Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

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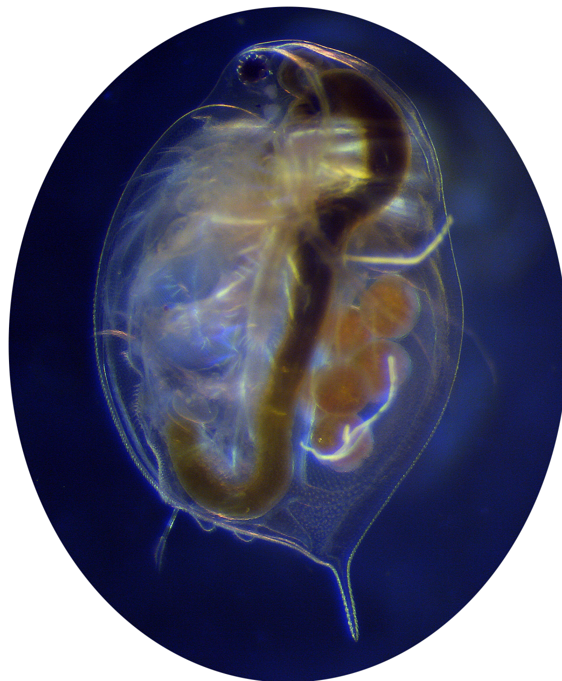
Investigation of the effect of cohabitation on the microbiome and fitness of two *Daphnia* species.

Master's thesis in MSBIOTECH
Supervisor: Olav Vadstein
Co-supervisor: Sigurd Einum
May 2022



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Abstract

The purpose of this project was to investigate the effects of cohabitation of *Daphnia magna* and *Daphnia pulex* on the microbiome and fitness of the respective species. The study used laboratory techniques on *D. pulex* which have not been applied to this species before. Specimens were cultured in shared and separate aquaria for three weeks. Their microbiome was transferred to aposymbiotic juveniles in six treatment groups (3 per species, endogenous, co-culture and foreign microbiota). Fecundity and body length was measured. Gut and water samples were taken from the animals and their aquaria. Due to mortalities in the *D. pulex* cultures, the three treatments on *D. pulex* did not yield enough data to analyze statistically.

The bacterial community in the samples was analysed with 16s metagenomics. Significant differences in microbial community was detected based on treatment. No significant difference in microbial community was found based on fecundity. Treatment could not be correlated to changes in fecundity.

Sammendrag

Formålet med denne oppgaven var å undersøke effekten av kohabitering av *Daphnia magna* og *Daphnia pulex* på mikrobiomet og levedyktigheten til de respektive artene. Studien benytter labteknikker som ikke er anvendt på *D. pulex* tidligere. Dyrene ble kultivert i kohabiterende og separate akvarier i tre uker. Etterpå ble mikrobiomet deres overført til aposymbiotiske juveniler i seks behandlinger (3 for hver art, endogent, cohabitat og fremmed mikrobiom). Fekunditet og kroppslengde ble målt. Tarm og vannprøver ble tatt fra dyrene og deres akvarier. På grunn av høy dødelighet blandt *D. pulex* kan prøvene fra disse tre behandlingene ikke analyseres statistisk.

Bakteriesammensetningen i prøvene ble analysert med 16s metagenomikk. Signifikante differanser i bakteriesammensetningen ble funnet når prøvene ble gruppert etter behandling. Ingen signifikant differanse ble funnet når prøvene ble gruppert etter fekunditet. Behandling kunne ikke korreleres til fekunditet.

Acknowledgements

First, I would like to thank my supervisors Olav Vadstein and Sigurd Einum, for providing me with interesting challenges, and giving me the independence to fail or succeed on my own. I would then like to thank the group members of ACMS, who always answered my questions, and helped me to find the knowledge I needed. To the wonderful students in Volvox & Alkymisten, and the faculty of IBT and IBI, I raise my glass to you, your companionship and support has kept me going to the end. Finally I would like to thank my parents. You have always let me be myself, instead of pushing me into the mould of society.

Abbreviations

Abbreviation	Meaning
16s rRNA gene	The DNA sequence coding for the 16s Rrna subunit
ASV	Amplicon Sequence Variant
CBD	Centre For BioDiversity at the institute of biology NTNU
CFU	Colony Forming Unit
DNA	DeoxyriboNucleicAcid
dNTP	Deoxyribonucleotide triphosphates
dph	days post hatching
GA	GlutarAldehyde
GI	Gastrointestinal
NGS	Next Generation Sequencing
nt	nucleotide (usually used in reference to length)
NTC	non-Template Control
OTU	Operational Taxonomic Unit
PC	Positive Control
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
rDNA	ribosomal DNA
rRNA gene	The DNA sequence coding for a Ribosomal RNA subunit
Taq polymerase	*Thermophilus aquaticus* polymerase
TOC	Total Organic Carbon
V3	Variable region 3 in the 16s Rrna gene
V4	Variable region 4 in the 16s Rrna gene

Disambiguations

The following terms are ambiguous or used in an ambiguous fashion in this thesis, and their usage is explained.

Metagenomics: unless stated otherwise the term metagenomics will be used to refer to amplicon sequencing of V3-V4 16s region.

Sequence identity: The proportion of nucleotides that match exactly when two genetic sequences are aligned.

Taxonomic distance/percent identity will be used to refer to the sequence identity percent of the V3-V4 16s rDNA fragments. ie. ”has a taxonomic distance of 3 percent” will mean that two species or OTUs have 97% sequence identity in their V3-V4 regions.

High fidelity polymerase (HF-pol) will be used to refer to recombinant polymerases with proof reading activity.

The term aposymbiotic will be used to refer to animals which have been treated with a disinfectant, and therefore have no symbionts attached to them.

Parent microbiome is used as a descriptor for a microbiome which is transferred from one culture/habitat to aposymbiotic animals or otherwise moved from one culture/habitat to another, with the intent of colonizing the new culture/habitat with the microbiome in question.

Absolute/relative quantification. In molecular biology, many analyses that use PCR yield data where you cannot count the absolute abundance of a gene or organism. With these analysis you instead analyze the relative abundance of gene/organisms X vs. gene/organism Y. This is known as relative quantification.

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1 Introduction

Microbiology and freshwater ecology are two fields of study which are converging as methods to analyze microbiomes progress. With 16s metagenomics, it is possible to characterize the bacterial composition of the microbiome from environmental samples. This information can be used to study how host-microbe interactions influence the populations in a habitat, and how or if the microbiomes of one species affect other species.

1.1 Daphnids as model organisms

The species *Daphnia magna* Straus 1820 and *Daphnia pulex* Leydig 1860 are two cladoceran freshwater zooplankton in the genus *Daphnia*. Both species are naturally occurring in large parts of Northern Europe (de Jong et al., 2014). Despite broad geographical coverage, they rarely cohabit. In the cases where the two species have been found to cohabit, it is due to unstable conditions that overturn competition and allows periodic recolonization (Hanski and Ranta, 1983).

The composition, stability and change in the microbiome of *Daphnia* species; primarily *Daphnia magna*, has been the object of study with different techniques, including 16s metagenomics (Freese and Schink, 2011) (Peerakietkhajorn et al., 2015b) (Callens et al., 2020). These changes may be spontaneous (Obrestad, 2020), or induced (Motiei et al., 2020). Compositional changes occurring in the gut after death have also been studied (Freese and Schink, 2011), in an attempt to study the power of active symbiont selection in live organisms.

The effect of different perturbations on the microbiome and health of Daphnids have been studied. This includes the effects of diet (Taipale et al., 2012), antibiotics (Motiei et al., 2020) and the effects of microbiome origin, when foreign microbiomes are introduced to bacteria-free daphnids (Callens et al., 2020). These studies pave the way for understanding the mechanisms behind host-microbe interaction, and the resulting effect on animals and the biome. Further studies of how microbiomes change and behave in perturbed systems may allow elucidation of second-order effects of changes in environment on the ecosystem as a whole.

1.2 Knowledge gap

Food competition (Kreutzer and Lampert, 1999) and different predation patterns (Milbrink and Bengtsson, 1991) (Dawidowicz and Wielanier, 2004) (Leibold, 1991) have been offered as explanations for why *Daphnia* species rarely coexist. Previous cohabitation experiments indicate that competition occurs even in food replete conditions with minimal predation pressure (unpublished data Sigurd Einum), but it is not well understood how the species compete in such conditions.

Research has shown that some organisms are capable of exerting very strict control over microbiome colonization (Byndloss et al., 2018) (Schluter and Foster, 2012). The microbiota of *Daphnia* has also been demonstrated to change in response to environmental factors such as temperature (Sullam et al., 2017) or the physical origin of growth media in lab cultures (Callens et al., 2020). This indicates that the microbiome adapts in response to altered conditions. Presumably this adaptation is caused by changes in both host selection and the competitive balance in the bacterial community. It is however, not understood if microbiota plays a role in the interactions between coexisting *Daphnia* species, or if the microbiota is influenced by cohabitation. Answering these questions could help explain inter-species competition that cannot be ascribed to currently known mechanisms.

1.3 Aim

In this study, the two species will be made to cohabit purpose made aquaria, in food-replete conditions. Investigating the effects of this cohabitation on the fitness of each species, and the effects on their microbiomes is the primary goal of the study.

The microbiota of *D. pulex* has seen some study, but as far as I know the production of aposymbiotic *D. pulex* eggs has not taken place. An additional goal of the study is to optimize procedures for microbiome studies that have been used on *D. magna* for use on *D. pulex*.

The following questions are investigated in this project.

- Can *D. pulex* be used as a model organism for microbiome studies where production of aposymbiotic juveniles is required.
- Does cohabitation of *D. pulex* and *D. magna* cause significant changes in microbiome composition of the gut and/or habitat.
- Does cohabitation of *D. pulex* and *D. magna* cause significant changes in the fitness of either or both species.
- Can any observed changes in fitness and microbiome composition be correlated.

2 Theory

In this chapter the theoretical background for techniques and methods is described, including background knowledge on daphnids. Information and knowledge that informs the methods and materials choices are given.

2.1 Known occurrence of induced dysbiosis by cohabitation

The competitive advantages of tolerating a microbe that negatively affects your competitor has been seen with the artificial introduction of signal crayfish *Pacifastacus leniusculus* to Sweden (BACK, 1995) and Norway (Strand et al., 2019). Signal crayfish are resistant to *Aphanomyces astaci*, a small protist which is pathogenic to the naturally occurring noble crayfish *Astacus astacus* (ALDERMAN et al., 1987). As a result there is a major selective advantage for the invasive species and a rapid extirpation of noble crayfish ensues in most waterways infested with signal crayfish. Analogous mechanisms may exist in most, or all ecosystems. And the competitive advantage they lend may take a multitude of forms.

2.2 Antibiotics study on *D. magna*

Assumptions are sometimes made about the correlation between fitness and diversity. Some studies have approached the issue by treating *Daphnia* cultures with antibiotics to reduce diversity (Motiei et al., 2020). The expectation was a reduction in fitness. Somewhat surprisingly, treatment with antibiotics resulted in increased fitness (Motiei et al., 2020).

2.3 Major symbiont

Metagenomic analysis on *Daphnia* microbiomes has resulted in the identification of the genus *Limnohabitans* (Hahn et al., 2010), and especially the species *Limnohabitans planktonicus* as a major symbiote of *D. magna*, and the determination of its essential role in *D. magna* reproductive ability and longevity (Freese and Schink, 2011) (Peerakietkhajorn et al., 2015a) (Peerakietkhajorn et al., 2015b). Aposymbiotic *D. magna* do not reproduce successfully (Peerakietkhajorn et al., 2015a). *D. magna* which has been colonized with cultured *Limnohabitans spp.* only, grow and reproduce well (Peerakietkhajorn et al., 2015b). This indicates that fecundity may be closely tied to symbiosis with a single species of bacteria.

2.4 Gnotobiotic systems

A gnotobiotic system is an isolated habitat where all organisms have been identified (Basic and Bleich, 2019). This can be a single species, or multiple known species. Gnotobiotic systems are used for studies of specific interactions between species. The idea was first introduced in the late 1800s as a theoretical concept. By 1946, germ-free life cycle completion for mammals and birds had been reported (Reyniers et al., 1946) (REYNIERS et al., 1949). This discovery opened up the possibilities for performing research on animals with controlled microbiomes.

Gnotobiotic systems are useful for elucidating the effect of single symbiont colonization, or specific microbiome perturbations. The controlled environment removes parental effects of the microbiome. That means that the fitness of the experimental animals that have been introduced to new microbiomes is technically independent of the origin culture of the microbiome.

2.4.1 Preparing a gnotobiotic system of daphnid cultures

Disinfection procedures of *D. magna* eggs with glutaraldehyde allow for the production of bacteria-free neonates (Callens et al., 2015) (Obrestad, 2020) (Peerakietkhajorn et al., 2015b). These neonates have no microbiome, and can be colonized with a microbiome that has been recovered from or perturbed with specific conditions. Treatment groups in the current study are separated by the origin of the parental microbiome that was used to inoculate the neonates.

Experimental individuals are assigned randomly to a treatment protocol from a common pool of bacteria-free neonates and then inoculated. The effect of different microbiomes on fitness markers can thus be isolated and studied. Characterization of the induced microbiomes can be used to correlate the compositional changes in microbiome due to treatment, with fitness changes in the *Daphnia* populations. Disinfected animals from separate stock cultures are assigned to treatment groups at random. Therefore, parental effects are randomized, both those from microbiome and other sources.

The optimal GA concentrations, contact period, and egg development stage for producing bacteria-free *D. magna* neonates have been investigated by among others, Obrestad (2020) and Callens et al. (2015). Their findings indicate that adequate disinfection is reached by submerging eggs in 0.025% GA for 30 minutes. They also prove a strong correlation between egg developmental stage and hatching rate. Eggs that are either less than 12 hours or between 12 and 24 hours are best suited for disinfection. When visually screening eggs for disinfection, the optimal egg developmental stage is right after the chorion begins to visibly detach from the egg (Obrestad, 2020).

2.5 16s Metagenomics

Carl Woese and colleagues are considered pioneers in the field of microbial phylogenetics. By analyzing 16s rRNA genes they were able to determine that archae was a separate domain from bacteria (Woese and Fox, 1977). Their work was later refined by Pace and colleagues at Indiana University, who began using 16s rRNA for phylogenetic studies on bacteria (Lane et al., 1985). Their protocols allowed them to investigate evolutionary relationships between cultured bacteria, and eventually uncultured bacteria. Sequencing technology developed to the point where environmental DNA could be isolated and analyzed directly without culturing, this is what we now call 16s metagenomics (Schmidt et al., 1991).

The 16s rRNA gene (Figure 2.1) is present in all bacteria (Woese, 1987). It is the most common target of sequencing analysis intended for phylogenetic mapping of a sample. The gene encodes a subunit of the ribosome which binds the Shine-Dalgarno sequence (Shine and Dalgarno, 1975). Due to its role as promoter binding unit, the 16s sub-unit has a number of very highly conserved sequences for which ”universal bacterial primers” may be constructed (Woese, 1987). Between the conserved sequences, there are variable regions that mutate at higher speeds. Similarities and differences between these variable regions are used to determine or evaluate the phylogeny of bacteria present in the sample (Edgar, 2016a).

2.5.1 DNA purification

Traditionally, DNA extraction for PCR was performed with phenol-chloroform based protocols (Tan and Yiap, 2009), these are time-consuming and require toxic reagents (Psifidi et al., 2015) (Tan and Yiap, 2009). Phenol-chloroform extraction has now largely been replaced by silica membrane and paramagnetic bead-based protocols (Berensmeier, 2006) (Gaget et al., 2016). These are faster, and have fewer toxic components (Psifidi et al., 2015). Paramagnetic bead extraction is better suited than silica-based extraction for high trough-put workflows because it can be performed on a 96-well plate, as opposed to using individual tubes. When working with difficult samples, proprietary kits are available which remove PCR inhibitors such as humic acid in soil samples. These new extraction methods yield high-purity DNA from low biomass samples (Gaget et al., 2016) (Psifidi et al., 2015). To make study designs with gut samples from single individuals of *Daphnia* possible, the advantages of bead-based extraction methods are important.

2.5.2 PCR amplification of V3-V4 rRNA DNA with universal bacterial primers

In 16s metagenomics, multiple aspects of the chemistry in a PCR reaction may affect the downstream results. The primers used are "universal bacterial primers" which are designed to broadly target many bacterial genomes at the 16s V3-V4 region. When analyzing gut samples, due to the highly conserved nature of the target regions, host DNA may be compatible with the primers and cause contamination. This has been observed with the 18s rRNA genes from salmon (personal communication, Amalie Mathiesen NTNU). This has not been observed with *D. magna* or *D. pulex* samples, but is an issue that must be dealt with when applying 16s metagenomics to new sample types.

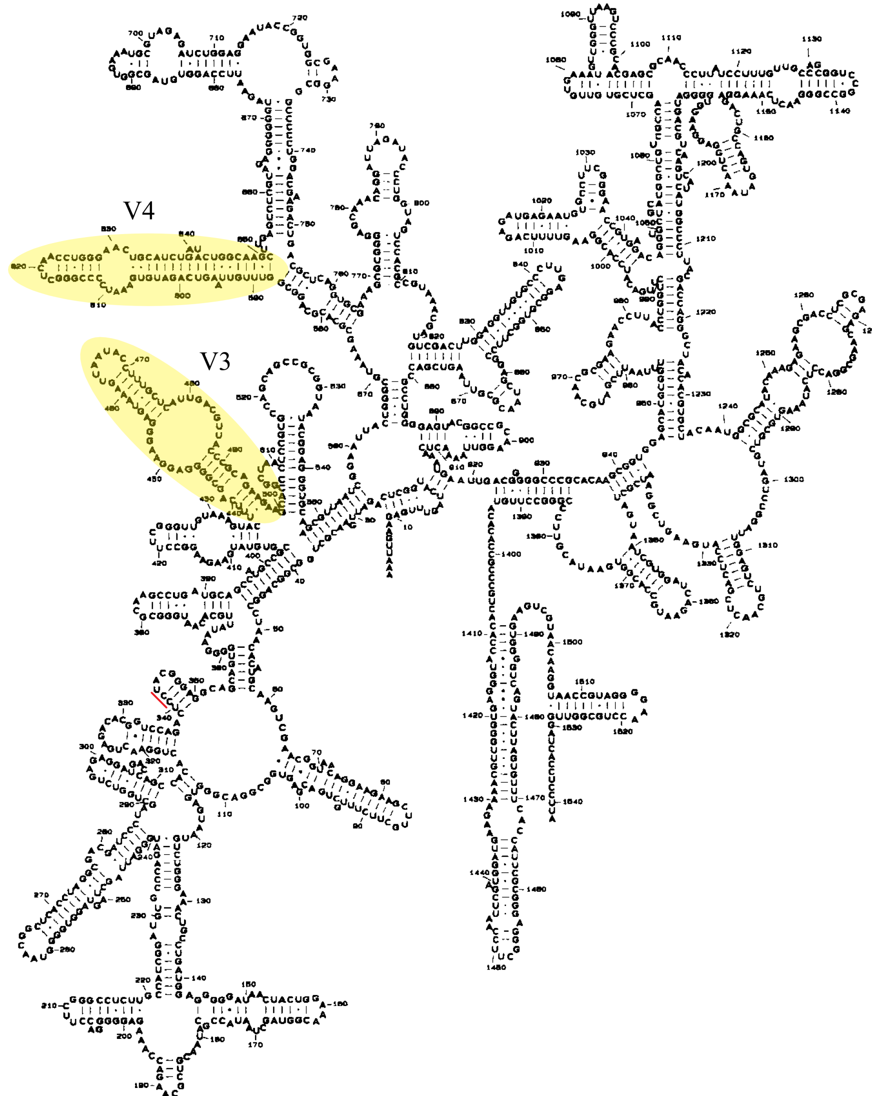


Figure 2.1: The secondary structure of the *E. Coli* 16s rRNA subunit. The position of the V3 and V4 regions have been highlighted (Madigan et al., 2017). The figure has been adapted from Gutell et al. (1985).

Not all bacteria can be targeted by a single primer set. All primers used for 16s metagenomics have their own inherent bias (Brooks et al., 2015) (Nearing et al., 2021). This means that entire taxonomic classifications like phyla or orders may be absent from data due to primer incompatibility. The primers used in this study are designed to have the widest possible coverage of known aquatic bacteria (personal communication Ingrid Bakke). A search was performed of the primer pair 341f-k, 805r in the RDP (Wang et al., 2007) database for probe matches with 0 diffs. The results from this search indicate that the primer pair has good coverage for phyla relevant to aquatic samples (personal communication Ingrid Bakke). The primer pair covers 62% of good quality entries in the RDP database, and 96% of the type strain entries. The primer pair seems to cover all major phyla in the RDP database. For investigating changes over time or differences in composition between sites, primers with high type-strain coverage is a sensible choice. Type-strains are often well understood in their metabolic profiles and ecological preferences. Therefore much information can be gathered from the data.

Other genetic factors also influence the PCR efficiency of individual species 16s rRNA genes. Targeted PCR amplification can be seen as an immensely complex multiplex PCR. In the competitive environment of a PCR reaction, minor differences in efficiency compound. Proportional abundances of different bacteria in the sample, therefore, do not correspond well to the proportional abundances of their sequences in the dataset (Brooks et al., 2015) (Nearing et al., 2021).

