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# A field experiment reveals seasonal variation in the *Daphnia* gut microbiome

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The gut microbiome is increasingly recognized for its impact on host fitness, but it remains poorly understood how naturally variable environments influence gut microbiome diversity and composition. We studied changes in the gut microbiome of ten genotypes of water fleas *Daphnia magna* in submerged mesocosm enclosures in a eutrophic lake over a period of 16 weeks, from early summer to autumn. The microbial diversity increased when *Daphnia* were reintroduced from the laboratory to the lake, and the composition of gut microbes drastically changed. Both gut microbiome diversity and composition continued to change over the 16-week period, with alpha diversity peaking in late summer. The gut microbiome community was clearly distinct from that of the surrounding water, and temporal changes in the two communities were independent of each other. There were no consistent differences in the gut microbiomes among *Daphnia* genotypes in the lake environment. The change in gut microbiome over the season was accompanied by a decline in reproductive output and survival. There were weak, but statistically supported, effects of microbiota composition on *Daphnia* fitness, but there was no evidence that natural variation in microbiome diversity or composition was associated with tolerance to the cyanotoxin microcystin. We conclude that the gut microbiome of *Daphnia* is highly dynamic in a natural lake environment, but that host genetic effects on microbiome diversity and composition between genotypes within a population can be vanishingly small. These results emphasize that establishing the ecological effects of gut microbiota will require large-scale experiments under natural conditions.

Keywords: microbiota, plasticity, seasonal adaptation

## Introduction

The gut microbiome – the community of bacteria that inhabits the gastrointestinal tract – is an important component of animal physiology. The presence and composition of gut symbionts are increasingly recognized for their association with the function of their animal hosts, and may influence for example digestion (Groussin et al. 2017), pathogen resistance (Ubeda et al. 2017) and energy metabolism (Sommer et al. 2016). The gut microbiome has also been linked to detoxification of harmful substances and



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thus an increased ability of the host to consume toxic foods (Kohl et al. 2014, Shiffman et al. 2017). As a result, the diversity and composition of the gut microbiome can be critical for an individual's biological fitness and may contribute to adaptation (Alberdi et al. 2016, Moran et al. 2019, Moeller and Sanders 2020).

The recruitment and maintenance of gut symbionts are largely influenced by the diet or the surrounding environment, and the community of gut microbes can therefore rapidly change in response to environmental conditions (David et al. 2014, Hicks et al. 2018, Youngblut et al. 2019). Provided that the host is able to utilize beneficial microbes, such changes may enable organisms to persist in otherwise hostile environments (Alberdi et al. 2016). For example, animal gut microbiomes can change over the season, both in invertebrates (Ferguson et al. 2018, Subotic et al. 2019) and vertebrates (Maurice et al. 2015, Hicks et al. 2018). However, it is poorly understood if seasonal dynamics of microbiome composition is associated with changes in the biology of hosts, and in particular their fitness.

The water flea *Daphnia* spp. offers a good opportunity to investigate temporal dynamics in gut microbiome and its impact on reproduction and survival. Many freshwater environments are highly variable in terms of nutrients, temperature and other biotic and abiotic factors that can impact fitness. In temperate regions such as Scandinavia and northern America, the biomass of *Daphnia* (and other zooplankton) can vary substantially across the season (Hansson et al. 2007, Sommer et al. 2012). In addition, seasonal changes in the environment are also expected to have direct negative effects on fitness, and reasons for this could include exposure to stressfully high temperatures (Yampolsky et al. 2014) or to harmful algal blooms that usually appear between mid to late summer in eutrophic lakes and ponds (Hansson et al. 2007). These blooms are commonly dominated by cyanobacteria that produce cyanotoxins, such as different variants of microcystins (Carmichael 1994, Dawson 1998), which can have large effects on the entire freshwater community (Paerl and Otten 2013).

An experimental laboratory study suggested that the gut microbiome can be involved in adaptation or acclimation to seasonal stressors like cyanotoxins (Macke et al. 2017). The authors demonstrated differences in the gut microbiome composition between two toxin-tolerant and two toxin-sensitive genotypes (i.e. *Daphnia* clones), and that the sensitive clones could be made more tolerant when receiving gut transplants from the tolerant clones. While this suggests that gut microbiome is an important component of fitness in *Daphnia*, little is known about how the gut microbiome community varies across the season under natural conditions, and how consistent clone effects are in the wild. Furthermore, it remains unknown if variation in the gut microbiome caused by naturally fluctuating environments has any effect on fitness of *Daphnia* in the absence or presence of toxins like microcystin.

In this study, we investigated 1) if and how the gut microbiome of water fleas *D. magna* in lake mesocosms changes over the summer season, 2) if this variation is associated with the microbiome of the water, 3) if different clones from a single lake differ consistently in microbiome diversity or composition and 4) if the diversity or composition of gut microbes can be linked to reproductive output or survival in the absence or presence of microcystin-producing cyanobacteria.

To test this, we reintroduced individuals of ten *D. magna* genotypes back into their native lake in submerged mesocosms in the beginning of summer, with each clone (i.e. genotype) housed separatedly in two enclosures, resulting in a total of twenty experimental units. We sampled individuals and water from each enclosure at four different time points, with four-week intervals, and analysed the gut microbiome of each clone as well as the microbiota of the water inside each enclosure using 16S rRNA amplicon sequencing. In addition, we quantified survival and reproductive output of *Daphnia* collected from the enclosures in a microcystin tolerance assay, in which we exposed individuals to two strains of cyanobacteria, one that produces microcystin (hereafter: toxic) and one that does not produce microcystin (hereafter: non-toxic). This allowed us to assess if seasonal changes in gut microbiome composition is accompanied by seasonal changes in fitness, and to establish statistically if individuals with higher fitness came from enclosures housing *Daphnia* with particular gut microbiomes.

