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Temporal and Ecological Community Dynamics of Water-Cooling Tower Associated *Legionella* spp.

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TEMPORAL AND ECOLOGICAL COMMUNITY DYNAMICS OF WATER-COOLING TOWER ASSOCIATED *Legionella* spp.

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ABSTRACT

Man-made water systems can have complex microbial communities. *Legionella* spp. are ubiquitous in these environments and, with favorable conditions, can lead to outbreaks of Legionnaires disease. Included in these water systems are free living amoebas (FLA) and potential other eukaryotes that may act as a reservoir for *Legionella* survival and replication. Associations with these hosts can play a role in Legionnaires' disease outbreaks. A water cooling tower in Aikens, SC associated with *L. pneumophila* was measured every month for environmental parameters (temperature, bromine (Br), chlorine (Cl), pH, and dissolved oxygen). Water and sediment samples were collected every week for six months (March-August 2016). Samples were sequenced using 16S rRNA and 18S rRNA gene primers and analyzed to understand the bacterial and eukaryote community dynamics and their association with environmental parameters and correlations among bacteria and eukaryotic taxa.

The genus *Legionella* was found to be positively correlated in microbial association networks with *Vermamoeba vermiformis, Vannella,* Bilateria*,* other eukaryotic groups (Streptophyta, ConTHREEp and Gregarinasina), and numerous bacterial genera including *Halomonas, Candidatus Protochlamydia,* and *Candidatus Xiphinematobacter.* These correlations could indicate a commensal or predator-prey relationship among the eukaryotes and a similar host/lifestyle mechanism as *Legionell*a. However, there was no correlation with *Acanthamoeba,* a common host of *Legionella* spp., potentially due to the crash in this amoeba population after April. The main environmental parameter that was correlated with changes in the bacterial and eukaryotic community composition was temperature. The bacterial community showed a stronger response to the environmental

parameters than the eukaryotic community as seen by the changes in community richness as well as the significant differences between the bacterial communities in spring and summer months. In response to seasonal changes, there were major seasonal taxonomic changes from spring months (March-April) to summer months (May-August). Beta diversity was significant between summer and spring months for the bacteria but not significant for the eukaryotes. Though, Streptophyta (plant clade) and *Pseudomonas* spp. dominated in the spring months but were much less abundant in the summer months when temperature increased and Cl/Br levels decreased below 0.5 ppm. *Legionella* was relatively low in abundance compared to the rest of the bacterial community, but the relative abundance increased with increasing temperature and decreasing Cl/Br levels. There were also significant differences in the beta diversity between sediment and water samples in the summer months for both communities, however, no differentially abundant taxa were identified among sample type. Since these water systems are complex, different community dynamics and mechanisms could be occurring within the water and sediment. For instance, *Legionella* was most prevalent in the dark sediment during the spring, but transitioned to being most prevalent in the water during the summer. Whereas, majority of the eukaryotes were most prevalent in the water during the spring and became more prevalent in the sediment during the summer. Further understanding of the different dynamics between water and sediment should be explored.

This study, to our knowledge, provides the first characterization of the bacterial and eukaryotic community of a cooling tower known to have *L. pneumophila* in relation to environmental parameters. Understanding the community dynamics of water cooling tower associated *Legionella* spp. can provide insight into the development of Legionnaires' disease by characterizing new host associations and bacteria with similar lifestyles.

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1. INTRODUCTION

Man-made water systems, such as cooling towers, municipal drinking water systems, and domestic hot water systems can serve as reservoirs for potential pathogens such as *Legionella pneumophila, Mycobacterium avium, Salmonella enterica,* and *Pseudomonas aeruginosa* (5, 25, 39, 43, 44). Cooling towers, in particular, have been a target of concern in outbreaks of Legionnaires' disease, a severe form of pneumonia caused by *Legionella* spp. (35, 45). *Legionella* spp. are gram-negative, aerobic, motile rod-shaped bacteria that can be found in freshwater environments (5, 25). Currently, there are around 60 known species of *Legionella* with over 70 serogroups, where *Legionella pneumophila,* specifically serogroup 1 (SG1), is the main pathogen causing Legionnaires disease in North America (17, 25, 33). However, serogroups 2-14 of *L. pneumophila* contain virulence genes (17), *Legionella longbeachae, Legionella bozemanii, Legionella micdadei, and Legionella dumoffii* are species linked with disease in other parts of the world (4, 33), and *Legionella*-like pathogens have been associated with Legionnaires disease, but they haven't successfully been cultured *in vitro* (20).