2.5.3 Polymerase errors in PCR

It has become customary to use polymerase with proofreading activity when preparing genetic material for sequencing. These proofreading polymerases have lower error rates than Taq polymerase, and give higher fidelity libraries (McInerney et al., 2014) (Ahn et al., 2012). McInerney et al. (2014) investigated the fidelity of multiple polymerases, and compared them to each other and their marketed performances. Their conclusions indicate that proofreading polymerases generally have an error rate one order of magnitude lower than Taq polymerase.

Ahn et al. (2012) investigated the behaviour of polymerases in 16s targeted amplification of bacterial DNA with mock communities. Their findings indicate that Phusion polymerase reaches PCR saturation earlier than Taq polymerase, and that PCR experiments run past the saturation point may experience an elevated number of chimeric sequences.

Their findings give a strong imperative to maintain vigilance and not forget that PCR fidelity is a multi-factor issue, and cannot be solved with higher fidelity polymerases alone. They suggest restricting PCR cycle number and to calculate OTUs with a minimum evolutionary distance of 0.03 (97% sequence identity). This was the point in which no mis attribution of OTUs occurred due to chimerism in their experiment, in which phusion polymerase PCR was run for 15 cycles. (Ahn et al., 2012) These results are valid only for their specific workflow. However the article demonstrates a clear need for in-house optimization of 16s metagenomics protocols. Based on the findings of Ahn et al. (2012), OTUs in the current study are clustered at 3 percent evolutionary distance, to avoid ambiguity about methodological stringency.

2.5.4 16s gene copy number variation

Bacteria have different gene copy numbers of the 16s rRNA gene (Farrelly et al., 1995). Which means that bacteria of species (A) may have one single copy of the gene, while bacteria of species (B) may have 10 copies of the gene. In a sample with equal numbers of bacteria A and B, the relative amount of 16s rRNA gene copies in the sample from A and B would be 1:10, thus significantly distorting the relative abundances as measured by 16s metagenomics. Attempts have been made to correct for this by different methods (Farrelly et al., 1995) (Kembel et al., 2012) (Louca et al., 2018). According to Louca et al. (2018) a satisfying solution has not been found. This precludes the accurate estimation of real abundance based on sequencing reads alone, and affects how 16s data must be analyzed.

2.5.5 DNA normalization

DNA normalization is the process of diluting or otherwise manipulating the DNA concentrations in a range of samples, to obtain comparable amounts of input from each sample into the next step of a workflow. Normalization after targeted amplification corrects for technical variance in PCR efficiency. The normalization of DNA after indexing ensures that a comparable amount of input from each sample is supplied to the sequencing experiment. This prevents marginalization of low input samples in the sequencing process.

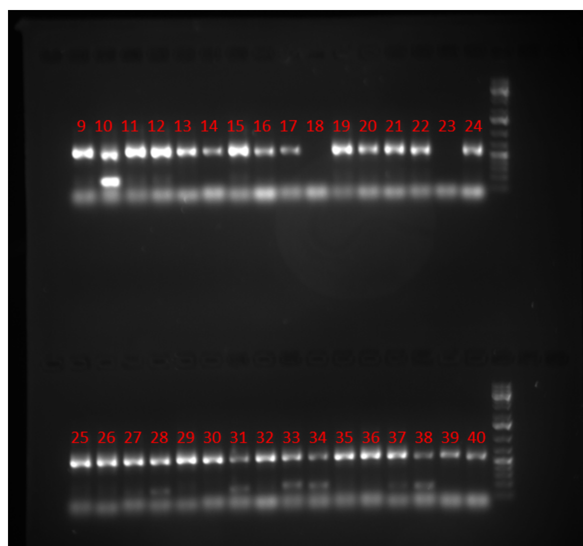


Figure 2.2: Indexing PCR with Illumina nextera V2 set D primers. Sample numbers in red. Primer-dimer formation can be seen in multiple wells, most prominent in sample nr. 10.

The realities of the bacterial DNA, and sample preparation workflow, influence the analysis of the data obtained from 16s sequencing. Due to the non-correlative relationship between the number of bacteria in a sample, and the number of reads obtained from a sequencing run, quantification cannot be performed on 16s metagenomics data. For this reason analysis of 16s metagenomics data rely on differences in abundance between samples rather than counts of individual bacteria. It is important to state that even without the normalization process, read counts from the sequencing would not correlate to CFU count in the input (Brooks et al., 2015). The distribution of read counts per sample would be significantly more skewed, in ways that cannot be compensated for in data analysis.

2.5.6 Indexing and sample pooling

Indexing was performed with Illumina nextera XT v2 indexing primers. The chemistry is a PCR. In the indexing PCR, a barcode sequence is ligated to each end of the Illumina tagged amplicons. The barcodes are supplied in two series of 8 and 12 primers, giving 96 unique combinations. This enables samples to be multiplexed during sequencing. The sequencing reads contain the barcodes at either end, and are separated by sample origin during the data processing.

Each combination of barcodes has different chemical characteristics in a PCR reaction: PCR efficiency is different for each combination, Some combinations have a tendency to form primer-dimer (Figure 2.2). This results in uneven amplification profiles, and is the primary reason for normalizing DNA again after indexing.

2.6 Data processing

The data analysis of large microbiome datasets consists of computational processes that compile large amounts of raw data (15 giga-base pairs for Illumina MiSeq sequencing lanes) into a human-readable format. In this process, errors can be made. Many of these errors are "invisible" because the researcher cannot verify or peruse the input and output manually.

A tremendous amount of work is being laid down on the task of designing and quality ensuring the bioinformatic pipelines used in metagenomics data processing (Prodan et al., 2020). With the use of mock communities, pipelines can be rigorously tested and prepared for use in real analysis. While this work is ongoing, researchers must use the best tools currently available to process their data. At ACMS metagenomic data is processed with the Usearch pipeline, which was determined by Prodan et al. (2020) to perform well compared to other availible pipelines in testing with mock communities.

2.6.1 Alpha diversity

The diversity of a community can give important information about how the community is structured. Analysis of diversity is performed with Hill numbers (Hill, 1973). Lucas et al. (Lucas et al., 2016) recently argued that a unified system of alpha diversity metrics should be used. They propose Hill numbers or "diversity of order q" as a method for diversity measurement. Hill numbers of each order are all calculated with a single equation, where the exponent q is the order. This creates a coherency between the different metrics, and permits an intuitive understanding of how each analysis weighs dominance against richness.

Equation 2.1 describes the calculation of diversity with Hill numbers. The proportional abundances (p) of each species to the power of q are summarized for the number of species present (R). Diversity of order q is then ^{q}D, the reciprocal of the q weighed mean relative abundances (Lucas et al., 2016).

For $q = 0$ Equation 2.1 returns the number of species, or richness. Richness is the number of species present in the sample. For $q = 2$ Equation 2.1 returns the reciprocal of Simpsons index (Lucas et al., 2016). This index weighs species abundance n, for the species $1 \rightarrow i$ as $n_i(n_i - n)$ or the square of abundance minus one degree of freedom.

$$ {}^{q}D = \frac{1}{{}^{q}\bar{p}_i} = \left(\sum_{i=1}^{R} p_i^q \right)^{1/(1-q)} \tag{2.1} $$

2.6.2 UniFrac distance

Using a phylogenetic tree of the species in a dataset, with abundances, UniFrac attempts to quantify the evolutionary distance between two or more sites/samples (Lozupone and Knight, 2005). This is done by dividing the sum of shared branch lengths for two samples, by the sum of all branch lengths in the phylogenetic tree. Weighted UniFrac is weighted by abundance for each taxa, unweighted takes only presence/absence into consideration. As the analysis requires a rooted tree, the phylogenetic tree in the current study was rooted by the longest terminal branch.

2.6.3 Bray-Curtis dissimilarity

Bray-Curtis dissimilarity (Bray and Curtis, 1957) is a method for describing the difference in the abundance of shared species between two sites/samples. The method describes the ratio of the difference of abundance between sites with the sum of abundance in both sites according to Equation 2.2. In which C_{ij} is the "sum of lesser counts" if the number of observations of a species N at sites a and b is $N_a < N_b$ then C_{ij} is $N_a + |N_a - N_b|$. S_i and S_j are the total number of observations of species N at sites a and b. BC_{ij} is the Bray-Curtis dissimilarity.

$$BC_{ij} = 1 - \frac{2C_{ij}}{S_i + S_j} \tag{2.2}$$

2.6.4 Sørensen-Dice dissimilarity

Sørensen-Dice index (Dice, 1945) is a measure of the number of species that are present in one sample/community but not in another. It measures only presence/absence and does not take differences in abundance into account, no matter how large. This index is useful to describe microbiomes with discrete differences in microbiome composition. It is calculated by the Equation 2.3, where X and Y are the number of species in each sample.

$$DSC = \frac{2|X \cap Y|}{|X| + |Y|} \tag{2.3}$$

2.6.5 PCoA plotting and PERMANOVA

When computing the beta diversity indices described in the preceding section, a distance matrix is made with multiple orthogonal axes. To visualize the differences among samples, a PCoA plot is made.

The primary benefit of the PCoA plot method for visualizing data from 16s metagenomics studies is that the plots only visualize internal dataset variation. When working with 16s data it is important to be careful before drawing conclusions based on data that originates from two different studies. The PCoA plots do not display absolute numbers, and only account for the differences between samples in one dataset. Therefore, the plots are well suited to analyze 16s data, without making an external comparison tempting or easy.

In a PCoA plot, each sample is represented with a point. The distances between the points correspond to the dissimilarity of the samples. Samples that are more different, lie further apart. The proportion of variability that is expressed by a given axis of a PCoA is called an eigenvalue, this value is annotated on the figure axis. The distance matrix for a dataset with many samples cannot be described on a two-dimensional plane. Therefore, the significance of any patterns cannot be tested with the plot alone. When patterns are seen in a PCoA plot, the significance of this pattern can be assessed with PERMANOVA (Anderson, 2017).

PERMANOVA uses the distance matrix that is underlying a PCoA plot directly. It is a semiparametric method to analyze variance. It applies a permutational variance analysis to a dataset of two or more subsets, testing if the centroid (geometric center) of each subset is significantly different. The output is a p-value. If clustering trends are observed in a PCoA, PERMANOVA can be used to verify this.

3 Materials and methods

When working with naked DNA, it is important to describe any equipment which comes into contact with your samples in as great a detail as possible. It has been reported that laboratory reagents used in metagenomics sometimes contain DNA (Salter et al., 2014a) (Voirol et al., 2020). Due to this, a detailed list of equipment is provided in Appendix B.

3.1 Experimental design

3.1.1 Culturing conditions

Daphnia cultures were maintained in ADaM medium (Klüttgen et al., 1994) which was changed every 7 to 9 days. The recipe for the medium is given in Appendix C. The cultures were kept at 19 °C with a 16:8 hour light-dark cycle. Each *Daphnia* culture was fed live, axenically grown *Raphidoceles subcapitata* (Suzuki et al., 2018), corresponding to 0.2mg TOC per day per individual, on Mondays, Wednesdays, and Fridays[1].

3.1.2 Pilot experiment

The purpose of the pilot experiment was to test the protocols and create reference data for fecundity measurements. *D. magna* eggs were extracted and disinfected. After hatching, the aposymbiotic juveniles were transferred to two 800 ml beakers. 16 and 17 individuals were added to the cultures, which were named 3.1 and 3.2 respectively. They were inoculated with their parent microbiome through the addition of 200ml culture water, and one egg-free adult that was allowed to cohabitate for 48 hours. Stocking density was 1 *Daphnia* per 47 ml media. Water was changed every 7 days. The *Daphnia* were fed 3ml "pool solution" (2.5 parts Shellfish Diet 1800™ to 100 parts water) daily throughout the experiment. Fecundity data from this experiment is shown in Table 4.1. How many animals in stock cultures are required to obtain a given number of eggs for disinfection was also informed by the results of the pilot experiment.

[1] Pilot experiment cultures were fed Shellfish Diet 1800™

3.1.3 Main experiment

The main experiment was subdivided into two phases. Phase 1 where microbiomes for the single species and cohabitation conditions were established, and phase 2 where the isolated effect of the different microbiome treatments on *Daphnia* was tested.

Phase 1:

In phase 1 the following fifteen cultures were established with ≈ 24 hour old juveniles:

5x 200 ml aquaria with 5 *D. pulex* each.
5x 200 ml cultures with 5 *D. magna* each.
5x 400 ml cultures with 5 *D. magna* and 5 *D. pulex* each. The two species were separated by a 90 µm plankton screen.
Figure 3.1 show how the cultures were set up and named.

Phase 1 cultures were maintained under stable conditions for 21-25 days to stabilize the microbiome/conditions. During this period, offspring were counted and removed. At the termination of this phase of the experiment: the body length of the experimental animals was measured, animals were dissected to take gut samples, and water samples were taken from the aquaria. The method for measuring body length is shown in Figure 3.2. The sample taking method for microbial analysis of samples is explained in Section 3.3.2.

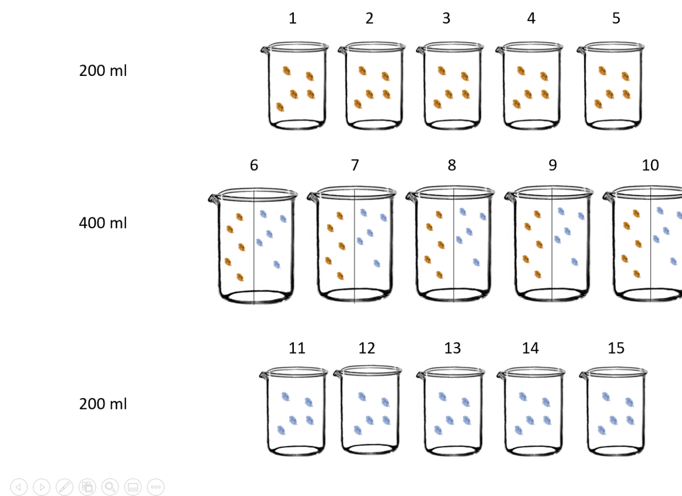


Figure 3.1: Culture setup for phase 1 cultures. *D. magna* in red and *D. pulex* in blue.

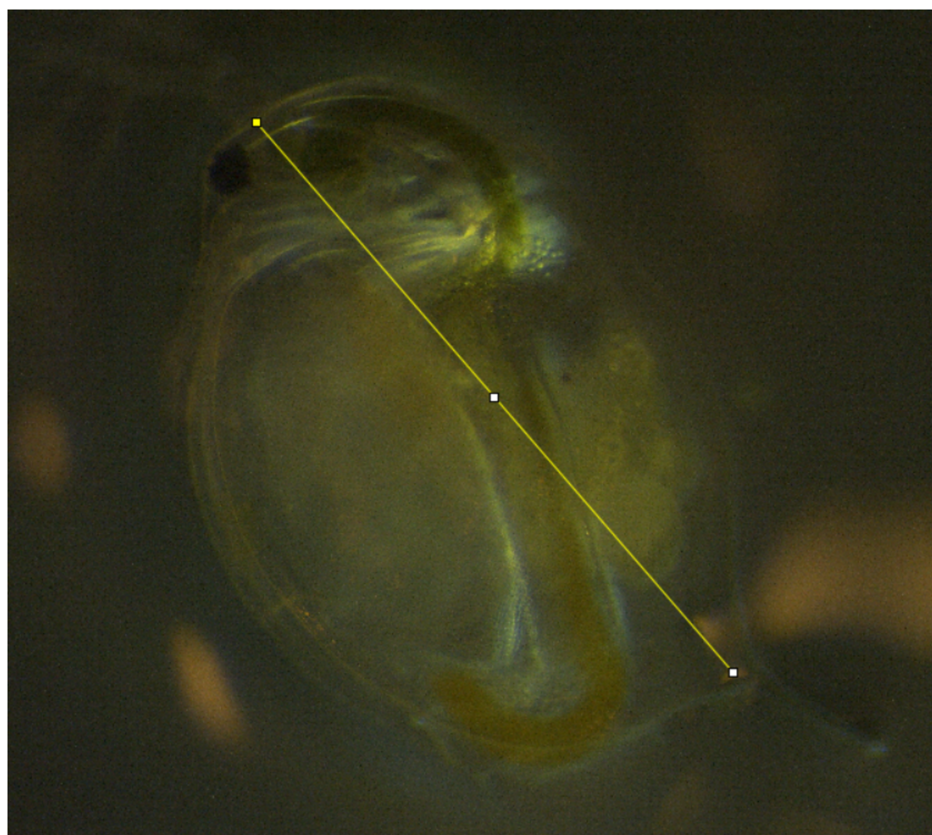


Figure 3.2: Example of how body length was measured from the base of the tail-spine to the apex of the head.

Phase 2:

In phase 2. cultures of five aposymbiotic juveniles of either *D. magna* or *D. pulex* were prepared as seen in Figure 3.4. The resulting array of experimental phase 2 cultures and their names are given in Table 3.1. The names will be used when referring to the cultures later. These cultures were then inoculated with 20ml water from the parent culture. Additionally, two adult *Daphnia* from the parent culture was added to the cultures inside a modified 50 ml Falcon tube with two rectangular windows, clad in 90 µm plankton screen (Figure 3.3). During phase 2, offspring was counted and removed. At the conclusion of the experiment, the body length of all experimental animals was measured. Gut samples were extracted from the animals, and water samples were taken from the aquaria.

Table 3.1: Which phase 1 culture microbiomes were transferred to which phase 2 cultures. Phase 1 cultures 1-5 contained *D. magna*, 6-10 are co-cultures, and 11-15 contained *D. pulex*. x indicates planned cultures which were not made due to insufficient juveniles

Culture names		
Phase 1	Phase 2	
	magna	*pulex*
1	1.2m	x
2	2.2m	x
3	2m-m3	2m-p3
4	4.2m	4.2p
5	2m-m1	2m-p1
6	6.2m	x
7	7.2m	7.2p
8	2mp-m3	2mp-p3
9	9.2m	x
10	2mp-m1	2mp-p1
11	11.2m	x
12	12.2m	x
13	2p-m3	2p-p3
14	14.2m	x
15	2p-m1	2p-p1

3.2 Sourcing and setup

Here follows a short explanation of how the *Daphnia* specimens and the live feed was obtained for use in the laboratory setup. I found it best to explain this before describing the procedures that were used in the experiment and in analyzing the samples.

Figure 3.3: *Daphnia* holding cage manufactured from a Falcon tube and algae net.

3.2.1 Obtaining *Daphnia* specimens

D. pulex specimens were obtained by disinfecting eggs sourced from stock cultures in the CBD *Daphnia* lab. The disinfection procedure is described in Section 3.3.1. They were then moved to a new lab area[2], and inoculated with the parental microbiome. Inoculant material consisted of a 50 ml sample of used culture media. This media was filtered, first with 3 µm, and then with 1 µm polycarbonate filters. Filtration was allowed to proceed with no application of vacuum.

D. magna specimens were obtained from the CBD *Daphnia* lab culture, clone 7A. They were introduced to the lab in the same manner as the *D. pulex* specimens. Due to lower than expected growth and lack of egg production the *D. magna* were re-inoculated with 200 ml used M4 medium obtained from an environmental toxicology lab which maintains OECD compliant *D. magna* cultures. After transferring specimens into the new lab, the cultures were allowed to adapt and stabilize for approximately a month. During this time the cultures were monitored closely to check for cyanobacterial blooms. Cyanobacteria form visible spots on the sides of the aquaria, as seen in Figure 3.5. Experimental specimens were chosen from 2nd clutch juveniles of animals born in the lab.

[2]Henceforth ”the lab”

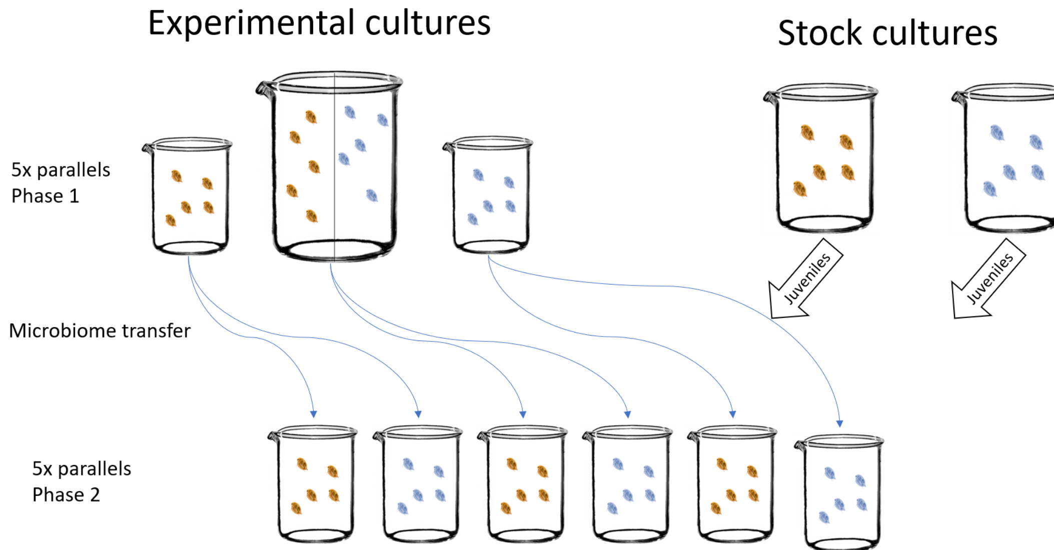


Figure 3.4: Flow chart representation of the cultures involved in both experimental phases. *D. magna* in red and *D. pulex* in blue. Microbiome transfer between phase 1 and phase 2 indicated by line-arrows. Juveniles for phase 2 were obtained by disinfecting eggs from stock cultures

3.2.2 Feed

Algae feedstock was obtained from Gabriël Olthof at the Department of biology NTNU. Algae were grown in the setup pictured in Figure 3.6. It consisted of two "towers" with a volume of approximately 1.5 l. Filtered air was pumped to the bottom of the cultures through a long glass tube submerged in the algae. Illumination was provided by a sunlight mimicking fluorescent light. Backup stock cultures of algae were kept in Erlenmeyer flasks, agitated by a shaking plate. Algae cultures were diluted as needed with OECD medium as described in Appendix F. The protocols were adapted as needed from those described in OECD test 201 (OECD, 2006).