## Material and methods

Ten clones of *Daphnia magna* were used in the study, all originating from Lake Bysjön in Southern Sweden (55°40′28.5″N, 13°32′45.4″E), a small (0.2 km$^2$), shallow (mean depth: 3.6 m) and hypereutrophic lake that frequently experiences algal blooms (Hansson et al. 2007, Schwarzenberger et al. 2013). The clone lines were sampled in spring 2015, genotyped using microsatellites, and cultivated under standardized conditions (Radersma et al. 2018 for more details on the clones). In brief, each clone line was housed in two separate jars filled with 500 ml tap water and fed green algae (*Scenedesmus obliquus*; NIVA CHL-6) (Culture collection of Algae, Norwegian Inst. for Water research). The lines were kept at 18°C, with a 14:10 light:dark cycle. Water was changed approximately every 1–2 months and green algae was added ad libitum.

### Lake mesocosm experiment and sampling

On 6 June 2018, the clones were reintroduced into their native lake Bysjön where they were kept in mesocosms for 16 weeks with the aim to study changes in their gut microbiome, as well as reproduction and survival (for an illustration of the experimental design, Supporting information). The *Daphnia* were housed in 15 l cylinder-shaped plastic enclosures (d = 25 cm, h = 30 cm) with five 15 × 10 cm windows in the sides and lid that were covered with a 150 µm mesh net. These mesocosms allowed lake water and phytoplankton to enter the enclosure through the mesh while at the same time preventing individuals from escaping. A total number of 40

individuals were added to each enclosure, and two replicate enclosures were created for each clone line, resulting in a total of twenty experimental units (Supporting information). The clone replicates were placed at two different sites in the lake (250 m apart), submerged approximately 10 cm below the water surface and securely attached to wooden posts.

We sampled *Daphnia* by filling up 1 l of water from each enclosure at four time points with regular intervals after the start of the experiment; 4 weeks (4 July), 8 weeks (1 August), 12 weeks (29 August) and 16 weeks (26 September). In addition, we also collected 250 ml of water (to be used for microbiome analysis) from each enclosure and these samples were kept under cool conditions (5–10°C). This water was first filtered through a 150-µm mesh net in order to remove potential zooplankton or larger algae. Subsequently, we collected the sample on a 0.2-µm Supor 200 membrane filter (Pall Corporation, USA), which was placed in a sterile 1.7 ml tube fully submerged in TE buffer (1×) and then stored at −80°C until further processing.

The collected *Daphnia* were allowed to acclimatize to laboratory temperature (18°C) for at least one hour before being handled. Ten individuals from each mesocosm were selected for the microcystin tolerance assay, and 20–25 individuals were selected for the microbiome analysis. These individuals were transferred to fresh, sterile (i.e. autoclaved) ADaM medium (Klüttgen et al. 1994) and kept for 24 h in order to remove most algae particles and bacteria associated with the food (following Macke et al. 2017). Individuals were then frozen in this medium (−20°C) until further processing. When thawed, the gut from each individual *Daphnia* was carefully extracted using a pair of tweezers and dissection needles under a stereomicroscope. In total, twenty guts from each sample were placed in 50 µl of deionized water (MilliQ) and frozen at −80°C before DNA extraction.

Lake Bysjön regularly experiences algal blooms, usually in late summer (Hansson et al. 2007, Schwarzenberger et al. 2013), and as a result we expected *Daphnia* to experience a peak of the cyanotoxin microcystin in August. We therefore measured the microcystin concentration within each enclosure, as well as outside in the lake water by collecting 15 ml of water at each sampling date. Each sample was kept cool until frozen (−20°C), after which samples were analysed for total microcystin (free and cell-bound) using an enzyme-linked immunosorbent assay (ELISA) kit (Eurofins Abraxis, USA).

### Microcystin tolerance assays

At each of the four lake sampling dates (4, 8, 12 and 16 weeks), and once before individuals were transferred to the mesocosms (0 weeks), we assessed survival and reproductive output of a subsample of individuals from each enclosure (i.e. five identical experiments were run at week 0, 4, 8, 12 and 16). From each enclosure, ten female *Daphnia* that did not carry eggs, but judged to have reached the minimum size for being reproductively active (ca 1.6–2.0 mm from eye to base of spine), were selected. Each individual was washed in clean ADaM medium for a few minutes before being transferred to either 'toxic' (microcystin producing; NIVA CYA-228/8) or a 'non-toxic' (non-microcystin producing; NIVA CYA-143) strain of the cyanobacterium *Microcystis aeruginosa* (for a more detailed desciption of the strains, Lürling 2003, Gustafsson et al. 2005, Radersma et al. 2018). Throughout the duration of these four experiments, single individuals were kept in a 100 ml jar filled with ADaM medium containing 120 000 cells $ml^{-1}$ of frozen green algae (CHL-6), and 35 000 cells $ml^{-1}$ of either toxic or non-toxic cyanobacteria (CYA 228/8 or CYA-143). We transferred each individual to a new jar containing fresh medium and algae every other day. At the same time, mortality was recorded and produced offspring were counted and removed. Frequent medium changes were made to keep toxicity and food levels approximately constant throughout the duration of the experiments. Each experiment lasted for 18 days.