Legionella spp. within these man-made water systems can survive in a variety of states, including within biofilms, a viable but not culturable (VBNC) or planktonic states. Another key state of *Legionella* spp. in these systems is the ability to naturally replicate inside amoebas, such as *Acanthamoeba, Hartmanella, Vahlkampfia, Vermamoeba,* and *Naegleria* (6, 9, 11, 13, 23, 25, 28, 29). Once inside the host cell (i.e. amoeba or macrophages in the case for human infection) *Legionella* replicate within a membrane structure called a Legionella-containing vacuole (LCV) and produce a high number of mature infectious cells (122). This host-symbiont relationship can play a role in the survival

of *Legionella* and is an area of interest that is still not well understood. Studies have shown that *Legionella* infect most amoeba *in vitro* (29), while only a few environmentally isolated species have been naturally infected with *Legionella* (9, 29). While the full natural host range of amoeba or other eukaryotes remains unclear, there is an extensive potential host repertoire, including the phyla Amoebozoa, Percolozoa, Ciliophora and Opisthokonta (52). Protozoa will graze and feed on biofilms within the environment, however, the exact mechanism that triggers entry of *Legionella* into a host is uncertain (29, 122). While not mutually exclusive, possible explanations include low nutrient levels, proximity within biofilms, and environmental stress such as chlorination (29). Under these many conditions, the bacteria may attach and enter the amoeba, surviving inside until proper conditions for replication have returned (37).

Since *Legionella* can reside in amoeba, these eukaryotes can serve as protective barrier for *Legionella* due to amoeba having a greater resistance to disinfection methods (6, 7, 12, 20). Disinfection methods tend to only be effective against planktonic *Legionella* cells and relatively ineffective against sessile *Legionella* (cells within biofilms), VBNC cells and cells within a host, i.e. amoeba (52, 53, 58). In part, intracellular *Legionella* are not exposed to the same degree of disinfection as planktonic *Legionella* cells. Inherent cellular changes, such as biochemical and physiological, cause released intracellular *Legionella* cells to have increased resistance to chemical disinfection, pH, temperature, oxidizing agents and also an increased infectivity in mammalian hosts (20, 52, 53, 105).

Understanding the community ecology and dynamics of water systems is important to gain insight into the possible species, serogroups and hosts playing a role in Legionnaires

disease outbreaks. More studies are now utilizing Next Generation Sequencing to more efficiently identify different species and serogroups of *Legionella* within microbial communities (33, 34, 35, 64). One study showed that high throughput amplicon sequencing could detect low abundance of *L. pneumophila* Philadelphia-1 when mixed in with human DNA and other bacterial DNA mock samples, suggesting that sequencing is a better detection method in metagenomic samples than the commercialized kits, such as ones that test for SG1 antigens (34). Another study successfully utilized 16S rRNA gene and 18S rRNA gene amplicon sequencing together with shotgun sequencing to characterize bacterial and amoeba species in different watersheds (33). This provided information on different eukaryote species associated with *Legionella* spp. in natural aquatic environments and how *Legionella* is most abundant in cleaner water sites, rather than agricultural sites. A different study assessed bacterial community dynamics using 16S rRNA gene amplicon sequencing with respect to environmental conditions in a water tower from Germany (35). A diverse community profile was observed and fluctuations in the taxonomic profile were correlated with key environmental factors, such as temperature. This provided a better understanding of how the cooling tower system/community changed with exposure to different environmental parameters. They also found multiple potentially pathogenic genera present in the cooling tower such as *Legionella, Sphingomonas,* and *Mycobacterium.* Another study utilized 16S rRNA gene and 18S rRNA gene amplicon sequencing in combinations with environmental parameters and microbial co-occurrence networks to observe the community dynamics associated with free-living amoeba (64). *Vermamoeba* and *Pseudomonas* spp. were highly prevalent in the community and there were 21 newly identified correlations among the amoeba and bacteria. However, *Legionella* spp. was not isolated.