To prepare the feed for consumption, the algae were transferred to 50ml falcon tubes, which were centrifuged at 3500 rpm for 3:30 seconds, in a Thermo-Fischer Scientific Heraeus Multifuge X1R. Supernatant was discarded, and pelleted algae was resuspended in ADaM. To estimate the TOC per ml of suspension, ABS at 440 nm was measured in a Hitachi U-5100 spectrophotometer. Inferences of TOC per volume of feed concentrate was made according to a standard curve constructed by Gabriël Olthof. The equation and standard curve is given in Figure 3.7. Ready for use feed was kept at 4 °C for up to a week. The tubes were reused and cleaned with alcohol between uses.

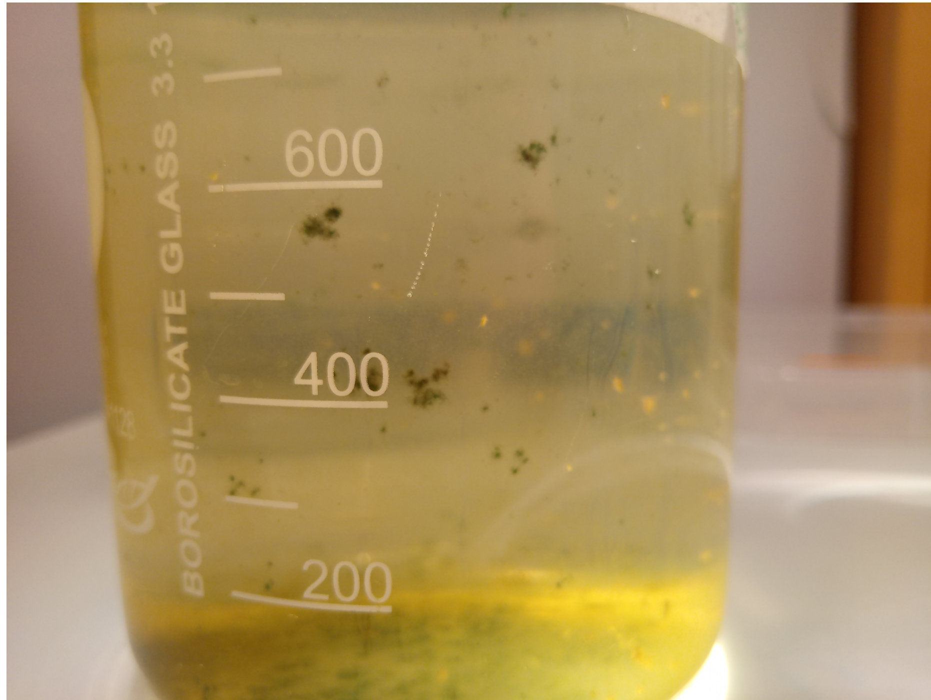


Figure 3.5: Visible spots on glass is cyanobacteria plaques. The cyanobacteria also form plaques on the water surface and incorporate themselves into the biofilm on the container walls. There are at least two species, one with a green macroscopic appearance and one with a black macroscopic appearance. The black species does not incorporate into the other biofilm but outcompetes it.

3.3 Laboratory procedures

3.3.1 Obtaining bacteria-free *Daphnia*

To obtain a large number of robust eggs deposited less than 24 hours in advance of the disinfection procedure, adult females with no eggs in the egg pouch were transferred into a separate jar and fed to saturating amounts of algae. Usually, this was done two days in advance, and after 1 day any egg-carrying females were removed from the holding aquaria and moved into the main aquaria. Then the egg-free adults from the main aquaria were transferred into the aquaria with egg-free adults. This increase how many eggs < 24 hours old can be obtained at once.

To disinfect the eggs, adult *Daphnia* carrying eggs <24 hours after ovulation was transferred into a dry petri dish. Excess media was removed with a fine-tipped pipette. The head of the individual was grabbed with forceps. Another forceps was used to grab the tail or back shield, and the carapace separated from the body. If the eggs did not separate from the carapace, a pipette with some media was used to shake the carapace up and down. Eggs were transferred to a 1.5 ml Eppendorf tube containing $\approx 0.5\,\text{ml}$ ADaM.

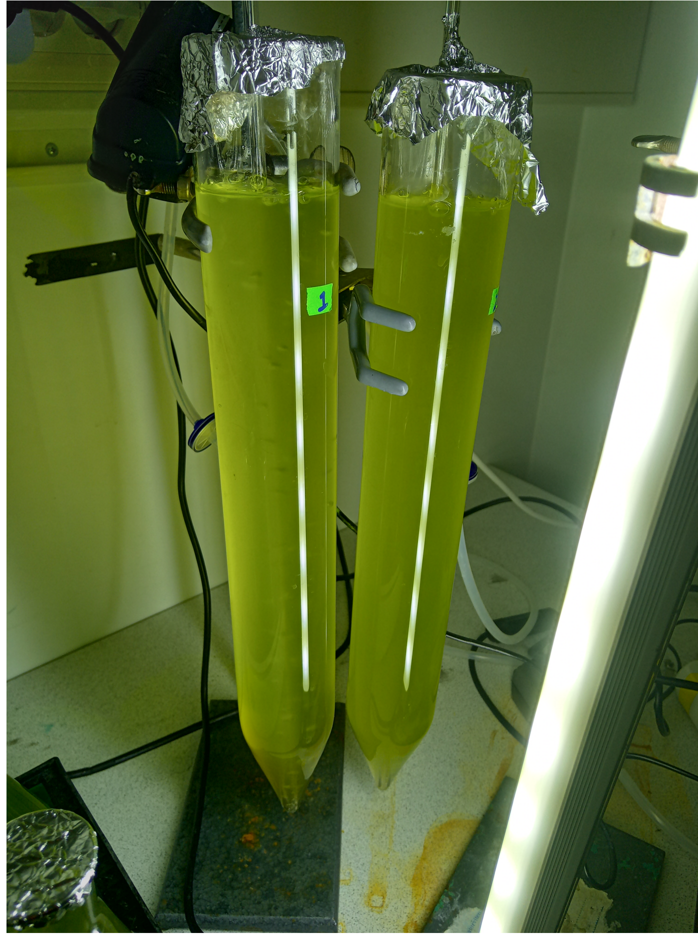


Figure 3.6: Growth tubes for algae. Filtered air is provided by a pump, and light is provided by a fluorescent light armature.

Eggs were pipetted into a cell culture dish containing ≈ 10 ml 0.025% GA solution. The eggs tended to stick to the side of the pipette and holding tube, especially *D. pulex* eggs. To reduce the number of eggs lost, the Eppendorf tube was flushed with GA solution as needed.

The eggs were incubated in the GA solution for 30 minutes. After the incubation in GA, eggs were washed with sterile ADaM. This was done by removing the GA solution and adding 10 ml sterile ADaM. The washing process was repeated five times, retaining the eggs in the same dish during all washing steps.

Cell culture dishes containing eggs and media were covered with parafilm and incubated at 19 °C for 72 hours. After hatching, the juveniles were counted and used in experiments. When opening the cell culture dishes, 100 µm of the incubation media was spread on TSA plates which were incubated for another 72 hours. This verified suitably bacteria-free conditions in the media as previously established by Obrestad (2020).

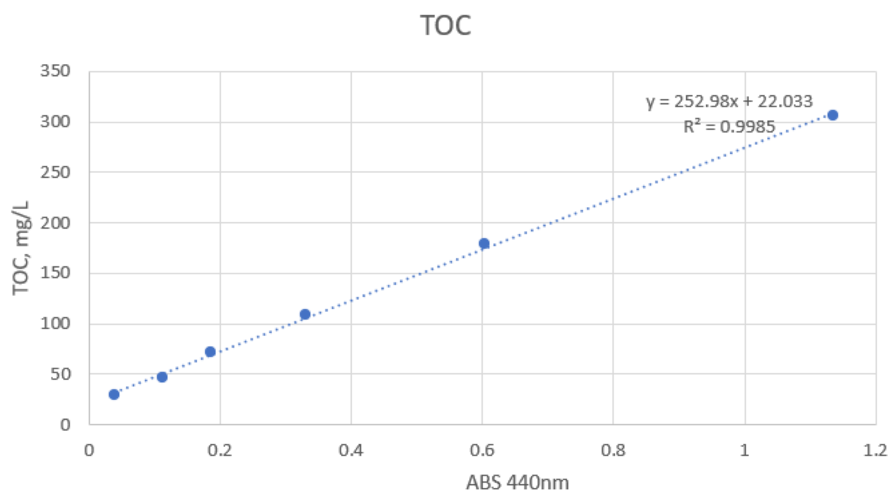


Figure 3.7: Standard curve for correlating ABS at 440 nm with TOC.

3.3.2 Sampling for microbial analysis

On termination of phase 1 and phase 2 cultures, water samples were collected for analysis. The aquaria were prepared for the sampling of water after removal of the adult individuals, any juveniles present were left in the aquaria. The biofilm was agitated with a sterile disposable plastic transfer loop. 100ml of the water/biofilm suspension was transferred to a stomacher bag and homogenized in a stomacher for $2 * 30$ seconds at 24 RPM. All preparations were done in a laminar flow hood with sterile equipment.

10 ml filtrate was collected from the filter compartment of the stomacher bag and vacuum filtered on a 0.2 um poly-carbonate membrane. The filters were stored in cryotubes and immediately frozen in liquid nitrogen. Water samples were initially collected with Sterivex filters, but difficulties with freezing the sample and processing the membrane prompted a change to Poretics membrane filters. The membranes were assembled on a 12-filter parallel vacuum line (Figure 3.8).

To extract gut samples, a live daphnid was taken from the aquaria and placed in a dry and cleaned glass petri dish. A forceps was used to grab the head. Another forceps was used to grab the tail meat, in a manner that avoided pinching the gut. The head and gut was then removed from the thoraxial and tail segments. By pinching the head correctly, the gut is freed from the head and can be pulled away carefully. Images of guts with and without a head can be seen in Figure 3.9 and Figure 3.10.

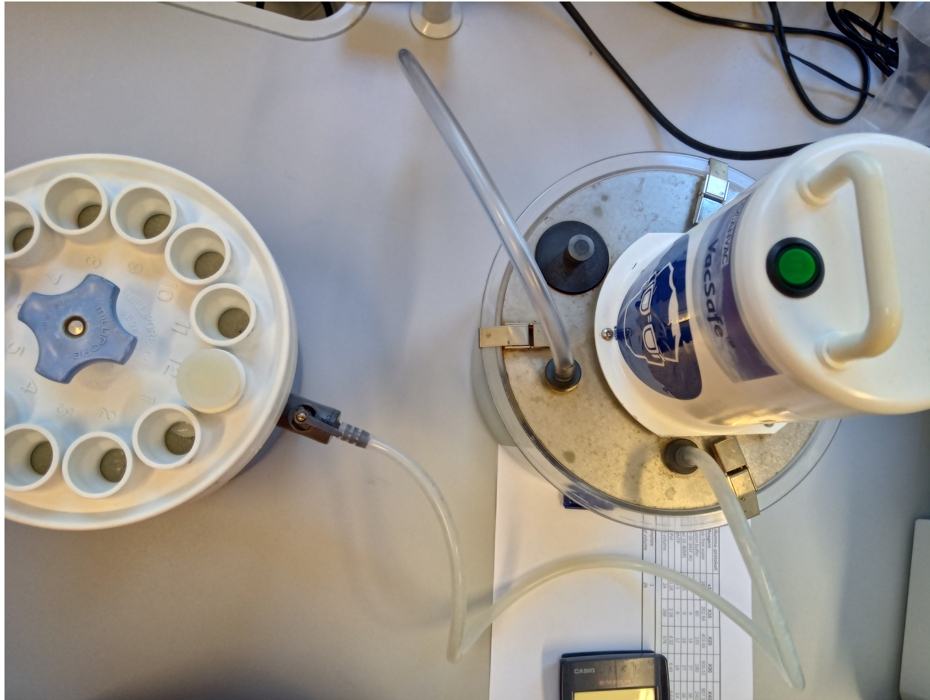


Figure 3.8: The 12 parallel vacuum filtration setup used in water sample preparation.

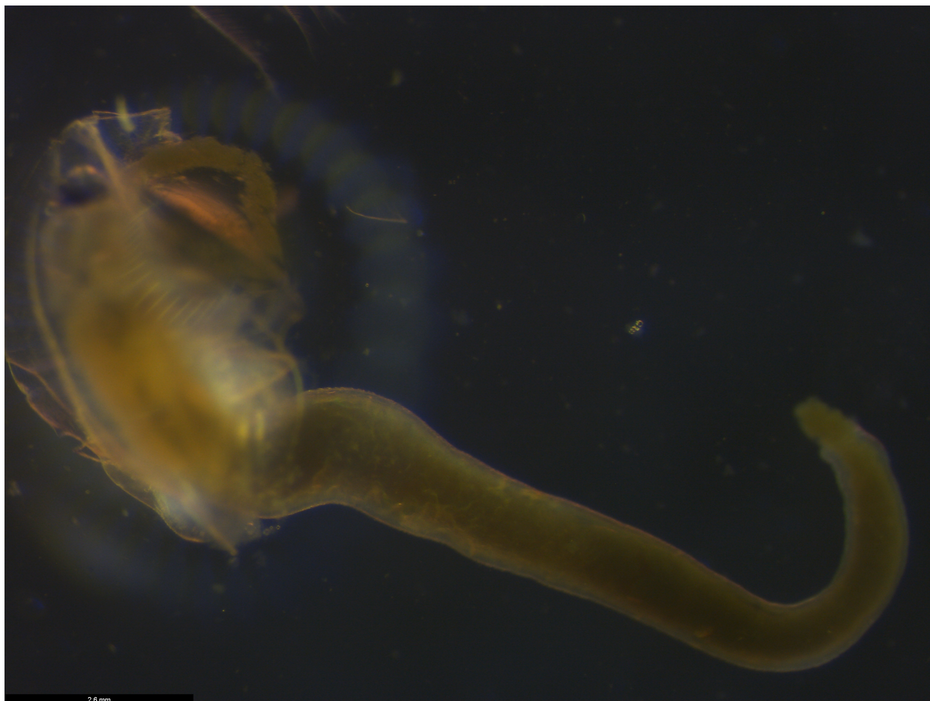


Figure 3.9: *D. magna* gut with the head still attached.

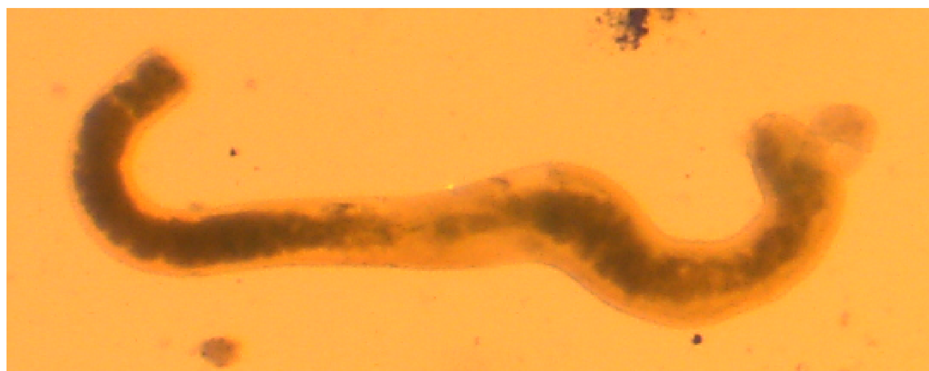


Figure 3.10: *D. magna* gut.

The extracted guts were moved to cryotubes with a disposable pipette and snap-frozen in liquid Nitrogen. To minimize cross-contamination between samples, the forceps and petri dish were cleaned with 96% ethanol and wiped down with tissue paper between uses. The technical blank for the sample extraction process was assembled with distilled water that had been washed over the cleaned petri dish. Some smaller individuals were sampled whole, to avoid risk of rupturing the gut (sample numbers 11,12,13,14). After snap-freezing the samples were stored at -18 °C

3.3.3 DNA Extraction and PCR

The Qiagen MagAttract Powersoil Pro DNA kit was used for DNA extraction. Manufacturers protocol can be found in the supplimentary information. Lysis was performed with a Precellys 24 with 1.4 mm Zirconium oxide beads. Homogenization was run for 2 x 30 seconds at 5500 RPM. DNA extraction and purification was performed on a Thermo scientific Kingfisher Flex.

Universal bacterial primers Ill341F-k1 (Muyzer et al., 1993) and Ill805R targeting the (V3-V4 region of 16s rDNA) was used to amplify the bacterial DNA marker region. The sequence of the primers is shown in Table 3.2. 2 µl of template was added to the reactions. Successful amplification was verified with gel electrophoresis, an example gel can be seen in Figure 4.1

Table 3.2: Primers for 16s V3-V4 targeted PCR (Sigma-Aldrich). The primers consist of an Illumina tag region, an N-linker region, and a bacterial 16s rDNA probe region in **bold**.

Forward primer	Ill341f-k1
5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNN**CCTACGGGNGGCWGCAG** 3'	

Reverse primer	Ill805R
5' TCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNN**GACTACNVGGGTATCTAAKCC** 3'	

Table 3.3: Master mix recipe for the targeted PCR with illumina adapter.

Reagent	Final concentration	Amount 1x
PRC grade water		16.82 µL
5x Phusion buffer HF	1x	5.0 µL
Ill341F_kI (10 µM)	0.3 mM	0.75 µL
Ill805R (10 µM)	0.3 mM	0.75 µL
dNTP (10mM each)	200 µM each	0.5 µL
Phusion HF-pol (2 units/ µL)	0.02 units/µl	0.18 µL
Template		2 µL

Typical cycling conditions (optimization may be needed for other primers)

98°C 2min

98°C 15 sec

55°C 20 sec X 36 cycles

72°C 20 sec

72°C 5min

4°C ∞

Figure 3.11: The cycling conditions used for targeted PCR.

3.3.4 DNA quantification with NanoDrop

After a trial extraction of 6 gut samples with kingfisher flex, double stranded DNA (ds-DNA) was quantified with NanoDrop. The samples were determined to have too low DNA concentrations for quantification with NanoDrop (< 20 ng DNA ml^{-1}). However, they are readily amplified with the intended PCR procedure (Figure 3.12). Therefore, ds-DNA in experimental samples was not quantified after extraction in the current study.

3.3.5 Normalization of amplicon concentration

Between PCR and indexing, and between indexing and sendoff, the amplicon amount in each sample was normalized. This was done with the Applied Biosystems™ SequalPrep™ Normalization Plate Kit according to manufacturers protocol, which can be found in the supplementary information. 15 µl of amplicon was transferred from the PCR tube to the normalization plate. According to the documentation supplied by the manufacturer the output from the plate was on average 20 ng DNA at 1 ng µl^{-1}, with a maximum threefold difference in concentration.

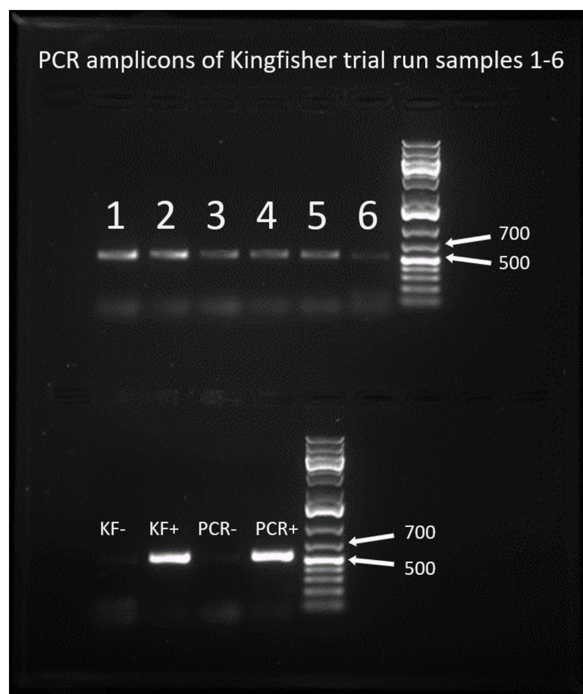


Figure 3.12: Gel image taken with 400ms exposure.

3.3.6 Indexing

The normalized amplicons were indexed with a unique combination of indexing primers. The primers were supplied by Illumina. Sample identities and the index pairs used to index the sample is given in Appendix A. An adapted protocol was used for the master mix in the indexing to save materials, as seen in Table 3.4. The cycling conditions during indexing PCR are given in Figure 3.13.

Table 3.4: Reaction mix for indexing PCR program.

Reagent	**Final concentration**	**Amount 1x**
PRC grade water		11.81 µL
5x Phusion buffer HF	1x	5.0 µL
dNTP (10mM each)	200 µM each	0.5 µL
Phusion HF-pol (2 units/ µL)	0.02 units/µl	0.19 µL
Index 1 (orange lid, N series, 8 unique)		2.5 µL µl
Index 2 (white lid, S series, 12 unique)		2.5 µL µl
Template		2.5 µL

Cycling conditions

98°C	2 min	
98°C	15 sec	8 – 12 cycles
50°C	20 sec	
72°C	20 sec	
72°C	5min	
4°C	1min	
10°C	∞	

Figure 3.13: Typical cycling conditions for indexing PCR.

