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Larval *Culex* Mosquitoes Increase Primary Production and Decrease Bacterial Diversity in Aquatic Mesocosms

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To my husband James, for always being there for me.

You are the best.

ABSTRACT OF THE THESIS

Larval *Culex* Mosquitoes Increase Primary Production and Decrease Bacterial Diversity in Aquatic Mesocosms

by

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Mosquitoes are important vectors of many devastating human diseases. Much of the research to date has focused on their adult biology as vectors, but the field of microbiome research is rapidly developing. Commensal and symbiotic bacteria may play important roles in nutrition, digestion, or chemical defense for mosquitoes. The effects of larval mosquito presence and nutrient input on aquatic and larval mosquito microbiomes and on ecosystem structure and function were studied in $1\,m^2$ mesocosms. Three levels of enrichment with organic and inorganic nutrients were cross-classified with larval mosquito presence or absence. Water quality variables (total nitrogen, nitrates, nitrites, ammonium, total phosphorous, and chemical oxygen demand), mosquito oviposition rates, invertebrate abundance, phytoplankton biomass, planktonic particle size spectra, primary production, community metabolism, and microbial diversity were measured during the 1-month study. The oviposition rate of *Culex* mosquitoes was directly related to nutrient enrichment. Mosquito presence significantly influenced phosphorus concentration, but did not significantly affect the concentration of other water quality variables. The presence of larval mosquitoes with

high levels of nutrient input increased the photosynthetic productivity of aquatic mesocosms, with a corresponding decrease in bacterial diversity. Conversely, when mosquitoes were prevented from ovipositing in mesocosms, water column bacterial diversity increased at high levels of nutrients. This work shows that larval mosquitoes have profound impacts on aquatic ecosystems, especially when those ecosystems are affected by high nutrient input.

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Chapter 1

1.1 Introduction

Mosquitoes (Culicidae) are well-known vectors of disease-causing pathogens, such as *Plasmodium*, West Nile virus, and dengue virus. Previous work on mosquitoes has primarily focused on mosquito biology, vector competence, and mosquito control (Tempelis et al. 1965; Beerntsen et al. 2000; Fradin & Day 2002; Goddard et al. 2002; Reisen et al. 2006; Wang et al. 2017). Effective control of larval mosquitoes can be accomplished using toxins derived from strains of *Bacillus thuringiensis* var. *israelensis* and *Lysinibacillus sphaericus* (Sanahuja et al. 2011; Berry 2012). Much work has been done on the biochemical nature of the insecticidal toxins, potential nontarget effects on other organisms, and potential evolution of resistance (Tabashnik 1994; Hershey et al. 1995; 1998; Schnepf et al. 1998; Duguma et al. 2015b). It has been shown that these toxins are very selective, and direct nontarget negative effects of *Bti* and *L. sphaericus* have not been seen. However, the application of these insecticides may have indirect effects on aquatic ecosystems through the suppression of larval mosquito populations.

Most mosquito larvae filter-feed on bacteria and microeukaryotes such as protists and rotifers (Wallace & Merritt 1980; Merritt et al. 1992). This means mosquito larvae may regulate microbial populations when mosquito larvae occur in an area. When mosquitoes colonize a new area, they may disrupt existing aquatic food webs. This has been studied in pitcher plant habitats (Addicott 1974), detrital communities (Kaufman et al. 1999), and artificial aquatic mesocosms (Duguma et al. 2015a). The role of protists in regulating mosquito larval populations has also been studied in a diverse array of habitats, from tree holes to flooded rice paddies (Amarasinghe & Rathnayake 2014).

A significant component of any food web is primary production. In terrestrial and shallow aquatic habitats, primary production occurs through photosynthesis, while in deep marine areas primary production occurs through chemosynthesis (Pennisi 2017). In aquatic environments, primary production occurs through phytoplankton suspended in the water column or periphyton on the benthos. Previous studies have found a positive correlation between larval mosquitoes and phytoplankton biomass, but have not quantified production directly (Duguma et al. 2013; 2015b; 2017).

The discovery of next-generation sequencing as a way to identify previously-unculturable microbes, including phytoplankton such as *Cyanobacteria*, initiated an explosion of research into microbiomes in various habitats and hosts, especially using the 16S rRNA marker gene (Broderick et al. 2004; Clarridge 2004; Xiang et al. 2006; Lundgren et al. 2007; Sun et al. 2010; Grubbs 2013; Hu et al. 2013; Kaltenpoth & Steiger 2013; Bansal et al. 2014; Duguma et al. 2015a; Creer et al. 2016; Pennington et al. 2016). This work is showing that prokaryotes have been underappreciated in their diversity, abundance, and ecological roles. For

organisms such as mosquitoes that feed on prokaryotes, the role of microbes is especially important.

High-throughput sequencing initially focused on prokaryotes, but has been applied to eukaryotes as well. Much work focused on fungi due to their prominent ecological roles as decomposers and pathogens (Huffnagle & Noverr 2013; Lindahl et al. 2013; McGuire et al. 2013). However, the field is expanding rapidly to include microbial eukaryotes such as ciliates, dinoflagellates, and other protists (Bik et al. 2012; Hugerth et al. 2014; Stoeck et al. 2014).

Microbial communities of environmental habitats can be very diverse, even in unexpected places such as desert soils of South Africa (Elliott et al. 2014), seawater from the Baltic Sea (Hu et al. 2016), wastewater sludge (Riviere et al. 2009), and moose rumen content (Hugerth et al. 2014). Microbial communities vary based on environmental conditions and available nutrients, and may have impacts on primary production if nutrients are limiting (Bratbak & Thingstad 1985; Falkowski et al. 1998). Conversely, algal production may influence microbial populations, since many bacteria utilize excreted organic carbon compounds from algae as carbon sources (Cole et al. 1982). Production of terrestrial plants may also control microbial physiological processes, such as methane emission in wetlands (Whiting & Chanton 1993).

While microbes found in waters and soils are important for ecosystem functioning, host-associated microbes also have important roles. Many insects harbor beneficial bacterial endosymbionts in their guts, most likely to aid digestion of difficult substrates such as cellulose or nutrient-poor phloem (Slaytor 1992; Douglas 1998; Kikuchi et al. 2007;

Morales-Jimenez et al. 2012; Peterson et al. 2015). Other symbiotic bacteria provide defensive services to inhibit opportunistic pathogens like fungi (Cardoza et al. 2006; Adams et al. 2008). There have been many studies documenting the antibacterial and antifungal properties of *Actinobacteria* associated with a wide variety of organisms, from marine sponges to leaf-cutter ants (Cardoza et al. 2006; Zhang et al. 2006; Adams et al. 2008; Sun et al. 2010; Six 2013).

Host-associated microbes have been characterized for several mosquito species in various life stages, including *Anopheles coluzzi* (Gimonneau et al. 2014), *Anopheles gambiae* (Wang et al. 2011; Coon et al. 2014; Gimonneau et al. 2014), *Anopheles stephensi* (Chouaia et al. 2012), *Aedes aegypti* (Coon et al. 2014), *Culex quinquefasciatus* (Pennington et al. 2015), and *Culex tarsalis* (Duguma et al. 2013). Many of these studies focus on mosquito gut endosymbionts only, while others included microbes from the whole organism. The present study focuses on larval *C. quinquefasciatus* and *C. tarsalis*, which are known to be abundant in the study location (inland southern California).

Previous studies on larval mosquito microbiomes show that microbial symbionts are required for proper development in multiple mosquito species (Chouaia et al. 2012; Coon et al. 2014). Without these symbionts, larvae either do not develop beyond first instars or take much longer times to develop compared to controls. Furthermore, the composition of the microbiota is affected by environmental factors such as water contaminated with pharmaceutical products (Pennington et al. 2016). It is currently unclear which specific benefits larval mosquito microbial symbionts provide for their hosts.

In this experiment, I sought to study the influence of larval mosquitoes on primary production in semi-natural habitats. Since increases or decreases in primary production can have cascading effects on ecosystems, mosquito larvae may have indirect effects on aquatic food webs. Furthermore, I sought to characterize the microbial communities of both the water column and the mosquito gut. This is important to determine how mosquito larvae change prokaryotic and eukaryotic communities in their habitat.

To study these topics, I used a variety of methods. Environmental analysis was carried out using chemical tests and continuous monitoring of certain variables such as temperature and dissolved oxygen. Organisms were identified using morphological methods (eukaryotes) and high-throughput sequencing with two different molecular markers (prokaryotes and eukaryotes).

1.2 Methods

1.2.1 Nutrient enrichment

This project was conducted at the Aquatic Research Facility at UCR's Agricultural Experiment Station during summer 2016. Twelve fiberglass tubs ($1\,m^2$) were filled with water from an irrigation reservoir to 30 cm deep. The fauna in the water column was homogenized by placing 1 L of water from each tub into a large bucket and thoroughly mixing the contents. One liter of the homogenized water was then transferred back to each of the 12 tubs. A 1 L sample of the homogenized water was taken from one tub for chemical analysis prior to enrichment.

Tubs were enriched with organic and inorganic nutrients (alfalfa rabbit food pellets (Sun Seed Company, Inc., Bowling Green, OH) and ammonium sulfate (Lilly Miller Brands, Walnut Creek, CA) respectively) to attract mosquitoes for oviposition, and to determine the influence of increased nutrients on bacterial diversity in the water column. The "high" enrichment group received 20 g ammonium sulfate and 30 g rabbit food pellets. The "low" enrichment group received 4 g ammonium sulfate and 6 g rabbit food pellets. The "no" enrichment group did not receive any additional nutrient input. Each enrichment group consisted of 4 tubs, 2 of which allowed mosquito access and 2 of which prevented mosquito access (6 treatment groups total). Each treatment group was given a unique label, which will be used throughout the text to avoid confusion (see Table 1.1 for the treatment labels).

The experiment was initiated on 27 June 2016 (day 0) by filling the tubs with water and carrying out the enrichment. Tubs were randomly assigned to treatments prior to enrichment. Water quality measurements were taken every week for 4 weeks, on days 8, 15, 22, and 29 of the experiment. DNA extractions of mosquito larvae and water samples were done every week for 3 weeks, on days 9, 15, and 22 of the experiment. DNA extractions were not done during week 4 (day 29) due to very low abundance of mosquito larvae in the no (N1) and low (L1) enrichment treatments.

1.2.2 Mosquito populations

Mosquito access was controlled through the use of 1 m^2 gray fiberglass mesh window screens placed on each tub above the water surface. All tubs had mesh screens to control for the effect of the mesh on light availability to phytoplankton. Screens were attached to

Table 1.1. Treatment groups used in the analysis.

Group number	Enrichment level	Mosquito access	Tubs
N0	None	No	06, 25
L0	Low	No	16, 22
H0	High	No	13, 23
N1	None	Yes	11, 17
L1	Low	Yes	09, 21
H1	High	Yes	18, 24

Each treatment group consisted of 2 replicate mesocosms, for a total of 12 mesocosms.

19 mm diameter PVC pipe. Six of the screens additionally had 19 mm x 11 mm thick black weatherstripping foam applied to the lower edges of the frame to exclude mosquitoes in the "no mosquito access" groups (N0, L0, and H0). Screens were shifted to leave a gap ∼3 cm wide between the PVC pipe and the tub edge in the "mosquito access" groups (N1, L1, and H1).

Egg raft censuses were conducted every two days for the duration of the study through visual examination of the total water surface. After new egg rafts were counted, they were transferred to a Styrofoam arena floating on the water surface. A plastic container inverted over the top of the arena prevented mosquito oviposition into the arena. This setup allowed larvae to hatch from the egg rafts into the water while ensuring that the next census would include only new rafts laid within the previous 48 hours. Egg rafts were transferred from the high enrichment tubs to the other treatment groups in order to equalize mosquito oviposition into the high enrichment tubs and to maintain similar but low levels of mosquito oviposition among low and no enrichment tubs.

Fourteen days into the experiment, the tubs were supplemented with egg rafts from laboratory-reared *Culex quinquefasciatus* to increase larval mosquito densities. Low

and no enrichment tubs each received 4 egg rafts, while the high enrichment tubs each received 8 egg rafts.

1.2.3 Immature mosquito and microinvertebrate abundance

Dip samples were taken using a standard 350 mL dip cup to quantify the abundance of larval mosquitoes and other aquatic invertebrates. Three dip samples per mesocosm were filtered through a plankton net (mesh opening: 64 µm) and preserved by adding 95 % ethanol to a final concentration of ∼50 % ethanol. The three dips were aggregated together prior to organism counting and identification. Data are reported using these aggregate values (dip volume of 1050 mL).

1.2.4 Water quality

One liter samples of water from each tub were collected into opaque brown bottles and transported to the laboratory on ice. Bottles were kept on ice throughout the day until all analyses were completed. Nutrient concentrations (total nitrogen (TN), ammonium nitrogen (NH_4−N), nitrite nitrogen (NO_2−N), nitrate nitrogen (NO_3−N), total phosphorus (TP), and chemical oxygen demand (COD)) in the water column were measured colormetrically using a Hach DR^{TM} 2800 spectrophotometer (TNT Plus tests, Hach Chemical Co., Loveland, CO).

Nitrogen:phosphorus ratios were calculated using molar ratios of each nutrient. Quantities were converted from milligrams to moles per tub, and the ratios were then averaged per treatment at each time point.

1.2.5 Chlorophyll biomass

Chlorophyll biomass was quantified using acetone extractions of vacuum-filtered water samples (Wetzel & Likens 1991). Vacuum-filtration was done using 50–300 mL of water onto Millipore membrane filters (pore opening: 0.2 µm or 0.45 µm). Filters were wrapped in aluminum foil and frozen at −20 °C for at least 48 hours prior to extraction. After freezing, filters were placed in alkaline acetone (90 % acetone, 10 % water, 3 drops L^{-1} NH_4OH) for 1.5–2 hours in foil-covered centrifuge tubes on ice. Extracts were then analyzed at 665 nm with a BioSpec-1601 spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD) and corrected for turbidity.

1.2.6 Primary productivity and community metabolism

Water column primary productivity was measured as the change in dissolved oxygen concentration using 250 mL light and dark glass BOD bottles (Wetzel & Likens 1991). The bottles were incubated *in situ* for 2–4 hours between approximately 11:00 and 14:00. Initial oxygen concentration and post-incubation dissolved oxygen concentration in the bottles were measured using a stirring oxygen electrode (model 9708, Thermo Orion, Boston, MA). Gross photosynthesis (mg O_2 L^{-1} d^{-1}), net photosynthesis (mg C m^{-3} h^{-1}), and respiration (mg O_2 L^{-1} d^{-1}) were calculated using formulae in Wetzel and Likens (1991).

Primary productivity can occur in the water column and on the sides of shallow mesocosms. Community metabolism was measured in representative mesocosms from each treatment (n = 1 to 3 mesocosms at various time points for each treatment). The net O_2 change per day resulting from biological activity (net daily metabolism, NDM) was

calculated as the difference between gross primary productivity (GPP) and daily community respiration (CR_{24}). Pressure-compensated dissolved oxygen concentration was measured at 10 min intervals using an optical dissolved oxygen sensor (mini DO_2T; PME, Vista, CA). Water temperature was measured concurrently by the sensor during 3–5 day deployments.

Changes in dissolved oxygen concentration were computed hourly. Average hourly respiration rate was estimated from the mean change in dissolved oxygen concentration hourly between sunset and sunrise. This value for each day was multiplied by 24 to obtain daily community respiration (CR_{24}; Bott 2007). The hourly rates of net O_2 change were summed over the photoperiod and added to the absolute value for respiration rate during the same period to estimate GPP. Oxygen diffusion rates from the water surface into the atmosphere were negligible and assumed to be zero for calculations.

1.2.7 Particle counts

Sestonic particle size distribution (equivalent spherical diameter [ESD]: 0.4 to 224 µm) was enumerated using a Multisizer 4 Particle Analyzer (Beckman Coulter Inc., Brea, CA). The particles in bulk water samples were quantified in 3–8 replicate samples using 100 and 280 µm apertures. Approximately 10 mL of bulk water was filtered through 10 µm aperture mesh and the particle distribution (0.4 to 10 µm ESD) in the filtered water was determined in three replicates using a 20 µm aperture. Controls for background particle spectra consisted of either unfiltered (for 100 and 280 µm apertures) or filter-sterilized (0.2 µm pore size; for the 20 µm aperture) electrolyte (ISOTON II).

Particle counts were binned by diameter into three categories: small (0.4–1 µm), medium (1.01–10 µm), and large (>10 µm). Data were log-transformed to account for the differences in bin ranges prior to analysis.

1.2.8 Microbiome Sample Collection

Water microbiome samples were taken by dipping sterile 50 mL Falcon tubes approximately 2 cm beneath the water surface at the tub center and collecting 15–50 mL of water (most samples contained 30–40 mL of water). Tubes were then transported back to the laboratory on ice, and frozen at −20 °C for at least 24 hours prior to DNA extraction. DNA extractions were done on 1 mL samples from each Falcon tube after thoroughly mixing the contents by inversion.

Mosquito samples were collected by sweeping a small fish net through the tub or by targeting visible larvae with the fish net. Four large (late instar) larvae were taken from the net with forceps, placed into 1.5 mL tubes containing 95 % ethanol, and transported on ice to the laboratory. When late instar larvae were not available, early instar larvae were used instead (tub 11 [N1] during week 1 and tub 9 [L1] during week 3). Only 2 larvae were available in tub 11 [N1] during week 2. Larvae were not present in the following tubs: tubs 9 [L1] and 21 [L1] during week 2, tub 17 [N1] during week 3.

1.2.9 DNA Extraction

DNA extractions were performed using the DNeasy® Blood & Tissue Kit by Qiagen. Mosquito samples were prepared for extraction by sonicating in a mixture of equal parts 10 % bleach (NaOCl) solution and 10 % Tween® 80 (Fisher Scientific, Fair Lawn, NJ)

for 3 min, followed by sonication in 0.2 µm filter-sterilized H_2O for 2 min. Clean larvae were placed on ice in sterile 1X phosphate-buffered saline (PBS, pH 7.4, Life Technologies Corp., Grand Island, NY) for immediate use or stored at −20 °C in sterile 1X PBS for 24 hours. Prior to extraction, clean larvae were ground with sterile glass pestles. Mosquito DNA was then extracted according to the "Animal Tissue" protocol provided by the manufacturer.

Water samples were prepared for extraction by centrifuging 1 mL aliquots at 300 rpm for 5 min to pellet all organisms. After removing the supernatant, the pellet was resuspended in sterile 1X PBS. DNA was then extracted according to the "Cultured Cells" protocol provided by the manufacturer. Isolated DNA was stored at −20 °C until analyzed with the Thermo Scientific Nanodrop^{TM} 2000c Spectrophotometer at the IIGB Core of the University of California, Riverside.

1.2.10 Library Preparation

Illumina library preparation of the DNA extractions was done using a two-step PCR protocol (Bybee et al. 2011; Ionescu et al. 2015). Briefly, the first step involved template-specific primers with "common sequence" (CS) linkers at the 5' ends to generate PCR amplicons containing the CS linkers. The second step, performed by the DNA Services Facility at the University of Illinois at Chicago (UIC), used primers containing Illumina adapters and sample-specific Fluidigm barcodes. These primers targeted the CS linker sequences on the amplicons from the first step. Combined, these steps generated amplicons with the required adapters and barcodes necessary for Illumina sequencing and indexing.

Two sets of template-specific primers were used in this study. Set 1 targeted the bacterial 16S marker gene using primers 341F and 806R (Ionescu et al. 2015). Set 2 targeted

the eukaryotic 18S marker gene using primers 574*F and 1132R (Hugerth et al. 2014). See Table 1.2 for a list of primer sequences and CS linker sequences. Both sets of primers were used with all DNA extractions. However, the 18S sequencing was only successful on 12 samples (11 from larval mosquitoes, 1 from environmental water).

