0.38 | 0.93 | **-1.88** | **0.05** | 1.64 | 0.19 | -0.25 | 0.92 | 1.03 | 0.57 |
| Sphingobacteriales | -2.11 | 0.08 | 1.08 | 0.37 | -0.60 | 0.97 | -2.64 | 0.19 | -0.41 | 0.68 | 0.65 | 0.71 |
| Sphingomonadales | 1.30 | 0.12 | 0.11 | 0.97 | 0.11 | 0.98 | -0.29 | 0.89 | -0.04 | 0.92 | 0.04 | 0.96 |
| Verrucomicrobiales | -0.82 | 0.55 | -0.89 | 0.41 | -0.68 | 0.98 | 0.92 | 0.44 | 0.88 | 0.34 | 1.22 | 0.57 |

**Table 2**. Wald test for differential abundance of dominant bacterial orders in infected vs uninfected *Daphnia* (done separately for each year). p-values were adjusted for multiple comparisons using the "fdr" method. Significant **p*-adj <0.05 are shown in bold. "Log2Change" column indicates log2 fold change in taxon abundance; negative numbers indicate an increase in *Caullerya*-infected *Daphnia*, positive numbers indicate an increase in uninfected *Daphnia*, absolute values indicate the magnitude of change.

**Bray-Curtis dissimilarity**

| **Variable** | **r-squared** | ***p*-adjusted** |
|---|---|---|
| Temperature (°C) | 0.1325 | **0.001*** |
| Dissolved oxygen (mg/L) | 0.2069 | **0.001*** |
| Cyanobacterial density | 0.0378 | **0.001*** |
| *Daphnia* density | 0.0436 | **0.001*** |
| Green algal density | 0.1649 | **0.001*** |
| Infection prevalence | 0.0456 | **0.011*** |
| Protist density | 0.0756 | **0.029*** |
| Gold algal density | 0.061 | 0.376 |
| Diatom density | 0.0566 | 0.696 |
| Total zooplankton density | 0.0089 | 1 |

**Unifrac distance**

| **Variable** | **r-squared** | ***p*-adjusted** |
|---|---|---|
| Temperature (°C) | 0.2776 | **0.001*** |
| Dissolved oxygen (mg/L) | 0.1376 | **0.001*** |
| Cyanobacterial density | 0.2209 | **0.001*** |
| *Daphnia* density | 0.1102 | **0.001*** |
| Green algal density | 0.2771 | **0.001*** |
| Infection prevalence | 0.0705 | **0.001*** |
| Protist density | 0.1718 | **0.001*** |
| Gold algal density | 0.0087 | **0.001*** |
| Diatom density | 0.023 | 1 |
| Total zooplankton density | 0.0208 | **0.001*** |

**Table 3** Correlation of *Daphnia* bacterial community beta diversity indices (Bray-Curtis dissimilarity and Unifrac distance) with abiotic (temperature, dissolved oxygen) and biotic (*Caullerya* infection prevalence,

density of phytoplankton and zooplankton groups in lakewater) using vegan::*envfit* (9999 permutations). *P* values were adjusted for multiple comparisons using the Bonferroni method. Significant *$p < 0.05$ are indicated in bold.

| **Jaccard dissimilarity** | | |
|---|---|---|
| **Taxonomic group** | **r** | ***p*-value** |
| Cyanobacteria | 0.31 | 0.21 |
| Green algae | 0.27 | 0.22 |
| Gold algae | -0.13 | 0.58 |
| Protists | -0.15 | 0.66 |
| Diatoms | -0.15 | 0.59 |
| Zooplankton | -0.09 | 0.55 |

**Table 4.** Mantel tests correlating presence-absence community composition (Jaccard dissimilarity) between *Daphnia*-associated bacteria and phytoplankton taxa (*Daphnia* diet) in lake water, across years. ZOTUs of individual *Daphnia* bacterial communities were pooled for each year. Microbial community composition of dominant taxa in lake water was determined with microscopy.

# Acknowledgement

We would like to thank past and present members of the Spaak group at Eawag for experimental support as well as collecting long-term plankton samples data from Greifensee. We also thank the Genetic Diversity Center (GDC) at ETH Zürich for extensive support with 16S rRNA library preparation and sequencing. We give a special thanks to Cansu Çetin and Kristel Fernanda Sánchez for stimulating discussions during manuscript preparation, and also to Eva Cereghetti for her constructive comments on the manuscript. This work was funded by Eawag (Swiss Federal Institute of Aquatic Science and Technology) and a joint "lead agency" grant from the German Science Foundation (WO 1587/6-1 to JW) and Swiss National Science Foundation (310030 L 166628 to PS).


# Author Contribution

**Amruta Rajarajan**: Conceptualization (equal); Data curation: (lead); Formal analysis (lead); Investigation (lead); Writing – original draft preparation (lead); Writing – review & editing (equal). **Justyna Wolinska**: Conceptualization (equal); Formal analysis (supporting); Writing – original draft preparation (supporting); Writing – review & editing (equal); Funding acquisition (equal); Supervision (equal). **Jean-Claude Walser**: Data curation (supporting); Formal analysis (supporting); Methodology (equal); Writing – review & editing (supporting); **Nadine Tardent**: Investigation (supporting); Writing – review and editing (supporting); **Silvana Käser**: Investigation (supporting); Methodology (equal); Writing – review and editing (supporting). **Esther Keller**: Investigation (supporting); Methodology (equal); Writing – review and editing (supporting). **Piet Spaak**: Conceptualization (equal); Formal analysis (supporting); Writing – original draft preparation (supporting); Writing – review and editing (supporting); Funding acquisition (equal); Supervision (equal).

# Data availability statement

16S rRNA gene sequence data will be made available in the European Nucleotide Archive (ENA) and R code to reproduce graphs and analyses will be deposited in Dryad upon manuscript publication.