3.4 Sequencing and processing of sequence data

Sequencing was performed on Illumina MiSeq by the Norwegian sequencing centre at the Department of Medical Genetics Ullevål. Sequencing data analysis was performed with the Usearch v11 software package (Edgar, 2013) (Edgar, 2010) (Edgar, 2016b) (Edgar, 2021).

Primers and the N-linker sequences were stripped from the reads, 21 bases for the forward reads (ill341f-k1), and 25 for the reverse reads (ill805R). The reads were paired, with a maximum difference threshold of 10 and de-replicated. 114 000 unique reads were clustered into OTUs, with an OTU radius percent of 3, singletons were excluded. Before chimera filtering, 12000 putative OTUs with 97 percent identity thresholds to the centroid sequences was obtained.

Unoise 3 was used to filter chimera sequences and 503 OTUs were retained. Low quality or irrelevant data was removed manually in Excel. The removed data was: any OTU with 8 or fewer occurrences in the total dataset, any OTU classified as a chloroplast, and any sample with fewer than 1500 accepted reads. After curating, the dataset contained 127 samples and 435 OTUs.

3.4.1 Taxa summary

Taxa summary files were constructed in Usearch with the ”sintax_summary” command. This command outputs a file containing the proportional abundances of every OTU classified at the relevant taxonomic rank, in each sample of the dataset. The command was repeated for the phylum, order, class, family, and genus level of classification. The taxa summary files were exported to Rstudio, and bar plots showing relative abundances for taxa present in the different samples were made.

3.4.2 Phylogeny

Taxonomy of the centroid sequences was analysed with sintax (Edgar, 2016a), using the RDP 16S v18 database (Wang et al., 2007).

Centroid sequences from the Usearch output were exported to MEGA 11 (Tamura et al., 2021). All OTUs were aligned in MUSCLE (Edgar, 2004), 500 maximum iterations. A maximum-likelihood tree was constructed in MEGA 11 with the Tamura-Nei model (Tamura and Nei, 1993), and 50 bootstrap replications.

3.5 Statistical analysis and graphing in R studio

Data from Usearch and MEGA was imported into Rstudio and processed with the Phyloseq (McMurdie and Holmes, 2013a), Vegan (Oksanen et al., 2020) and Ape (McMurdie and Holmes, 2013b) packages.

A phyloseq object was made containing:

- The OTU table.
- Sintax file with phylogenetic information, at 0.8 confidence cutoff.
- Metadata with sample identities and various grouping information.
- The phylogenetic tree.

3.5.1 Dissimilarity/diversity analysis as PCoA plots

Using the phyloseq and vegan packages in Rstudio, dissimilarity matrices were calculated. These were used in an ordination to produce PCoA plots with the ggplot (Wickham, 2016) package displaying the two axes with highest eigenvalues. This was repeated for different subsets of the samples, and with UniFrac, Bray-Curtis, and Sørensen-Dice methods. Plots of subsets that could be used to investigate the aims of the current study was selected and further analyzed.

3.5.2 PERMANOVA

PERMANOVA analysis was performed on the dissimilarity/distance matrices for plots selected in the previous step. This analysis was run for 9999 permutations, with the seed "5846763".

4 Results

4.1 Pilot experiment

During the spring of 2021, the disinfection procedure was optimized for use on *D. magna* clone A7 at the ACMS laboratory. Hatching rates improved from 65% to around 80% over the period of experimentation. The disinfected juveniles in pilot experiment displayed low mortality, and acceptable reproduction rates (Table 4.1).

Table 4.1: Results from the fecundity monitoring of disinfection run 3 in spring 2021. DPH: Days Post Hatching, ind: individuals, juv: juveniles. The number of juveniles removed from each culture on a given day is shown. Where no removal of juveniles took place, this is either due to no births or because no culture maintenance was performed that day. The "juv. each" column has the number of juveniles divided by the number of adults. At the bottom of each table, the total number of juveniles per individual is given, rounded. *This number is weighted to account for the mortality mid-experiment.

Culture 3.1				Culture 3.2			
DPH	ind.	juv.	juv/ind	DPH	ind.	juv.	juv each
10	16	81	5.1	10	17	106	6.2
11	16	26	1.6	11	17	18	1
12	16	24	1.5	12	17	80	4.7
13	16	0	0	13	17	0	0
14	16	59	3.7	14	17	20	1.2
15	16	0	0	15	17	0	0
16	16	0	0	16	17	0	0
17	16	214	13.4	17	17	233	13.7
18	16	0	0	18	17	0	0
19	15	90	6	19	17	148	8.7
20	15	22	1.5	20	17	20	1.2
Total:		516		Total:		625	
Total each:			32.7*	Total each:			36.8

4.2 Main experiment

The different treatments in the current study are named as such: "phase 1 culture type" - "phase 2 culture type", magna-magna is therefore a phase 2 *D. magna* culture inoculated with the microbiome from a phase 1 *D. magna* culture. Fecundity scores were assigned to each aquaria, as fecundity was only measured per aquaria. In the data, each sample from an aquaria will have the same fecundity score, but ordinations by fecundity are still made on a by sample basis, and not for averages by aquaria. The scores used are absent (no juveniles), low (less than 4 juveniles per individual), medium (4 to 7 juveniles per individual) and high (more than 7 juveniles per individual). Scores were assigned in a way that made it possible to separate the different levels of fecundity that was observed in a good way, and that would permit subsetting when analysing the metagenomics data.

4.2.1 Sample processing and library preparation

Some samples could not be satisfyingly amplified in the PCR. Material from these samples was indexed and sent for sequencing. These samples are (56, 72, and 121). Sequences obtained from these samples must be interpreted with suspicion. A table describing sample amplification behaviour, and which includes the index sequences used on each sample is found in Appendix A.

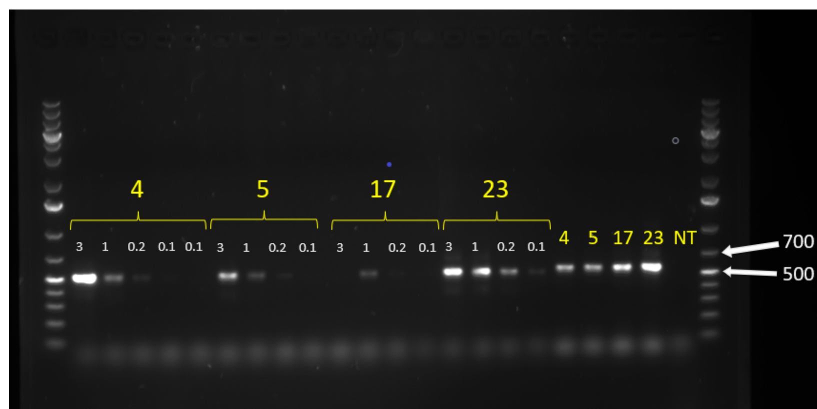


Figure 4.1: Gel image taken with 400ms exposure.

4.2.2 Computational processing of sequencing reads

The sequence data contained 10 737 126 paired reads, each consisting of two sequences. As seen in Table 4.2 the reads have a median length of 468 base pairs. The shortest read is 381 base pairs. A distribution of read lengths is expected due to biological and experimental variance. When aligning the paired reads, some data attenuation is expected due to sequencing errors. Normally around 80% of the reads in an Illumina library can be aligned (personal communication Ingrid Bakke).

Table 4.2: The length distribution of merged pairs from the illumina sequencing data. The shortest read is above the nominal cutoff for V3-V4 reads which is 370nt.

Merged length distribution:	
381	Min
468	Low quartile
468	Median
473	High quartile
536	Max

The reads were merged into 114 189 unique pairs with the ”fastq_mergepairs.pl” command. 21 and 25 nt were stripped for the forward and reverse reads respectively, to remove the Illumina tag and n linker sequences.

By applying the quality filtering command ”fastq_filter.pl fastq_maxee” with an expected error threshold of 1, the accepted reads were reduced to 95 995 unique sequences.

These were pooled, de-replicated, and sorted by ”size” (number of reads for each unique). The FASTA files with the unique sequences were clustered into OTUs at 3 percent OTU radius, which yield OTUs containing unique reads with more than 97% sequence identity. This was done in Usearch with the ”cluster_OTUs.pl” command. After chimera filtering with Uchime (Edgar et al., 2011), 503 OTUs were maintained to be real reads. A significant amount of chimera sequences were removed in this process. As the samples were low biomass samples run through a high number of PCR cycles, this was expected.

Taxonomy was assigned to the centroid sequences with the sintax command in Usearch (Edgar, 2016a). A cutoff confidence score of 0.8 (OTU groups with the taxa in question for at least 80 out of 100 replications) was used.

4.2.3 Phase 1

For phase 1, very high mortality was observed, especially in the *D. pulex* cultures. After 7 days the cultures were restarted with new juveniles in the same aquaria. Significant improvement in fitness was seen after rebooting. Some mortalities still occurred, especially in the co-culture aquaria.

Because phase 2 was initiated after two disparate runs of egg disinfection, Phase 1 aquaria 3, 8, 13, 5, 10, and 15 were terminated on day 21 and the aquaria 1, 2, 4, 6, 7, 9, 11, 12, and 14 were terminated on day 25. The number of offspring per animal at 21 DPH in phase 1 cultures are found Table 4.3.

Table 4.3: Average number of cumulative offspring per animal for phase 1 cultures at 21 DPH. Cultures 1-5 are *D. magna* alone, cultures 6M-10M are *D. magna* in coculture, 6P-10P are *D. pulex* in coculture, and 11-15 are *D. pulex* alone.

Culture name	Juveniles
1	6.2
2	5.2
3	7.0
4	6.0
5	7.2

Culture name	Juveniles
6M	6.7
7M	7.1
8M	4.6
9M	6.0
10M	3.0

Culture name	Juveniles
6P	23.3
7P	9.8
8P	20.1
9P	15.6
10P	24.8

Culture name	Juveniles
11	18.6
12	17.8
13	5.4
14	12.8
15	11.8

Samples were taken of guts from animals used to inoculate the phase 2 cultures which were made after 25 days. PCoA plots of the beta diversity of these samples were made, in order to investigate if the distances and dissimilarities between phase 1 culture types and phase 2 treatments are similar or comparable. Semidistinct grouping is observed, especially between *D. magna* and *D. pulex* cultures (Figure 4.2). The distances/dissimilarities are not statistically significant by PERMANOVA analysis.

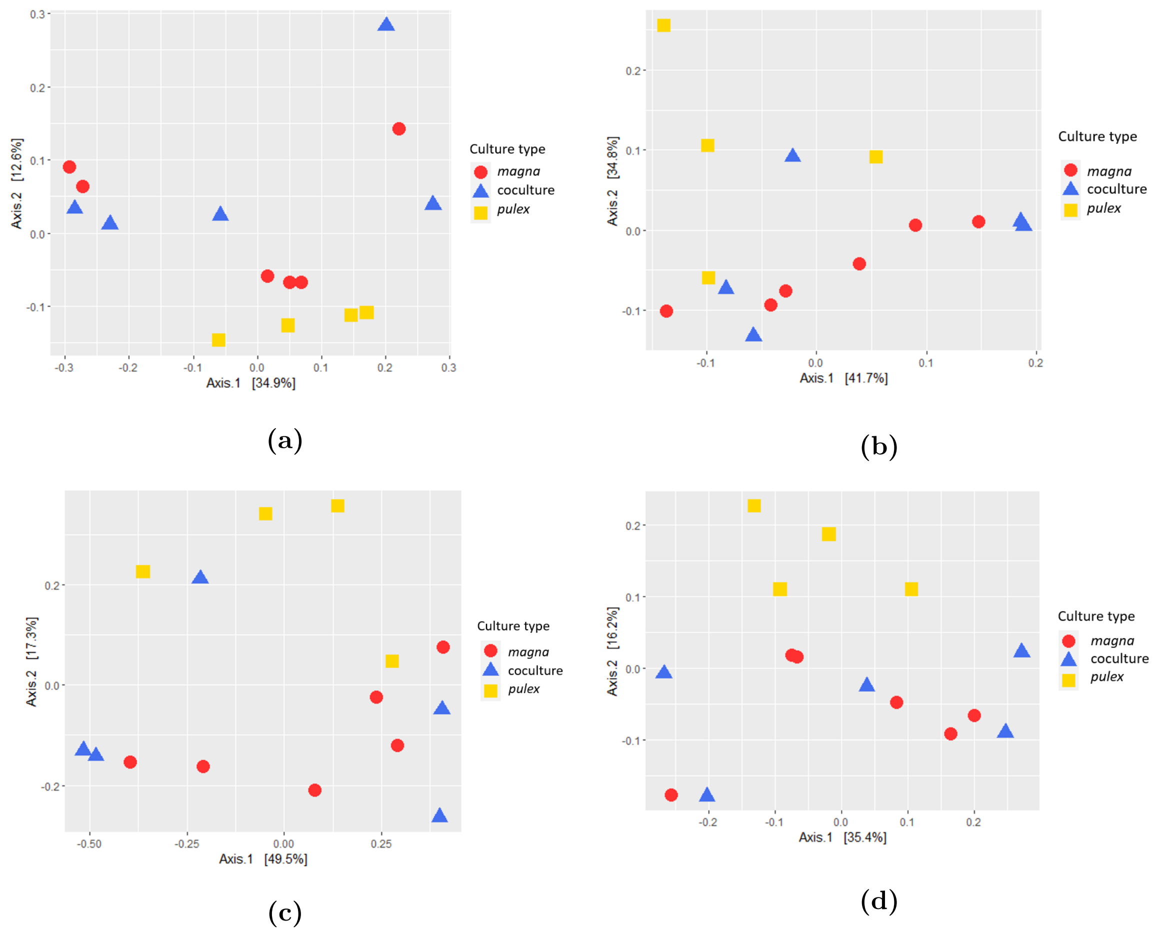
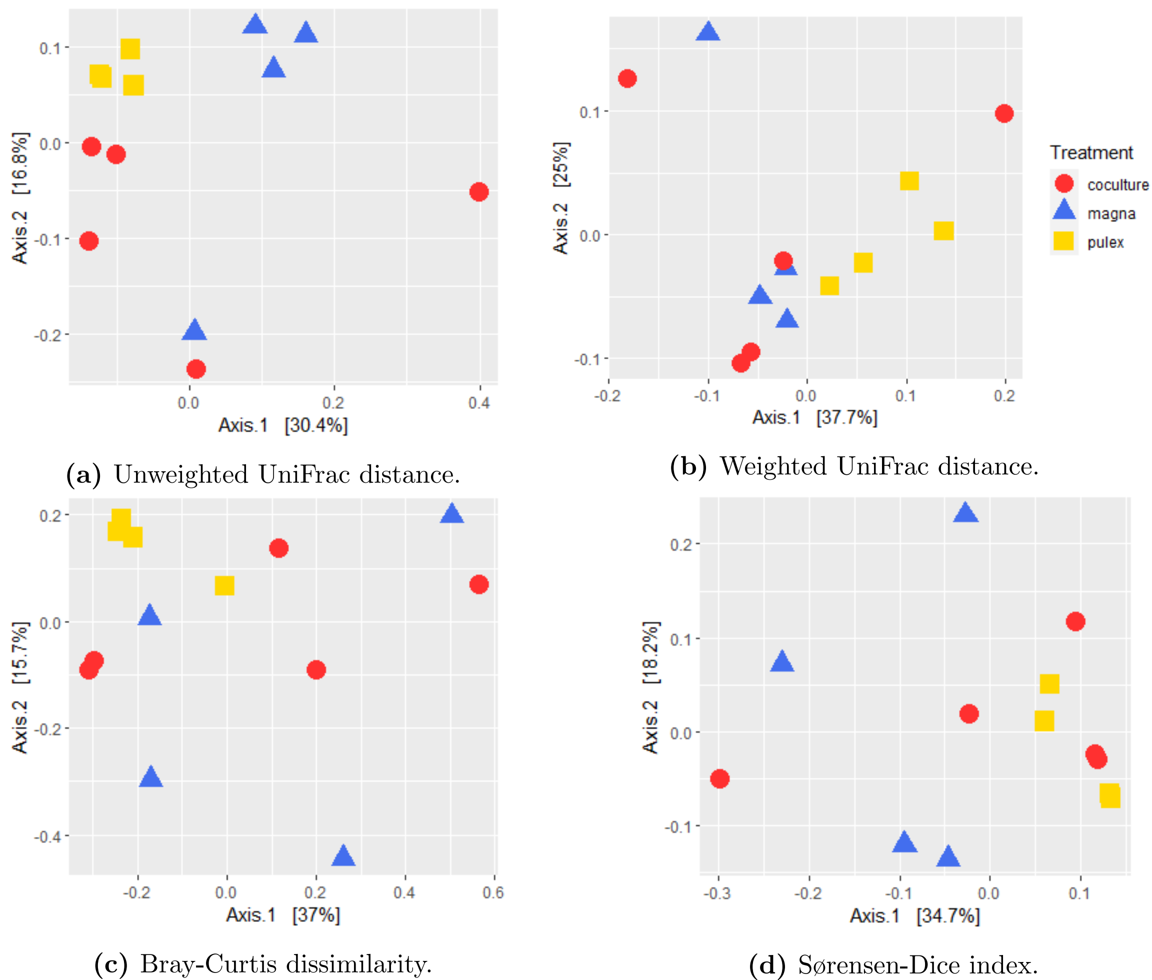


Figure 4.2: Gut samples from phase 1 animals, sorted by the type of culture they came from. (a) Unweighted UniFrac distance, (b) weighted UniFrac distance, (c) Bray-Curtis dissimilarity, (d) Sørensen-Dice dissimilarity.

Water samples from the phase 1 aquaria has sufficiently broad representation from both species that a between-species comparison seems reasonable to make. Fecundity of phase 1 cultures is accounted for and displayed in Table 4.3. Beta diversity PCoA plots of water samples from phase 1 cultures are shown in Figure 4.3. The populations are statistically significantly different by unweighted UniFrac, but not for other measurements.



(a) Unweighted UniFrac distance.

(b) Weighted UniFrac distance.

(c) Bray-Curtis dissimilarity.

(d) Sørensen-Dice index.

Figure 4.3: Water samples from phase 1 cultures plotted with different distance metrics. (a) Unweighted UniFrac centroids are statistically significantly different $p = 0.0267$, (b) Weighted UniFrac, (c) Bray-Curtis dissimilarity, (d) Sørensen-Dice dissimilarity.

4.2.4 Phase 2

For the second phase of the experiment, aposymbiotic juveniles were prepared on two separate days. In the first run of disinfection, sufficient juveniles were obtained to make three parallels of the six treatments for phase 2. Only two were made as some of the *D. pulex* juveniles appeared to be in poor health. They exhibited typical behaviour of moribund *D. pulex* by sticking to the surface of the water by surface tension interaction.

The hatching rates were 81% for *D. pulex* and 93% for *D. magna.* These were higher than previously attained. Despite the apparently good hatching rates the disinfected *D. pulex* continue to be plagued with post-hatching mortality. The surviving *D. pulex* were determined to consist of males and non-reproducing females. Throughout phase 2, only two *D. pulex* juveniles were observed.

The second round of egg disinfection produced 73 *D. magna* juveniles with a hatching rate of 67% and 15 *D. pulex* juveniles with a hatching rate of 38%.

Some cultures of *D. pulex* survived the experiment (4.2p, 7.2p) and some metagenomic samples were sent in from these. However, due to the overall poor health of the animals and lack of reproduction, results of the microbiome experiments are inconclusive and not fit for scientific discussion. In most statistical analyses only the three treatments of *D. magna* juveniles will be used.

4.2.5 Fecundity and body size measurements

Fecundity was monitored for 21 days and body sizes were measured before sampling. Sizes and cumulative offspring are detailed in Table 4.4. The fecundity varied considerably between cultures, and was overall lower than expected. Some cultures had no juveniles at all.

4.3 Community composition

As demonstrated by the bar plots in Appendix E, the measured composition of the microbial community varied within and between aquaria and treatments. PCoA plots ordinated by various beta diversity metrics did not consistently cluster different treatments or sample types. This indicates that the variance in the microbiome composition is not fully systematic.

The most abundant phylum by far for *D. magna* gut samples is Proteobacteria, as seen in Appendix E.5.

Subsets of the normalized OTU table were made. Each contains gut samples from one of the tree populations of phase 2 *D. magna.* These subsets were sorted by which taxa were present in the highest percentage of samples for each subset. The 20 most broadly present taxa for each subset, the number of reads (post normalization), and the percentage of samples the taxa is present in is shown in Table 4.5. This gives human-readable data about the bacterial species present in the samples.

Table 4.4: Body length parameters and cumulative offspring per individual at day 21 post hatch for phase 2 *D. magna* cultures. N= number of animals present at the termination of the culture. *Cumulative offspring calculated as the number of juveniles removed divided by the number of animals present each day and summarized.

(a) *D. magna* with coculture origin microbiome.

Culture name:	2mp-m3	2mp-m1	6.2m	9.2m	7.2m
mean body length	2.89	2.92	2.64	2.98	2.93
SD	0.12	0.10	0.21	0.18	0.08
N=	6.00	6.00	5.00	6.00	5.00
Fecundity*	3.67	4.50	4.00	5.00	7.80

(b) *D. magna* with *D. magna* origin microbiome.

Culture name:	2m-m3	2m-m1	1.2m	4.2m	2.2m
mean body length	2.92	2.90	2.81	2.92	2.96
SD	0.10	0.06	0.11	0.10	0.06
N=	5.00	4.00	5.00	4.00	5.00
Fecundity*	0.20	0.00	5.37	1.00	5.90

(c) *D. magna* with *D. pulex* origin microbiome.