### DNA extraction, library preparation and amplicon sequencing

All samples were randomized before processing to avoid bias caused by batch effects. Gut and water samples were thawed, and DNA was extracted using a DNeasy PowerSoil Pro DNA kit (Qiagen, Sweden). DNA yield was assessed using a Qubit dsDNA HS assay. We applied a nested PCR to increase specificity and amplicon yield (Bakke et al. 2011, Berg et al. 2016, Macke et al. 2017). First, the full length 16S rRNA was amplified with the primers 27F (5 µM) and 1492R (5 µM) on 10 ng of template using GoTaq polymerase for 30 cycles (94°C–30 s; 50°C–45 s; 68°C–90 s). The PCR products were subsequently purified using ExoSAP-IT (Thermo Fisher Scientific, USA). To obtain dual index amplicons of the V4 region, a second amplification was performed on 8.5 µl of PCR product using primer 515F and 806R (Kozich et al. 2013, Apprill et al. 2015) for 30 cycles (98°C–10 s; 55°C–30 s; 72°C–45 s). Both primers include an Illumina adapter and a unique 8-nucleotide (nt) barcode at the 5′-end. A final purification step using AMPure beads (Beckman Coulter, USA) was performed before samples were diluted and pooled in equimolar concentrations. The amplicons were sequenced using a MiSeq Reagent kit v2 (500-cycles) with custom primers (Kozich et al. 2013) on an Illumina Miseq platform at the DNA Sequencing Core Facility, Dept of Biology, Lund Univ., Sweden, producing 2 × 250-nt paired-end reads. In total, 200 samples (including gut, water and control samples) were sequenced in two separate sequencing runs of 100 samples each.

### Microbiome data processing

The 16S amplicon sequences were analysed using the DADA2 package (Callahan et al. 2016b), following a published pipeline (Callahan et al. 2016a). Sequences were trimmed (the first 10 nucleotides) and filtered (maximum of 2 expected errors per read) and sequence variants (ASVs, hereafter OTUs) were inferred using the DADA2 method (Callahan et al. 2016b) for each sequence run. The sequence tables of the two runs

were combined and PCR-originating chimeras were identified and removed from the data set.

Taxonomy was assigned with a naïve Bayesian classifier using the RDP v16 training set. OTUs that were not assigned to the kingdom bacteria, or which were assigned to 'chloroplast/mitochondria' or 'cyanobacteria' were subsequently removed from the data set. Low population size and mortality during acclimation prevented obtaining enough guts for some replicates, especially during late summer and autumn, and the sample size therefore varies across the study period (4 weeks: n = 19, 8 weeks: n = 18, 12 weeks: n = 10 and 16 weeks: n = 11). We compared the microbiomes of our gut and water samples with the control samples (either negative controls of MilliQ water added before the first PCR, or MilliQ samples collected during dissection of guts and filtering of lake water). As expected, these control samples contained a very different microbial composition compared to the actual gut and water samples, and were therefore not considered for further analysis. We tested for batch effects by comparing the relationship between read depth and OTU richness for both runs separately, and by comparing the microbiome composition of ten water samples that were sequenced twice (i.e. were included in both runs). No consistent difference in microbiome diversity and composition were detected between the runs and we therefore did not consider batch effects further. Finally, we removed low abundant taxa that only occurred in one sample and had a total abundance (read count) of less than 10.

## Statistical analyses

All data was statistically analysed using R ver. 3.6.2 (<www.r-project.org>). The microbiomes were analysed using the Bioconductor package phyloseq (McMurdie and Holmes 2013). α-Diversity was calculated as both OTU richness (number of taxa) and Shannon index, using the absolute abundance of reads. The difference in α-diversity (both OTU richness and Shannon index) between gut samples over the season was analysed using linear mixed models (lme4 package; Bates et al. 2015) with the α-diversity measure as the response variable, sampling date (four levels: 4, 8, 12, 16 weeks) and site (two levels; Supporting information) as fixed categorical factors, and both clone and enclosure as random effects. Pairwise differences between each sampling date were assessed with a post hoc Tukey's HSD, using the emmeans package (Lenth 2020). To investigate seasonal differences in community composition (β-diversity) between samples, we calculated Bray–Curtis dissimilarity (Bray and Curtis 1957), weighted and unweighted UniFrac distance metrics (Lozupone and Knight 2005). The effects of sampling date, site and clone was examined with a permutational multivariate analysis of variance (PERMANOVA) on both Bray–Curtis and UniFrac distances using the Adonis function in the vegan package with 999 permutations (Oksanen et al. 2019). Note that, in contrast to the mixed models, this analysis does not handle random effects and therefore treats clone as a fixed effect.

To identify how many, and what type of bacterial taxa that differed between gut samples from different sampling dates, we grouped OTUs at the family level and made pairwise comparisons between each sampling period. Differential abundance analysis was performed using the Bioconductor package DESeq2 (Love et al. 2014).

To investigate the relationship between the water and gut microbiome we tested for a correlation in α-diversity (both OTU richness and Shannon index) between water and gut samples collected from the same mesocosms at each time period. In addition, to test if the two microbial communities (i.e. water and guts) varied independently, we performed separate symmetric co-correspondence analyses (using the relative abundance of reads) for each sampling period using the cocorresp package (Simpson 2009), following Alric et al. (2020).

Reproduction and survival over the course of the microcystin tolerance assays were measured as the total number of offspring produced and the number of days survived for each individual, respectively. Because our primary interest was in the association between these estimates of fitness and variation in the gut microbiome (where the latter is an estimate at the level of enclosure, not individual), we averaged the reproductive output and survival of individuals per enclosure and sampling date in both the toxic and non-toxic treatment. We first fitted a linear mixed model with the average total number of offspring produced (log-transformed) as the response variable, and date, treatment and their interaction as fixed effects, and clone as a random effect. We also fitted the corresponding generalized linear mixed model for survival, with the response variable being the proportion of survivors, using a binomial distribution and a logit link function.

Following these results, we then investigated the association between the variation in the two fitness variables (number of offspring and survival) and variation in the gut microbiome. Given the results of the analysis mentioned above, and to prevent overly complex models, we fitted separate models for the toxic and non-toxic treatments. Average total reproductive output in the non-toxic treatment was fitted using a linear model. Since reproductive output was low in the toxic treatment, and many individuals failed to reproduce, we modelled reproduction in the toxic treatment using a binomial generalized linear model (GLM) with the proportion of reproducing individuals for each mesocosm and date as the response variable. Similarly, we modelled survival in the toxic treatment using a binomial GLM but with proportion of surviving individuals as the response variable. Since almost all individuals in the non-toxic treatment survived, we were not able to test whether the microbiome was associated with survival in that treatment.