25 µL polymerase chain reactions were done in duplicate for every sample. Reaction mixes for all water samples consisted of: 10.3 µL nuclease-free water, 2.5 µL 10X ThermoPol® Buffer (New England BioLabs, Ipswich, MA), 5 µL dNTP/dUTP mix (1 mM dATP, dCTP, dGTP, 2 mM dUTP) (Thermo Scientific, Waltham, MA), 4 µL 25 mM $MgCl_2$, 0.5 µL of each primer (final concentration: 200 µM), 0.2 µL *Taq* polymerase (New England BioLabs), and 2 µL DNA template. Reaction mixes for the 16S mosquito samples were as above, except the $MgCl_2$ was reduced to 1 µL and the nuclease-free water increased to 13.3 µL. Reaction mixes for the 18S mosquito samples were as above but contained 3 µL $MgCl_2$ with a corresponding reduction of water to 11.3 µL.

Thermocycling was done in a T100™ thermocycler (Bio-Rad Laboratories, Foster City, CA). Cycling conditions for all samples were as follows: initial denaturation at 95 °C for 2 min, 28 cycles of 95 °C for 30 s, 50 °C for 30 s, 68 °C for 90 s, with a final extension at 68 °C for 5 min. Amplification was verified with gel electrophoresis using 1.2 % agarose gels. Samples with strong bands of the expected size (∼450 bp for 16S, ∼650 bp for 18S) were shipped to the DNAS Facility at UIC for sequencing on an Illumina MiSeq instrument using a MiSeq v3 reagent kit (read length 2x300 bp).

Table 1.2. Sequences of primers used for Illumina library preparation.

Gene	Primer	Sequence
16S	341F	CCTACGGGAGGCAGCAG
16S	806R	GGACTACNVGGGTWTCTAAT
18S	574*F	CGGTAAYTCCAGCTCYV
18S	1132R	CCGTCAATTHCTTYAART
Forward linker (5')	CS1	ACACTGACGACATGGTTCTACA
Reverse linker (5')	CS2	TACGGTAGCAGAGACTTGGTCT

Forward linkers were used only on forward primers. Reverse linkers were used only on reverse primers. Note: F = forward primer, R = reverse primer.

1.2.11 Bioinformatics Pipeline

Paired-end reads for the 16S samples were merged using the program PEAR (Zhang et al. 2014). Fragment lengths prevented merging for the 18S samples, so only the forward reads were used for analysis. All sequences were further trimmed to remove primers, adapters, and chimeric sequences, and quality-checked using QIIME (Caporaso et al. 2010). Sequences were clustered into Operational Taxonomic Units (OTUs) at 97 % identity with QIIME using the Greengenes database (2013 revision, 16S samples) or the Silva 119 database (18S samples) and organized into a BIOM file (McDonald et al. 2012). The BIOM file was filtered to exclude low-abundance OTUs (abundance $\leq$ 3 total sequences) and OTUs that were tagged as "Chloroplast". This new BIOM file was used to filter the set of fasta sequences and to generate a phylogenetic tree for Unifrac distances. Alpha diversity using the Chao1 index was plotted using data from the filtered BIOM table.

The dataset was further trimmed and analyzed using the R package *phyloseq*, version 1.20.0 (McMurdie & Holmes 2013; R Core Team 2015). All taxa in "Unassigned" or ambiguous domains were removed, leading to a reduction of 7.9% of the dataset (removal

of 410 taxa). A prevalence threshold of 3% of samples was set using a function coded by Callahan et al. (2016). This prevalence threshold means an OTU must be present in at least ∼2 samples to avoid being removed from the dataset. The prevalence threshold pruned 640 taxa, with 4158 taxa remaining in the dataset. After pruning, absolute abundances were transformed to relative abundances to stabilize variances across samples. Samples were not rarefied to the lowest sampling depth in order to preserve differences in community structure (McMurdie & Holmes 2014). *Phyloseq* was then used to compute PCoA ordinations using weighted Unifrac distances (16S samples) or Bray-Curtis distances (18S samples) and graphed with the R package *ggplot2* (Wickham 2009). The phylogenetic trees used for the Unifrac calculations were rooted randomly by the R package *ape*, version 4.1 (Paradis et al. 2004).

Further analyses were conducted using subsets of the main dataset, split by the factor DNA Source ("water" or "larval mosquito"). Taxa that were not present in each subset were removed, leading to a final count of 3705 OTUs in the "water" subset, and 2083 OTUs in the "larval" subset.

1.2.12 Statistical Analysis

All non-microbiome data were analyzed using linear mixed effects models using the *lme4* package in R (Bolker et al. 2009; Barr et al. 2013; Winter 2013; Bates et al. 2015; R Core Team 2015). Data were pooled over Week and modeled using Treatment as the only fixed effect. Treatment here refers to the six treatment groups (N0, N1, L0, L1, H0, H1), which combines the effects of Mosquito Access (presence/absence) and Nutrient Enrichment (no/low/high), since both factors were tested simultaneously in each mesocosm.

Random effects were set using Week and Tub as random intercepts. Mosquito abundance data (egg raft counts) was tested using only Nutrient Enrichment as a fixed effect and Tub as a random intercept, removing data from N0, L0, and H0 from the dataset prior to analysis since it was not necessary to test Mosquito Access (all values were zero in treatments without mosquitoes). Chemical oxygen demand data was additionally modeled using both Treatment and Week as fixed effects and Tub as random intercepts to examine interactions between the fixed effects.

Models were analyzed using likelihood ratio tests to compare full models against the mean of the data (null models). Models with interaction terms were analyzed in the same manner, comparing the full model to reduced models with only one fixed effect. When fixed effects were significant, *post hoc* Tukey tests were done at a 95 % confidence level, using the *multcomp* package in R (Hothorn et al. 2008). When significant interactions were present, *post hoc* Tukey tests were done on one factor against each level of the other factor (i.e. Treatment at each level of Week, and Week at each level of Treatment). Normality and homoscedasticity assumptions were checked visually with QQ plots and residual plots. Statistical tests are reported as significant if $p \leq 0.05$ and marginal if $0.051 < p < 0.085$.

Microbiome data were analyzed using PERMANOVA implemented in the "adonis" function in the R package *vegan*, version 2.4.3 (Anderson 2001; Oksanen et al. 2016). Tests were done with 999 permutations on the trimmed dataset transformed with relative abundances mentioned above, with "Unassigned" taxa excluded. Bray-Curtis distances were calculated for three datasets: the trimmed dataset as a whole, a subset of the data with only water samples included, and a subset including only larval mosquito samples.

"Adonis" was then run on each of the three datasets independently, testing the following factors: Treatment, Nutrient Enrichment, and Week. Mosquito Access was tested for the water samples only, and Source was tested for the whole dataset only. Tests of homogeneity of variance were done using the "betadisper" function in *vegan*, using 999 permutations. Each of the factors tested in "adonis" was tested separately with "betadisper", for each of the three datasets. Results are reported as significant if $p \leq 0.05$.

Core microbiomes were examined using the R package *microbiome*, version 0.99.52 (Leo Lahti 2017). OTUs were defined as part of the "core" if they occurred with $> 0.1\,\%$ relative abundance per sample, and were present in $> 0.99\,\%$ of all samples within a treatment (Hu et al. 2013). Data were pooled across time points.

Differential abundance was examined using the R package *DESeq2*, version 1.16.1 (Anders & Huber 2010; Love et al. 2014; McMurdie & Holmes 2014). This package models OTU count data on the negative binomial distribution and shrinks OTU dispersion estimates based on the average dispersion of OTUs at the same read depths. See Appendix B for dispersion plots of the water and mosquito data (Figures B.1 and B.2). Alpha was set to 0.05, Cook's cutoff set to False, and interactive filtering set to True when analyzing the results. P-values were obtained through a Wald test on the logarithmic fold changes of OTU abundance, using the Benjamini-Hochberg method for adjusting p-values. Summary plots of genera that showed significant p-values (alpha = 0.05) are presented in the Results section. Points above zero indicate increases in abundance in the first listed treatment compared to the second listed treatment, while points below zero indicate decreases in abundance in the first listed treatment.

Table 1.3. Oviposition rates and egg raft loads for each treatment.

Treatment	Daily Average Oviposition Rate	Total Egg Rafts Laid	Daily Average Egg Load	Total Egg Load
N0	0.00	0	0.00	0
L0	0.00	0	0.00	0
H0	0.00	0	0.00	0
N1	0.16	9	0.50	28
L1	0.36	20	0.64	36
H1	2.77	155	2.71	152

"Daily Average Oviposition Rate" is the average mosquito oviposition rate in each treatment ($n = 2$ replicate tubs per treatment) for the 28 days of the experiment, *before* redistribution of egg rafts. "Total Egg Rafts Laid" is the sum of all egg rafts laid within treatment replicates after 28 days. "Daily Average Egg Load" is the average egg raft load in each treatment per day, *after* redistribution of egg rafts. "Total Egg Load" is the sum of all egg rafts in each treatment after 28 days, *after* redistribution of egg rafts.

1.3 Results

1.3.1 Mosquito abundance

There was a significant effect of Nutrient Enrichment on mosquito egg raft abundance ($X_2^2 = 7.8344, p = 0.0199$). Oviposition rates into the high enrichment treatment were 7.7 times that into the low enrichment treatment and 17.3 times that in unenriched tubs, differing significantly from the low and no enrichment ($p = 0.002$ for H1 $\geq$ L1, $p < 0.001$ for H1 $\geq$ N1, with no difference between L1 and N1).

No egg rafts were collected in treatments that excluded mosquitoes (see Table 1.3). This finding indicates that the mesh screens with weatherstripping were largely effective against mosquito entry (but see the dip sample data for exceptions).

1.3.2 Dip Samples

Only two mosquito species were present in the dip samples: *Culex tarsalis* and *Culex quinquefasciatus*. Mean abundances of mosquito larvae and pupae for each treatment are presented in Table 1.4 (see Figure A.1 for boxplots showing abundances of selected organism groups in each treatment). Pupae were much less abundant than larvae, so statistical analyses were done on mosquito larval abundances only.

Mosquito larvae showed a significant effect of Treatment ($X_5^2 = 13.569, p = 0.0186$), with a mean of 6.2 larvae per 1050 mL across mesocosms and a maximum of 70 larvae per 1050 mL in H1. *Post hoc* Tukey tests showed 2 significant pairwise comparisons: H1 $\geq$ L1 and H1 $\geq$ N1. This indicates that high enrichment was able to support larger populations of mosquito larvae compared to low and no enrichment. L0 and N0 each had a single larva in the dip samples (L0: week 3, N0: week 2). While this might indicate that the mesh screens were not completely effective in excluding mosquitoes, no egg rafts were counted in these treatments, and larval mosquito abundance remained at zero for the other dip samples. H0 never showed any indication of mosquito colonization. It is likely that the presence of larvae in the dips is due to carryover of larvae from other mesocosms, since the same plankton net was used to filter all dips.

Enrichment and Mosquito Access (together grouped as "Treatment") did not affect microinvertebrate population densities. Total rotifer abundance ($X_5^2 = 4.8443$, $p = 0.4352$) had a mean of 319.5 rotifers per 1050 mL across mesocosms and a maximum of 2216 rotifers per 1050 mL in H1. Copepod nauplii ($X_5^2 = 8.3261, p = 0.1392$) had a mean of 222.3 nauplii per 1050 mL and a maximum of 1632 nauplii per 1050 mL in H1. Planktonic cladocerans

Table 1.4. Mean mosquito abundance in dip samples (larvae and pupae, counts/1050 mL).

Week	H0	H1	L0	L1	N0	N1
1	0	10.5	0	0.5	0	3.5
2	0	45.0	0	15.5	0.5	1.5
3	0	32.5	0.5	4.5	0	6.5
4	0	28.5	0	4.5	0	1.0
Mean	0	29.1	0.1	6.3	0.1	3.1

Data shown are mean abundances for 3 dip samples pooled together per sampling date per treatment (3 dips = 1050 mL).

($X_5^2 = 6.4716, p = 0.263$) had a mean of 34.83 cladocerans per 1050 mL across mesocosms and a maximum of 296 cladocerans per 1050 mL in L0.

1.3.3 Primary production and community metabolism

Treatment had a statistically significant effect on community net daily metabolism (NDM) from data obtained from the water column and sides of the mesocosms ($X_5^2 = 17.232, p = 0.0041$). *Post hoc* Tukey tests indicate 3 significant pairwise comparisons: H0 $\leq$ H1, H0 $\leq$ L0, and H0 $\leq$ N0 (see Figure A.2).

Treatment was also statistically significant for gross primary production (GPP) ($X_5^2 = 13.352, p = 0.02$) and daily community respiration (CR_{24}) ($X_5^2 = 15.64, p = 0.0080$) (Figure A.2). *Post hoc* Tukey tests indicate that 2 pairs were significant for both GPP and CR_{24}: H1 $\geq$ L1, N1 for GPP, H1 $\leq$ L1, N1 for CR_{24} (see Figure A.2). Two other pairs were also significant for CR_{24}: H0 $\leq$ L0, N0.

These results show that the presence of mosquito larvae only affected NDM in high enrichment mesocosms. NDM values were higher when mosquito larvae were present

in the mesocosms compared to those that had no mosquito larvae. Enrichment levels also affected NDM, with high enrichment leading to lower NDM when mosquitoes were absent. Enrichment was the primary driver of differences between the treatments for GPP and CR_{24}. High enrichment when mosquitoes were present caused increases in GPP but decreases in CR_{24}.

Mosquito larvae strongly increased net photosynthesis in the water column of highly-enriched tubs. Treatment affected net photosynthesis in the water column of the mesocosms ($X_5^2 = 19.778, p = 0.001$). H1 had higher values for net photosynthesis compared to H0 (Figure A.3). Net photosynthesis in H1 was significantly higher than in L1 and N1, indicating that high enrichment leads to higher net photosynthesis only when mosquito larvae were present. There was no significant effect of Treatment on net respiration in the water column ($X_5^2 = 7.68, p = 0.175$) since all mesocosms had negative net production by the end of the study (Figure A.3).

1.3.4 Water Quality

Nitrogen

For the four nitrogen species analyzed (total nitrogen, $NH_4{-}N$, $NO_2{-}N$, and $NO_3{-}N$), no significant effect of Treatment was found. However, certain trends in the data were not captured by the statistical analysis (Figures 1.1 and A.4).

Total nitrogen (TN) was initially high in treatments H0 and H1 due to enrichment with ammonium sulfate. TN was also high in treatment N0 overall, starting at $3.62\,\mathrm{mg\,L^{-1}}$

in week 1 and reaching a maximum of $11.2\,\mathrm{mg\,L^{-1}}$ in week 3. Across the remaining three weeks of the study, TN was similar across the other five treatments.

Ammonium-nitrogen (NH_4−N) was greatest in the high enrichment treatments in week 1 (H1 mean: $7.34\,\mathrm{mg\,L^{-1}}$, H0 mean: $6.81\,\mathrm{mg\,L^{-1}}$) but rapidly declined by week 2 (H1 mean: $0.05\,\mathrm{mg\,L^{-1}}$, H0 mean: $0.06\,\mathrm{mg\,L^{-1}}$). Values for all other mesocosms at all other time points were near zero.

Nitrite-nitrogen (NO_2−N) was highest in the low and no enrichment treatments (N0 mean: $0.48\,\mathrm{mg\,L^{-1}}$, N1 mean: $0.19\,\mathrm{mg\,L^{-1}}$, L0 mean: $0.15\,\mathrm{mg\,L^{-1}}$, L1 mean: $0.18\,\mathrm{mg\,L^{-1}}$, H0 mean: $0.07\,\mathrm{mg\,L^{-1}}$, H1 mean: $0.06\,\mathrm{mg\,L^{-1}}$).

Nitrate-nitrogen (NO_3−N) was highest in the low and no enrichment treatments, with higher levels in L0 and N0 (N0 mean: $1.83\,\mathrm{mg\,L^{-1}}$, N1 mean: $0.73\,\mathrm{mg\,L^{-1}}$, L0 mean: $1.64\,\mathrm{mg\,L^{-1}}$, L1 mean: $0.50\,\mathrm{mg\,L^{-1}}$, H0 mean: $0.28\,\mathrm{mg\,L^{-1}}$, H1 mean: $0.33\,\mathrm{mg\,L^{-1}}$).

Phosphorus

Treatment was statistically significant for the total phosphorus data ($X_5^2 = 19.528$, $p = 0.0015$; Figures 1.2 and A.5). Highly enriched mesocosms with larval mosquitoes contained significantly higher levels of water column phosphate than H0, L1 and N1 mesocosms.

Nitrogen:Phosphorus ratio

Treatment did not have a statistically significant effect on the molar ratios of nitrogen:phosphorus in the mesocosms ($X_5^2 = 8.667, p = 0.1231$). However, the N:P ratio of treatments N0 and N1 tended to be higher than the other four treatments (Figures 1.3

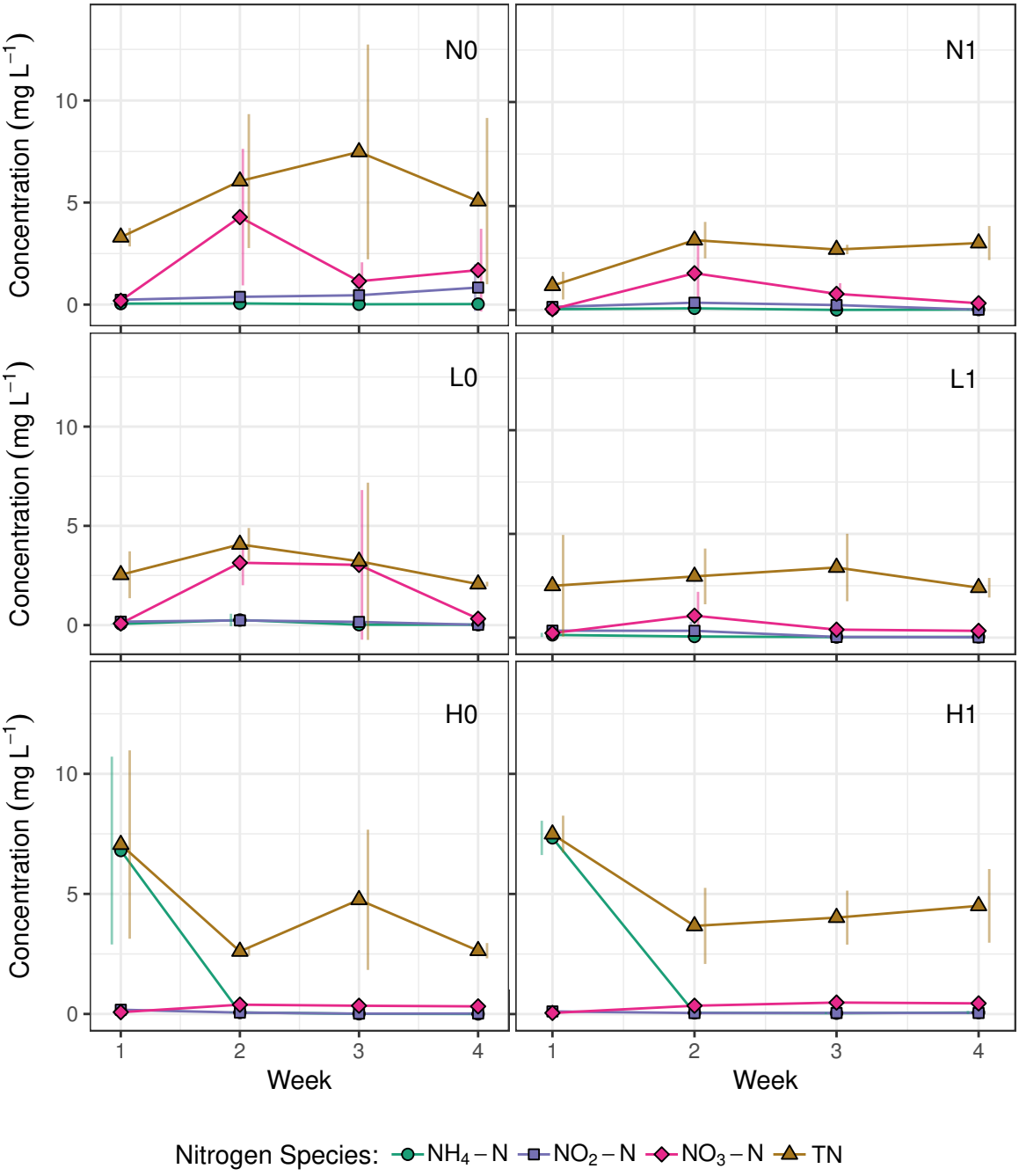

Figure 1.1. Nitrogen species in each treatment over time ($n = 2$ replicates per time point per treatment). Error bars showing standard deviation have been dodged to either side of the points for clarity.