Culture name:	2p-m3	2p-m1	14.2m	11.2m	12.2m
mean body length	3.06	3.10	3.10	3.04	3.00
SD	0.13	0.07	0.07	0.13	0.06
N=	4.00	4.00	5.00	2.00	5.00
Fecundity*	10.00	0.00	14.40	0.00	8.67

Table 4.5: The 20 taxa which were most broadly present in the sample data from *D. magna* inoculated with the three different microbiomes. The sum of reads is for the taxa in all samples of the subset. % indicates how many percent of the samples the taxa was present in. Most taxa are classified at the genus level, otherwise indicated by an f, o or p in superscript. * The two Rhizobiales are two distinct OTUs. † These taxa are represented in only one of the subsets.

Treatment = "coculture-magna", 24 samples

Classification	Sum	%
Bradyrhizobium	1983	92
Xanthobacteraceae ^{f}	9448	88
Aurantimicrobium	663	79
Limnohabitans	33433	75
Formosimonas	10218	75
Bacteroidetes ^{p}	22052	71
Phenylobacterium	30946	63
Daejeonella	28447	63
Cutibacterium	14169	63
Janthinobacterium ^{f}	5921	63
Microbacteriaceae ^{f}	40	63
Bosea	749	58
Comamonadaceae ^{f}	6365	54
Microbacterium	458	54
Microbacteriaceae ^{f}	330	54
Brevundimonas†	538	50
Devosia	178	50
Escherichia/Shigella	11608	46
Aquabacterium	4680	46
Xanthobacteraceae ^{f} †	1577	46

Treatment = "magna-magna", 15 samples

Classification	Sum	%
Xanthobacteraceae ^{f}	33081	100
Phenylobacterium	23157	87
Bradyrhizobium	1673	87
Pseudomonas	9694	80
Daejeonella	5273	80
Cutibacterium	3179	80
Caulobacter	1513	80
Rhizobiales ^{o} * †	1040	80
Caulobacteraceae ^{f} †	50	80
Bosea	7797	73
Escherichia/Shigella	4742	73
Reyranella	3115	73
Formosimonas	3078	73
Microbacterium	1340	73
Rhizobiales ^{o} *	34	73
Limnohabitans	16841	67
Sphingomonas	4948	67
Acidibacter	2534	67
Acidibacter ^{f}	484	67
Alphaproteobacteria	371	67

Treatment = "pulex-magna", 20 samples

Classification	Sum	%
Bradyrhizobium	1962	90
Phenylobacterium	29569	85
Microbacteriaceae ^{f}	2835	85
Microbacteriaceae ^{f}	163	85
Bacteroidetes ^{p}	23707	80
Aquabacterium	8640	75
Aurantimicrobium	4143	75
Cutibacterium	1804	75
Comamonadaceae ^{f}	12960	70
Daejeonella	6746	70
Limnohabitans	6147	65
Microbacterium	2267	65
Caulobacter	1947	65
Pseudomonas	24173	60
Xanthobacteraceae ^{f}	5755	60
Sphingomonas	8375	55
Escherichia/Shigella	6567	55
Devosia	628	50
Acidibacter	3892	45

4.4 Alpha diversity

The alpha diversity of all samples was calculated in R with hill numbers (Hill, 1973). The results can be seen in Appendix D.

Students t-test was used to investigate if the samples had significant differences in alpha diversity. The species richness of phase 2 *D. magna* gut samples were significantly lower than the water samples from their aquaria, $p = 2.7 * 10^{-11}$ (one tailed, equal variance). For gut samples $n = 60$, $\bar{x} = 38$ and $SD = 18$. For water samples $n = 15$, $\bar{x} = 77$ and $SD = 14$. Differences in abundance between the gut samples from different treatments could not be shown to be significant. For: magna-magna $n = 15$, $\bar{x} = 44$, $SD = 15.5$. For pulex-magna: $n = 20$, $\bar{x} = 37.3$, $SD = 19.4$. For coculture-magna: $n = 24$, $\bar{x} = 35$, $SD = 18.2$.

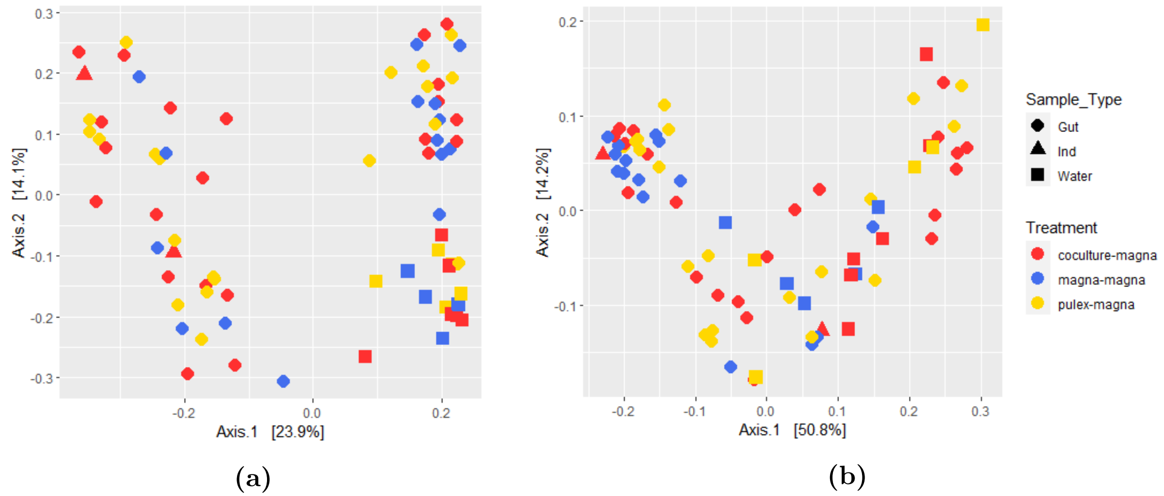
4.5 Beta diversity

Beta diversity distance plots of the phase 2 samples of Magna guts and phase 2 aquaria water samples were made. As can be seen in Figure 4.4, the water samples interlace with gut samples in weighted UniFrac ordination, but cluster alone in unweighted UniFrac (Figure 4.4).

In the Sørensen-Dice plot (Figure 4.8) a distinct cluster of gut samples form in the right side of the plot, above the water samples. All treatment groups and fecundity scores are represented in this cluster, as well as samples from both rounds of egg disinfection. The samples seem to have higher diversity than the other gut samples, but nothing else has been found that makes them biologically distinct.

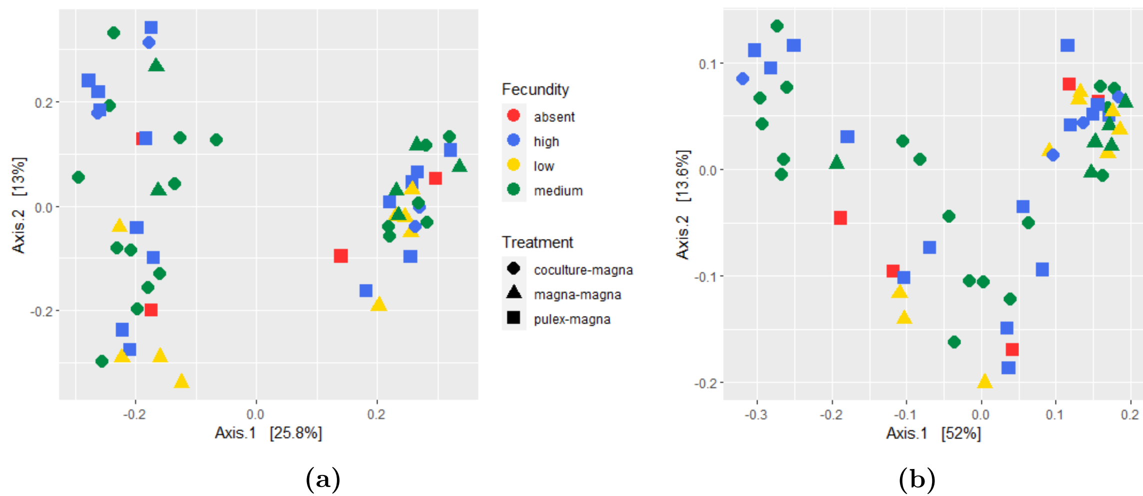
PERMANOVA analysis of the plots show that some statistically significant distances and dissimilarities exist between sample subgroups.

Significant differences are seen by treatment for weighted UniFrac when water and gut samples are analyzed together ($p = 0.028$), but not for unweighted UniFrac (Figure 4.4). When gut samples are analyzed alone, the situation is reversed and significant differences are detected in unweighted UniFrac ($p = 0.024$), but not weighted UniFrac (Figure 4.5). This is possibly detecting small differences which are statistically significant due to the sample number, but which has minimal biological importance. Another explanation is that qualitative differences between treatments are made harder to observe when water samples with higher richness are present. Finally it may be a result of contamination in the water samples from the filters which were used.



(a) (b)

Figure 4.4: Phase 2 *D. magna* gut samples and water samples from their aquaria. (a) Unweighted UniFrac, (b)weighted UniFrac, PERMANOVA by treatment, p=0.028.



(a) (b)

Figure 4.5: Phase 2 *D. magna* gut samples. (a) Unweighted UniFrac, PERMANOVA by treatment p= 0.0244, (b) Weighted UniFrac.

Bray Curtis dissimilarity of gut and water samples shows significant differences between fecundity scores ($p = 0.018$), treatments ($p = 0.0001$) and sample types ($p = 0.0029$), (Figure 4.6). Curiously the dissimilarity is more statistically significant between treatments than between sample types (though it does not follow that the dissimilarity is larger just because it is more statistically significant). For gut samples only, Bray-Curtis dissimilarity is only significant for treatment ($p = 0.0039$), not for fecundity (Figure 4.7). This dissimilarity seems to be mostly between ”magna-magna” and ”pulex-magna” treatments, while the co-culture samples interpolate both populations.

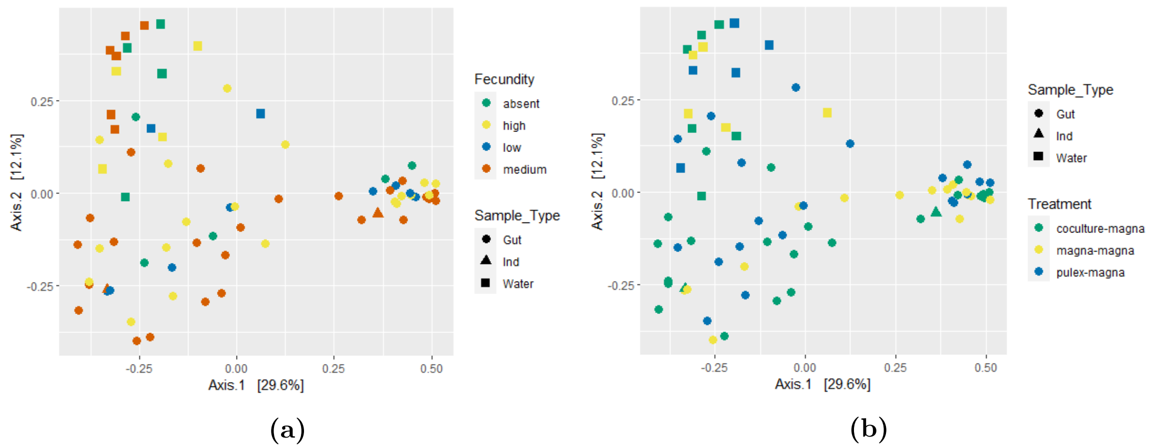


Figure 4.6: Phase 2 samples of *D. magna* guts and water samples from their aquaria. PCoA plot ordinated by Bray-Curtis dissimilarity. The different sample types are water, gut, and the two *D. magna* which were sampled as whole animals are marked ”ind”. (a) Shape indicate sample type, colour indicate fecundity score, (b) Shape indicate sample type, colour indicate treatment.

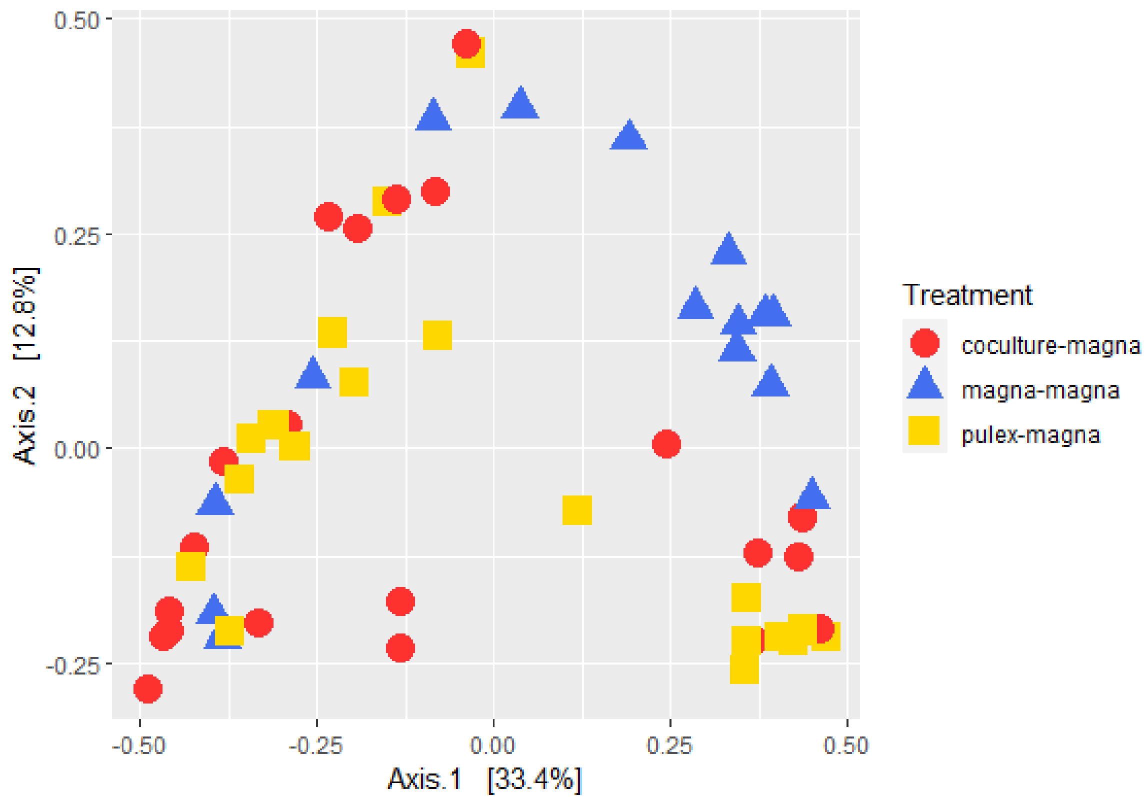


Figure 4.7: Second phase magna gut samples, Bray-Curtis dissimilarity. PERMANOVA by treatment p= 0.0039.

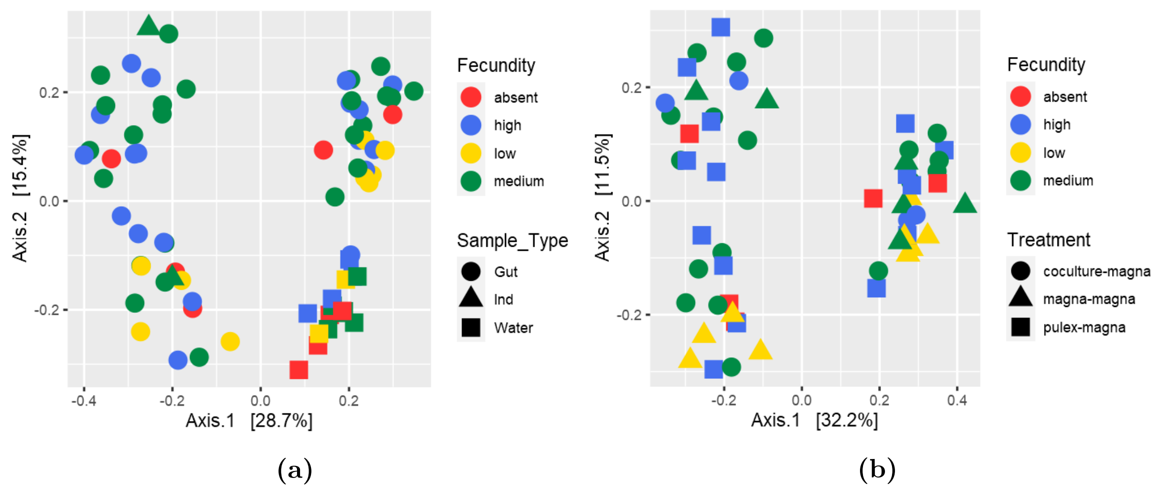


Figure 4.8: Sørensen-Dice distance of (a) Gut and water samples from phase 2 *D. magna* cultures. Fecundity score indicated by colour, sample type indicated by shape. (b) Gut samples from phase 2 *D. magna* cultures only. Fecundity score indicated by colour, treatment indicated by shape.

5 Discussion

5.1 Findings

5.1.1 Microbiome composition differs by treatment

Data analysis demonstrates that there is a difference in microbiome composition between magna-magna, pulex-magna, and coculture-magna cultures. It has been difficult to isolate what this difference consists of in a manner that maintains statistical integrity. From unweighted UniFrac analysis of magna guts, it seems like it may be attributable to binary differences in species composition.

Bray-Curtis dissimilarity also suggests that there exists a systematic difference in abundances for shared species of the three treatment groups.

5.1.2 Possible indications of these results

Differences in microbiome composition has not been linked to changes in fecundity. This may indicate that fecundity depends primarily on the presence of limnohabitans, as suggested by Peerakietkhajorn et al. (Peerakietkhajorn et al., 2015a). Because differences in body length and fecundity were not statistically significant, conclusions cannot be drawn without further research. On repetition of the experiment in the current study it may be possible to prove that differences in microbiome composition correlate to the treatments, and whether this influences fitness in *Daphnia*. Because the animals are inoculated with a microbiome many days before they begin producing offspring, the effect of inoculation source may not be strong enough to cause large changes in fitness later in life. If the experiment can be designed with a continuous inoculation pressure, this may be easier to test.

Some indications that cohabitation influences the fitness of *D. magna* and *D. pulex* was observed in the phase 1 cultures. The juveniles added to co-habitats had higher mortality rates than expected. This may have been caused by the physical environment, and not the cohabitation itself. Improved fitness of phase 1 *D. pulex* in cohabitation later in the experiment suggests that if the confounding effect of the altered habitat had not been present, increased fitness of *D. pulex* could have been seen. This corresponds with results obtained by Sigurd Einum where *D. pulex* appeared to out-compete *D. magna* in an open cohabitation situation (unpublished data). The altered fitness of *Daphnia* specimens in cohabitation environments may also be caused by differences in how well the respective species tolerate the physical environment of co-habitation aquaria. The silicone glue which was used to construct them is designed to have minimal impact on aquatic life, but does produce acetic acid during the curing process. This "offgassing" may influence cultures which are not adapted to the environment, even when the aquaria are cleaned well before use.

It is possible that the biological variation in microbiome is greater than the effect of the treatments, and that distinct microbiomes with a large dissimilarity simply do not follow from the inoculation with co-culture microbiome or a microbiome from the other species. This can be confirmed by a repetition of the experiment. If this is the case, it would suggest that the qualitative differences in *D. pulex* and *D. magna* microbiomes are small. When the qualitative differences are small, selective pressures cause bacterial abundances to re-stabilize at levels preferred by the species or individual in question, and large differences in microbiome based on treatment are not seen.

Comparison of the PCoA plots for phase 2 samples with the PCoA plots for phase 1 samples seem to indicate that the magnitude of biological (or individual) variation is not significantly different between the naturally colonized animals in phase 1 and the inoculated animals in phase 2. This supports the idea that in the current study, selection and individual variation dominates over the effect of treatment as determinants for microbiome composition.

Those taxa which were present in the greatest proportion of samples for the by-treatment subsets of phase 2 gut samples are shown in Table 4.5. The taxa in this table are present in all three treatment groups, and the differences are in how common or highly ranked the taxa are in each subset. This makes it reasonable to conclude that those species which are highly prevalent overlap significantly in the *D. magna* and *D. pulex* laboratory cultures used in the current study.

5.2 Limitations

5.2.1 Library preparation and sequencing workflow

Kitome contamination becomes significant at low biomass inputs, and has been shown to dominate the sequencing reads at 10^3 CFU inputs (Salter et al., 2014b). This causes an increased contamination risk with low bacterial biomass in samples. Samples in the current study have low biomass ($< 20\,\text{ng DNA µl}^{-1}$). Kitome contamination is most readily observed as high read conuts in the blanks, whit OTUs that do not occur at a high prevalence in the samples. For the two extraction blanks, one has almost no reads, and the other has a high number of reads from a few bacteria, consistent with sample cross contamination. The PCR blank has almost no reads. Kitome contamination may therefore be assumed to have had little effect on species diversity and read counts in the gut and whole individual samples.

The two water filter negatives had a large number of reads, of which 90% was accounted for by 9 OTUs. The OTUs which are prevalent in the negative control filters are not abundant in the water samples. The water samples may have a high amount of contamination originating from the membranes, but it is also possible that this contamination is "out competed" by the bacteria in the water samples to a sufficient degree that it influences analysis only minimally, as described by Salter et al. (2014a). Statistical analysis has been performed on the water samples, the results are presented, and they are discussed. I do however, not believe that any conclusions should be drawn from these samples.