To these models, we added both α-diversity and β-diversity measures as explanatory variables. Because of missing data for gut microbiome (above), this resulted in lower sample size. We included OTU richness (number of taxa), but also the first three axes of variation produced by the PCoAs of Bray–Curtis dissimilarity and weighted UniFrac distances. We selected the first three axes because those accounted for a substantial amount of variation in the microbiome composition (62.9%

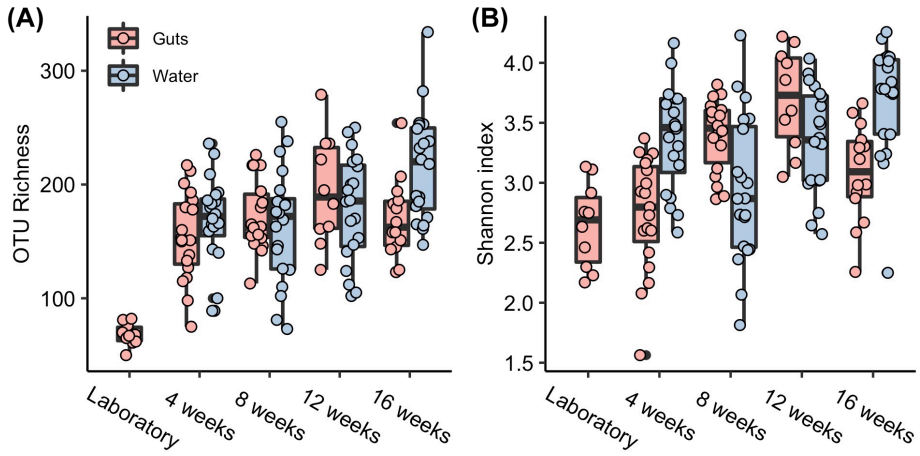


Figure 1. Difference in (A) OTU richness and (B) Shannon index in gut and water samples in the laboratory and after 4, 8, 12 and 16 weeks in the mesocosm enclosures. Each data point represents a single enclosure during one sampling event.

and 76.0%, respectively), and Bray–Curtis and UniFrac axes were only weakly correlated with each other. Together with date, these microbiome variables were added as fixed effects to create a full model. From this full model, we subsequently fitted all simpler models using the dredge function in the MuMIn package (Bartoń 2020) and ranked the top models based on Aikaike information criteria (AICc). Although we present and discuss the (three) best models in the results section, we also performed model averaging over the 'best' models ($\Delta$AICc < 2) (Burnham et al. 2002) to ensure that these models were representative of the variables with the most predictive power. We also tested the statistical significance of the variables present in the best models. Throughout, all statistical tests for ANOVA refer to type III (for models with interactions) or type II (without interactions). Figures were plotted using the ggplot2 package (Wickham 2016).

## Results

### Microbiome diversity and composition

The OTU richness of gut microbiomes increased dramatically following translocation from the laboratory to the lake enclosures (Fig. 1A). OTU richness of the guts also varied across the season in the mesocosms (date; Table 1), but according to the post hoc test, only week 4 and 12 were significantly different ($p < 0.01$). There was also a weak and marginally significant effect of site (Table 1, Supporting information). The results were similar for the Shannon index (Fig. 1B, Table 1, Supporting information). The diversity increased from June (4 weeks) until late August (12 weeks), but declined in late September (16 weeks) (Fig. 1B). The gut microbiome diversity (both OTU richness and Shannon index) was largely similar among the clone lines (Supporting information).

PCoA of Bray–Curtis dissimilarity, and weighted and unweighted UniFrac distances, showed that the microbiome composition of the gut samples clustered by sampling date (Fig. 2A). The microbiome composition of *Daphnia* kept in the laboratory prior to transfer to the lake were clearly differentiated from samples collected from the mesocosm experiment (Supporting information), but were marginally more similar to samples collected at the first sampling event (Supporting information). Furthermore, the β-diversity of the gut microbiomes within each sampling period were always more similar to each other than to the other sampling periods (Supporting information). According to the PERMANOVA, the microbiome composition of *Daphnia* kept in mesocosms varied significantly over the season and also between the two sites, although the latter effect was substantially weaker (Table 1). In contrast, clone identity did not have a significant effect on the microbiome composition (Table 1, Fig. 2B).

Overall, the gut samples were dominated by four different classes of bacteria: Planctomycetia, Alphaproteobacteria, Actinobacteria and Betaproteobacteria (Fig. 3, Supporting information). The relative abundance of both Planctomycetia and Actinobacteria increased following transfer from the laboratory into the lake, and subsequently over the season, while the abundance of Betaproteobacteria decreased to nearly undetectable levels after 16 weeks in the mesocosms (Fig. 3, Supporting information). The bacteria class Flavobacteria, a candidate for high tolerance to toxic cyanobacteria (Macke et al. 2017), were absent or uncommon in most gut samples, and abundance did not appear to change over the season (Supporting information). According to the differential abundance analysis, a greater number of bacterial families differed between the gut samples collected from lab-reared *Daphnia* (i.e. prior to reintroduction of the clones in the lake) and the gut samples collected during the first mesocosm sampling event (28 families), than between the first and the last mesocosm sampling event (17 families). This suggests that there is more change in the gut microbiome (at the family level) triggered by the translocation from the laboratory to the lake, than over the season in the lake itself (Supporting information).