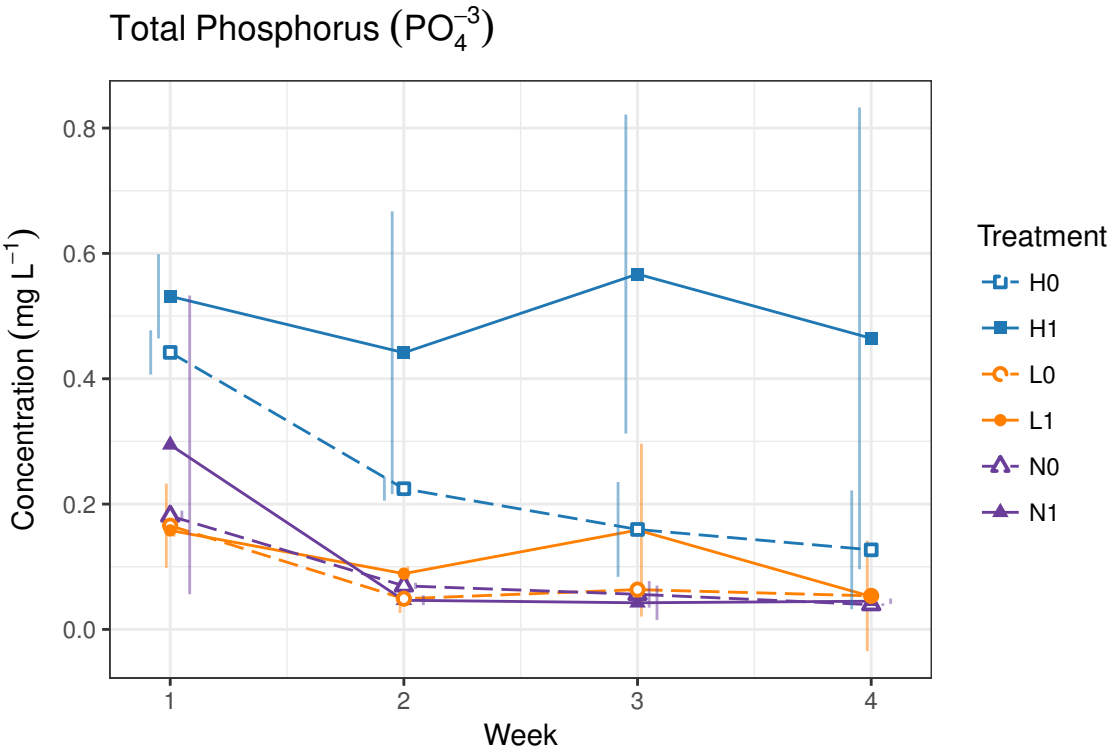

Figure 1.2. Total phosphorus (PO_4^{-3}) in each treatment over time. Open symbols with dashed lines indicate treatments without mosquitoes. Closed symbols with solid lines indicate treatments with mosquitoes. Error bars showing standard deviation have been dodged to either side of the points for clarity.

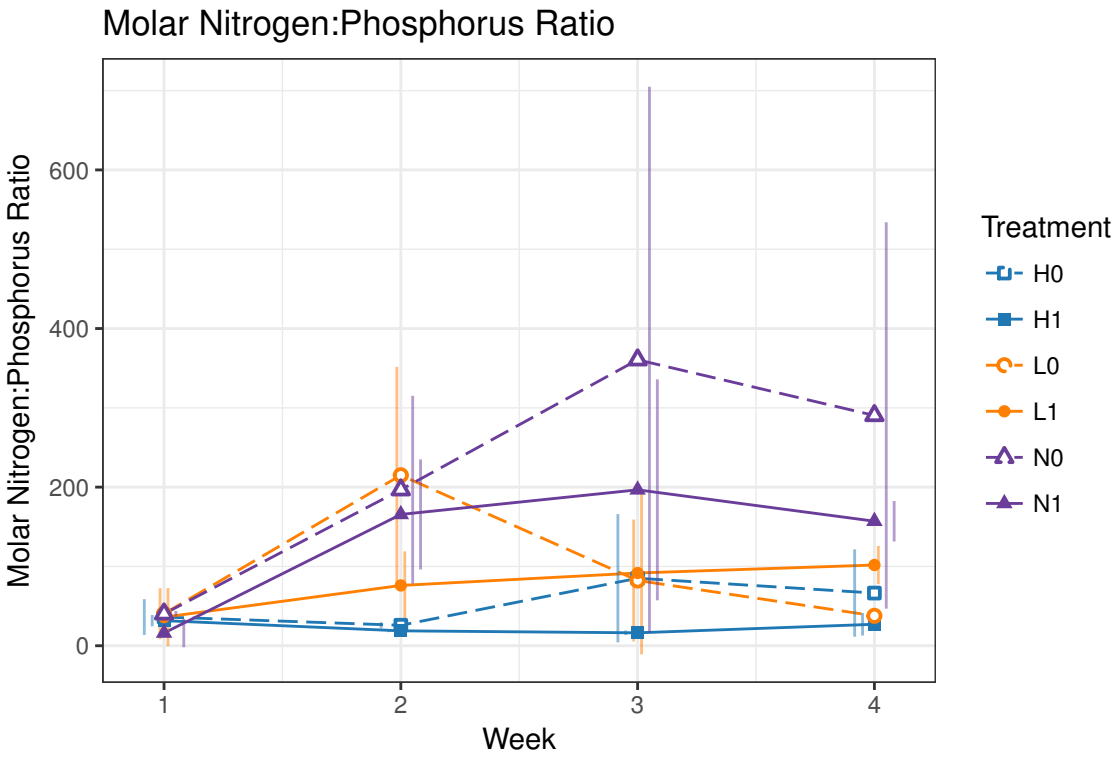

Figure 1.3. Molar nitrogen:phosphorus ratios over time. Open symbols with dashed lines indicate treatments without mosquitoes. Closed symbols with solid lines indicate treatments with mosquitoes. Error bars showing standard deviation have been dodged to either side of the points for clarity.

and A.5). In all cases, treatments that excluded mosquitoes (N0, L0, H0) had higher mean molar N:P ratios across 4 weeks compared to the corresponding treatment with mosquitoes (N0 mean: 222.0, N1 mean: 133.8, L0 mean: 101.9, L1 mean: 76.4, H0 mean: 53.4, H1 mean: 23.5).

Chemical oxygen demand

Treatment had a marginally-significant effect on chemical oxygen demand (COD) in the mesocosms ($X_5^2 = 9.6783, p = 0.0849$) when analyzed with Treatment as the only fixed effect (Figure A.5). When analyzed with both Treatment and Week as fixed effects, COD

showed a significant interaction between Treatment and Week ($X^2_{15} = 34.08, p = 0.003$). *Post hoc* Tukey tests indicated no treatment was significantly different from the others within each week. Within treatments, all groups showed a significant mean decrease from week 1 to week 2 of 250.3 ppm (starting values in week 1 ranged from 244–458 ppm, with a mean of 352.8 ppm). Treatments that allowed mosquito access (H1, L1, and N1) had significant increases from week 2 to week 4 (H1 week 2: 161.5 ppm, H1 week 4: 257.5 ppm (+ 96 ppm), L1 week 2: 95.5 ppm, L1 week 4: 161.0 ppm (+ 65.5 ppm), N1 week 2: 104.5 ppm, N1 week 4: 134.5 ppm (+ 30 ppm)). N0 showed a marginally-significant increase from week 2 to week 4 ($p = 0.067$), but L0 and H0 did not show this difference ($p > 0.1$).

1.3.5 Chlorophyll biomass

Chlorophyll biomass was significantly affected by Treatment ($X^2_5 = 23.703, p = 0.0002$) (Figure A.5). *Post hoc* Tukey tests indicated 3 significant pairwise comparisons: H0 $\leq$ H1, H1 $\geq$ L1, and H1 $\geq$ N1. Both the presence of mosquito larvae and high enrichment caused large increases in chlorophyll levels. Over time, all treatments showed a decrease in chlorophyll levels to near- or below-baseline levels, likely due to depletion of nutrients in the mesocosms (Figure 1.4).

1.3.6 Particle Counts

All three size classes of particles were significantly affected by Treatment (small: $X^2_5 = 23.14, p = 0.0003$, medium: $X^2_5 = 13.38, p = 0.0201$, large: $X^2_5 = 20.183, p = 0.0012$) (Figure A.6). *Post hoc* Tukey tests indicated that 2 pairs of treatments were statistically

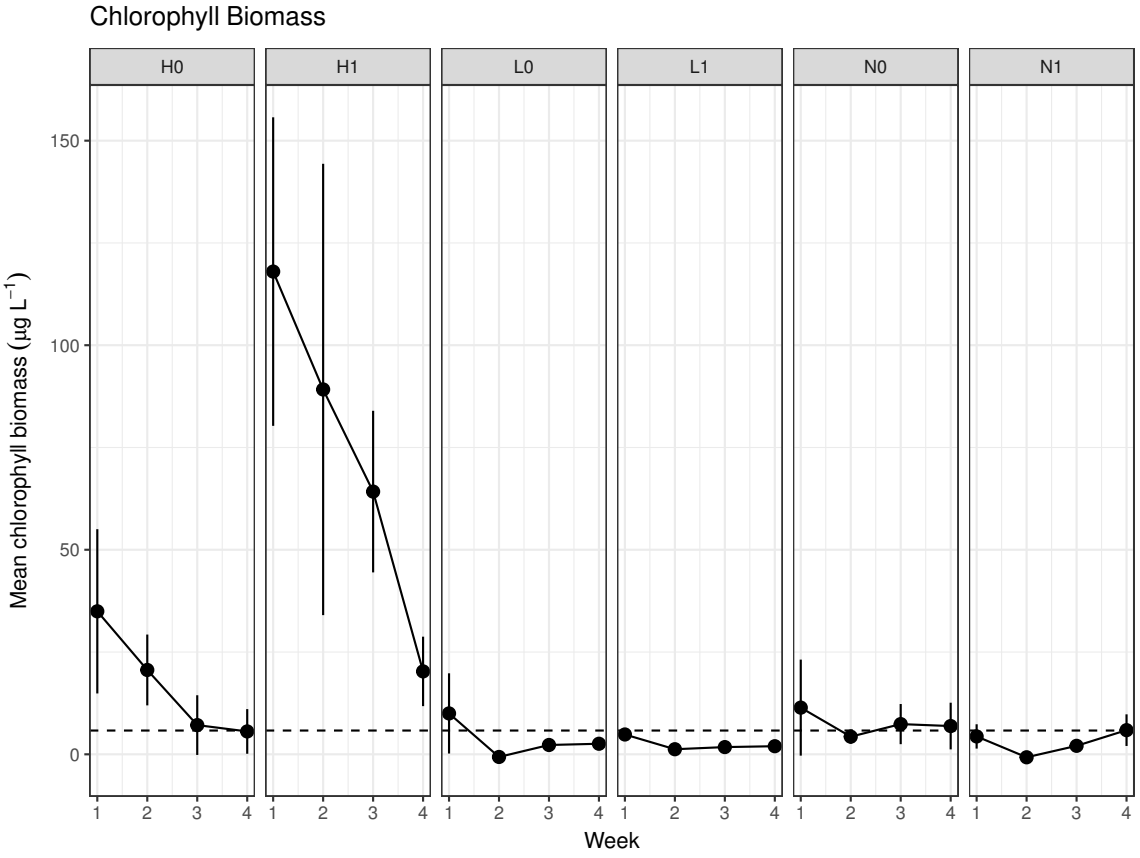

Figure 1.4. Average biomass of chlorophyll over the study period. Horizontal dashed line represents the baseline level of chlorophyll (5.81 ppm) in the source water.

different in all 3 size classes: H1 $\geq$ L1, N1. Additionally, within small particles, H0 $\geq$ L0, N0. Within large particles, H0 $\leq$ H1.

1.3.7 16S Microbiome analysis

Overview

A total of 1,656,147 sequences were obtained, clustered at 97 % similarity into 6528 OTUs (after filtering to exclude OTUs with less than 4 reads total). Counts per sample ranged from 7059 to 68,291, with a mean of $32,473.47 \pm 11,134.25$ sequences per sample.

In the water samples, 31 unique phyla were recovered, plus ambiguous/unclassified OTUs ("recovered" means the phylum occurred in at least one sample, even if average relative abundance was near zero). Taxa in the "Unassigned" category, which contained ambiguous/unclassified OTUs, were not included in phyla counts but are reported here for completeness. There were 16 phyla with at least 0.1 % average relative abundance, of which 5 phyla occurred with $\geq$ 1 % average relative abundance: *Proteobacteria* (42.51 %), *Bacteroidetes* (28.03 %), *Actinobacteria* (13.88 %), "Unassigned" (7.76 %), *Cyanobacteria* (2.51 %), and *Planctomycetes* (1.43 %).

At the species level, 566 unique taxa were recovered in the water samples (duplicates were pooled together). Most taxa were not identified to the species level, so they are reported to the most specific level available. Ninety-one taxa had at least 0.1 % average relative abundance, and 23 taxa occurred with $\geq$ 1 % average relative abundance (see Table 1.5 for a list of OTUs with $\geq$ 1 % average relative abundance).

Table 1.5. 16S OTUs with $\geq 1\,\%$ average relative abundance in the water samples.

Number	OTU	Phylum	Average RA
1	"*Candidatus* Aquiluna rubra"	*Actinobacteria*	10.96 %
2	*Sediminibacterium*	*Bacteroidetes*	8.45 %
3	"Unassigned"	"Unassigned"	7.80 %
4	*Rhodocyclaceae*	*Proteobacteria*	7.46 %
5	*Comamonadaceae*	*Proteobacteria*	4.55 %
6	*Flavobacterium*	*Bacteroidetes*	4.27 %
7	*Sphingomonadales*	*Proteobacteria*	4.21 %
8	*Cytophagaceae*	*Bacteroidetes*	3.56 %
9	*Polynucleobacter cosmopolitanus*	*Proteobacteria*	3.13 %
10	*ACK-M1*	*Actinobacteria*	2.35 %
11	*Cyclobacteriaceae*	*Bacteroidetes*	2.08 %
12	*Sphingomonadaceae*	*Proteobacteria*	2.03 %
13	*Sphingobacteriales*	*Bacteroidetes*	1.85 %
14	*Burkholderiales*	*Proteobacteria*	1.75 %
15	*Hydrogenophaga*	*Proteobacteria*	1.45 %
16	*Erythrobacteraceae*	*Proteobacteria*	1.32 %
17	*Pseudanabaena*	*Cyanobacteria*	1.23 %
18	*Saprospiraceae*	*Bacteroidetes*	1.14 %
19	*Chitinophagaceae*	*Bacteroidetes*	1.12 %
20	*Hyphomonadaceae*	*Proteobacteria*	1.11 %
21	*Sphingobacteriaceae*	*Bacteroidetes*	1.10 %
22	*Synechococcus*	*Cyanobacteria*	1.10 %
23	*MWH-UniP1*	*Proteobacteria*	1.06 %

"Average RA" = Average Relative Abundance.

In the mosquito samples, 23 unique phyla were recovered (plus ambiguous/unclassified OTUs). There were 11 phyla with at least 0.1 % average relative abundance, of which 7 phyla occurred with $\geq$ 1 % average relative abundance: *Proteobacteria* (48.35 %), *Cyanobacteria* (17.33 %), *Verrucomicrobia* (10.12 %), *Planctomycetes* (6.39 %), "Unassigned" (6.28 %), *Firmicutes* (5.44 %), *Actinobacteria* (2.41 %), *Bacteroidetes* (1.85 %).

At the species level, 427 taxa were recovered in the mosquito samples (duplicates were pooled together). Most taxa were not identified to species level, so they are reported to the most specific level available. Seventy taxa had at least 0.1 % average relative abundance, and 21 genera occurred with $\geq$ 1 % average relative abundance (see Table 1.6 for a list of OTUs with $\geq$ 1 % average relative abundance).

Alpha diversity (within-treatment diversity)

Most of the water samples showed a decreasing trend in alpha diversity (Chao1 Index) over time (see Figure 1.5), with little difference among treatments. The only exception was Treatment H0 (high enrichment without mosquitoes), which showed an increasing trend in alpha diversity over time.

The larval samples showed overall lower levels of alpha diversity compared to the water samples (maximum of $\sim$1050, while the water sample maximum was $\sim$1600). Each enrichment level varied in how alpha diversity levels changed over time (see Figure 1.6).

All phyla recovered in the two data subsets are shown in Figures B.3 and B.4. In general, OTUs with greater prevalence tended to be more abundant. The larval samples

Table 1.6. 16S OTUs with $\geq 1\,\%$ average relative abundance in the mosquito samples.

Number	OTU	Phylum	Average RA
1	*Rhizobiales*	*Proteobacteria*	11.23 %
2	"Unassigned"	"Unassigned"	10.40 %
3	*Microcystis*	*Cyanobacteria*	6.66 %
4	*Pirellulaceae*	*Planctomycetes*	6.42 %
5	"*Candidatus* Xiphinematobacter"	*Verrucomicrobia*	6.37 %
6	*Acetobacteraceae*	*Proteobacteria*	5.72 %
7	*Hydrogenophaga*	*Proteobacteria*	5.44 %
8	*Rhodobacter*	*Proteobacteria*	4.66 %
9	*Bacillales*	*Firmicutes*	2.88 %
10	*Synechococcus*	*Cyanobacteria*	2.69 %
11	*Leptolyngbya*	*Cyanobacteria*	2.61 %
12	*Roseococcus*	*Proteobacteria*	2.39 %
13	*Acinetobacter*	*Proteobacteria*	2.22 %
14	*Comamonadaceae*	*Proteobacteria*	2.00 %
15	*Anaerospora*	*Proteobacteria*	1.47 %
16	*Wolbachia*	*Proteobacteria*	1.43 %
17	*Pseudanabaena*	*Cyanobacteria*	1.30 %
18	*Cloacibacterium*	*Bacteroidetes*	1.22 %
19	*Gomphosphaeriaceae*	*Cyanobacteria*	1.13 %
20	*Actinomycetales*	*Actinobacteria*	1.06 %
21	*Rhodobacteraceae*	*Proteobacteria*	1.02 %

"Average RA" = Average Relative Abundance.

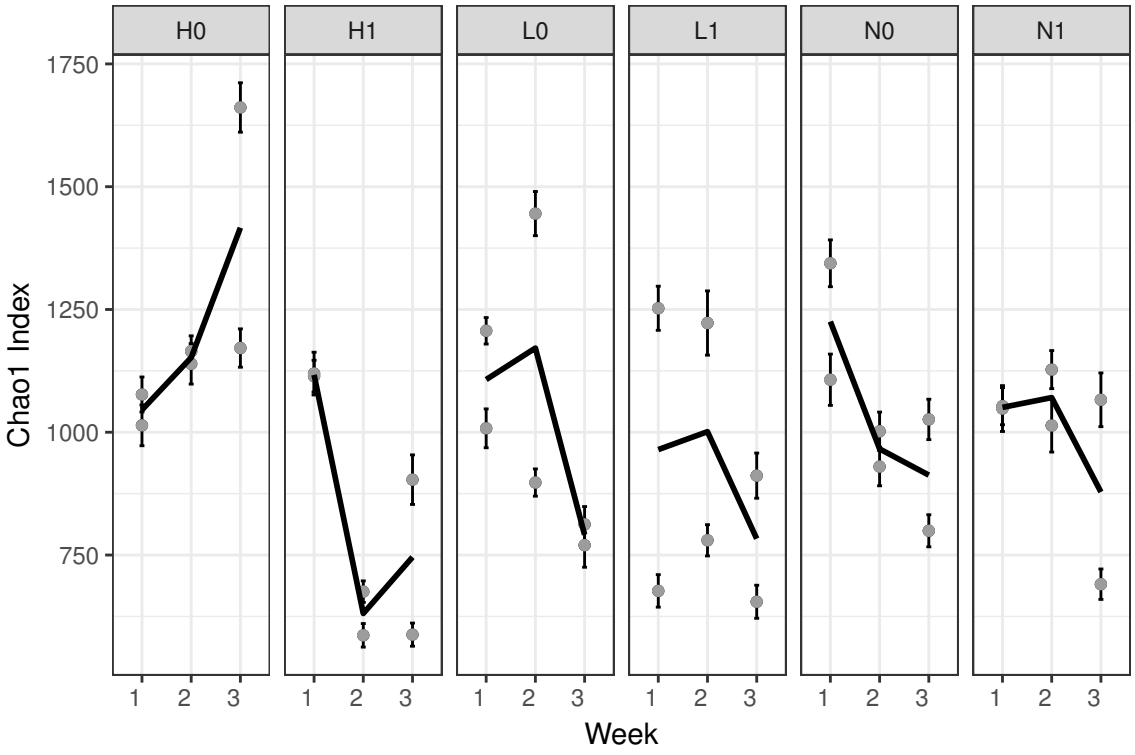

Figure 1.5. Chao1 indices for the 16S water samples. Thick black lines connect means between replicates at each time point.