This study aims to understand the microbial community within a water cooling tower, specifically the (i) community dynamics, (ii) relationships between *Legionella* and eukaryotic taxa and (ii) if there are biotic and abiotic factors that affect the community composition (40, 41, 42). It is surmised that new eukaryote associations with *Legionella* will be identified using this approach. Currently, legionellosis outbreaks are on the rise but overall knowledge on health risks relating to the disease and mechanisms of these outbreaks are limited (45, 54, 56). Increasing resistance problems with disinfection and unknown interactions between eukaryotic hosts emphasize the need to determine biological relationships between the water system's microbial community and *Legionella.* This could provide insights on what allows the bacteria to recover and survive. To our knowledge, this is the first study to assess the community composition (16S rRNA gene and 18S rRNA gene) over time and correlate environmental parameters within a water cooling tower associated with *Legionella spp.* to determine community profiles and predictive patterns of what allows *Legionella* to flourish in the community. Our results show correlations among *Legionella,* four eukaryotic taxa (including *Vermamoeba vermiformis*) and numerous bacteria (including *Halomonas* spp.), which could suggest symbiotic relationships and similar niches or host mechanisms. Major seasonal taxonomic changes were a result due to fluctuations in environmental parameters, especially temperature and bromine level. Significant differences in beta diversity and taxonomic differences among water and sediment samples also indicate the need to further explore the community dynamics within these different sample types.

2. MATERIALS AND METHODS

2.1 Water cooling tower sample collection and measurement of environmental parameters

Five water cooling towers (FA, HTF-1, HTF-2, FTF-1, FTF-2) used to cool building heat exchangers in Aikens, SC were sampled and analyzed due to prior detection of *L. pneumophila* SGs 1, 2, 4 and 6 by the Savannah River Research Laboratory (SRRL) using the Direct Fluorescent Antibody (DFA) method (Supplemental Table 1). The towers are located in different areas around Aikens, SC and the HTF and FTF towers have "sister" towers adjacent to them (i.e. HTF-1 and HTF-2). For these "sister" towers, only one tower was operating at once and when it needed to be cleaned, the other tower was turned on and sampled from, starting a new timeline for the new tower in use. Chemtreat, a solution containing both chlorine and bromine, was added continuously based on the volume within the tower. The recommended concentration to keep the Cl/Br was 0.5-1.0ppm. A corrosion inhibitor (Drew 2235 Cooling Water Treatment) was also added at a recommended level of 0.7-1.1ppm, to help reduce corrosive side effects of Chemtreat.

Water, bright sediment (sedimentation exposed to sunlight) and dark sediment (sedimentation not exposed to sunlight) samples were collected every week from March 2016 to November 2016. Thus, one bright sediment, one dark sediment, and one water sample were collected every week for the duration of time the tower was running. In addition, seven environmental parameters (temperature, dissolved oxygen, conductivity, pH, free chlorine concentration, bromide concentration, and turbidity) were measured once a month over the sampling period.

The environmental data collected by SRRL was graphed to visualize the fluctuations of these factors and concentration of *Legionella pneumophila* (cells/L) over time in each tower. Tower HTF-2 yielded the longest time series (25 weeks) that had time points before and after addition of Chemtreat. The tower also had large fluctuations in average *Legionella pneumophila* cells/L over time. Due to the dramatic changes in environmental data and cell counts, this tower was chosen as a starting point for further investigation of the microbial community dynamics. At this time, forty seven samples were used in the analysis.

2.2 DNA Extraction of water and sediment samples

The DNA was extracted from the sediment and water samples using the boil method as described previously with slight modifications (30, 31). In brief, the modified method added 0.5% tween 20 (serving as a detergent to help disrupt the cell membranes) to aliquots of samples and was mechanically disrupted in a bead beater for 5 minutes. Next, the samples were boiled for 10 minutes at 100°C to further disrupt the cell membrane. The boiled samples were then centrifuged for 10 minutes at 10,000g to separate the cell debris from the DNA in the supernatant. The supernatant was stored at -20°C until library preparation.

2.3 Illumina Library preparation and Sequencing

The 16S rRNA gene V4 region was amplified using universal bacterial primers from Kozich *et al.* (2013). The primer sequences are as follows: 16S rRNA V4 FWD 5‘-