The microbial communities of the water samples were highly distinct from that of the gut samples (Fig. 3, Supporting information). The water samples were dominated

Table 1. Results of the gut microbiome models. α-diversity models (both OTU Richness and Shannon index) include sampling date (Date; four levels) and site (Site; two levels) as fixed effects, and clone and enclosure as random effects. β-diversity models (PERMANOVA) include sampling date, site and as these analyses do not handle random effects, they also include clone (Clone; ten levels) as a fixed effect.

| | Effect | $R^2$ | df | F | p |
|---|---|---|---|---|---|
| α-Diversity | | | | | |
| OTU Richness | Date | | 3, 43 | 3.91 | 0.015* |
| | Site | | 1, 8.6 | 5.19 | 0.050* |
| Shannon index | Date | | 3, 43 | 19.16 | < 0.001*** |
| | Site | | 1, 8.6 | 4.91 | 0.055 |
| β-Diversity | | | | | |
| Bray–Curtis | Date | 0.46 | 3, 56 | 17.82 | < 0.001*** |
| | Site | 0.03 | 1, 56 | 3.87 | 0.004 ** |
| | Clone | 0.07 | 9, 56 | 0.92 | 0.636 |
| w. UniFrac | Date | 0.34 | 3, 56 | 10.78 | < 0.001*** |
| | Site | 0.05 | 1, 56 | 4.85 | < 0.001*** |
| | Clone | 0.09 | 9, 56 | 1.00 | 0.445 |
| unw. UniFrac | Date | 0.29 | 3, 56 | 8.17 | < 0.001*** |
| | Site | 0.04 | 1, 56 | 3.26 | < 0.001*** |
| | Clone | 0.16 | 9, 56 | 1.09 | 0.237 |

by Actinobacteria, Flavobacteriia, Betaproteobacteria and Planctomycetia (Fig. 3, Supporting information). Overall, the α-diversity indices were similar in water and gut samples, but there was no correlation between the microbial diversity (including both OTU richness and Shannon index) in the water of the enclosure and the gut microbiome of the *Daphnia* in the same enclosure (Supporting information). In addition, the co-correspondence analysis failed to support a relationship between the microbial community composition of the water within the enclosure and the gut microbiome ($p > 0.05$ for all comparisons; Supporting information).

The microcystin concentration in the mesocosm enclosures was very low during the entire period, ranging from about 0.15 µg $l^{-1}$ after 4 weeks of exposure, to under 0.10 µg $l^{-1}$ after 16 weeks (Supporting information). This corresponds to a concentration about ten times lower than what has been measured in the lake the previous year (Hegg 2020). Comparable low values were detected outside of the enclosures in the surrounding lake water, suggesting that there was no algal bloom dominated by microcystin-producing cyanobacteria in Bysjön during 2018.

## Reproduction and survival of *Daphnia* from the mesocosm enclosures

To test how the fitness of *Daphnia* varied across the season, we first fitted linear mixed models of the number of offspring produced and proportion of survivors in the microcystin tolerance assays, respectively. Sampling date (four levels: 4, 8, 12, 16 weeks), microcystin treatment (two levels: toxic versus

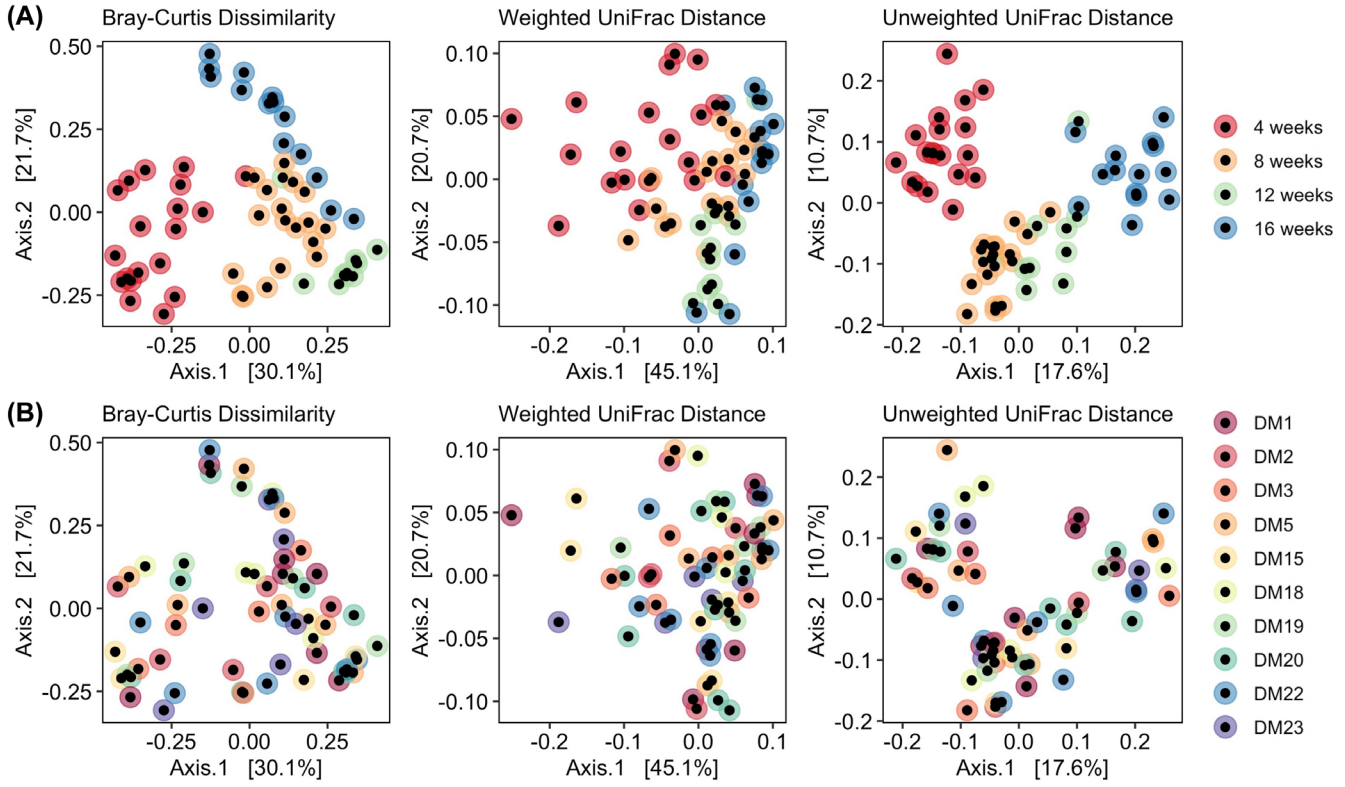


Figure 2. PCoA plots of Bray–Curtis dissimilarity (left) and weighted (middle) and unweighted UniFrac distances (right) between *Daphnia* gut samples. (A) (top row) show samples coloured based on sampling date (4, 8, 12 or 16 weeks), and (B) (bottom row) show the same data coloured by clone ID (DM1, DM2, DM3, DM5, DM15, DM18, DM19, DM20, DM22 or DM23). Brackets in the PCoA show the percent of variance explained by the first two dimensions.