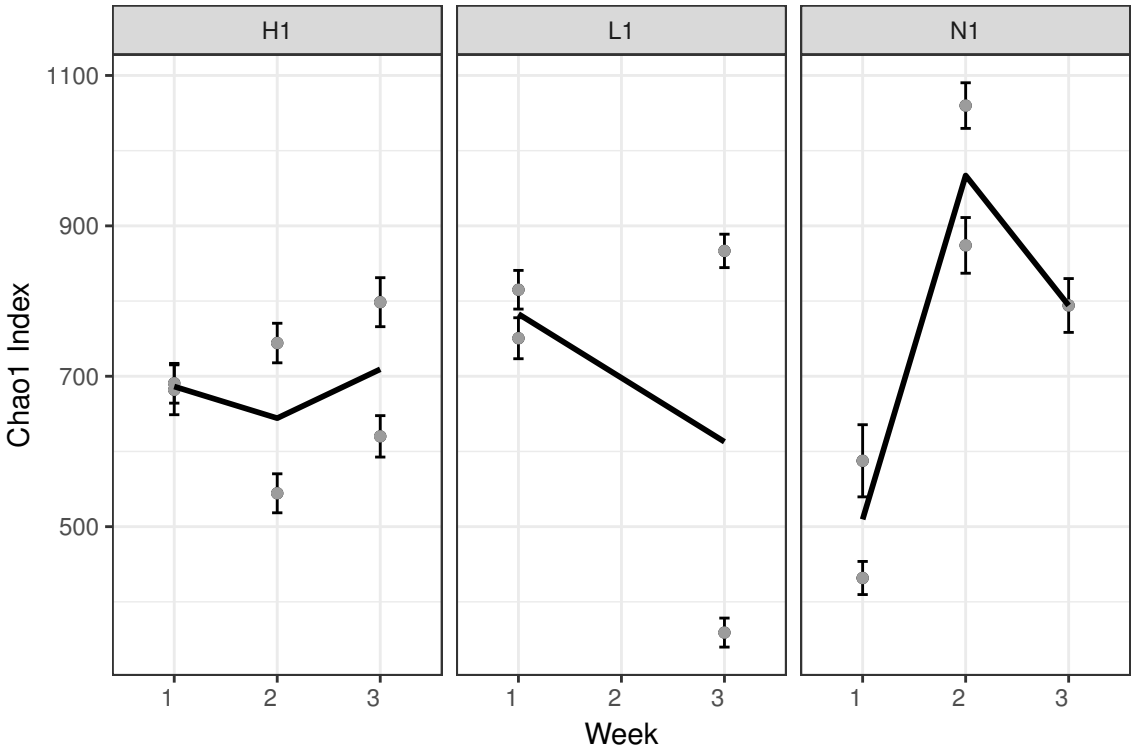

Figure 1.6. Chao1 indices for the 16S larval mosquito samples. Thick black lines connect means between replicates at each time point.

contained fewer phyla (23) compared to the water samples (30), with a corresponding decrease in overall numbers of OTUs (2083 taxa in mosquito larvae compared to 3705 taxa in water samples).

Beta diversity (between-treatment diversity)

PCoA plots based on weighted Unifrac ordinations indicated no major differences between treatments in water sample diversity (Figure 1.7A). Similar results were obtained when each factor (Mosquito Access and Nutrient Enrichment) was examined separately (Figure 1.7B, C). There was a minor shift in microbial communities based on nutrient enrichment (samples in high enrichment clustered somewhat separately from the no enrichment samples), but the difference was very slight.

Microbes from larval mosquitoes separated somewhat based on nutrient enrichment (Figure 1.7D). The high enrichment samples clustered together, separating slightly from the other two enrichment levels.

The phyla *Cyanobacteria* and *Actinobacteria* were examined individually by subsetting the water and mosquito datasets to include only the phylum of interest. Based on just *Cyanobacteria*, the water samples clustered slightly based on enrichment and were mostly mixed based on mosquito access (Figure 1.8A, B). The larval samples clustered slightly based on enrichment, although the low enrichment mesocosms scattered widely, obscuring the pattern (Figure 1.8C).

Based on just *Actinobacteria*, the water samples had clear separation based on mosquito access, with samples originating from mesocosms with mosquitoes clustering together, with some overlap from mesocosms without mosquitoes (Figure 1.9A). Based on

Table 1.7. Number of taxa in each 16S water core microbiome by treatment.

Treatment	Total taxa	Phyla	Genera
H1	7	4	5
H0	16	4	6
L1	17	5	8
L0	16	6	9
N1	21	4	7
N0	10	3	4

Taxa tagged as "Not Assigned" or duplicates are included in "Total taxa" counts but not in "Genera" counts. Data are pooled across 3 time points.

enrichment, the clusters overlapped and had more outliers, but the low enrichment mesocosms clustered together most closely, followed by high and then no enrichment (Figure 1.9B). The larval samples clustered slightly based on enrichment, with the no enrichment samples clustering the tightest (Figure 1.9C).

Core Microbiome

The number of core taxa varied according to treatment (Table 1.7 and 1.8). Treatment H1 harbored the fewest numbers of phyla or genera in both aquatic and larval mosquito core microbiomes. Low and no enrichment samples were similar to each other in numbers of core taxa.

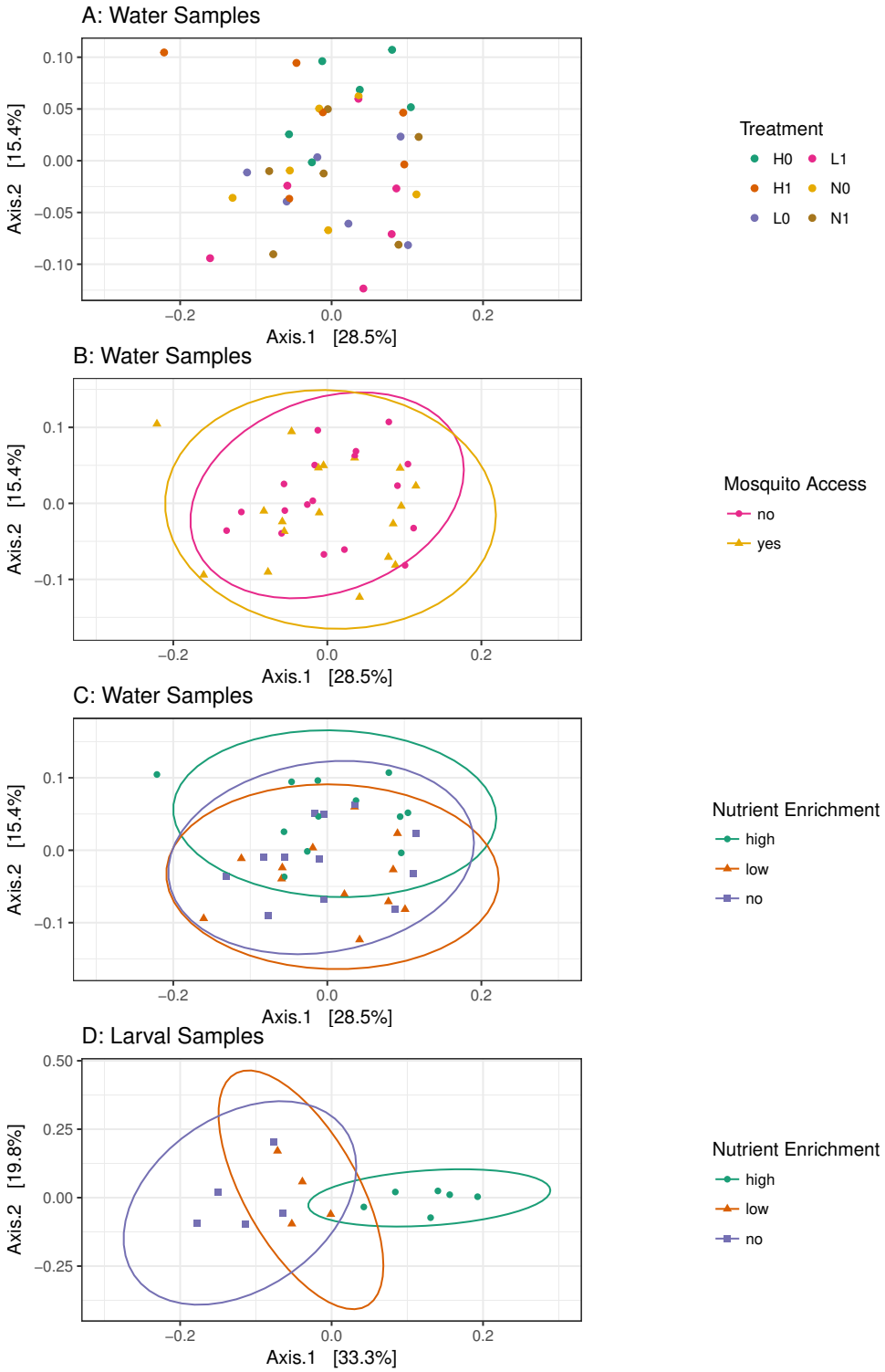

Figure 1.7. PCoA plots based on weighted Unifrac distances. Panels A-C show water samples. Panel D shows larval mosquito samples. Ellipses indicate 95 % confidence intervals.

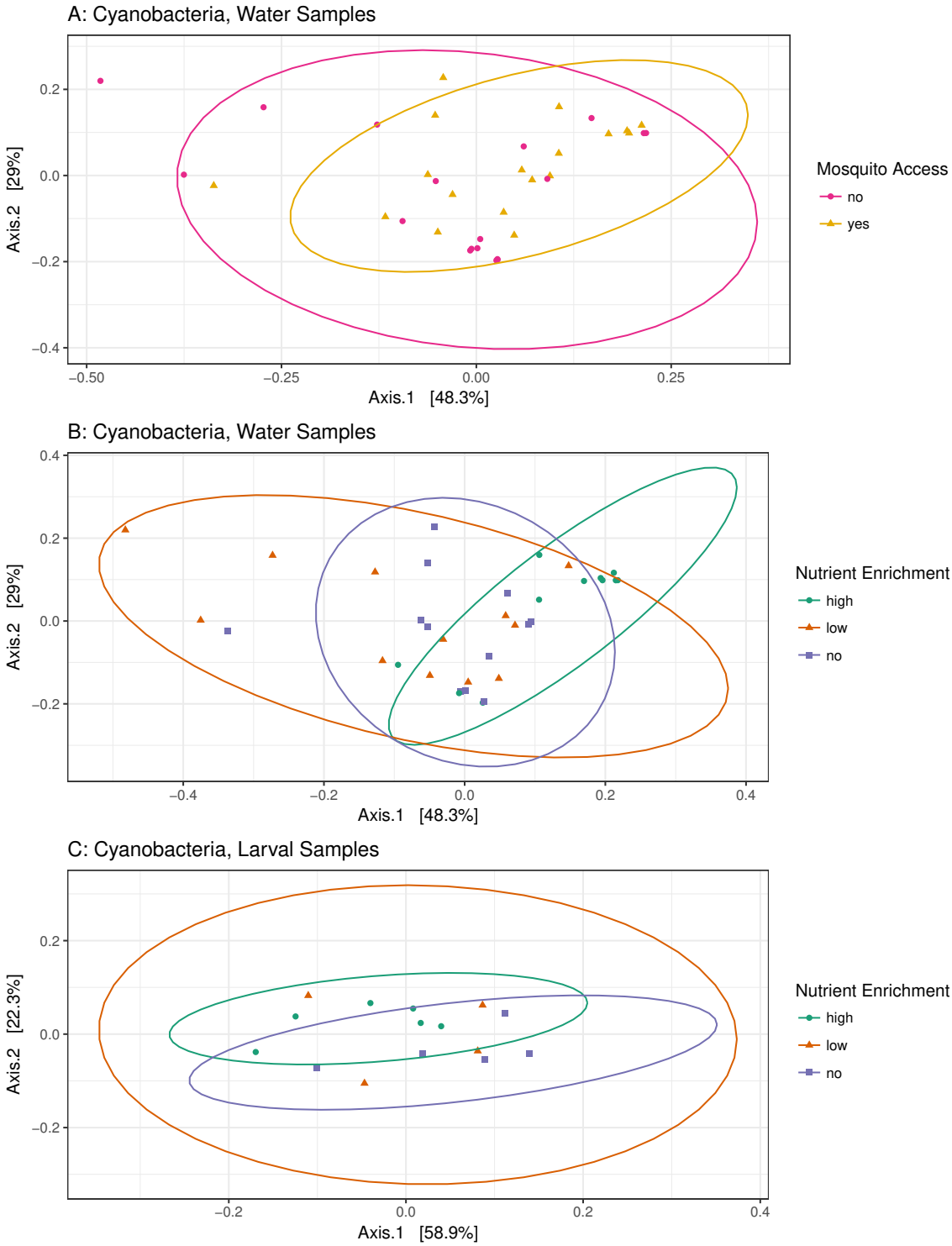

Figure 1.8. PCoA plots based on weighted Unifrac distances of data subsetted by the phylum *Cyanobacteria*. Panels A and B show water samples. Panel C shows larval mosquito samples. Ellipses indicate 95 % confidence intervals.

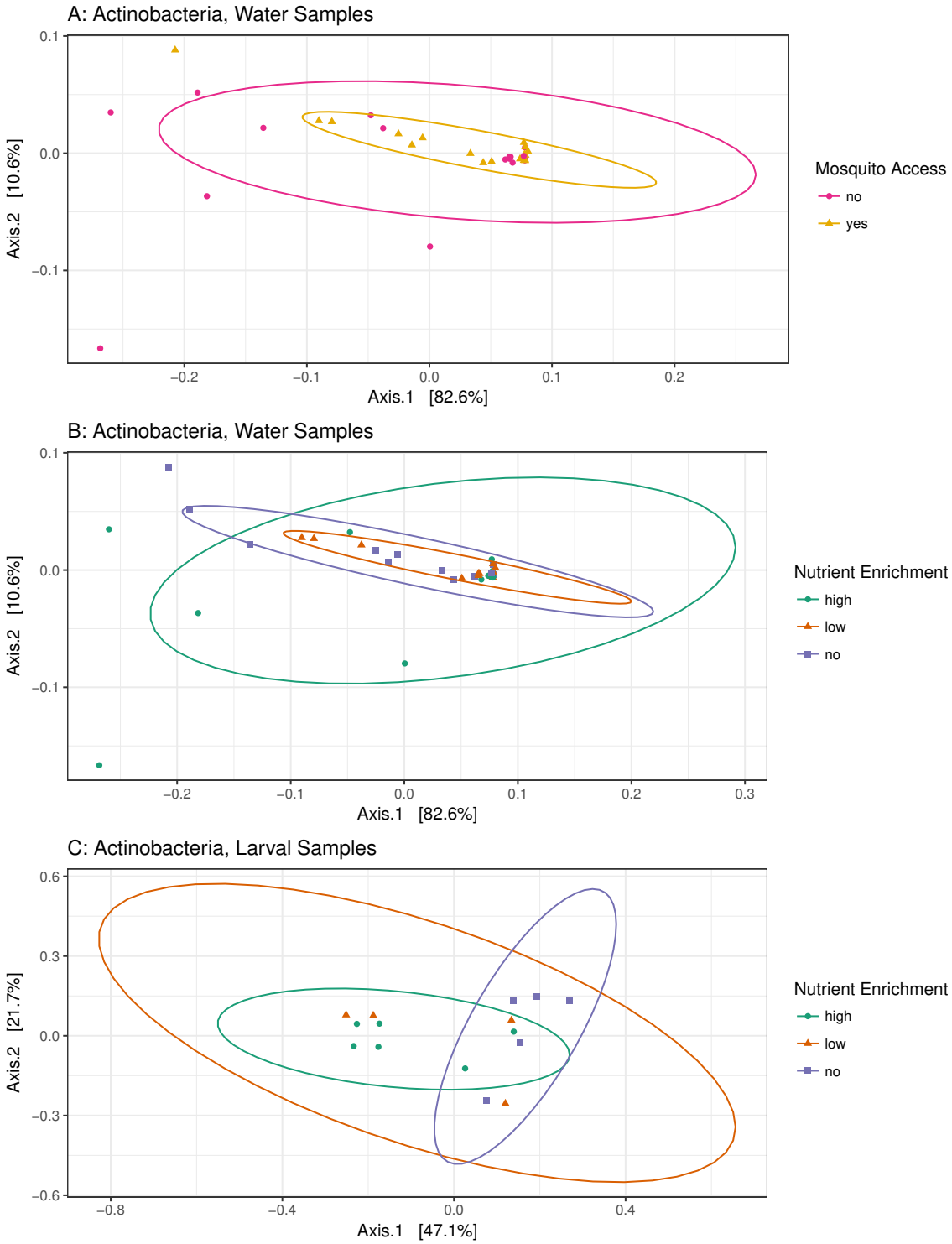

Figure 1.9. PCoA plots based on weighted Unifrac distances of data subsetted by the phylum *Actinobacteria*. Panels A and B show water samples. Panel C shows larval mosquito samples. Ellipses indicate 95 % confidence intervals.

Table 1.8. Number of taxa in each 16S larval core microbiome by treatment.

Treatment	Total taxa	Phyla	Genera
H1	15	4	8
L1	22	5	10
N1	22	6	7

Taxa tagged as "Not Assigned" or duplicates are included in "Total taxa" counts but not in "Genera" counts. Data are pooled across 3 time points.

PERMANOVA

The global adonis test for the factor Source (water versus larval source DNA) was significant but not due to dispersion differences ("adonis": $F_{1,49} = 15.932$, $p = 0.001$, "betadisper": $F_{1,49} = 0.3808$, $p = 0.544$), indicating that environmental water and larval mosquitoes harbored different communities of microbes. Data were split into two subsets based on the DNA source ("water" and "larva") in order to examine each microbial community separately.

"Adonis" results indicated significant differences in Treatment in both data subsets tested (water: $F_{5,30} = 1.6874, p \leq 0.001$, larval: $F_{2,12} = 2.6078, p = 0.003$). "Betadisper" results for Treatment were not significant in either data subset (water: $F_{5,30} = 0.8771$, $p = 0.501$, larval: $F_{2,12} = 0.1835$, $p = 0.83$). Nutrient Enrichment was significant in both subsets tested, but not due to dispersion differences ("adonis" water: $F_{2,33} = 2.5073, p \leq 0.001$, "betadisper" water: $F_{2,33} = 1.739$, $p = 0.17$, "adonis" larval: $F_{2,12} = 2.6078, p = 0.002$, "betadisper" larval: $F_{2,12} = 0.1835$, $p = 0.842$). Significant differences across time (factor Week) were found in the water samples, but not the larval samples ("adonis" water: $F_{2,33} = 2.3688, p \leq 0.001$, "betadisper" water: $F_{2,33} = 0.3837$, $p = 0.671$, "adonis" larval:

$F_{2,12} = 1.4775, p = 0.071$, "betadisper" larval: $F_{2,12} = 1.1443$, $p = 0.375$). Mosquito Access was not significant for the water samples ("adonis": $F_{1,34} = 0.8916, p = 0.593$, "betadisper": $F_{1,34} = 0.613$, $p = 0.445$), and there was no significant interaction between Mosquito Access and Nutrient Enrichment in the water samples ("adonis": $F_{2,30} = 1.1923, p = 0.167$).