Some samples had inconsistent amplification behaviour during PCR, statistical analyses were run with and without these samples (56, 72, and 121). Their inclusion did not appear to influence results at all. Therefore, they are included in the analysis.

Index hopping is an error that can happen in pooled samples that are sequenced on a patterned flow chip such as is used in Illumina sequencing. It results in reads being assigned to the wrong sample (Costello et al., 2018). The consequence of index hopping on a multi-sample experiment will be increased correlation between samples in downstream statistical analysis. This can attenuate the change/difference signal. Index hopping does not occur at a consistent magnitude (Costello et al., 2018), and is difficult to account for (Costello et al., 2018). Therefore it would not be rigorous to compare sequencing samples with another library. When analyzing a single library, index hopping makes significant differences more difficult to detect, and high similarity more likely to be seen. This bias towards high similarity should be kept in mind.

5.2.2 Phylogeny analysis

Phylogenetic analysis of metagenomic sequences with sintax (Edgar, 2016a) is performed by selecting 32 k-mers, 8 nt in length by default, from a centroid sequence at random. These 8-mers are matched to sequences in a database of known sequences. The taxonomic group with the highest number of matches in the selection is assigned to the centroid sequence. This process is looped, with 100 iterations by default. And the taxonomic group most frequently assigned to the centroid sequence is outputted as the assigned taxonomy. The reported taxonomic classification is annotated with a confidence score. This confidence score is the proportion of analysis repeats in which the reported taxa is assigned. For example, if an OTU is assigned to *Limnohabitans* 97 out of 100 times, the confidence score is given as 0.97.

With this system, unknown and novel species are often classified as their nearest neighbor (Edgar, 2016a). This is known as over-classification. Due to over-classification, novel strains may go undetected. The confidence scores are troublesome because they are simply the proportion of hits from 100 iterations, which means that the confidence score is normally distributed, with an unknown population average and standard deviation. This method of assigning taxonomy to metagenome data is used for a lack of more satisfying methods. The classification is the best that can be obtained, but it is important to remember that it is not flawless.

5.2.3 Possible biomass limitation of gut samples

Higher richness in water samples may indicate biomass limitation in the gut samples. Logically most or all taxa present in the water should be present in the gut samples, as *Daphnia* readily ingest bacteria (Taipale et al., 2012). Some bacteria evade digestion and colonize the guts of *Daphnia* (Callens et al., 2020) (Callens et al., 2015). By colonizing the gut of a single individual, a strain or consortia of bacteria may out-compete the other species, and cause a low diversity ”polydominance” situation. In such a situation the low abundance gut bacteria (often transient) may become undetectable.

5.3 Evaluation of methods and protocols

Optimization of the protocols was an important goal of the study. These protocols make it possible to execute microbiome studies with multiple species of *Daphnia* in a laboratory environment. Recommendations for protocol adaptations are detailed in this section.

5.3.1 Egg screening and selection

Choosing eggs for disinfection is a critical step in the procedure. For *D. magna* eggs, consistently high hatching rates was obtained with the methods described in Section 3.3.1. When attempting to apply this protocol to *D. pulex*, poor results were obtained. To improve the success rate and consistency of hatching rates for *D. pulex* eggs, verification of egg suitability by microscopy was implemented. Success rate when disinfecting eggs is primarily correlated to the chorion being unperforated (Callens et al., 2015) (Obrestad, 2020). By visually inspecting the eggs, it can be verified that the chorion has not separated from the egg, which is the point where perforation of the chorion becomes prevalent. Egg screening by visual inspection was tested in March 2022, a hatching rate of 90.5% with 19 hatched eggs out of 21 was obtained (unpublished data).

5.3.2 Age synchronized cultures

In the current experiment, age-heterogeneous stock cultures was used. Eggs for disinfection, and juveniles for the first phase of the experiment were obtained from aquaria with between 200 ml and 500 ml volume, and an age heterogeneous population. The stocking density varied between 3 to 10 adult individuals per 200 ml aquaria. This model was adopted from Sigurd Einum and the CBD *Daphnia* lab. It has been my experience that the method works poorly for the type of experiment that has been performed here.

The use of first clutch juveniles as experimental animals is disencouraged by OECD guidelines for reproduction tests on *D. magna* (OECD, 2012). With age heterogeneous cultures, it is cumbersome to preclude first clutch juveniles from the experimental workflow. Production of ephippia and a shift to sexual reproduction is not induced solely by photo-period signalling, but may also happen in response to food limitation, and high stocking density (CARVALHO and HUGHES, 1983). With high density age-heterogeneous cultures it becomes difficult to maintain the cultures in a parthenogenetic reproduction state. Reproduction by parthenogenesis is required when disinfecting eggs for further study. I therefore suggest that age synchronized cultures with more than 66 ml media volume per animal is used for further studies which include production of aposymbiotic juveniles.

5.3.3 Vitamin deficiency

After the conclusion of the main experiment, while re-reading articles, it became obvious that many of the issues with culture performance could be caused by a vitamin deficiency. The original publication of ADaM (Klüttgen et al., 1994) specifies that the natural occurrence of B-vitamins in seawater is important for its ability to sustain cultures. E-mail correspondence with Tetra Marine confirmed that their synthetic SeaSalt does not contain any vitamins.

Vitamin-B solution for M4 media (OECD, 2012) was sourced from Gabriel Orthof at NTNU and added to the ADaM media in an attempt to improve culture fitness. This caused immediate restitution of culture performance. For further experiments, the updated media protocol will be used. Fecundity has been monitored under several different conditions. For disinfected *D. magna* juveniles, the highest fecundity was observed when feeding *R. subcapitata*, and adding vitamin solution to the media. Fecundity measurements for this condition are shown in Table 5.1. Fecundity measurements for other conditions are shown in Table 4.1, Table 4.3 and Table 4.4.

Table 5.1: Four parallel cultures of disinfected *D. magna* monitored for fecundity. Work was performed by Jenny Poppe under supervision.

Date	Culture identities			
	1D	2D	3D	4D
	Juveniles per adult			
08-Apr	3.7	11.0	8.7	15.5
11-Apr	6.3	16.0	6.3	0.0
13-Apr	9.3	0.0	5.7	14.0
15-Apr	4.7	11.0	16.3	5.5
18-Apr	8.7	0.0	5.0	6.0
20-Apr	8.3	0.0	7.3	11.5
Total	41.0	38.0	49.3	52.5
Average:		45.2		

5.3.4 Microbiome transfer

Poor correlation was found between the microbiome of phase 1 cultures, and the phase 2 cultures they were used to inoculate. It is not known if this is due to biological variation, a methodological weakness, or a combination of both. Assuming a methodological weakness, adaptations must be made so that the microbiome composition is more even between subjects, more conserved after inoculation, and more representative of the source community.

For further experiments, an updated protocol should be considered for inoculation. A method adapted from Callens et al. (Callens et al., 2015) may be a sensible choice. Animals from the first phase of the experiment are pooled by treatment group and homogenized. Juveniles for phase 2 are inoculated in a suspension of homogenized adults. A sample of the inoculant is retained for metagenomics analysis. When inoculating all phase 2 juveniles for each treatment group with a common suspension of inoculant, the resultant microbiomes should have higher similarity within each treatment group. This would give more statistical power to observed changes in fitness.

5.3.5 Implementation

Disinfection of *D. pulex* eggs was performed in spring 2022 with the reevaluated protocols for culture stocking, media composition, and egg screening procedures described in Section 5.3. Hatching rates of 96% was obtained, with 15% mortality after 21 days. This is a major improvement in the suitability of the protocols.

5.4 New methods/kits providing new possibilities

New methods continuously push the boundaries of what can be constructively analyzed with metagenomics. Ten years ago, the first metagenomics study on *D. magna* required 15 guts to be pooled, in order to successfully analyze the microbiome (Freese and Schink, 2011). In the current study, samples of single guts are successfully analyzed. Below are descriptions of methods and reagents which have permitted more ambitious study designs in microbiome analysis to be executed.

5.4.1 DNA purification with PowerSoil Pro

Testing indicated that a high amount of template can be used from the PowerSoil Pro extracted samples without significant issues with PCR inhibition, as can be seen in Figure 4.1. This indicates that the kit removes PCR inhibitors efficiently. Using DNA extracted with this or comparable kits can permit analysis of lower biomass samples, by increasing the input template amount to PCR. This will decrease the influence of "kitome" OTUs (Salter et al., 2014a) (Voirol et al., 2020). It will allow a reduction in PCR cycle number, reducing chimera formation. Additionally, it will provide deeper coverage in low biomass samples. The use of more advanced PCR inhibitor removal techniques may alleviate many of the issues with the 16s metagenomics workflow.

5.4.2 PERMANOVA

Analysis of PCoA ordination by PERMANOVA detected multiple statistically significant differences between populations. Despite this, the PCoA plots in question do not seem to contain meaningful patterns. In terms of assessing statistical significance, PERMANOVA outperforms the human eye. No assumptions are made about the distribution of data, but the populations must have the same dispersion. Therefore PERMANOVA can be applied to data that is difficult or cumbersome to test for normality of distribution. This provides a distinct advantage when analyzing microbiome data.

5.5 Outlook

The current study has aided the production of institutional knowledge, and contributed to the establishment of functional protocols for microbiome studies on *Daphnia* species. It is now up to the next generation of students to further the applications of metagenomic studies in freshwater ecology, and to gain an increased understanding of host-microbe interactions in an ecological context.

Repetition of the experiment in the current study is underway, using the reevaluated protocols. This may permit rigorous testing of the central hypothesis of the current study: that *D. magna* and *D. pulex* influence the fitness of each other, trough their microbiomes.

Investigations of interactions between microbiomes in laboratory cultures can be contextualized with studies of the microbiome of wild-caught *Daphnia*. This could identify new research questions that are suitable for investigation with the protocols herein for microbiome studies on *Daphnia* species. A comparison between lab culture microbiome and wild type microbiomes may also strengthen or weaken the assumption that mechanisms observed in the lab can be transferred to wild ecosystems.

6 Conclusions

Suitable protocols for use of *D. pulex* for microbiome experiments in the lab was eventually established.

It was demonstrated that statistically significant differences existed between the microbiomes of different treatment groups. A consistent pattern or causative mechanism was not observed. This limits the ability to draw further conclusions with this particular dataset.

The differences in microbiome may or may not correlate with fitness. Measurements of fitness had insufficient statistical power to draw satisfying conclusions.

It could not be demonstrated that differences in microbiome caused differences in fitness.

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A PCR results

Table A.1: All samples, both accepted and rejected, and the results from tageted PCR and indexing PCR. Solid band is indicated with (+), poor but visible bands with (/) and no band with (-). Samples which had poor or no bands after targeted PCR but visible bands after indexing PCR are bolded, these are assumed to be suspect.

sample identity	sample number	targeted PCR	Indexing PCR	Indexes
2mp-m32	1	+	+	n715-s502
2mp-m33	2	+	-	n715-s503
2m-m33	3	+	+	n715-s505
2p-m14	4	+	+	n715-s506
2p-m11	5	+	+	n715-s507
2p-m13	6	+	+	n715-s508
2p-m33	7	+	-	n715-s510
2m-m32	8	+	+	n715-s511
2p-m31	9	+	+	n716-s522
2mp-p11	10	+	pd. +	n716-s521
2mp-m16	11	+	+	n716-s520
2mp-p33	12	+	+	n716-s518
2mp-p32	13	+	+	n716-s517
2mp-m12	14	+	+	n716-s516
2mp-m11	15	+	+	n716-s515
2mp-m34	16	+	+	n716-s513
2mp-m13	17	+	+	n718-s522
2p-m12	18	+	-	n718-s521
2mp-m15	19	+	+	n718-s520
2mp-p14	20	+	+	n718-s518
2m-m34	21	+	+	n718-s517
2p-m32	22	+	+	n718-s516
2mp-m14	23	+	-	n718-s515
2mp-m36	24	+	+	n718-s513
2mp-m31	25	+	+	n719-s522
2mp-p12	26	+	+	n719-s521
2mp-p31	27	+	+	n719-s520
2m-m31	28	+	+	n719-s518
2mp-m35	29	+	+	n719-s517
2p-m34	30	+	+	n719-s516
7.2p3	31	+	+	n719-s515

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Table A.1 – continued from previous page

sample identity	sample number	targeted PCR	Indexing PCR	Indexes
7.2p1	32	+	+	n719-s513
14.2m4	33	+	+	n720-s522
9.2m6	34	+	+	n720-s521
1.2m4	35	+	+	n720-s520
9.2m2	36	+	+	n720-s518
12.2m5	37	+	+	n720-s517
12.2m2	38	+	+	n720-s516
9.2m5	39	+	+	n720-s515
14.2m2	40	+	+	n720-s513
9.2m1	41	+	+	n722-s522
1.2m3	42	+	+	n722-s521
12.2m4	43	+	+	n722-s520
7.2m1	44	+	+	n722-s518
7.2m4	45	+	+	n722-s517
2.2m4	46	-	-	n722-s516
1.2m1	47	+	-	n722-s515
7.2p2	48	+	-	n722-s513
1.2m6	49	+	-	n721-s522
Empty/neg ctrl	50	+	-	n721-s521
2m-p3w	51	+	\	n701-s502
2m-m1w	52	+	+	n701-s503
2mp-p1w	53	+	+	n701-s505
2p-m1w	54	+	+	n701-s506
2mp-m1w	55	+	+	n701-s507
2p-m3w	56	-	+	n701-s508
2mp-m3w	57	+	+	n701-s510
2m-m3w	58	+	+	n701-s511
2mp-p3w	59	+	+	n702-s502
14w	60	+	-	n702-s503
11w	61	+	+	n702-s505
4w	62	-	-	n702-s506
12w	63	+	+	n702-s507
7w	64	+	+	n702-s508
1w	65	+	+	n702-s510
2w	66	-	-	n702-s511

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Table A.1 – continued from previous page

sample identity	**sample number**	**targeted PCR**	**Indexing PCR**	**Indexes**
6w	67	+	+	n703-s502
9w	68	+	+	n703-s503
neg1	69	-	+	n703-s505
2.2m	70	+	+	n703-s506
12.2mw	71	+	+	n703-s507
4.2mw	72	-	+	n703-s508
7.2pw	73	+	+	n703-s510
4.2pw	74	\	+	n703-s511
1.2mw	75	+	+	n704-s502
neg2	76	-	\	n704-s503
7.2mw	77	\	+	n704-s505
9.2mw	78	+	+	n704-s506
14.2mw	79	+	+	n704-s507
6.2mw	80	+	+	n704-s508
11.2mw	81	+	+	n704-s510
3w	82	+	+	n704-s511
5w	83	+	+	n705-s502
8w	84	+	+	n705-s503
15w	85	+	+	n705-s505
10w	86	+	+	n705-s506
13w	87	+	+	n705-s507
12.2m1i	88	+	+	n705-s508
11.2m1i	89	+	+	n705-s510
4.2m1i	90	+	+	n705-s511
7.2p2i	91	+	+	n706-s502
14.2m1i	92	+	+	n706-s503
2.2m1i2	93	+	+	n706-s505
1.2m2i	94	+	+	n706-s506
2.2m2i	95	+	+	n706-s507
4.2m2i	96	+	+	n706-s508
4.2p1i	97	+	+	n706-s510
7.2p1i	98	+	+	n706-s511
6.2p1i	99	+	+	n707-s502
12.2m2i	100	+	+	n707-s503
6.2m2i	101	+	+	n707-s505

Continued on next page

Table A.1 – continued from previous page

sample identity	sample number	targeted PCR	Indexing PCR	Indexes
1.2m1i	102	+	+	n707-s506
7.2m1i	103	+	+	n707-s507
7.2m2i	104	+	+	n707-s508
9.2m4	105	+	+	n707-s510
4.2p2i	106	+	+	n707-s511
12.2m3	107	+	+	n710-s502
9.2m3	108	+	+	n710-s503
2.2m1i	109	+	+	n710-s505
7.2m5	110	+	+	n710-s506
4.2m3	111	+	+	n710-s507
4.2m2	112	+	+	n710-s508
12.2m1	113	+	+	n710-s510
6.2m3	114	+	+	n710-s511
11.2m1	115	+	+	n711-s502
unknown	116	+	+	n711-s503
6.2m2	117	+	+	n711-s505
12.2m6	118	+	+	n711-s506
14.2m1	119	+	+	n711-s507
11.2m2	120	+	+	n711-s508
2.2m2	121	-	\	n711-s510
6.2m4	122	+	+	n711-s511
4.2m5	123	+	+	n712-s502
4.2p4	124	+	\	n712-s503
4.2m1	125	+	+	n712-s505
2.2m5	126	+	+	n712-s506
4.2p3	127	+	+	n712-s507
14.2m3	128	+	+	n712-s508
2.2m3	129	+	+	n712-s510
7.2m3	130	+	+	n712-s511
4.2m4	131	+	+	n714-s502
14.2m5	132	+	+	n714-s503
6.2m2	133	+	+	n714-s505
1.2m2	134	+	+	n714-s506
11.2p2	135	+	+	n714-s507
4.2p2	136	+	+	n714-s508

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Table A.1 – continued from previous page

sample identity	sample number	targeted PCR	Indexing PCR	Indexes
KF-(51-136)	137	\	\	n714-s510
KF-(1-50)	138	\	-	n714-s511

B Equipment list

A complete list of equipment used

Environmental cabinet
The environmental cabinet in which the Daphnia were cultured is a Memmert IPP 260 plus serial number: V614.0031. Which was programmed with the AtmoControl v2.9.2.0 software.

Distilled water supply
Distilled water for the medium was obtained from an ELGA water filtering stack. the 18.2 MΩ producing unit was Model nr. PF2xxxxM1 serial no. FLB00008667.
Pre PCR lab equipment Precellys 24 (serial no 0002673)

Micro tube 2ml, PP [1]
Sarstedt AG & co. KG
reference number: 72.609.001

Precellys lysing kit "1.4mm Zirconium oxide beads"[1]
bertin instruments
Produkt-nr.: P000927
vwr catalog nr: 432-0356

MagAttract PowerSoil Pro DNA Kit[1]
QIAGEN GmbH, QUIAGEN Strasse 1 40724 Hilden Germany
Reference Number: 47109

SequalPrep™ Normalization Plate Kit, 96-well[1]
Applied Biosystems™
Catalog number: A1051001

Bio-Rad T100 tm thermal Cycler
Bio-Rad Laboratories, Inc
serial numbers: 621BR32651, 621BR3257 VWR International

10 µl poding loops catalogue number 612-9353

PCR strips
Multiply µStrip Pro 8-strip [1]

[1] In direct contact with DNA samples during laboratory workflow
[1] In direct contact with DNA samples during laboratory workflow

Sarstedt AG & co. KG
Reference number: 72.991.002
(contact)

Hard-Shell High Profile 96-Well Semi-Skirted PCR plates[1]
Bio-Rad Laboratories, Inc
Catalog Number: HSS9601
(contact)

Adhesive PCR sealing foil sheets[1]
Thermo Scientific
Catalog-number: AB-0626
(contact)
pcr cabinet

Filter Tip (various sizes)[1]
Sarstedt AG & co. KG
reference numbers: 70.3030.265, 70.1130.210, 70.3050.255

eppendorf tubes [1]
Micro Tube 1.5ml SafeSeal Sarstedt AG & co. KG
reference number: 72.706.400

Phusion Hot Start II High-Fidelity DNA Polymerase[1] Thermo Fisher Scientific
Balticus UAB
VA Graciuno 8, LT-02241 Vilnius, Lithuania reference number: F-549L

PCR Plates for storage of sample aliqoutes[1]
Thermo scientific Thermo-Fast 96 Skirted PCR Plate With Black Lettering
SKU: AB-0800/G-L

Syngene G:BOX
spenningskilde
loading dye
ladder

GelRed Biotium, Inc.
46117 Landing Parkway
Fremont, CA 94538
Catalog Number: 41003

Standard Agarose - Type LE
BioNordika
Catalog Number: BN50004 TAE buffer

Gel electrophoresis tubs

uncategorized
LAF bench

14000 g minimum rating benchtop centrifuge. VWR CT15E centrifuge

Hitachi Koki Co., Ltd.
ECN: 521-3600

VELP scientifica WIZARD Advanced Vortex Mixer

Leica stereolupe and LAS-X camera stack

nanodrop

Polycarbonate membrane filter 47 mm diameter 1.0 µm pore size Poretics corporation 151 I LINDBERGH AVE, LIVERMORE, CA 94550 (lotnr. AH72AO11B005). Polycarbonate membrane filter

25mm diameter 3.0 µm pore size
Poretics corporation 151 I LINDBERGH AVE, LIVERMORE, CA 94550

[1]In direct contact with DNA samples during laboratory workflow

catalog number:
(lotnr. AH62AF11A005).

polycarbonate membrane filter [1]
25 mm diameter 0.2 µm pore size
Poretics corporation 151 I LINDBERGH AVE, LIVERMORE, CA 94550
Catalog number: 11021
(lotnr. AG83BI21BV22)

Sterivex™
0.22 µm FILTER UNIT
Merck Millipore corporation, Darmstadt Germany

10ml (12ml) Henke-ject sterile syringes [1]
HENKE SASS WOLF
Reference number: 5100-000v0

25mm Syringe Filter w/0.2 µm Cellulose Acetate Membrane[1]
VWR international
catalouge number: 514-0060

falcon tubes

Illumina Nextera primers Set A and Set D[1]
catalouge numbers: FC-131-2001, and FC-131-2004 Amicon Ultra-0.5mL

Centrifugal Filters Ultracel -30k[1]
regenerated cellulose
Merk Millipore Ltd.
Reference Number: R0PB52303

Chemicals

SeO_2
$NaCO_3$
$CaCl_2$

Tetra marine sea salt

Glutaraldehyde 25%

Ethanol 96% VWR[1]

VWR international
PCR grade water[1]
ID: 5401000-5000
Batch: 21B0303

VWR International
dNTP mix[1]
ID: 5100850-0500
Batch: 21A1202

ill341f-ki Forward primer [1]
ill805R Reverse primer[1]

200 ml aquaria The 200 ml aquaria were cadmium and lead free tempered glass containers from Ikea, article nr. 602.797.11

Internal cages were manufactured from 50 ml Falcon tubes and 90 µm algae net to allow for inoculation of juveniles with microbiome from adult specimens. Windows were cut into the cylinder of the falcon tube, and a tunnel of algae net was inserted and glued to the tube with DL CHEMICALS Parasilico Aquarium glue (lotnr: 23121621). A picture of a cage is shown in Figure 3.3 . Forceps

- 5-Durax rubistech
- Bochem 18/10 glass petri dish Disposable pipette

- Sarstedt Transferpipette 3.5 ml (reference number 86.1171) (lotnr. 1054021)

C Buffer and media recipes

Table C.1: 50x TAE-buffer.