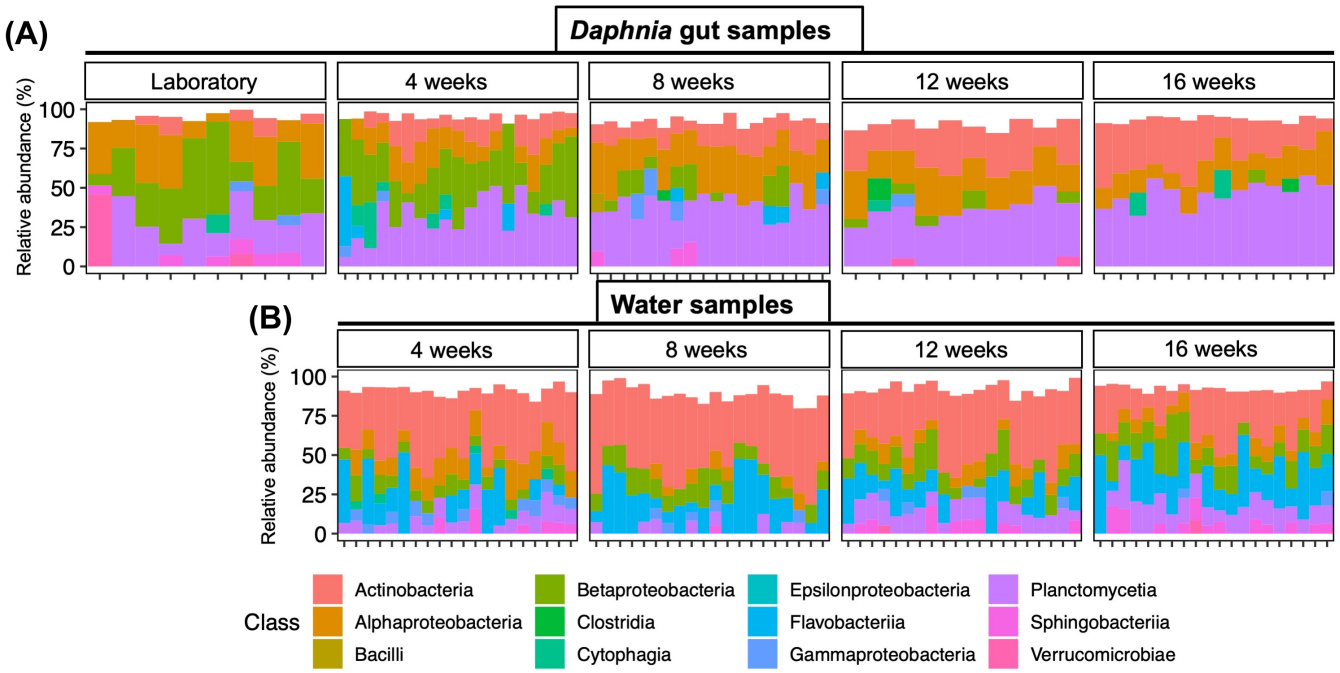


Figure 3. Relative abundance of OTUs grouped by bacterial classes of (A) *Daphnia* guts sampled before introduction to the mesocosms (Laboratory) and over the season (after 4, 8, 12 and 16 weeks of exposure in the mesocosms), and (B) water samples sampled from inside the mesocosms during the period (4–16 weeks). Each bar represents one sample. Bacterial classes with a relative abundance of < 5% are excluded from the plot.

non-toxic) and their interaction were entered as fixed effects, and clone as a random effect. This demonstrated a significant effect of microcystin treatment, sampling date and the interaction between sampling date and microcystin treatment on reproduction (Table 2). The proportion of *Daphnia* that reproduced in the microcystin-exposure experiments declined over the season (4 weeks: 98%; 8 weeks: 90%; 12 weeks 79.5%; 16 weeks: 86.5%), and those that did reproduce produced fewer offspring (Fig. 4A). Survival of *Daphnia* collected from the mesocosms was negatively affected by microcystin exposure, but did not differ between sampling dates (Table 2). There was, however, a significant interaction effect between sampling date and microcystin exposure, a result of a drop in survival in the microcystin exposure treatment in individuals collected at 16 weeks (Fig. 4B).

Table 2. Results of mixed effects models on reproduction and survival in the microcystin tolerance assay. Both models include the main effects of microcystin treatment (Treatment), sampling date (Date) and their interaction (Treatment × Date), and clone as a random effect.

| Response | Effect | df | F | $\chi^2$ | p |
|---|---|---|---|---|---|
| Reproduction | Treatment | 1, 127 | 116.86 | | < 0.001*** |
| | Date | 3, 127 | 19.16 | | < 0.001*** |
| | Treatment × Date | 3, 127 | 9.02 | | < 0.001*** |
| Survival | Treatment | 1, 145 | | 5.34 | 0.021* |
| | Date | 3, 145 | | 3.11 | 0.375 |
| | Treatment × Date | 3, 145 | | 14.62 | 0.0022** |

### Association between the gut microbiome and reproduction and survival

To test if the microbiome composition of individuals from the same enclosure explained any additional variance in offspring number or survival, we refitted the models for each treatment separately and added a measure of both α-diversity (i.e. OTU richness) and β-diversity (i.e. the three first PCoA axes of Bray–Curtis distances and weighted UniFrac), using model comparison to identify robust effects. This revealed that date was the overall best predictor of reproduction (in both treatments) and survival. However, models that included Bray–Curtis axis 2 explained significant amounts of variance in reproductive output, a result that was consistent for both treatments (Table 3). Survival in the toxic treatment also appeared to be explained by the clone line and microbiota composition (including weighted UniFrac axis 1; Table 3).