Differential abundance

Highly-enriched mesocosms showed differential abundance of 10 phyla between treatments H1 and H0, with the majority of phyla showing decreases in H1 (mosquito access) compared to H0 (no mosquito access) (Figure B.6). Mesocosms with low and no enrichment each showed 5 phyla with differential abundance, with treatment pairs (L1 and L0, N1 and N0) showing approximately equal levels of differentially-abundant taxa (Figures B.7 and B.8).

In mesocosms that allowed mosquito access, high enrichment (H1) had slightly more phyla than no enrichment (N1) (10 phyla with increases in H1 compared to 7 phyla with increases in N1) (Figure B.9). In mesocosms that prevented mosquito access, a similar pattern was found, with 11 phyla increased in H0 compared to 5 phyla in N0 (Figure B.10).

In larval samples, differentially-abundant phyla were approximately equally distributed across treatments H1 and N1 (5 phyla in H1 compared to 6 phyla in N1) (Figure B.11).

1.3.8 18S Microbiome analysis

Overview

A total of 450,667 sequences were obtained from sequencing the 18S gene, after applying the filter to remove OTUs with less than 4 reads across the dataset. These sequences clustered into 295 OTUs at 97 % similarity, using the Silva 119 database. Counts per sample ranged from 31,846.0 to 64,413.0, with a mean of $37,555.58 \pm 8500.74$ sequences.

Four major clades (plus ambiguous/unclassified taxa) were recovered from 18S sequencing: Opisthokonta (92.99 %), SAR (3.22 %), "Unassigned" (2.37 %), Archaeplastida (1.38 %), and Amoebozoa (0.04 %) (Figure B.5). The primary difference in phylum composition between the water community and the larval community was the inclusion of Amoebozoa in the water samples.

The dataset was split into two subsets based on DNA source (water and mosquito larvae) to examine each community type separately. The water sample had 38 unique taxon groups, of which 19 had ≥ 0.1 % relative abundance (see Table 1.9 for a list of taxa with $\geq$ 0.1 % relative abundance).

The larval samples, when combined, had 30 unique taxon groups, of which 5 had $\geq$ 0.1 % relative abundance (see Table 1.10 for a list of taxa with ≥ 0.1 % relative abundance).

Of the two native mosquitoes known to be present in the study area, only one (*Culex quinquefasciatus*) had an entry in the Silva database. The second species, *Culex tarsalis*, was shown to be present from morphological analyses of the dip samples. Sequences assigned to "Insecta" could not be classified any further in the database. These sequences

Table 1.9. 18S OTUs with $\geq 0.1\,\%$ average relative abundance in the water sample.

Number	OTU	Clade	Average RA
1	Spizellomycetaceae	Opisthokonta	68.90 %
2	"Unassigned"	"Unassigned"	8.60 %
3	Hypotrichia	SAR	5.11 %
4	Chlorophyceae	Archaeplastida	3.57 %
5	Nucletmycea fungus	Opisthokonta	2.99 %
6	*Paramecium*	SAR	2.33 %
7	uncultured Dikarya	Opisthokonta	2.18 %
8	*Telotrochidium*	SAR	1.90 %
9	*Amphileptus*	SAR	0.99 %
10	*Chilodonella uncinata*	SAR	0.98 %
11	*Didinium*	SAR	0.47 %
12	uncultured *Colpodella*	SAR	0.29 %
13	Bacillariophyceae	SAR	0.24 %
14	uncultured freshwater Opisthokonta	Opisthokonta	0.20 %
15	*Echinamoeba*	Amoebozoa	0.17 %
16	*Cyclidium*	SAR	0.15 %
17	*Nuclearia simplex*	Opisthokonta	0.13 %
18	uncultured Chytridiomycota	Opisthokonta	0.13 %
19	Eustigmatales	SAR	0.13 %

"Average RA" = Average Relative Abundance.

Table 1.10. 18S OTUs with $\geq 0.1\,\%$ average relative abundance in the mosquito samples.

Number	OTU	Clade	Average RA
1	*Culex quinquefasciatus*	Opisthokonta	80.70 %
2	Insecta	Opisthokonta	18.14 %
3	Chlorophyceae	Archaeplastida	0.43 %
4	"Unassigned"	"Unassigned"	0.27 %
5	*Elongatocystis ecballocystiformis*	Archaeplastida	0.22 %

"Average RA" = Average Relative Abundance.

could be a mixture of various Diptera since unidentified aquatic flies were seen resting on the water surface and walls of some mesocosms.

The water was fairly diverse, with a range of species from multiple clades. These data were based on a single sample (treatment H0, high enrichment without mosquitoes), so it was impossible to generalize to other mesocosms. However, the increased nutrient concentrations likely encouraged the growth of these microorganisms, due to the enhancement of algal primary producers and heterotrophic protists that feed on them. It is interesting to note that *Chilodonella uncinata* has been hypothesized to be a facultative parasite of mosquito larvae (Das 2003, but see Spring & Zufall 2013 for an opposing perspective). However, it was found in the water sample only, with zero reads for all larval samples, so it is unlikely to act as a pathogen in this case.

Alpha diversity (within-treatment diversity)

The 18S samples varied in terms of alpha diversity (Figure 1.10). Treatments L1 and N1 both showed decreasing trends of alpha diversity over time, but the variation in N1 was much larger than L1. Treatment H1 peaked in alpha diversity in week 2 before declining, but this peak was the result of a single data point with a much higher alpha diversity metric compared to the others in its group.

Beta diversity (between-treatment diversity)

PCoa plots based on Bray-Curtis dissimilarities indicated differences in community diversity from due to treatment in the larval mosquito samples (Figure 1.11A). All H1

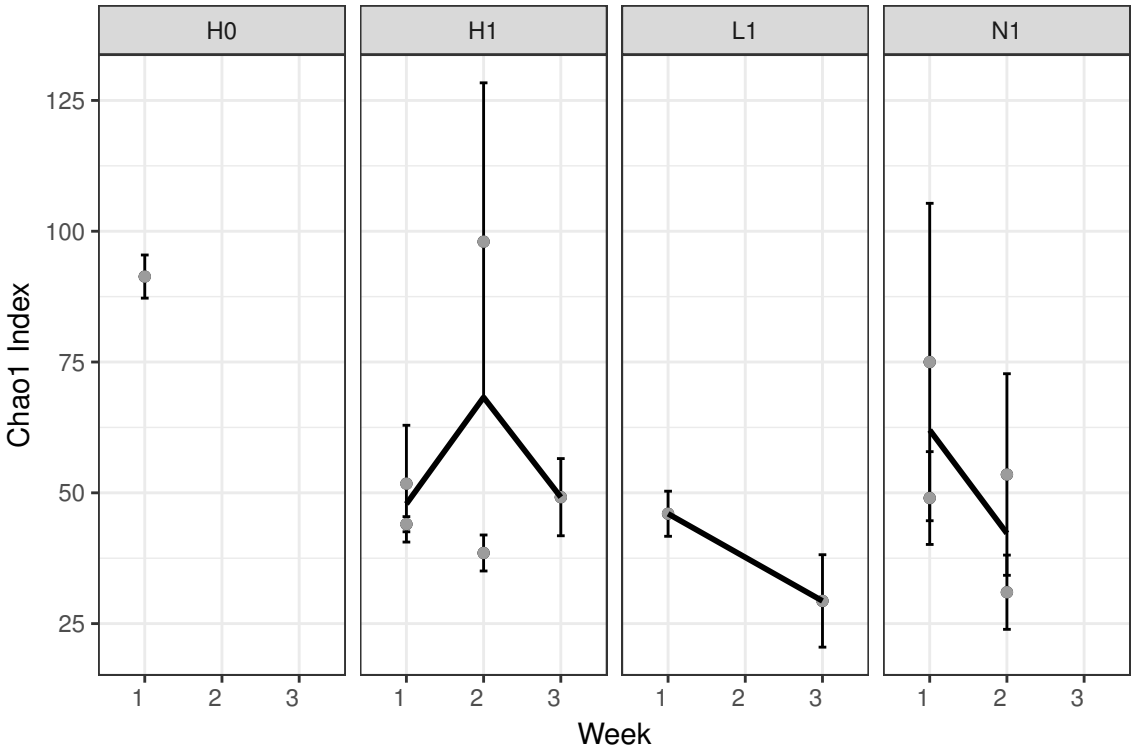

Figure 1.10. Chao1 indices for the 18S eukaryotic samples. Thick black lines connect means between replicates at each time point. Data for H0 came from environmental water, while data for the other 3 treatments came from larval mosquitoes.

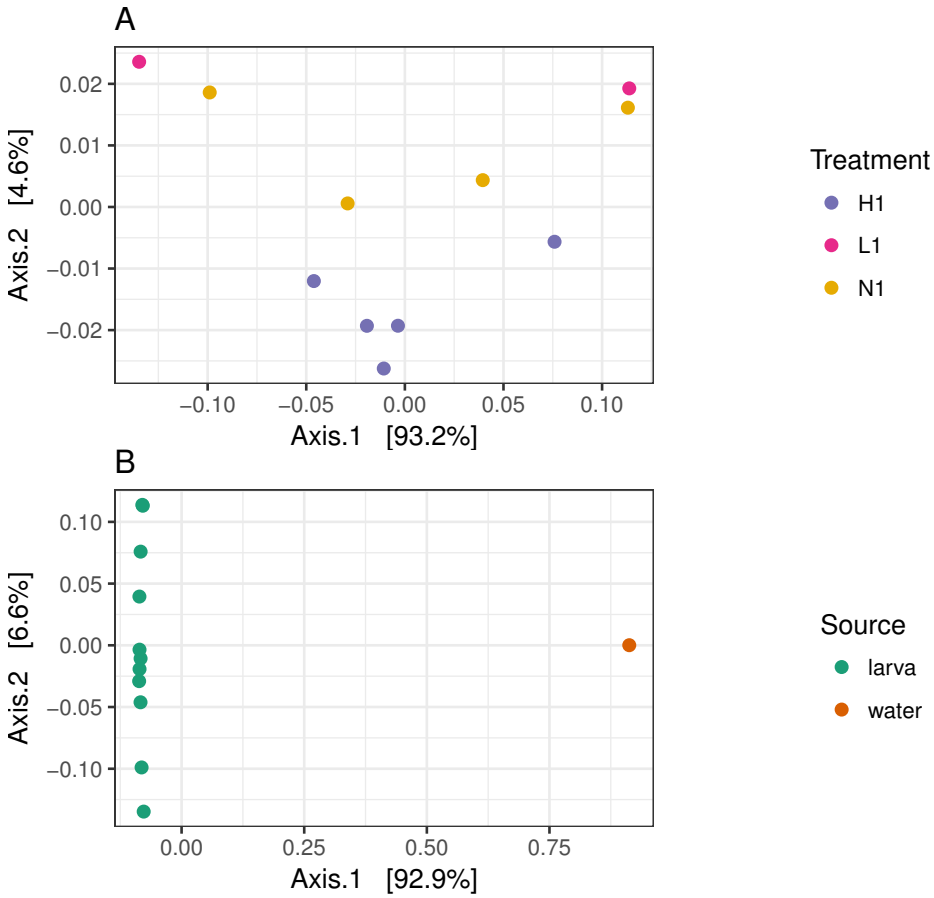

Figure 1.11. PCoA plots of 18S communities based on Bray-Curtis distances. Plot A contains only samples from larval mosquitoes. Plot B contains all 18S samples (1 water sample, 11 larval samples).

samples clustered together, followed by L1 and N1 samples clustering somewhat separately from the H1 samples.

When the larval samples were combined with the water sample, clear differences due to DNA source were visible (Figure 1.11B). All larval samples clustered together to the exclusion of the water sample.

1.4 Discussion

1.4.1 Effect of mosquito access

The presence of mosquito larvae affected five variables examined in this experiment: phosphorus, community net daily metabolism, net photosynthesis, chlorophyll biomass, and large particle abundance (particle size $> 10\,\mu m$). All five variables had significantly increased quantities in the high enrichment treatments that allowed mosquito access compared to those without mosquito access. Mesocosms with low and no enrichment levels never showed any significant differences for the duration of the experiment. This was likely because nutrient levels in the low enrichment groups declined to levels near the no enrichment groups within the first week of the experiment.

The increases in chlorophyll biomass and available phosphorus in the water column were surprising. It is probable that mosquito larvae prevented the phosphorus from settling to the bottom of the mesocosms due to their filter-feeding activities. Filtration rates could be as high or higher than 490–590 µL larva^{-1} h^{-1} (data for laboratory-reared *Culex quinquefasciatus*, Aly 1988). Extrapolating those data to the dip sample data in my experiment, the mosquito larvae could potentially filter 78–94 % of the 300 L mesocosms within a 24 hour period (using maximum larval density observed of 20,000 larvae mesocosm^{-1} in tub 24 during week 2 (treatment H1)).

Increased suspended nutrients would allow suspended algal cells and other planktonic photosynthetic organisms to grow and reproduce easily. The increase in chlorophyll was evident from visual inspection of the mesocosms. Treatment H1 contained very green, turbid water, while treatment H0 contained water that was less green and less turbid, even

though both treatments received identical amounts of nutrients at the start of the experiment. The dip samples did not have significant differences in rotifers, cladocerans, or copepod nauplii, meaning that the increases in net daily metabolism rates were not due to increases in population size for these organisms. The large particles were likely eukaryotic photosynthetic cells, which would contain the additional chlorophyll biomass and enhance community metabolism rates.

The weather was unusually hot and mosquito oviposition was lower than expected during the experiment. This created the need to supplement mesocosms with laboratory-reared *Culex quinquefasciatus*. This could have affected the results, especially the microbiome results (Xiang et al. 2006; Chandler et al. 2011; Colman et al. 2012). However, it is likely that the mosquito larvae guts were colonized by their native flora from the water column, since the water had already been exposed to mosquito larvae for 2 weeks.

Alpha diversity trends in the Chao1 indices showed that high enrichment lead to increasing bacterial diversity, due to high availability of nutrients necessary for growth and reproduction. When the high enrichment environment included mosquito larvae, alpha diversity decreased. Since larval mosquito food primarily consists of bacteria and suspended organic matter, decreasing alpha diversity was likely an effect of larval mosquito filter-feeding on microbes (Merritt et al. 1992).

1.4.2 Effect of nutrient enrichment

Enrichment levels greatly influenced most of the physicochemical variables examined. High enrichment tended to increase concentrations of phosphorus, gross primary production, net photosynthesis, chlorophyll biomass, and all three size classes of particles.

Some variables were highest in the low and no enrichment mesocosms, including NO_2−N, NO_3−N, total nitrogen (TN), molar nitrogen:phosphorus ratios, net daily metabolism, and CR_{24} (although the nitrogen data and molar nitrogen:phosphorus ratios were not statistically significant). There were no significant differences between low and no enrichment in any variable examined. Chemical oxygen demand and water column respiration were unaffected by the enrichment treatment.

Net daily metabolism (NDM) and community respiration (CR_{24}) were negative for all mesocosms, indicating the dominance of heterotrophy over autotrophy in the food web. Even though the presence of mosquito larvae increased primary production in H1 compared to H0, it was not enough to make the system autotrophic. Other studies have found similar results for lake environments, in that the lakes tended to be heterotrophic rather than autotrophic as had been previously assumed (Cole et al. 1994; Jansson et al. 2000). Heterotrophic systems rely on allochthonous import of organic carbon to support the food web (Cole et al. 1994; Jansson et al. 2000). Since the mesocosms were only enriched once at the start of the experiment, nutrients were quickly depleted from the low enrichment mesocosms to levels at or near those of the no enrichment mesocosms. Interestingly, NDM was only significantly different across enrichment levels when mosquitoes were excluded. Furthermore, NDM was lowest in the high enrichment mesocosms and highest in the no enrichment mesocosms. These results are unusual in that non-enriched mesocosms would be expected to have lower levels of NDM compared to the same treatment with higher nutrient levels. This can be explained by the higher respiration rates in the highly-enriched mesocosms and very low respiration rates in non-enriched mesocosms. Since NDM is the

result of respiration subtracted from gross primary production, the lack of respiration would artificially increase the value for NDM in the non-enriched treatments.

The remaining variables follow the same pattern as mentioned previously. Higher levels of phosphorus from enrichment sustained higher populations of photosynthetic organisms, leading to overall increases in chlorophyll biomass, gross primary production, and net photosynthesis in the water column.

1.5 Conclusions

Mosquito larvae affect aquatic ecosystems when high levels of nutrients are available in the environment. Mosquito larvae do not appear to affect community composition of heterotrophic planktonic and pelagic microeukaryotes, or microbial community composition. However, they have important effects on autotrophic planktonic primary production in aquatic habitats.

This has important implications for areas affected by urban or agricultural runoff, since they will provide high concentrations of nutrients in the water. Besides attracting gravid mosquitoes for oviposition, which may lead to higher instances of vector-borne diseases, these areas will also have higher than normal rates of primary production after larval mosquito populations are established. The long-term effects of high primary production were not studied in this experiment, but it has been shown in oceanic systems that phytoplankton and other microbes compete for resources and may have negative interactions (Bratbak & Thingstad 1985; Falkowski et al. 1998). The same is possible for freshwater systems.

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Appendix A

Environmental Variables

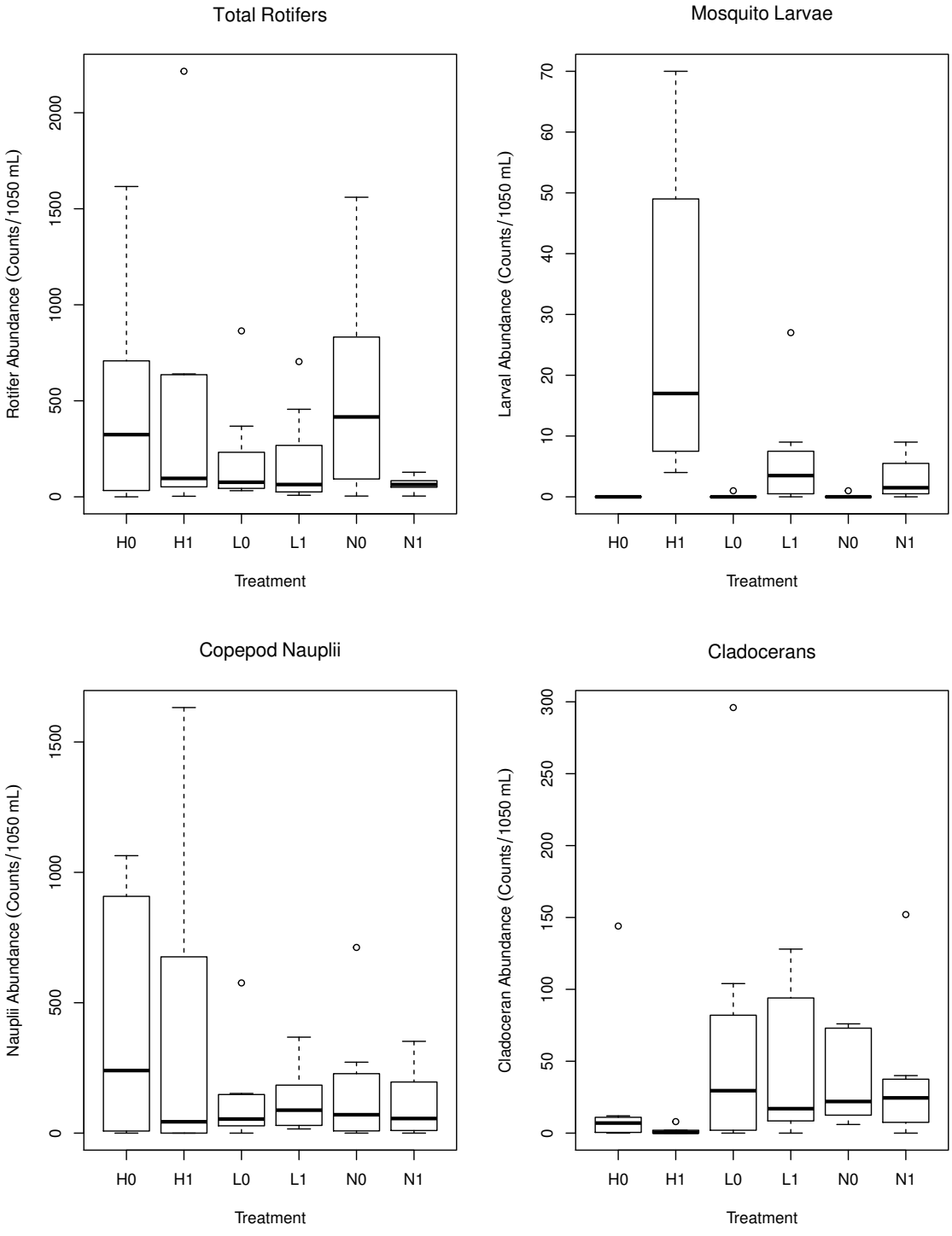

Figure A.1. Boxplots of selected organism groups from dip samples. Data are from 3 dips pooled together (sample = 1050 mL total). Thick black lines indicate median values.