Tris	242	g
Anhydrous acetic acid	57.1	ml
0.5M EDTA pH 8.0	100	ml
dH2O	Fill to 1 l	

Table C.2: Aachener Daphnien Medium (ADaM)

Synthetic sea salt (tetra marine)	$0.333\,g\,l^{-1}$
$CaCl_2$ solution 0.8 M	$2.3\,ml\,l^{-1}$
$NaHCO_3$ solution 0.3 M	$2.2\,ml\,l^{-1}$
SeO_2 13 mM	$0.1\,ml\,l^{-1}$

D Alpha diversity calculations

Table D.1: Alpha diversity calculations for all accepted samples. Column "Type" is gut, water or ind (which indicates those samples which were taken of whole individuals that were deemed too small to extract guts from). Column "Number" is the sample number assigned before DNA extraction. Column "Identity" is an identifyer that consists of the aquaria name, followed by either: the sample number from that aquaria for gut samples of phase 2 aquaria, w for water samples, the number 1 or 2 followed by i for inoculant individuals from phase 1 that were transferred to phase 2 aquaria, and then sampled after the 48 hour inoculation period. Columns "X0" "x1" "x2" and "inf" are Hill numbers of respectively order 0, 1, 2, and infinite.

Type	Number	Indentity	X0	X1	X2	Inf.
Gut	1	2mp-m32	39	7.0	4.5	2.6
Gut	3	2m-m33	49	3.4	2.7	1.9
Gut	4	2p-m14	44	5.6	3.3	2.0
Gut	5	2p-m11	41	4.9	2.9	1.8
Gut	6	2p-m13	18	3.5	2.4	1.6
Gut	7	2p-m33	29	8.3	5.0	2.6
Gut	8	2m-m32	33	5.4	3.4	2.1
Gut	9	2p-m31	26	2.0	1.4	1.2
Gut	10	2mp-p11	25	6.7	4.2	2.4
Ind	11	2mp-m16	32	4.4	2.7	1.8
Ind	12	2mp-p33	22	4.6	2.5	1.7
Ind	13	2mp-p32	14	3.5	2.2	1.5
Ind	14	2mp-m12	8	2.6	2.3	2.1
Gut	15	2mp-m11	33	5.1	3.8	2.7
Gut	16	2mp-m34	13	6.5	5.6	3.5
Gut	17	2mp-m13	23	5.0	3.4	2.1
Gut	19	2mp-m15	27	4.9	3.6	2.2
Gut	20	2mp-p14	14	1.3	1.1	1.0
Gut	21	2m-m34	26	3.0	2.0	1.5
Gut	22	2p-m32	32	8.7	6.1	3.7
Gut	24	2mp-m36	36	4.2	2.4	1.6
Gut	25	2mp-m31	40	8.2	5.1	3.1
Gut	26	2mp-p12	32	9.6	4.9	2.4
Gut	27	2mp-p31	19	4.0	2.4	1.6
Gut	28	2m-m31	13	5.2	3.3	2.0
Gut	29	2mp-m35	20	4.8	3.6	2.5
Gut	30	2p-m34	12	5.5	4.7	3.0

Continued on next page

Table D.1 – continued from previous page

Sample Type	Sample nr.	sample indentity	X0	X1	X2	Inf.
Gut	31	7.2p3	16	6.7	4.9	3.0
Gut	32	7.2p1	26	4.3	2.2	1.5
Gut	33	14.2m4	20	7.0	4.7	2.6
Gut	34	9.2m6	10	5.7	5.0	3.1
Gut	35	1.2m4	24	6.9	4.5	2.6
Gut	36	9.2m2	30	3.5	2.9	2.1
Gut	37	12.2m5	21	6.0	3.5	2.0
Gut	38	12.2m2	14	3.2	2.2	1.6
Gut	39	9.2m5	15	1.9	1.4	1.2
Gut	40	14.2m2	26	3.8	2.9	2.0
Gut	41	9.2m1	38	7.9	4.6	3.1
Gut	42	1.2m3	25	6.0	4.0	2.8
Gut	43	12.2m4	13	2.0	1.4	1.2
Gut	44	7.2m1	15	7.2	6.1	4.6
Gut	45	7.2m4	12	3.6	2.9	1.9
Water	52	2m-m1w	87	9.6	5.3	2.7
Water	53	2mp-p1w	70	5.4	3.4	2.2
Water	54	2p-m1w	67	2.5	1.8	1.4
Water	55	2mp-m1w	87	8.4	4.7	2.7
Water	56	2p-m3w	70	4.9	2.7	1.7
Water	57	2mp-m3w	93	8.8	4.2	2.5
Water	58	2m-m3w	74	11.0	7.2	4.4
Water	59	2mp-p3w	104	18.0	10.6	4.7
Water	61	11w	89	9.5	4.1	2.1
Water	63	12w	101	7.4	4.0	2.3
Water	64	7w	58	3.5	1.8	1.4
Water	65	1w	63	5.2	2.9	1.8
Water	66	2w	75	8.8	4.0	2.1
Water	67	6w	119	15.4	8.4	4.3
Water	68	9w	96	13.5	8.4	4.7
Water	69	neg1	32	7.5	4.2	2.2
Water	70	2.2m	80	9.5	5.3	2.7
Water	71	12.2mw	76	8.3	4.8	2.6
Water	72	4.2mw	67	13.2	8.0	3.9
Water	73	7.2pw	79	9.8	5.2	2.6

Continued on next page

Table D.1 – continued from previous page

Sample Type	Sample nr.	sample indentity	X0	X1	X2	Inf.
Water	74	4.2pw	77	8.0	3.8	2.1
Water	75	1.2mw	93	13.2	8.4	4.4
Water	76	neg2	45	6.2	3.8	2.3
Water	77	7.2mw	69	8.2	3.8	2.1
Water	78	9.2mw	76	13.1	8.7	4.5
Water	79	14.2mw	37	5.8	3.1	1.9
Water	80	6.2mw	96	10.3	5.7	3.1
Water	81	11.2mw	86	7.1	3.6	2.0
Water	82	3w	98	12.2	6.5	2.9
Water	83	5w	93	10.4	5.5	3.1
Water	84	8w	124	6.9	2.7	1.7
Water	85	15w	117	16.2	7.8	3.5
Water	86	10w	131	6.5	2.4	1.6
Water	87	13w	124	28.6	18.3	9.6
Gut	88	12.2m1i	51	10.2	6.2	3.0
Gut	89	11.2m1i	48	11.5	7.1	3.9
Gut	90	4.2m1i	53	4.6	2.7	1.7
Gut	91	7.2p2i	59	2.7	1.8	1.4
Gut	92	14.2m1i	60	6.9	4.2	2.7
Gut	93	2.2m1i2	57	7.6	4.8	2.8
Gut	94	1.2m2i	70	6.1	3.2	1.9
Gut	95	2.2m2i	43	8.8	5.3	2.9
Gut	96	4.2m2i	49	7.1	4.0	2.4
Gut	97	4.2p1i	44	5.7	3.5	2.1
Gut	98	7.2p1i	55	3.3	2.0	1.5
Gut	99	6.2p1i	79	12.4	8.6	5.0
Gut	100	12.2m2i	43	8.4	5.6	3.0
Gut	101	6.2m2i	44	8.5	5.6	3.6
Gut	102	1.2m1i	66	7.4	4.8	2.7
Gut	103	7.2m1i	64	12.1	7.1	4.0
Gut	104	7.2m2i	44	5.9	3.4	2.2
Gut	105	9.2m4	65	7.8	5.3	3.0
Gut	106	4.2p2i	53	6.3	5.1	3.8
Gut	107	12.2m3	58	11.7	6.2	2.9
Gut	108	9.2m3	58	12.6	6.5	2.9

Continued on next page

Table D.1 – continued from previous page

Sample Type	Sample nr.	sample indentity	X0	X1	X2	Inf.
Gut	109	2.2m1i	49	11.1	6.1	2.8
Gut	110	7.2m5	56	9.6	5.3	3.1
Gut	111	4.2m3	60	14.3	8.2	3.8
Gut	112	4.2m2	54	8.1	5.1	3.3
Gut	113	12.2m1	86	13.3	7.0	3.7
Gut	114	6.2m3	53	13.5	6.6	2.9
Gut	115	11.2m1	46	8.5	4.8	2.5
Gut	117	6.2m1	51	7.3	3.4	1.9
Gut	118	12.2m6	53	9.7	5.7	3.0
Gut	119	14.2m1	53	11.7	6.0	2.8
Gut	120	11.2m2	55	10.6	5.0	2.4
Gut	121	2.2m2	50	10.9	5.6	3.0
Gut	122	6.2m4	53	6.1	3.4	2.2
Gut	123	4.2m5	56	11.4	6.5	3.4
Gut	124	4.2p4	58	9.6	5.3	3.0
Gut	125	4.2m1	59	9.4	5.3	3.2
Gut	126	2.2m5	59	4.2	2.3	1.6
Gut	127	4.2p3	54	11.6	7.2	4.3
Gut	128	14.2m3	56	8.9	3.9	2.1
Gut	129	2.2m3	56	13.0	7.3	3.4
Gut	130	7.2m3	61	9.0	3.8	2.1
Gut	131	4.2m4	52	9.8	5.5	3.5
Gut	132	14.2m	43	8.0	3.9	2.1
Gut	133	6.2m2	58	9.8	6.8	4.1
Gut	134	1.2m2	45	8.3	4.2	2.2
Gut	135	11.2p2	57	11.3	6.6	3.7
Gut	136	4.2p2	53	4.5	3.1	2.1
none	137	KF-(51-136)	20	9.4	7.1	3.8

E Bacterial composition bar graphs

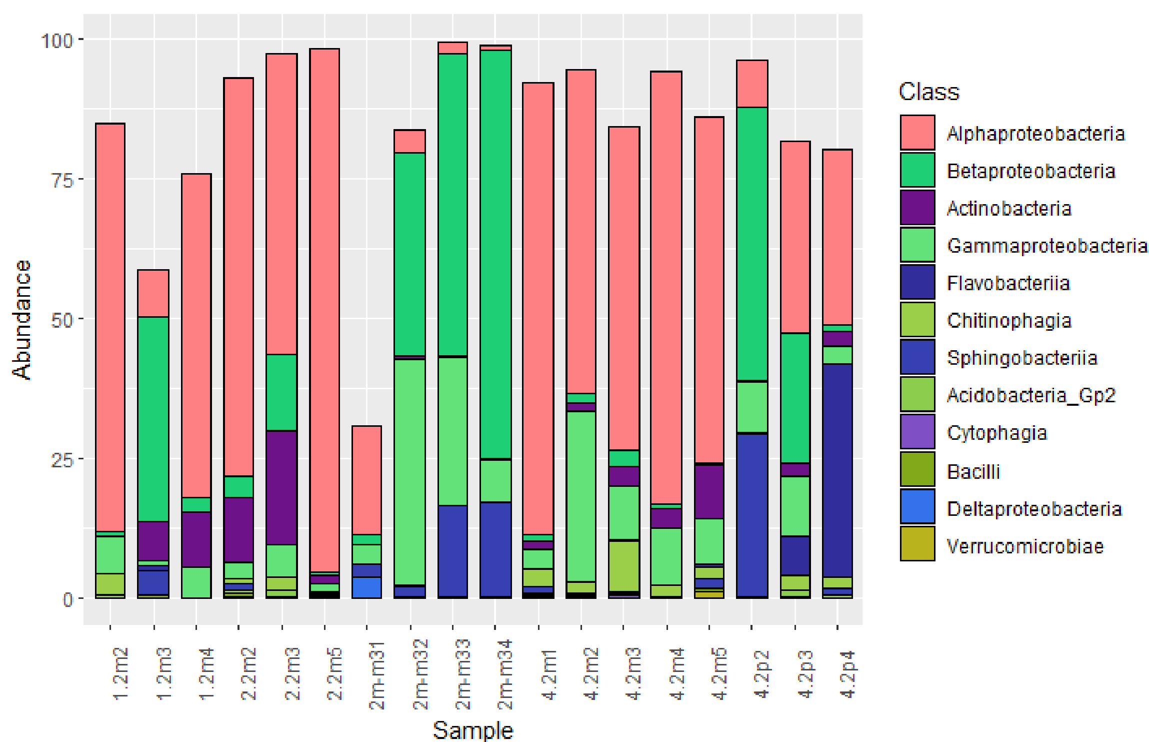

Figure E.1: The proportional abundances of all classes for *D.Magna* inoculated with *D. magna* microbiome.

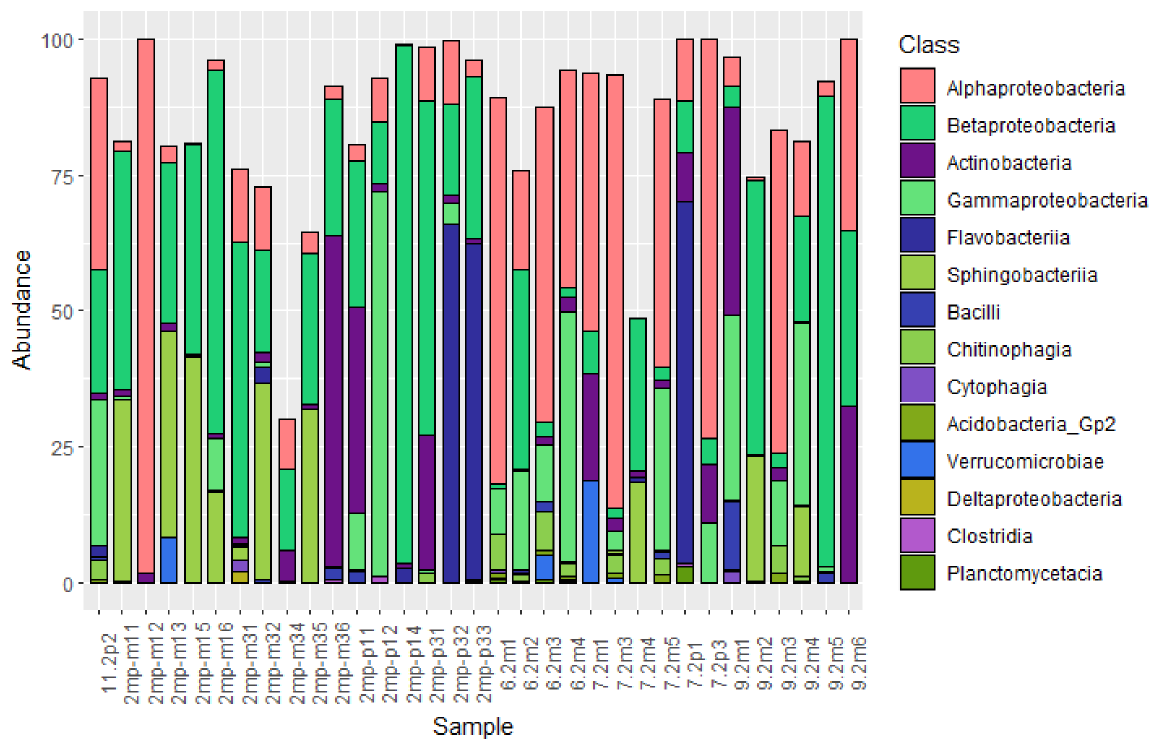

Figure E.2: The proportional abundances of all classes for *D.Magna* inoculated with co-culture microbiome.

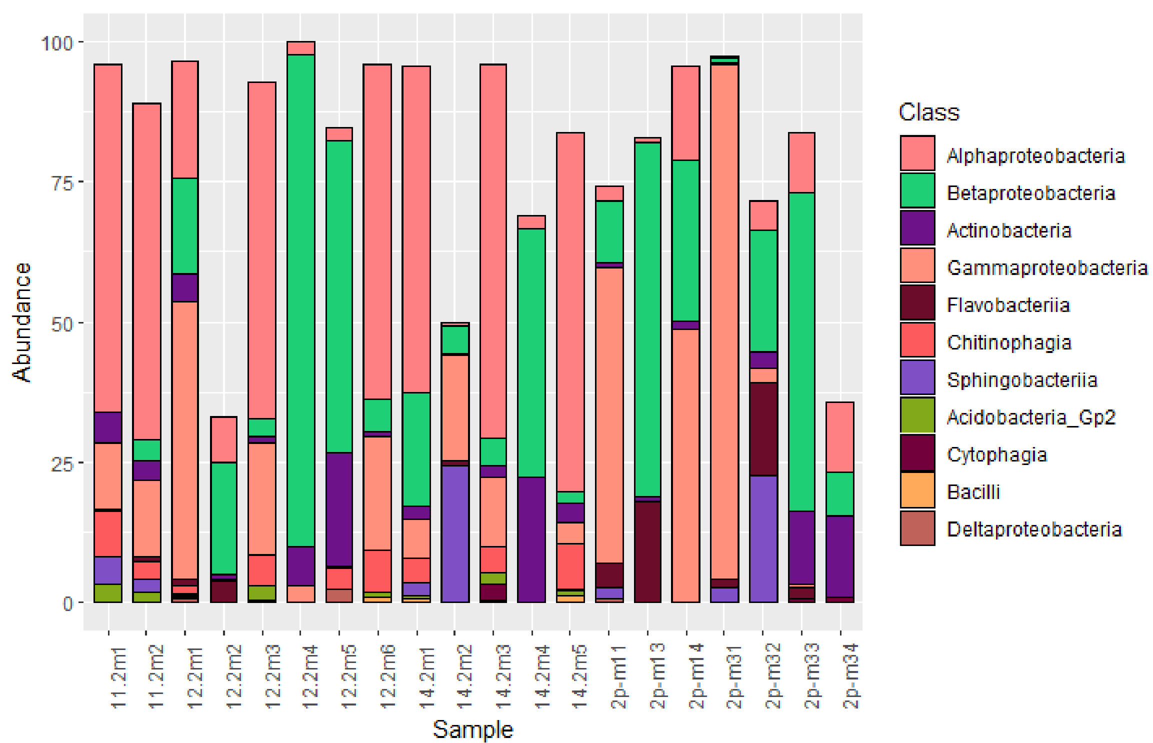

Figure E.3: The proportional abundances of all classes for *D.Magna* inoculated with *D. pulex* microbiome.

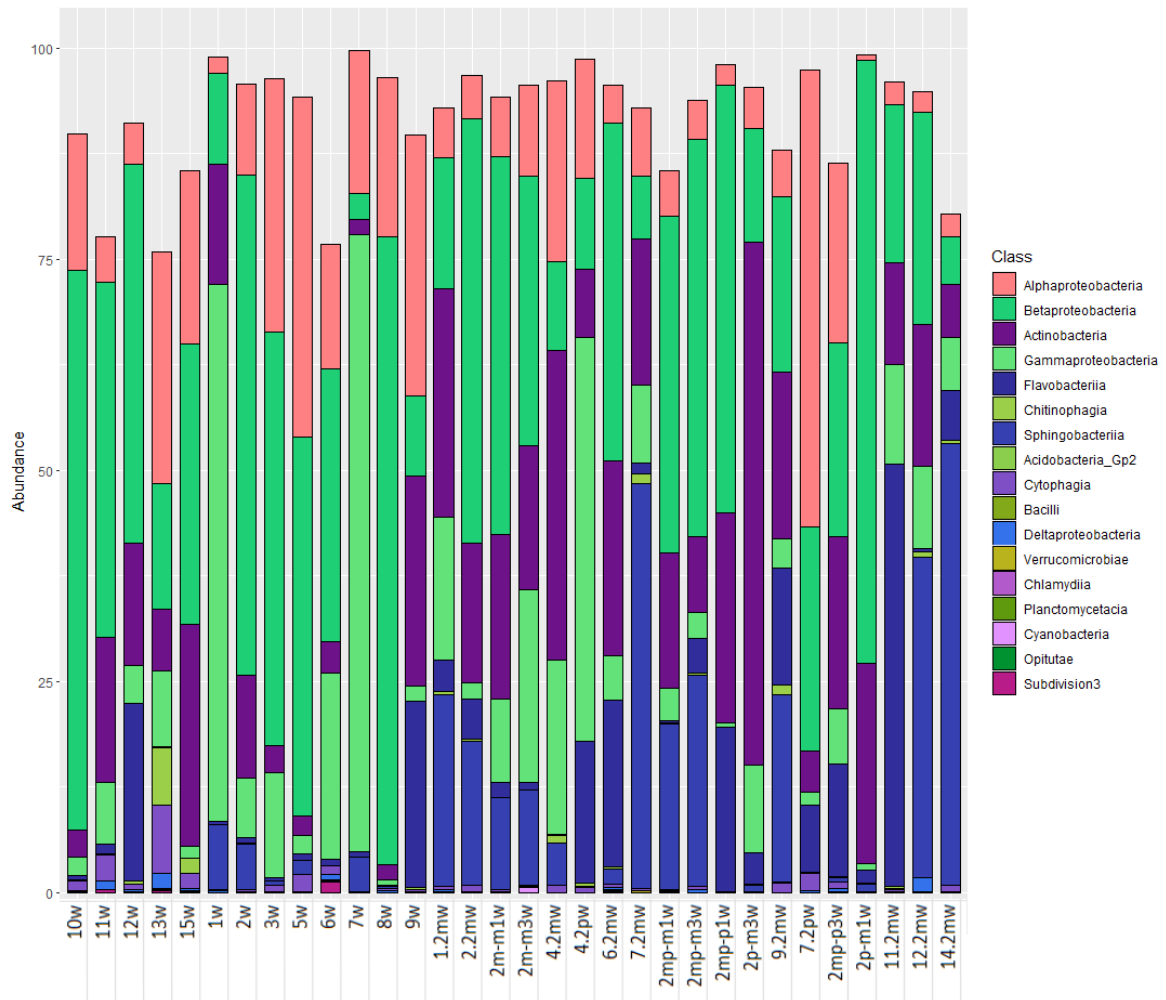

Figure E.4: The proportional abundances of all classes from water samples. The sample names have been edited because Rstudio sorts samples alphabetically, and did not put these samples in an order that was sensible for visualisation. Placeholder names were therefore used in making the figure.