## Discussion

This study reveals several sources of variation in the bacterial community in the guts of *Daphnia magna* exposed to a natural lake environment. The gut microbiomes changed substantially following transfer from a laboratory environment to the lake mesocosms, with guts being colonized by bacteria that were absent in the laboratory, leading to higher overall diversity. The relative abundance of gut microbes continued to change across the season, resulting in very different microbiomes in autumn compared to early summer.

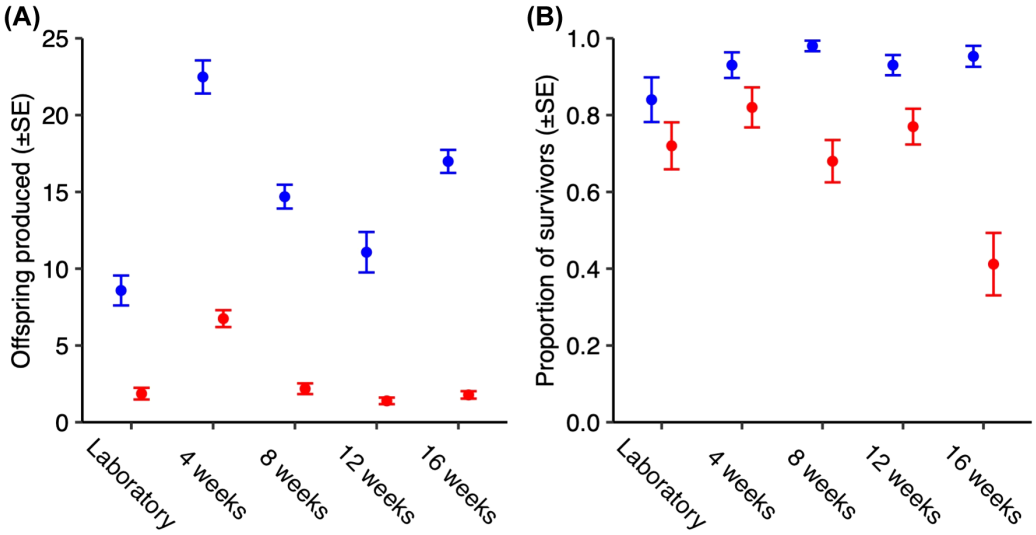


Figure 4. Fitness of individual *Daphnia* kept in the toxic (red) or non-toxic (blue) treatments. The individuals were sampled from the laboratory populations before introduction to the mesocosms, and after 4, 8, 12 and 16 weeks of exposure in the mesocosms. (A) Shows the average total number of offspring produced and (B) the average survival after 18 days. Unit of replication is experimental jar and error bars are ± SE.

The most abundant bacteria in the guts belonged to the class Planctomycetia, which also increased in relative frequency across the season. This class of bacteria was also reported to be one of the most abundant gut bacteria classes in a laboratory study using lake water (Houwenhuyse et al. 2021). The relative abundance of Betaproteobacteria, which is known to be a dominant group of bacteria inhabiting the *Daphnia magna* gut (Freese and Schink 2011, Frankel-Bricker et al. 2020, Motiei et al. 2020), declined from moderately high abundance in the laboratory and after 4 weeks, to very low abundance after 16 weeks in the mesocosms. Overall, these results demonstrate that the gut microbiome of genetically identical *Daphnia* in a natural environment can vary substantially from one point in time to another, and be very different from the microbiome under laboratory conditions.

The *Daphnia* gut microbiome is thus highly plastic, both in terms of diversity and composition, which is supported by several other recent studies (Sullam et al. 2018, Callens et al. 2020, Frankel-Bricker et al. 2020, Macke et al. 2020, Houwenhuyse et al. 2021). One reason for this plasticity is that bacteria can colonize the gut via the external environment, in this case the surrounding water. While both water and gut microbiota composition changed across the season, the two appear effectively decoupled. The lack of correspondence between the two communities (which was also found in a laboratory study; Massol et al. 2021) does not rule out

Table 3. The top three models for each fitness variable (reproductive output and survival) in both toxic and non-toxic treatments of the microcystin tolerance assay. AICc scores for each model and significance for each predictor is presented (NS, *, ** or ***). Model selection is based on comparing all simpler models of the global model: Response variable ~ Date + Clone + Richness + Bray axis 1 + Bray axis 2 + Bray axis 3 + Wuni axis 1 + Wuni axis 2 + Wuni axis 3.

| Response variable | Treatment | Model no. 1 | Model no. 2 | Model no. 3 |
|---|---|---|---|---|
| Reproduction | Toxic | Date (***) | Date (***) | Date (***) |
| | | Bray 1 (*) | Bray 1 (*) | Bray 2 (***) |
| | | Bray 2 (***) | Bray 2 (***) | |
| | | | Richness (NS) | Wuni 2 (NS) |
| | | AICc = 152.0 | ΔAICc = 1.41 | ΔAICc = 1.45 |
| Reproduction | Non-toxic | Date (***) | Date (***) | Date (***) |
| | | Bray 2 (*) | Richness (NS) | |
| | | Bray 3 (NS) | | |
| | | AICc = 350.7 | ΔAICc = 0.45 | ΔAICc = 0.79 |
| Survival | Toxic | Date (***) | Date (***) | Date (**) |
| | | Clone (***) | Clone (***) | Clone (***) |
| | | Bray 2 (**) | Bray 3 (*) | Bray 2 (NS) |
| | | Richness (*) | Wuni 1 (***) | Wuni 1 (***) |
| | | Wuni 1 (***) | | |
| | | AICc = 182.5 | ΔAICc = 1.42 | ΔAICc = 1.62 |
| Survival | Non-toxic | NA | NA | NA |

that important bacteria were taken up from the surrounding environment, but it does show that *Daphnia* recruit and maintain a microbiome largely independently of what they are exposed to in the lake water. In contrast, recent laboratory experiments suggest that the microbiome of the medium can have a strong influence on the *Daphnia* gut microbiome (Callens et al. 2020), but also the opposite, that the gut microbiome can influence the microbiome of the surrounding water (Macke et al. 2020, Massol et al. 2021). Our results suggest that such effects are at best marginal within a larger body of water in a lake environment.