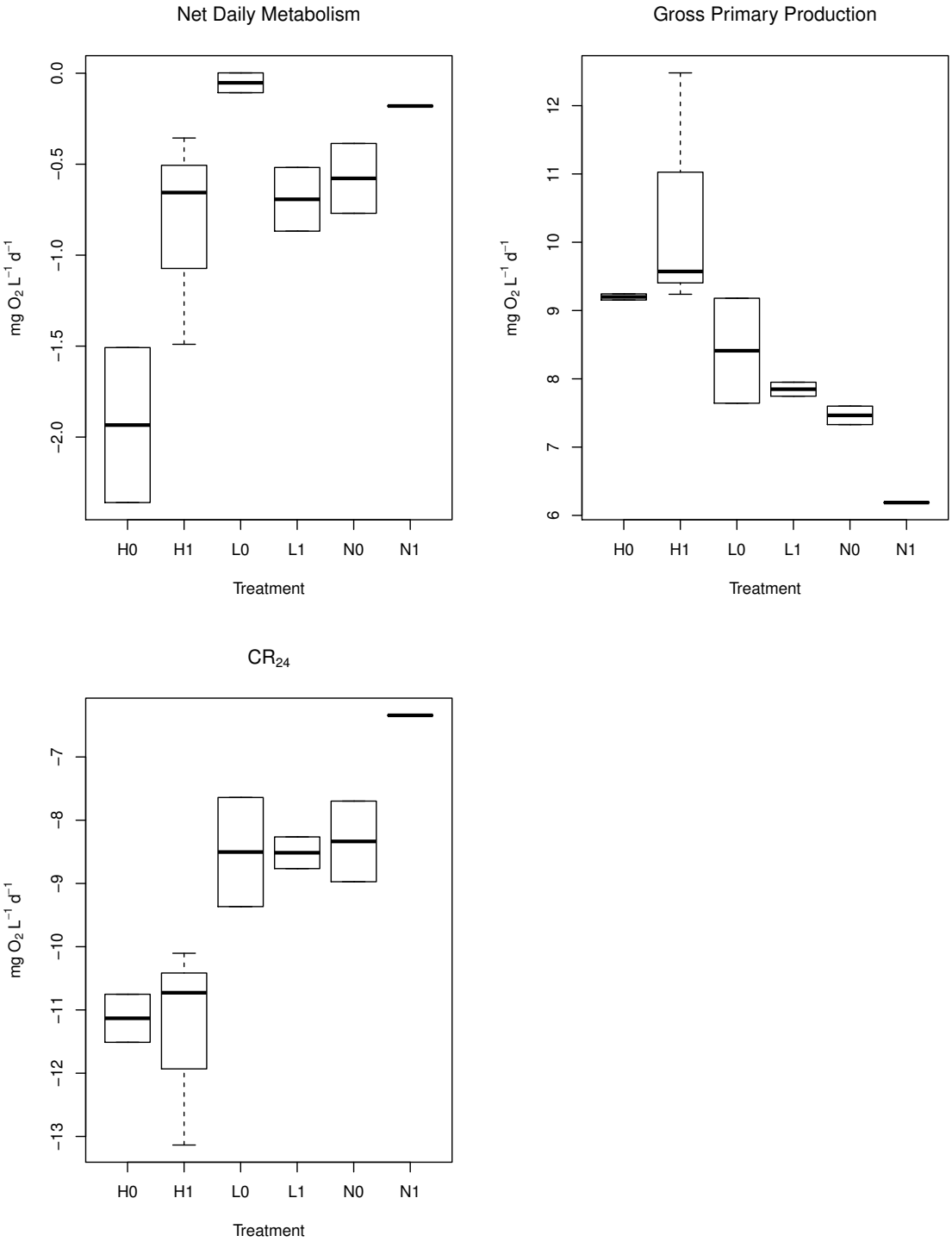

Figure A.2. Boxplots of net daily metabolism (NDM), gross primary production (GPP), and community respiration (CR_{24}). Thick black lines indicate median values. Note the negative axes for NDM and CR_{24}.

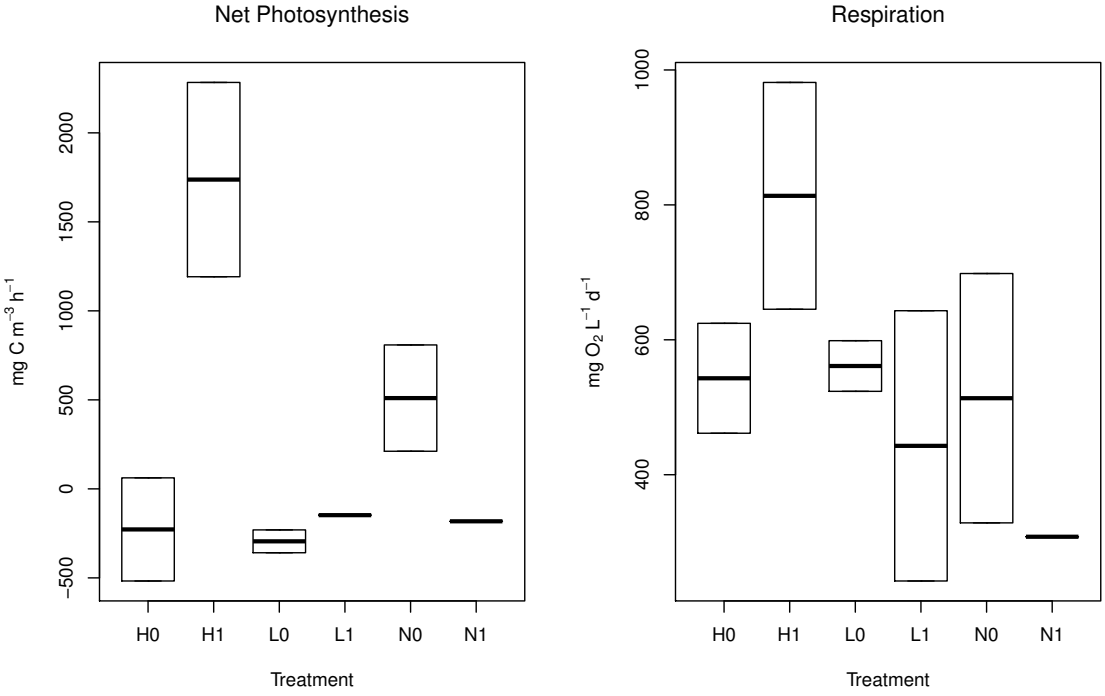

Figure A.3. Boxplots of net production and respiration. Thick black lines indicate median values.

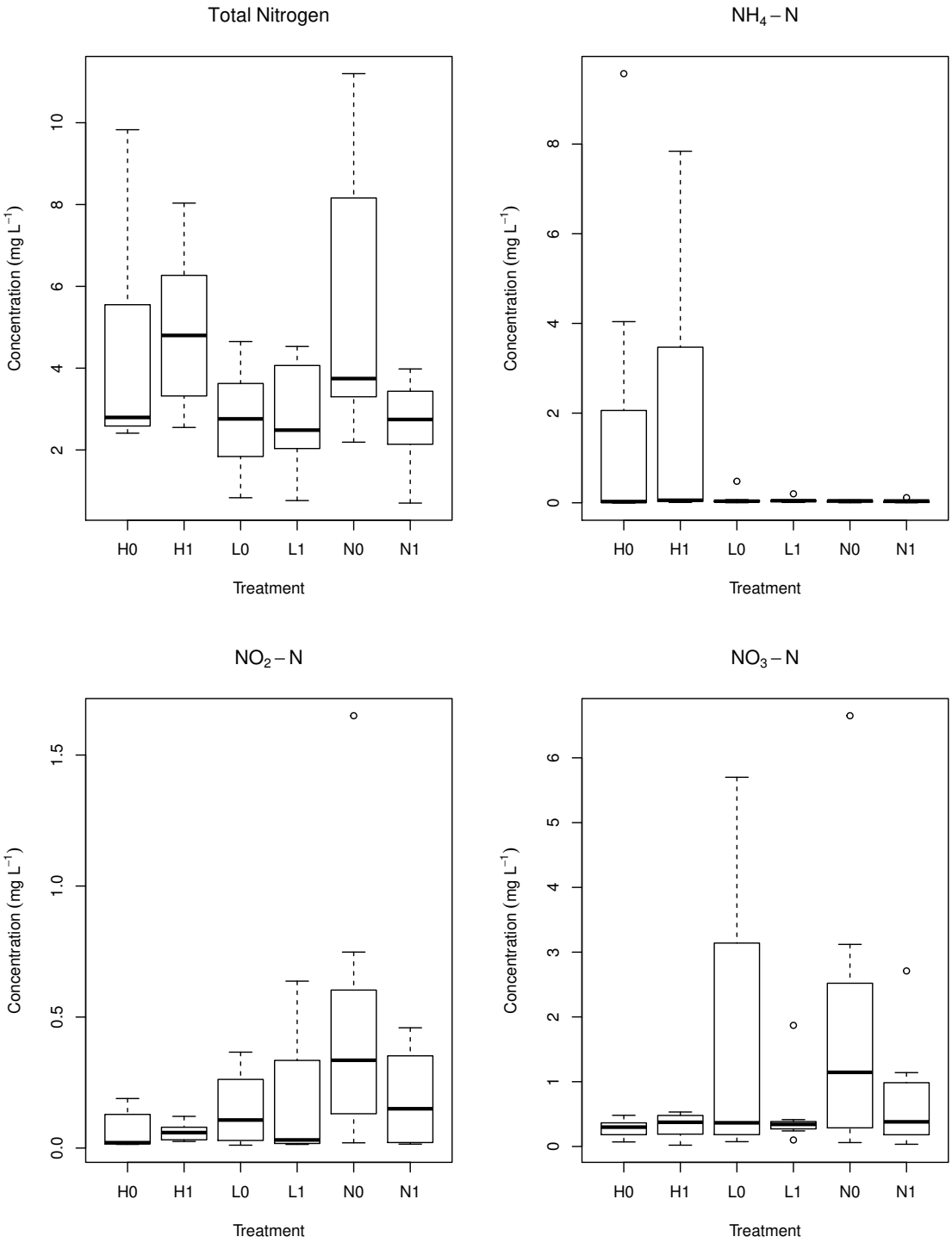

Figure A.4. Nitrogen species in each treatment. Data shown are pooled over time. Thick black lines indicate median values. Note the different intervals for the y-axes.

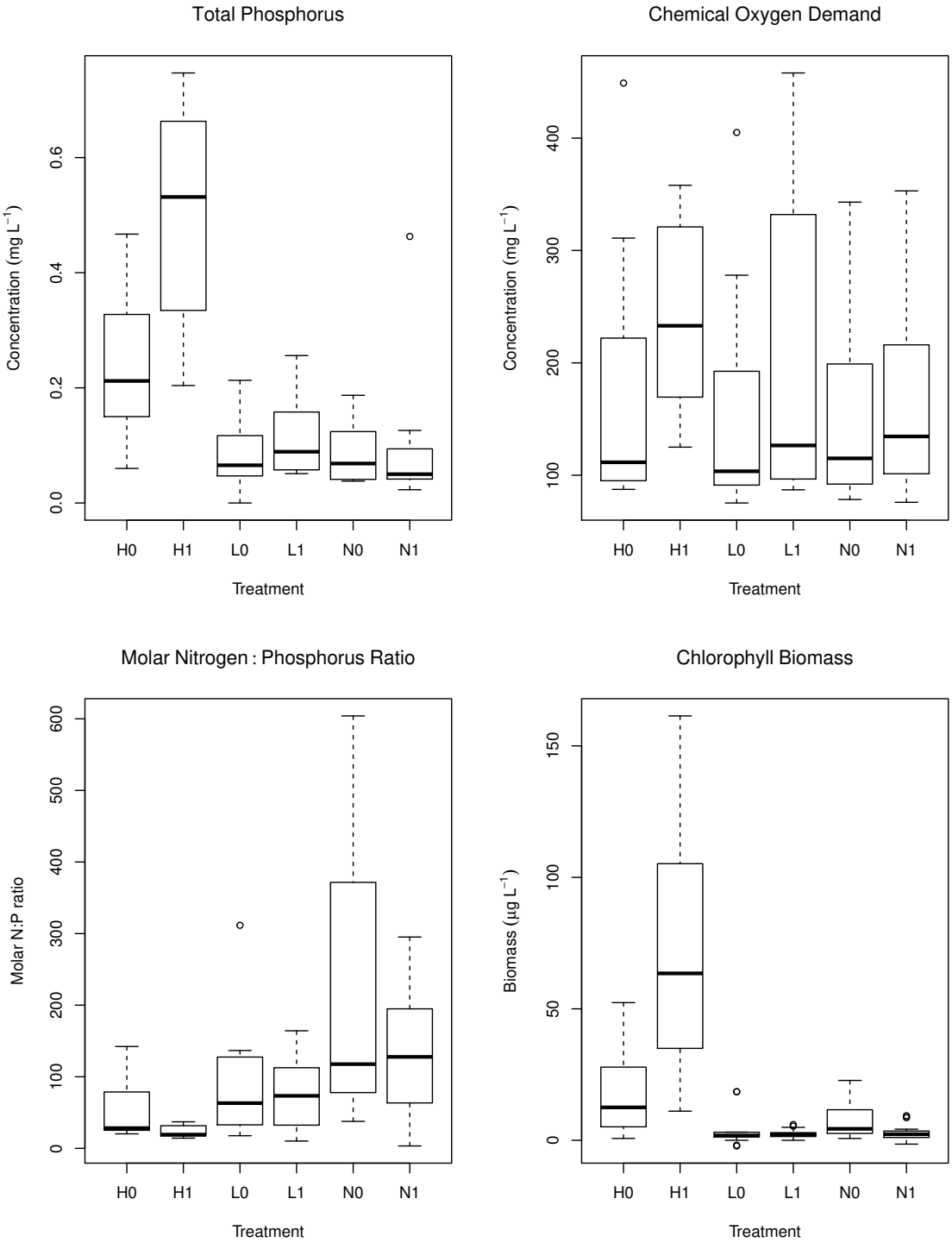

Figure A.5. Boxplots of total phosphorus (TP), molar nitrogen:phosphorus ratios, chemical oxygen demand (COD), and chlorophyll biomass in each treatment. Data are pooled over time. Thick black lines indicate median values.

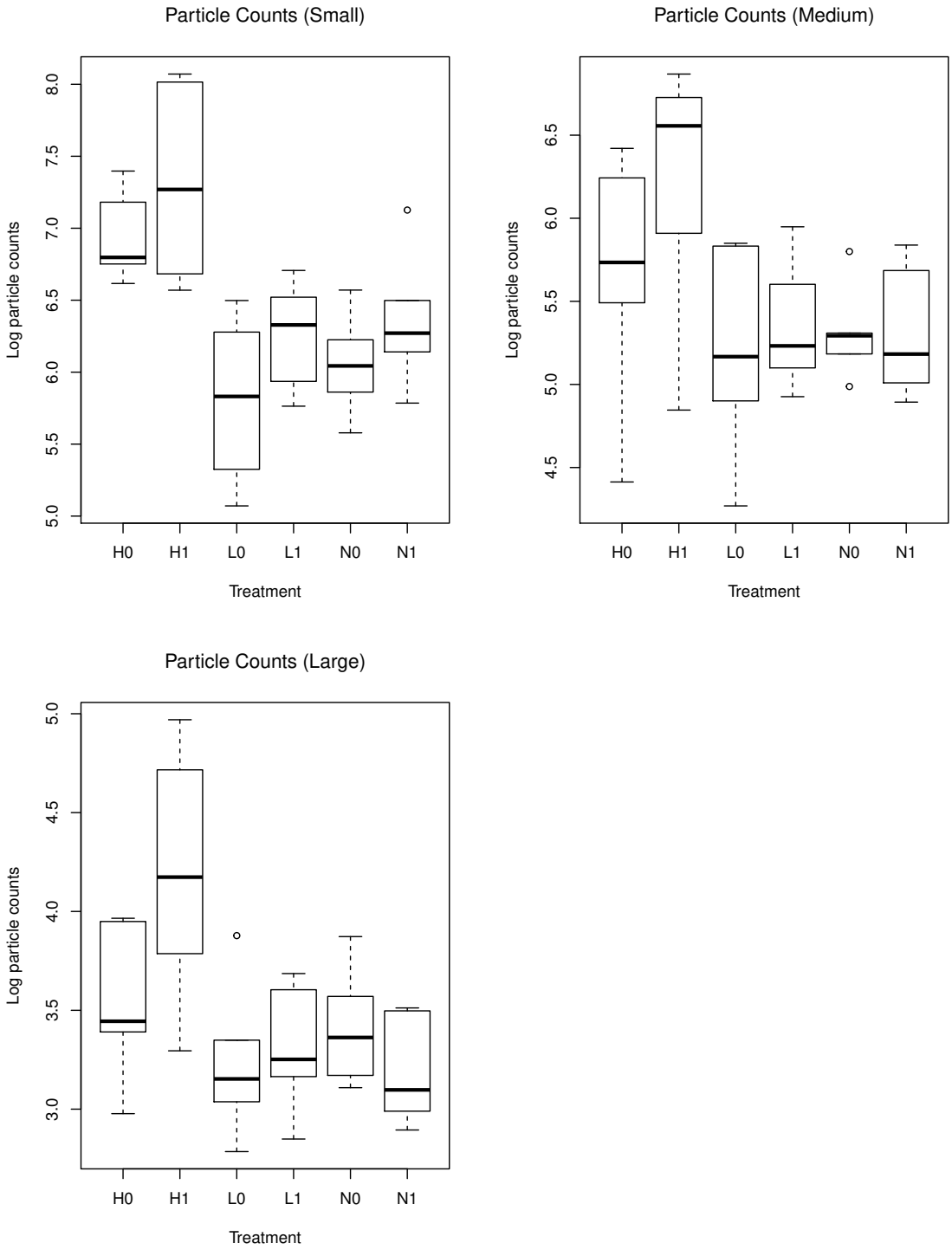

Figure A.6. Boxplots of particle abundance in each treatment, by size. Data are pooled over time. Thick black lines indicate median values.