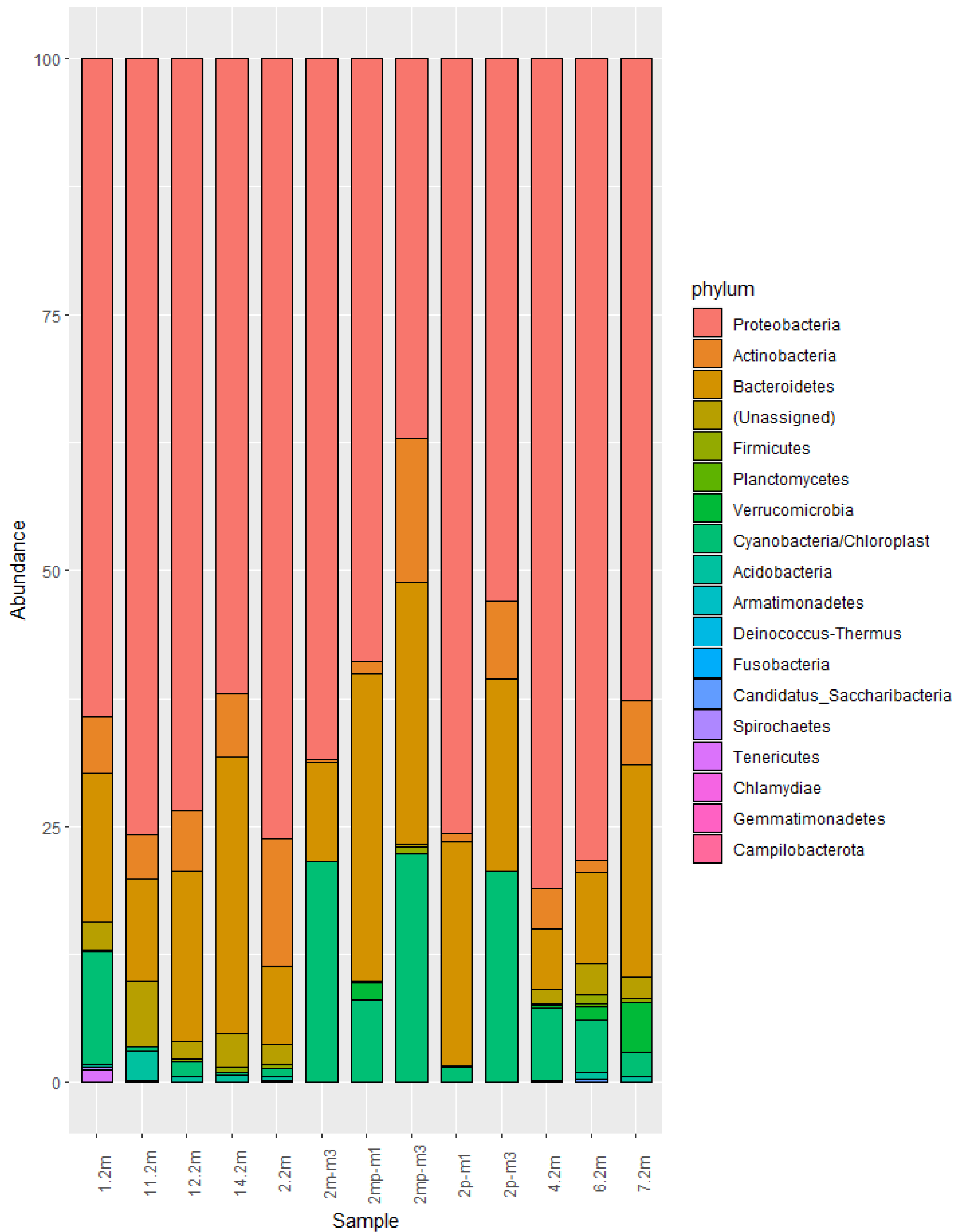

Figure E.5: The proportional abundances of all phylum present in the magna gut samples of the library. Samples of guts from the same aquaria have been averaged and represented in a single column. 14.2m 2p-m3, and 7.2m had the highest fecundity

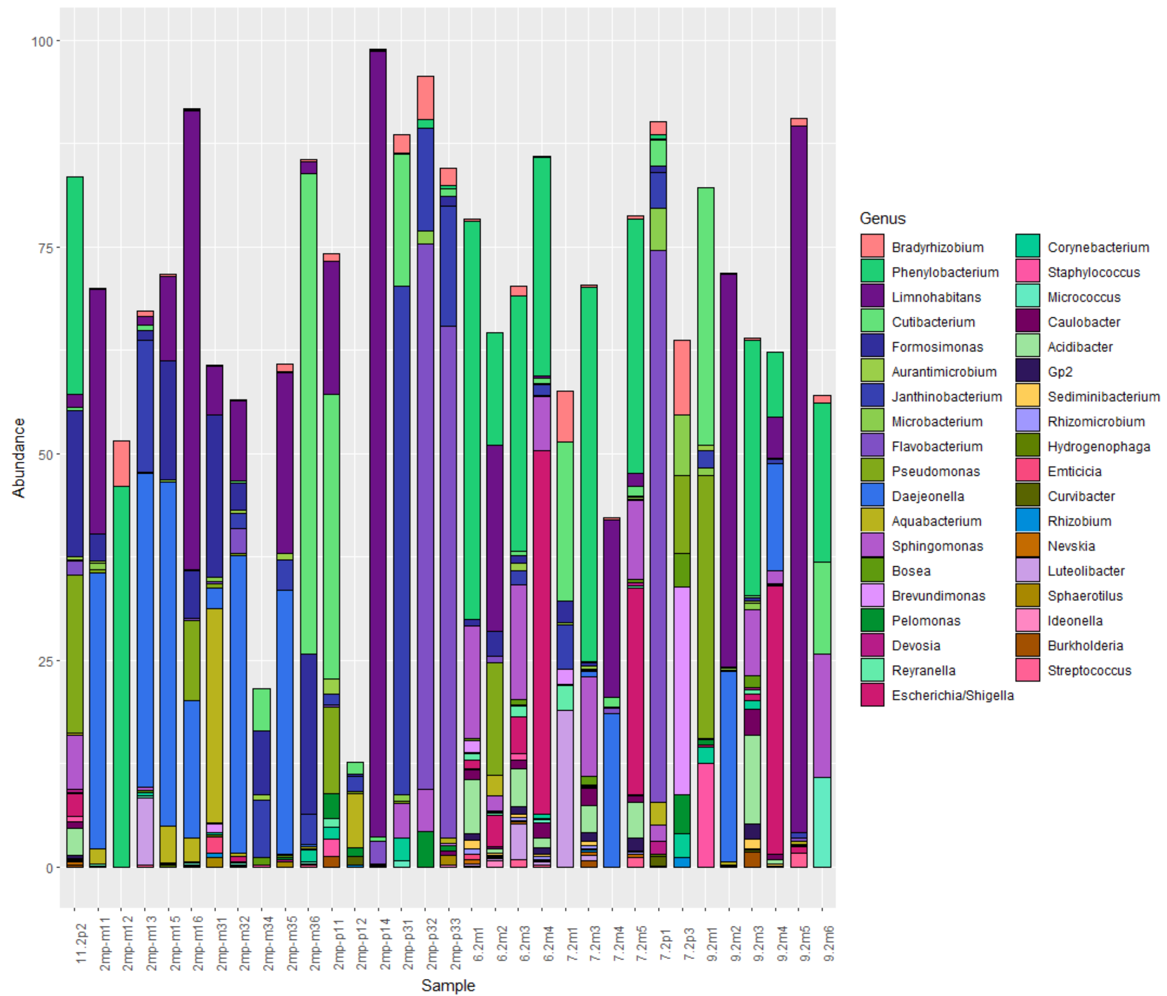

Figure E.6: The proportional abundance of reads classified at the genus level. From samples of phase two animal gut samples, inoculated with co-culture microbiome. The data has been filtered to remove taxa occurring in less than 6 of the samples in the selection. The proportion of unclassified reads has also been excluded.

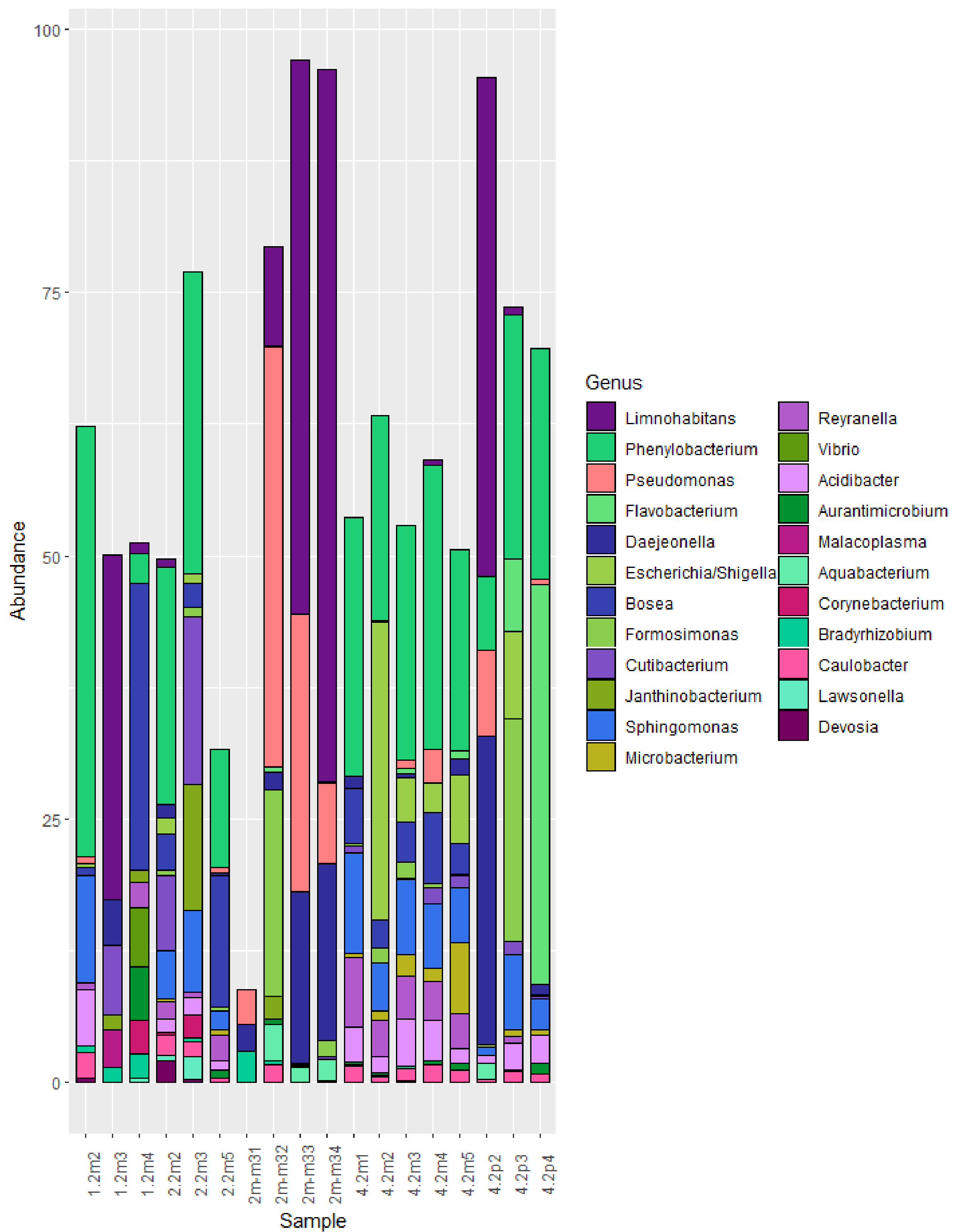

Figure E.7: The proportional abundance of reads classified at the genus level, from samples of *D. magna* and *D. pulex* inoculated with *D. magna* microbiome. Taxa with a maximal abundance of less than two percent in all samples in the selection have been excluded. The proportion of unclassified reads has also been excluded.

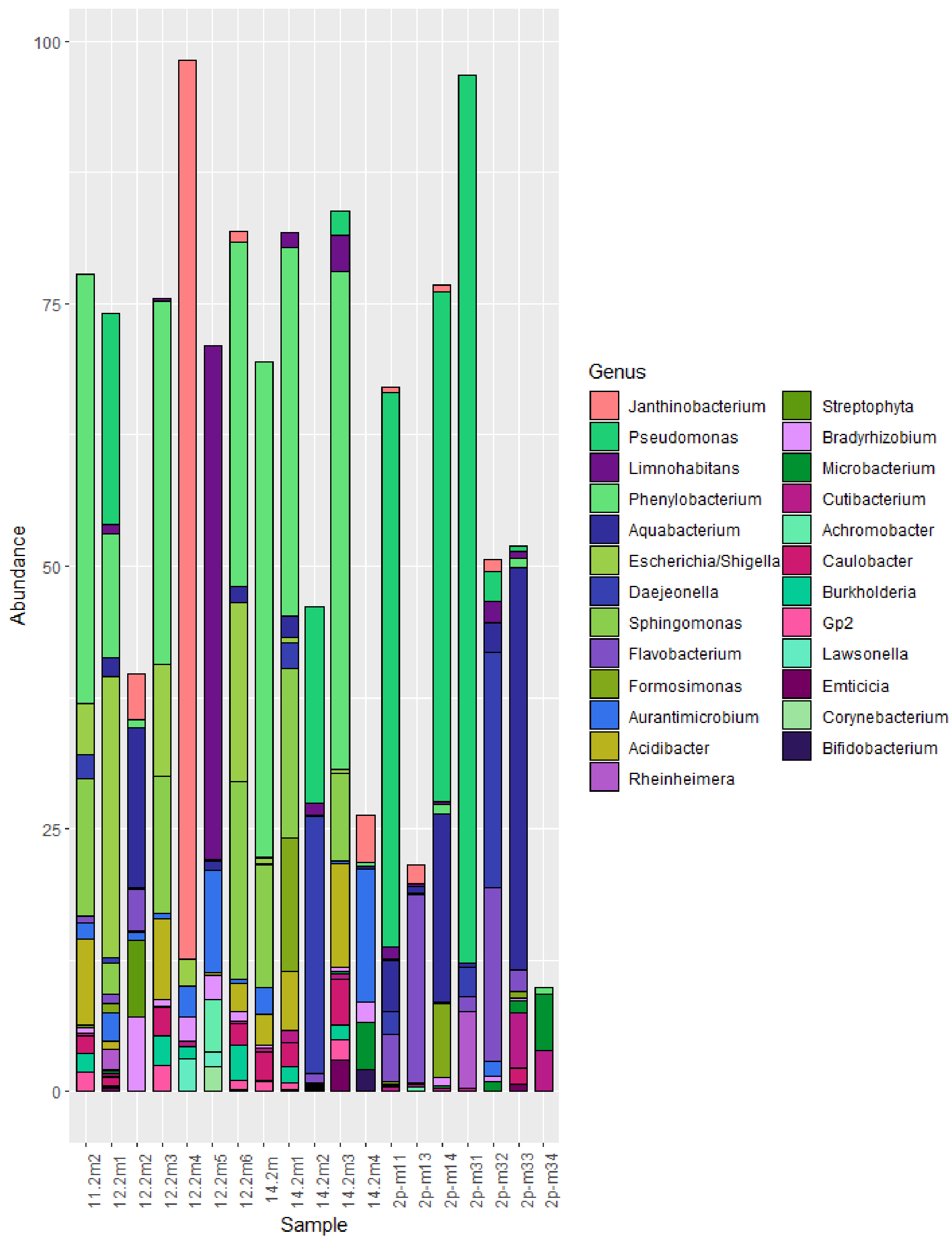

Figure E.8: The proportional abundance of reads classified at the genus level, from samples of *D. magna* inoculated with *D. pulex* microbiome. Taxa with a maximal abundance of less than two percent in all samples in the selection have been excluded. The proportion of unclassified reads has also been excluded.

F Algae media

ANNEX 3

GROWTH MEDIA

One of the following two growth media may be used:

OECD medium: Original medium of OECD TG 201, also according to ISO 8692
US. EPA medium AAP also according to ASTM.

When preparing these media, reagent or analytical-grade chemicals should be used and deionised water.

Composition of The AAP-medium (US. EPA) and the OECD TG 201 medium.

Component	AAP		OECD	
	mg/L	mM	mg/L	mM
$NaHCO_3$	15.0	0.179	50.0	0.595
$NaNO_3$	25.5	0.300		
NH_4Cl			15.0	0.280
$MgCl_2 \cdot 6(H_2O)$	12.16	0.0598	12.0	0.0590
$CaCl_2 \cdot 2(H_2O)$	4.41	0.0300	18.0	0.122
$MgSO_4 \cdot 7(H_2O)$	14.6	0.0592	15.0	0.0609
K_2HPO_4	1.044	0.00599		
KH_2PO_4			1.60	0.00919
$FeCl_3 \cdot 6(H_2O)$	0.160	0.000591	0.0640	0.000237
$Na_2EDTA \cdot 2(H_2O)$	0.300	0.000806	0.100	0.000269*
H_3BO_3	0.186	0.00300	0.185	0.00299
$MnCl_2 \cdot 4(H_2O)$	0.415	0.00201	0.415	0.00210
$ZnCl_2$	0.00327	0.000024	0.00300	0.0000220
$CoCl_2 \cdot 6(H_2O)$	0.00143	0.000006	0.00150	0.00000630
$Na_2MoO_4 \cdot 2(H_2O)$	0.00726	0.000030	0.00700	0.0000289
$CuCl_2.2(H_2O)$	0.000012	0.00000007	0.00001	0.00000006
pH	7.5		8.1	

- The molar ratio of EDTA to iron slightly exceed unity. This prevents iron precipitation and at the same time, chelation of heavy metal ions is minimised.

In test with the diatom *Navicula pelliculosa* both media must be supplemented with $Na_2SiO_3 \cdot 9H_20$ to obtain a concentration of 1.4 mg Si/L.

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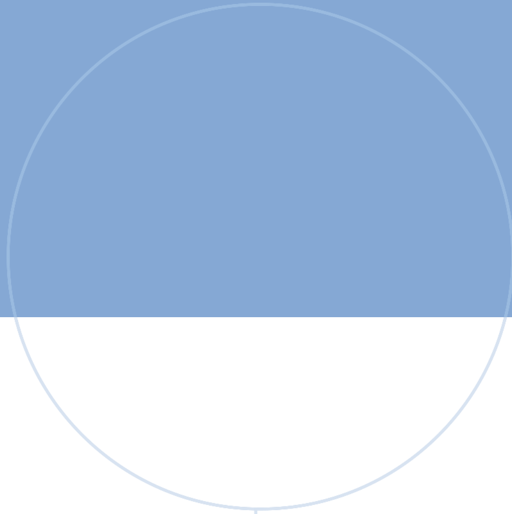
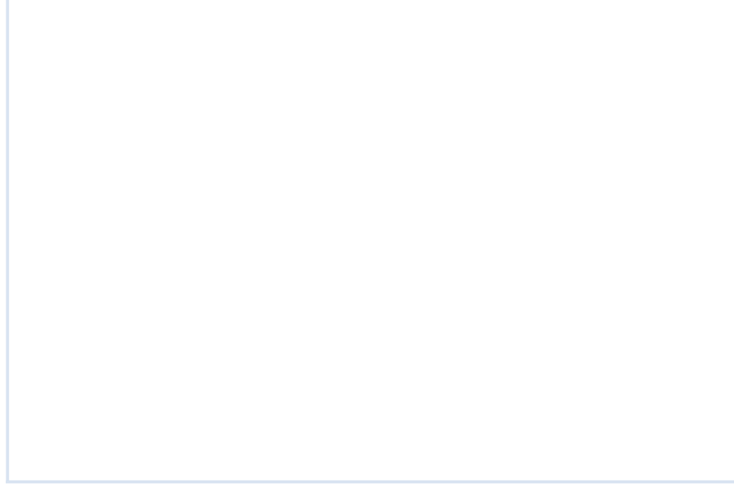

NTNU
Norwegian University of
Science and Technology