Another important reason for changes in gut microbiota is diet (David et al. 2014, Hicks et al. 2018, Youngblut et al. 2019), which has also been demonstrated in *Daphnia* (Callens et al. 2016, but see Macke et al. 2017, Akbar et al. 2020). *Daphnia* are filter feeders, and this likely means that the energy and nutrient content of their food is likely to vary across the season (Sommer et al. 2012). This can have consequences for the gut microbiome and may explain part of the seasonal variation. There are also associations between the phytoplankton community and the microbial community in the lake environment (Bunse et al. 2016), suggesting that the availability of bacteria that *Daphnia* can take up from the water can correlate with their diet.

One interesting hypothesis is that a change in gut microbiome composition can enable digestion of toxic cyanobacteria (Macke et al. 2017). Our study was designed to detect a shift in gut microbiome following elevated levels of the cyanotoxin microcystin in late summer when this eutrophic lake typically experiences bloom events (Hansson et al. 2007, Schwarzenberger et al. 2013). However, the year of study was unusual in that there was no algal bloom dominated by microcystin-producing cyanobacteria, resulting in very low microcystin concentrations across the season. Given previous studies on toxicity (Gustafsson and Hansson 2004, Sarnelle et al. 2010, Radersma et al. 2018), these low levels seem unlikely to have caused the observed effects on the gut microbiome following transition to the lake or across the season.

In addition to changes in diet, the gut microbiome may also be influenced by other environmental factors that have direct effects on the physiology of *Daphnia*. For example, changes in temperature can lead to significant changes in the gut microbiota (Sepulveda and Moeller 2020), and this appear to be the case also in *Daphnia*, even though the effects can vary among clone lines (Sullam et al. 2018, Frankel-Bricker et al. 2020). Mesocosm experiments should be useful to detect acute and chronic effects of temperature in a naturally variable environment, but it would require more extensive sampling of microbiomes. However, since many other factors covary with temperate in a lake environment (e.g. light and primary production; Brönmark and Hansson 2018), the experimental design used here should be complemented with experiments that directly manipulate environmental factors, like temperature.

In contrast to several laboratory studies (Macke et al. 2017, Sullam et al. 2018, Frankel-Bricker et al. 2020), we did not find any clear differences in the gut microbiome between the ten different *Daphnia* clones. Many laboratory studies use clone lines isolated from different environments or lakes, which likely means that the genetic variation among the genotypes in our study is comparably small (as they were isolated from a single lake in one year). However, the genotypes do show consistent differences in gut microbiome composition under laboratory conditions (Hegg 2020), so it is perhaps still surprising that there was no difference between these clones in the lake environment. Further studies are necessary to establish if very strong and consistent genotype–microbiome associations in the laboratory (Macke et al. 2017) are maintained in nature, and to identify associations between host genomic variation and gut microbiome.

The association between variation in gut microbiome and biological fitness is poorly understood. The reproductive output and survival of *Daphnia* collected from the lake enclosures was much reduced during the height of summer. Given that this seasonal pattern was striking despite that the microcystin levels remained low in the mesocosms, the decline in absolute fitness in the present study is unlikely to primarily be driven by acute toxicity. Whether or not other toxins, or abiotic stressors like temperature play important roles, or if a reduced reproductive output during summer is part of an adaptive life history, remains to be shown.

That both gut microbiomes and individual fitness varied dramatically from spring to autumn makes it difficult to establish if any particular microbiome features are associated with survival or reproduction. Nevertheless, the statistical analyses suggest that some variation in reproductive output and survival may be associated with microbiome composition. The fitness consequences of natural variation in microbiome composition in *Daphnia* warrants further investigation with experimental designs optimized for detecting fitness effects of the gut microbiome. In our study, the statistical association between microbiome composition and reproduction and survival appeared consistent in both absence and presence of microcystin-producing cyanobacteria (i.e. in both treatments). Thus, we conclude that there was no evidence that the natural variation in microbiome observed in this study provides tolerance to this toxin. This is not unexpected given that the year of study was unusual in that the lake did not experience a bloom of microcystin-producing cyanobacteria. Flavobacteria, a group demonstrated to provide tolerance to toxic cyanobacteria in *Daphnia* (Macke et al. 2017), were present but only at low levels, in particular late in the season when a peak in microcystin-producing cyanobacteria abundance normally occurs. Further studies are therefore necessary to test if clones with high levels of Flavobacteria, or other putative candidate gut bacteria, maintain tolerance to toxic cyanobacteria in a natural environment (Houwenhuyse et al. 2021).

In summary, the gut microbiome of *Daphnia* is highly variable over the season but, within populations, host genetic effects on microbiome diversity and composition can be vanishingly small. These results emphasize the importance of studying host–microbiome interactions in the field, and that establishing the ecological effects of the gut microbiome will require large-scale experiments under natural conditions.

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### Author contributions

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### Data availability statement

Data are available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.9s4mw6mg5> (Hegg et al. 2021). Raw sequences have been deposited in NCBI Sequence Reads Archive (SRA) with accession number PRJNA748877, <www.ncbi.nlm.nih.gov/bioproject/PRJNA748877>.

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