Appendix B

Microbiome Data

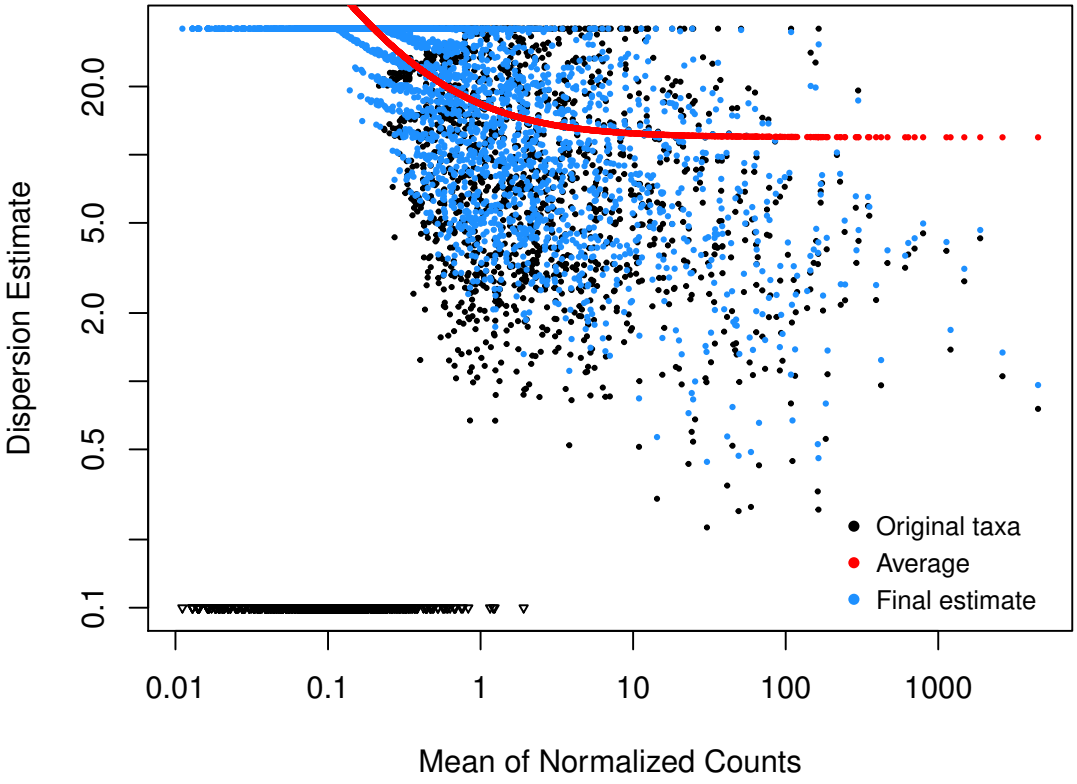

Figure B.1. Dispersion plot from *DESeq2* showing estimated and final (shrunk) dispersions of the OTUs from water samples. Small triangles indicate taxa that would be shown if the y-axis were extended below. Black dots indicate estimates calculated from the raw data. Red dots indicate the average dispersion estimate for all taxa at the same read depths (normalized counts). Blue dots indicate the final dispersion estimates for each taxon (see Love et al. 2014).

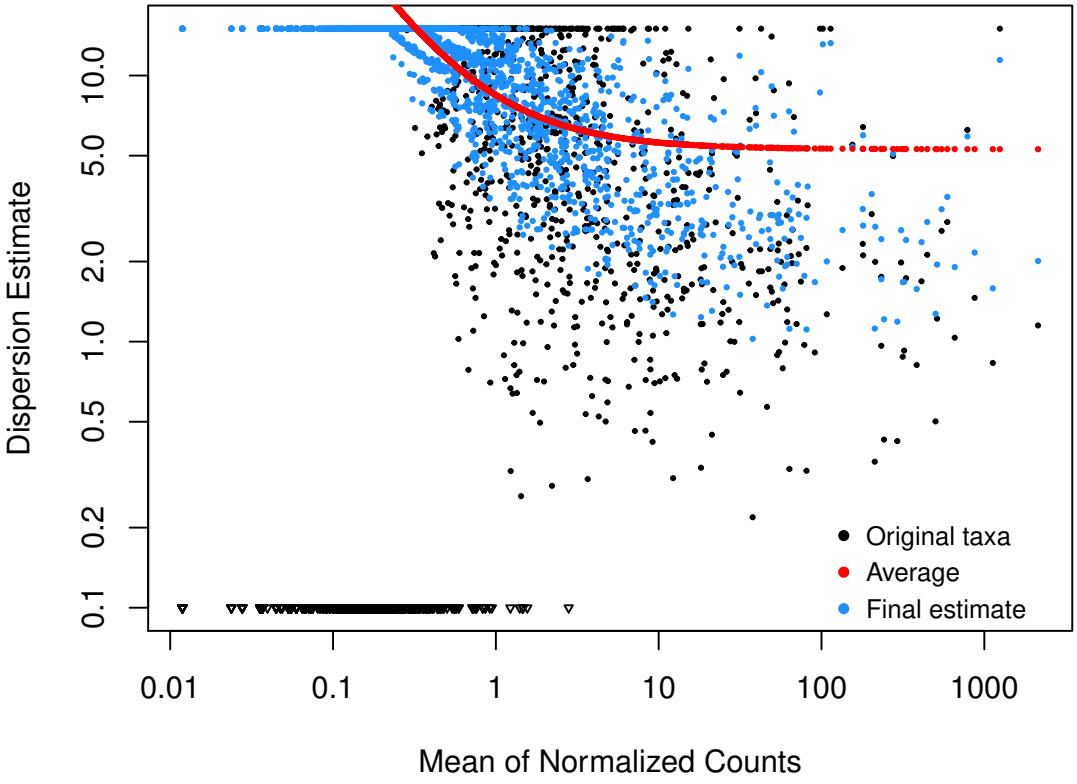

Figure B.2. Dispersion plot from *DESeq2* showing estimated and final (shrunk) dispersions of the OTUs from larval samples. Small triangles indicate taxa that would be shown if the y-axis were extended below. Black dots indicate estimates calculated from the raw data. Red dots indicate the average dispersion estimate for all taxa at the same read depths (normalized counts). Blue dots indicate the final dispersion estimates for each taxon (see Love et al. 2014).

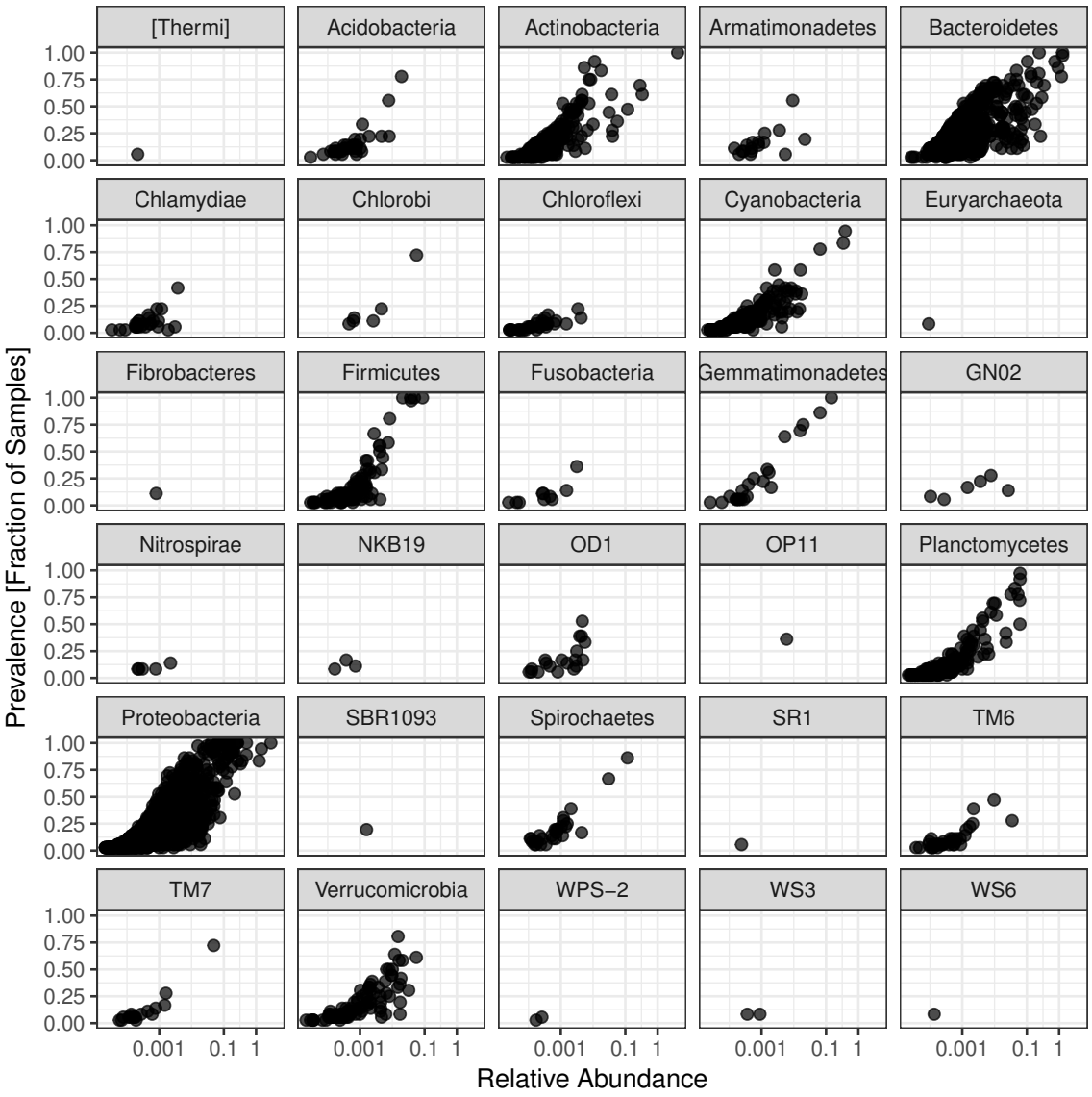

Figure B.3. Prevalence versus relative abundance for the 16S water samples. Data are plotted on a logarithmic scale (x-axis), showing 3705 taxa across 36 samples.

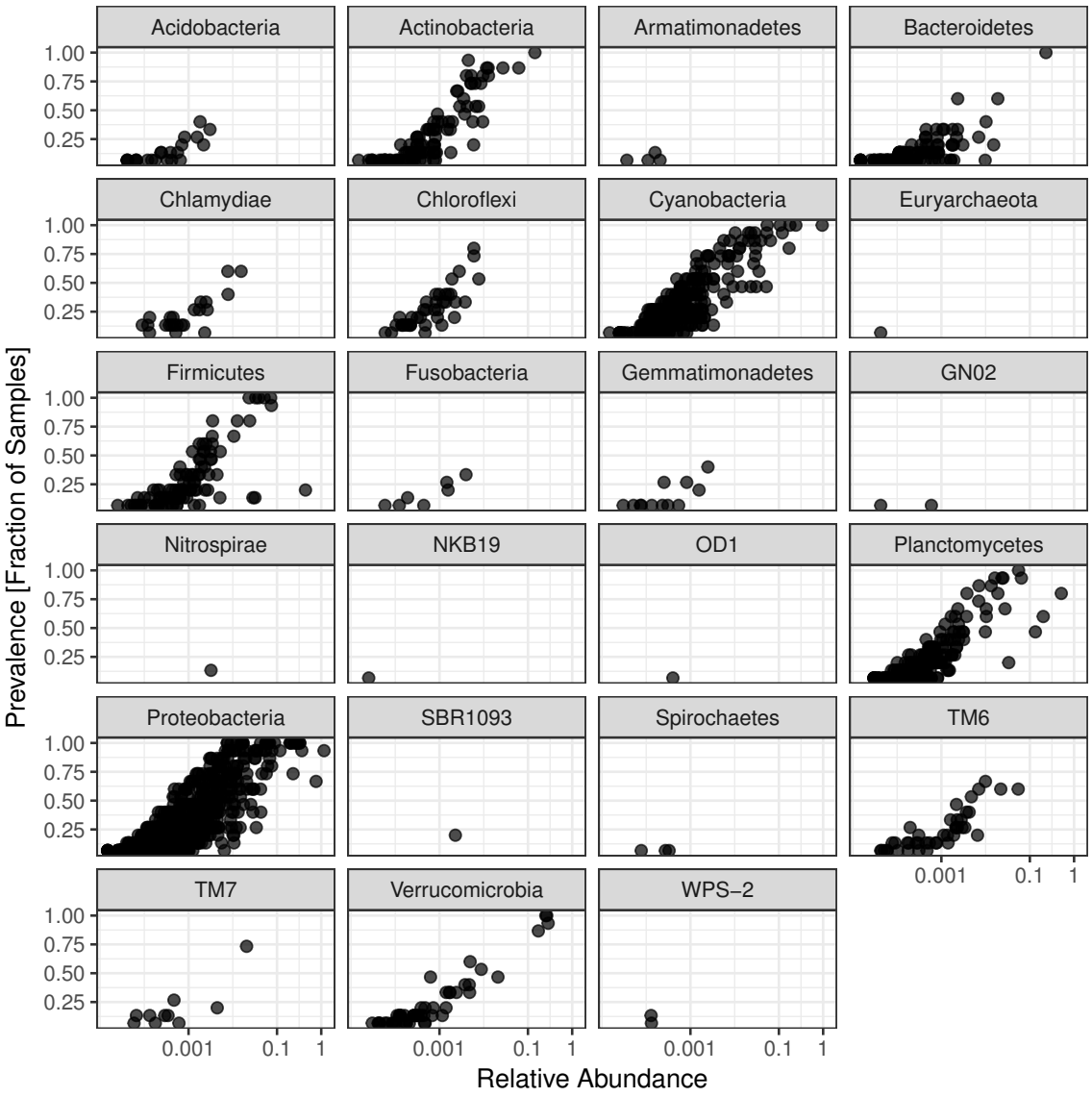

Figure B.4. Prevalence versus relative abundance for the 16S larval mosquito samples. Data are plotted on a logarithmic scale (x-axis), showing 2083 taxa across 15 samples.

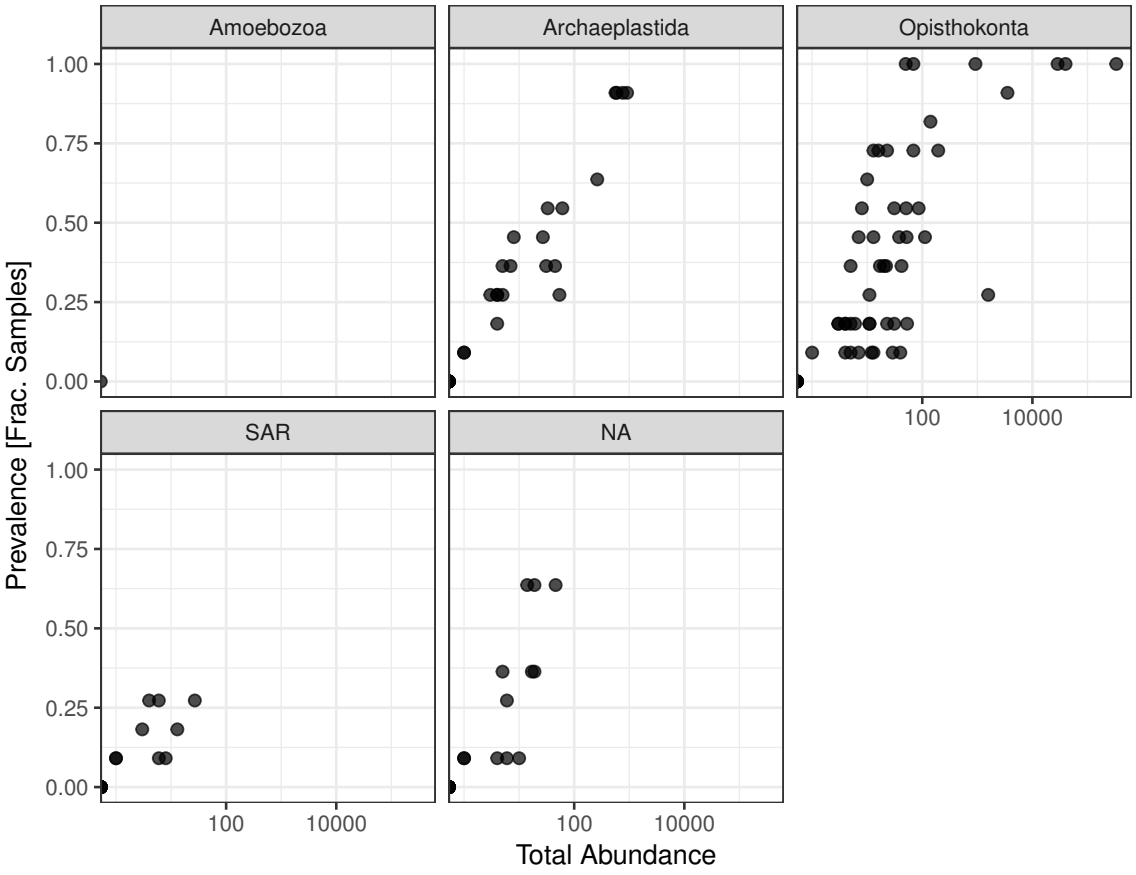

Figure B.5. Prevalence versus abundance for the 18S dataset (larval mosquito samples only), showing 147 OTUs grouped by clade across 11 samples. "NA" means taxa are "Unassigned" due to ambiguity or lack of matches in the database.

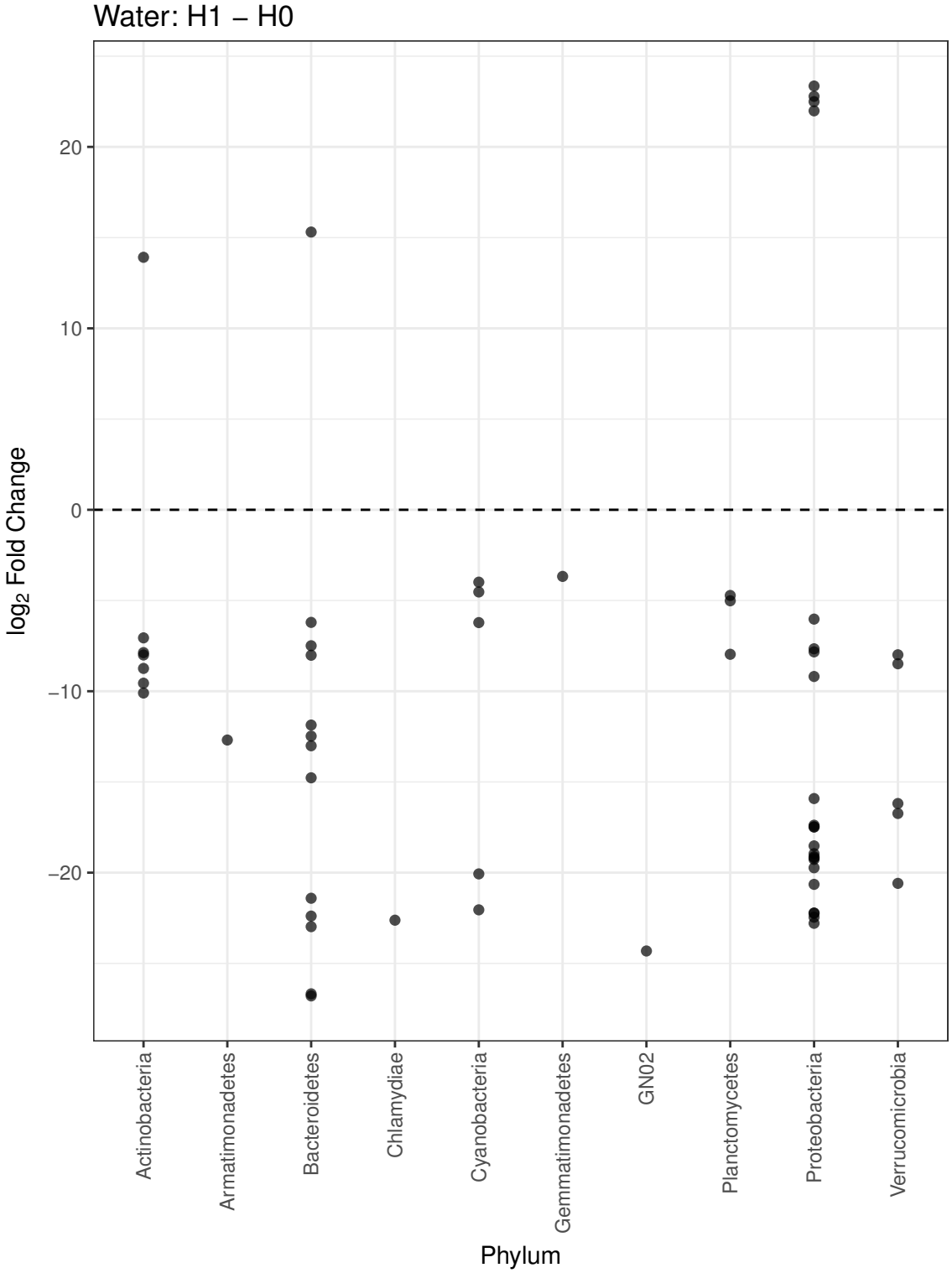

Figure B.6. Differential abundance of phyla in water samples, contrasting treatments H1 and H0 (high nutrient enrichment with and without mosquito access).

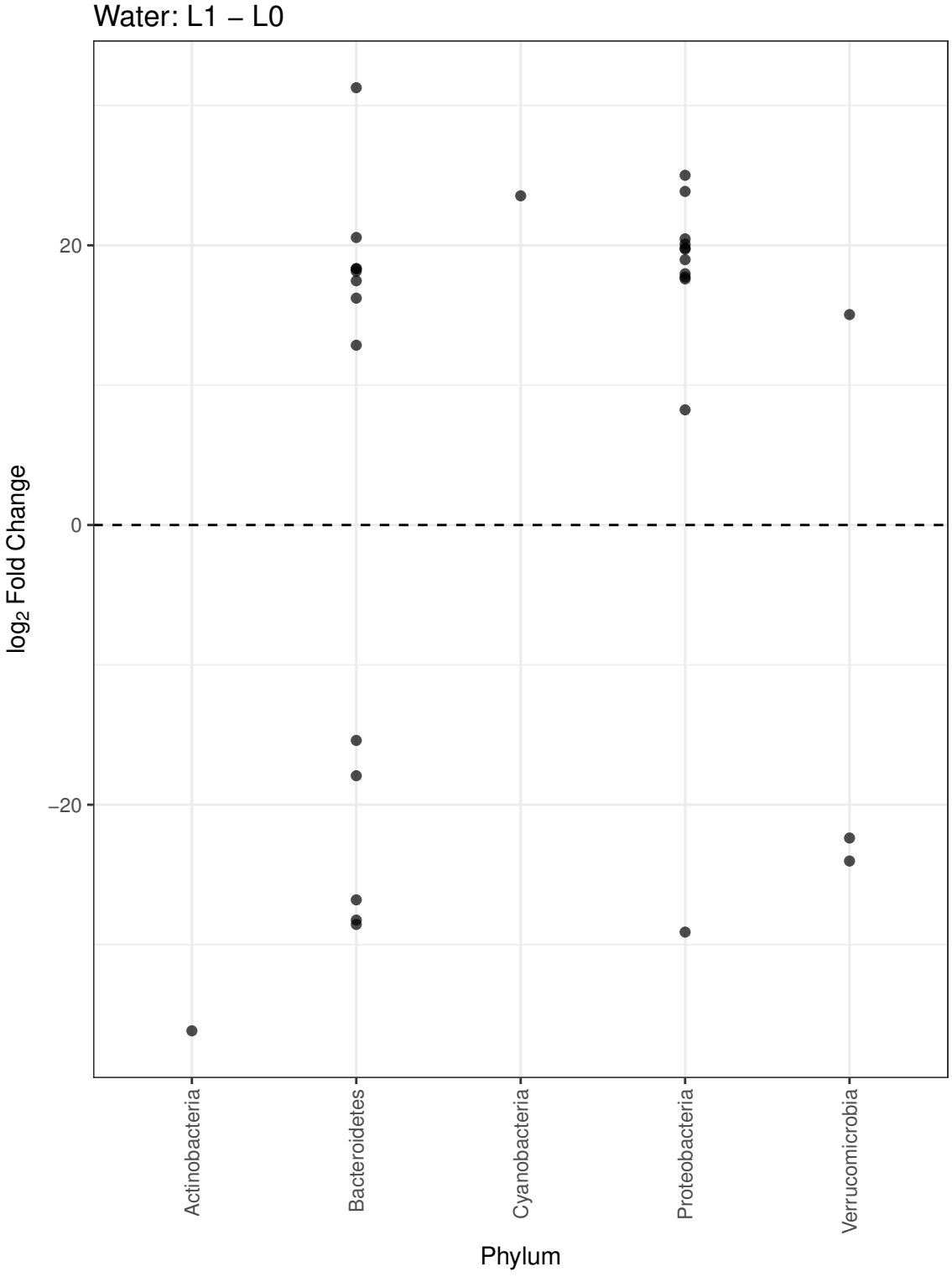

Figure B.7. Differential abundance of phyla in water samples, contrasting treatments L1 and L0 (low nutrient enrichment with and without mosquito access).

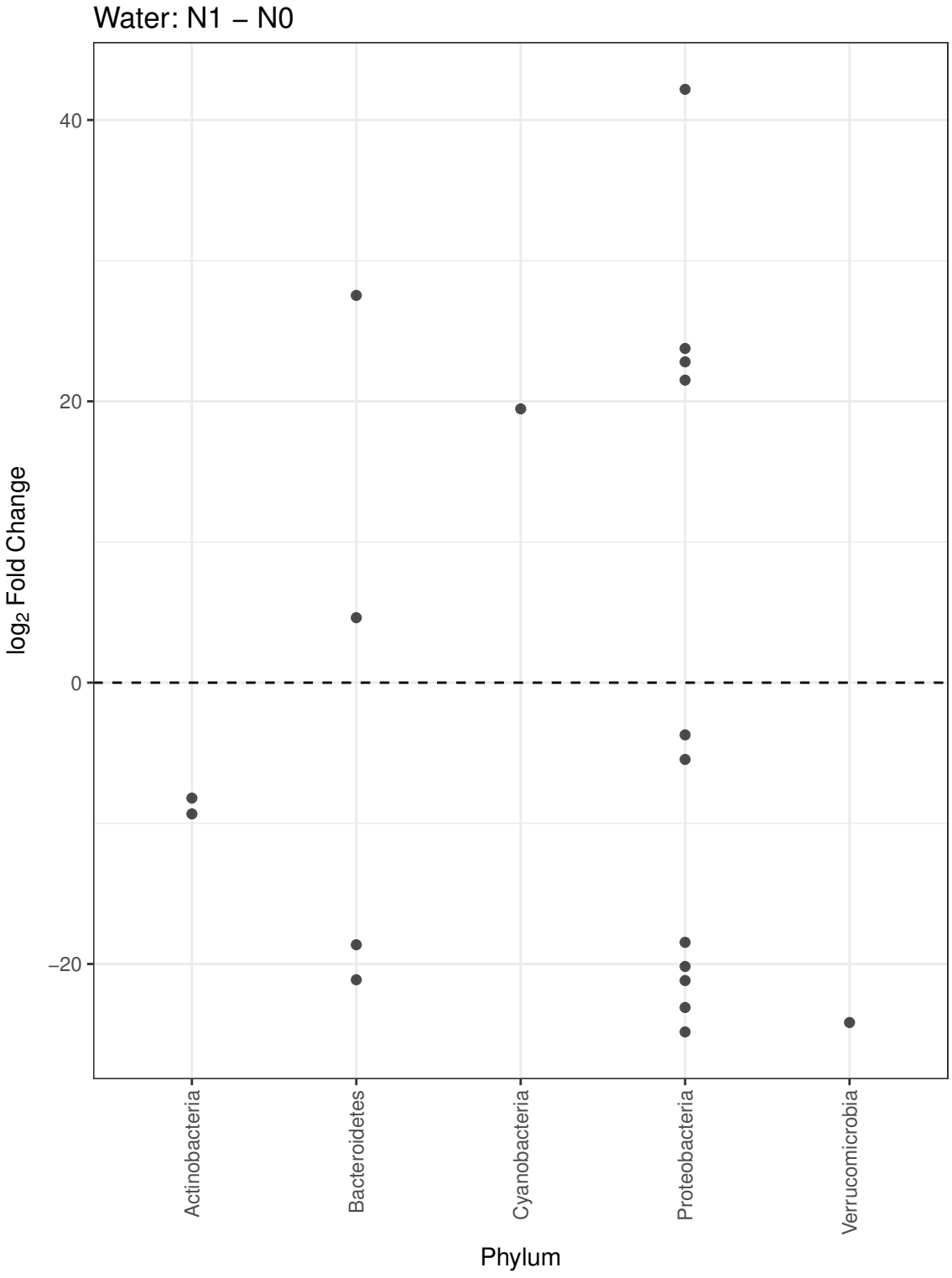

Figure B.8. Differential abundance of phyla in water samples, contrasting treatments N1 and N0 (no nutrient enrichment with and without mosquito access).

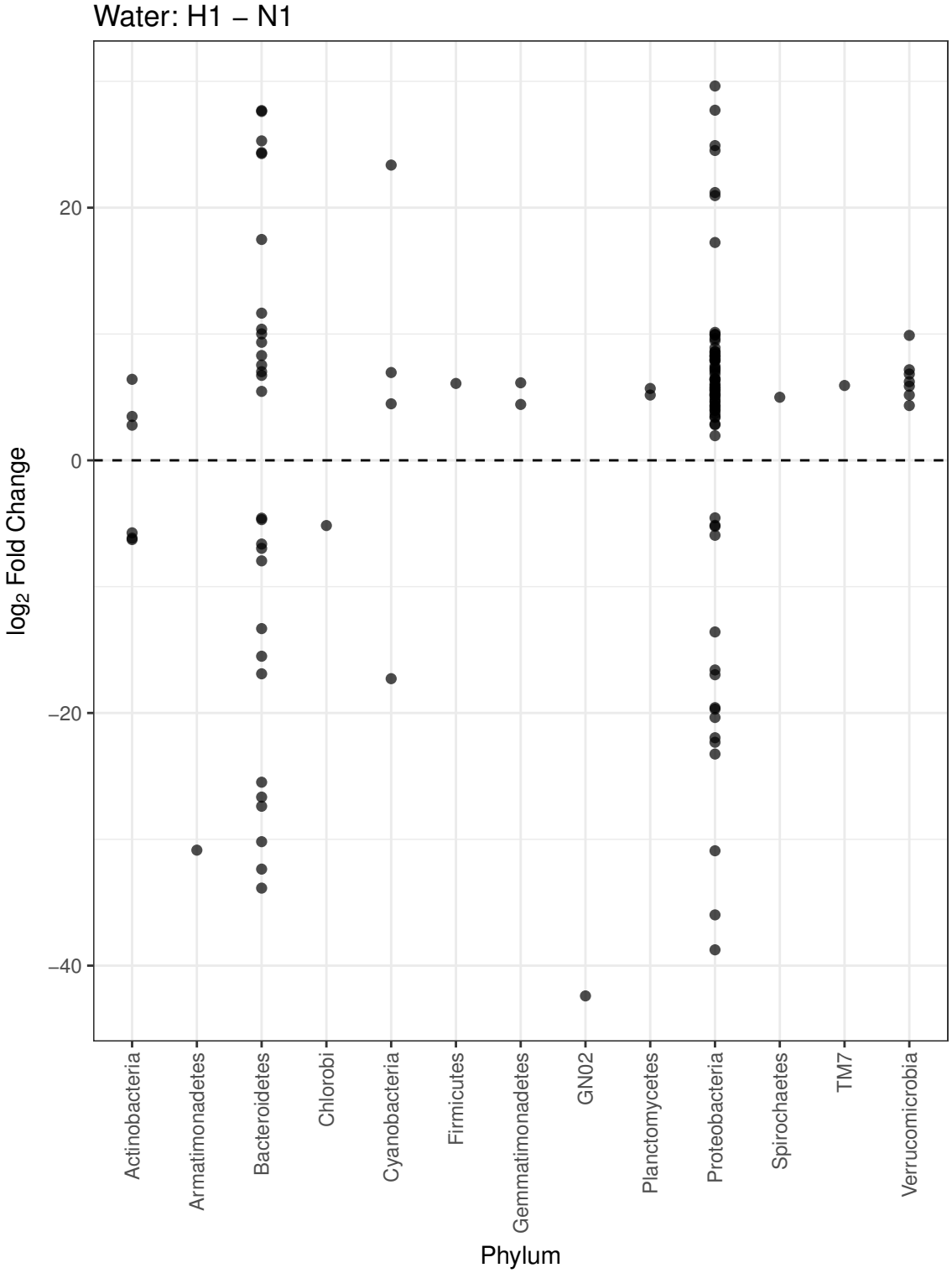

Figure B.9. Differential abundance of phyla in water samples, contrasting treatments H1 and N1 (high and no nutrient enrichment with mosquito access).

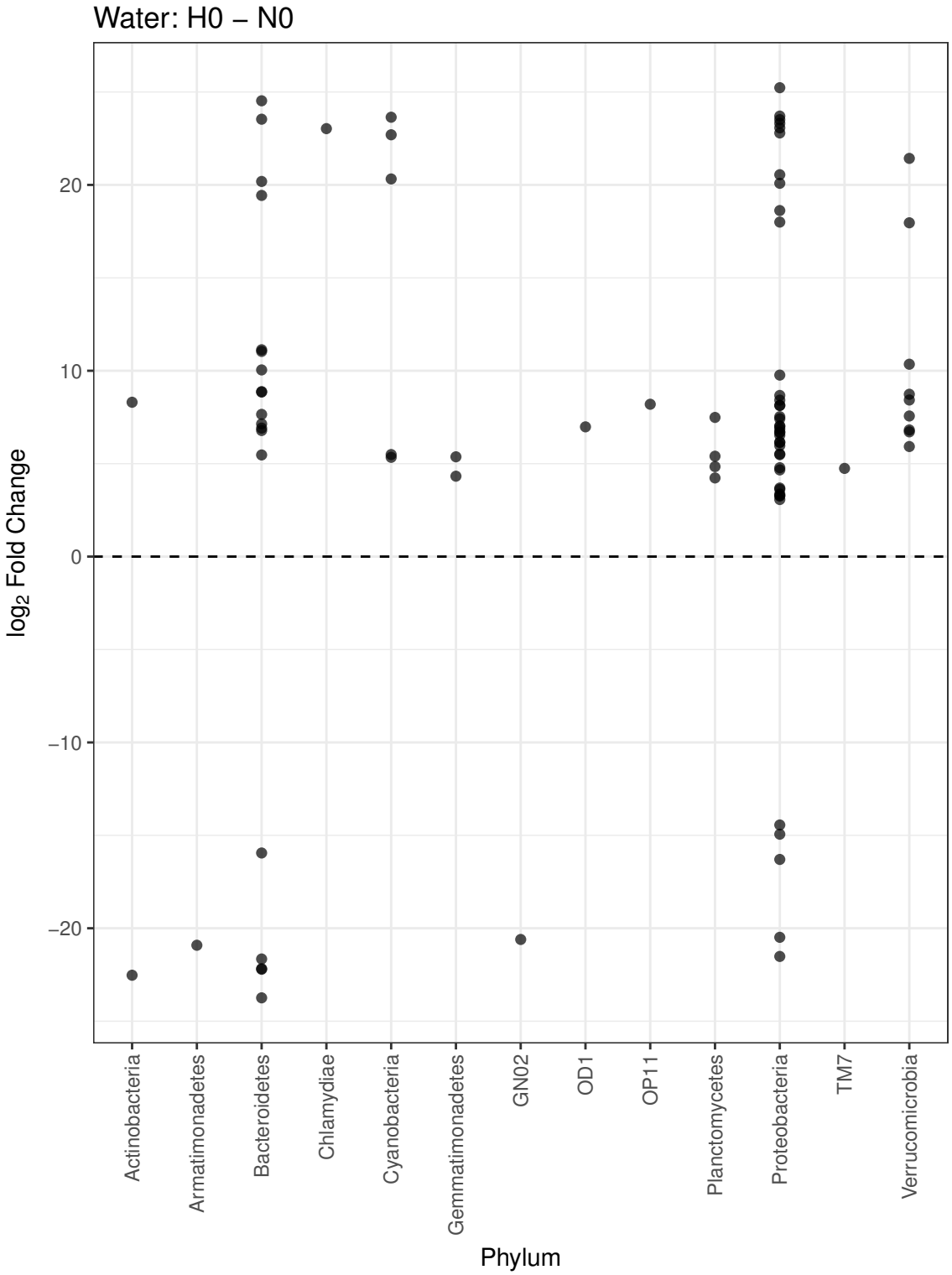

Figure B.10. Differential abundance of phyla in water samples, contrasting treatments H0 and N0 (high and no nutrient enrichment without mosquito access).

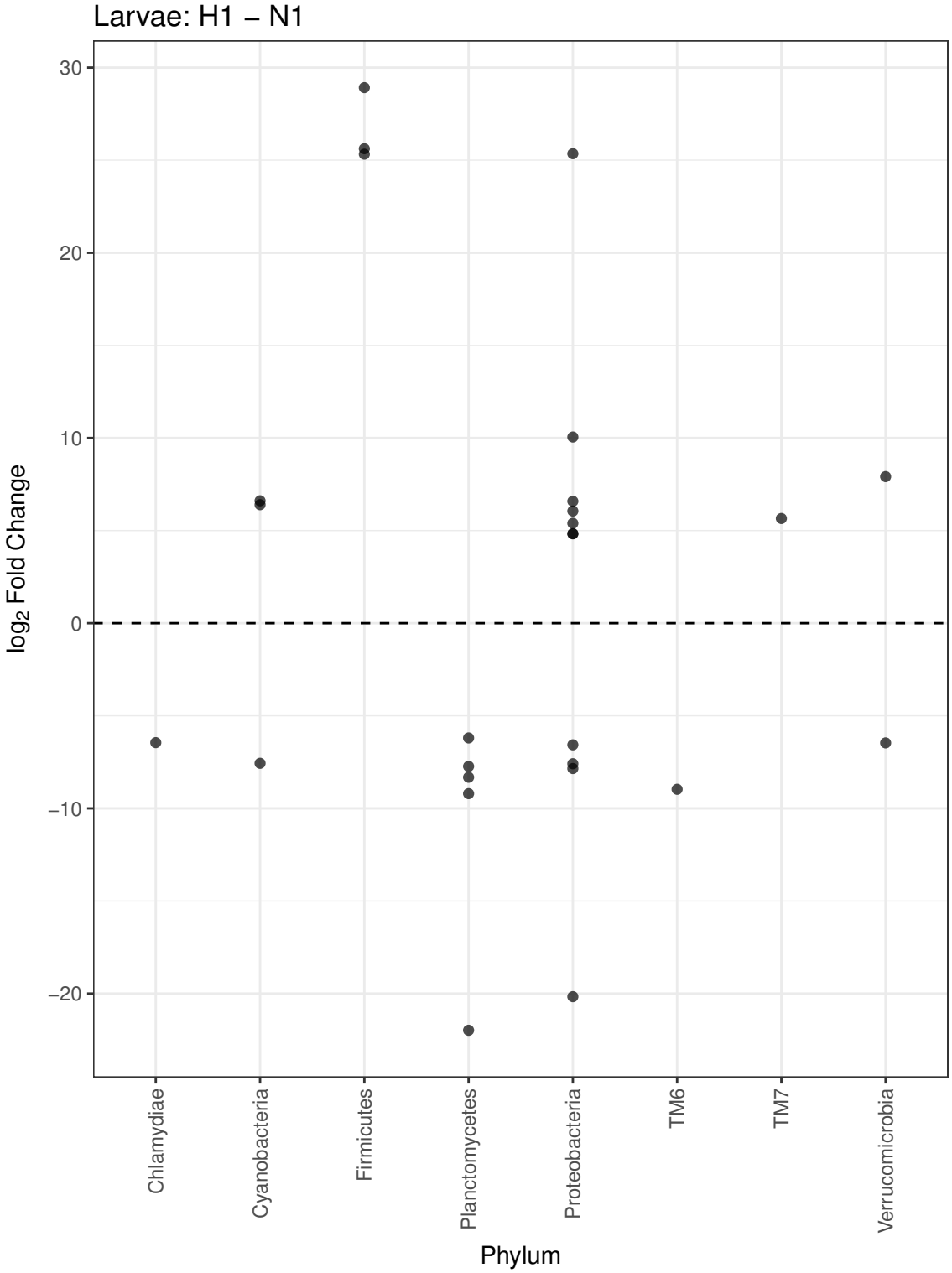

Figure B.11. Differential abundance of phyla in larval mosquito samples, contrasting treatments H1 and N1 (high and no nutrient enrichment with mosquito access).