GTGCCAGCMGCCGCGGTAA-3'; 16S rRNA V4 REV 5'-GGACTACHVGGGTWTCTAAT-3'. 18S rRNA gene primers from Delafont *et al.* (2013) were tested with samples and pure *Acanthamoeba polyphaga* DNA. PCR reactions yielded multiple bands with samples and the pure *A. polyphaga* DNA. Thus, custom primers were created from a conserved region of the 18S rRNA ribosome identified using a global nucleotide alignment of 18S rRNA sequences from amoeba species commonly known to be associated with *Legionella* (see below) (23, 52, 64, 65). The amplicon region spanned from -1391 bp to -1644 bp position in the 18S rRNA gene, which was a 253 base pair long region. The 18S rRNA gene sequences used included 2 phyla, 8 orders, 8 families 11 genera and 13 different species. Specifically, the amoebae genera included in the alignment were *Vanella* (2 18S rRNA sequences from two species, see Supp. Fig. 1)*, Diphylleia* (1)*, Echinamoeba* (4)*, Hartmannella/Vermamoeba* (4)*, Acanthamoeba* (2)*, Balamuthia* (2)*, Saccamoeba* (2)*, Neoparamoeba* (1)*, Valhkampfia* (2)*,* and *Naegleria* (1). The primer sequences are as follows: 18S rRNA FWD 5'-AGAYGATYAGATACCGTCGTAG-3' (22 bp with 2 degenerate bp); 18S rRNA REV 5' GGTGYCCYTCCGTCAATTCCTTT-3' (23bp with 2 degenerate bp). Since there were degenerate bases, primer sequences were blasted using the nr/nt database to determine the taxonomic capture of the primers. The primers hit 10,000 sequences each with 91% identity and 100% cover. The taxonomic range included a variety of protists, amoeba, fish and plants.

The 16S rRNA and 18S rRNA amplicon libraries were constructed in a single PCR using the primers described above with added base pairs consisting of the Illumina adapter sequence, barcode indices, pad sequences, and linker sequence as outlined in Kozich *et*

al. (2013). The libraries were then quantified using a Qubit 2.0 Fluorometer (Invitrogen) and pooled at an equimolar concentration of 2nm. Libraries were sequenced on the MiSeq platform using V2 chemistry (250bp, PE reads).

2.4 Bioinformatics and Statistical Analysis of microbial communities

The QIIME2 software pipeline (https://qiime2.org, 6) was utilized for microbiome analysis of the 16S rRNA and 18S rRNA amplicon data. The demultiplexed forward and reverse fastq files of each dataset were separately imported as Casava 1.8 paired-end, assembled reads and then demultiplexed. Chimeric and low quality sequences were removed using DADA2. Taxonomic assignment was achieved using the Naïve Bayesian classification system in combination with a Greengenes 13_8 99% trained classifier (16S rRNA) and a SILVA_128_SSU Ref_Nr 99% trained classifier (18S rRNA). Normalized taxonomic assignments were used to calculate alpha (Shannon) and beta (Bray Curtis) diversity. Rarefaction without replacement was conducted based on the number of sequence counts for each community (16S rRNA= 13,738; 18S rRNA= 7,700) as outlined in QIIME2 (6). Alpha rarefaction plotting was conducted using the rarefaction depth to determine if the richness of samples was fully observed (Supp. Fig. 2). The alpha rarefaction curves leveled off before the specified sampling depth, indicating the community diversity was represented in the analysis. Associations and correlations between environmental parameters and diversity measures were tested. Spearman correlation was conducted for alpha diversity correlations between environmental parameters and ANOSIM (compares beta diversity in terms of environmental parameters) in combination with the BIOENV test and a mantel test were used to find correlations between the beta diversity and environmental parameters. Differential abundance testing using ANCOM (Analysis of

Composition of Microbiomes), a method that is highly sensitive, has a good control of false discovery rate, and makes no assumptions on distribution (47, 61), was performed to determine OTUs that were differential abundant across sample groups. Next, OTU tables from QIIME2 were used for further analysis utilizing the MicrobiomeAnalyst software pipeline (38). Positive and negative correlations among taxa were determined using the Spearman correlation method and LEfSe LDA (Linear Discriminant Analysis Effect Size analysis) (61) (with default settings) was conducted to characterize taxa that explain differences between groups (i.e. different sample types).

To account for sequencing error, OTUs with less than five counts present in less than 10% of samples and having less than 5% variance within a sample were removed. Taxonomic assignments were normalized using rarefaction without replacement (47).

Microbial association networks (bacteria and eukaryote combined networks) were created using CoNet (48). To visualize these networks, Cytoscape 3.6.1 was used (48, 49, 50). In brief, networks were created by importing environmental parameters and an OTU table from each dataset into CoNet. Pearson, Spearman, Mutual Information, Bray Curtis, and Kullback-Leibler dissimilarity measures were used to calculate pairwise associations among the taxa and find the most agreed upon edge correlations (48, 100). The edge selection parameter was set to 1000 (top and bottom) for each method in the combination networks (bacteria and eukaryote). This produced the top 1000 positive and negative edges, or correlations among nodes/taxa. Compositional bias was controlled by performing permutations that shuffled and renormalized the vectors of each taxon pair (48, 100). Bootstrapping of p-values for each correlation methods was done by merging them

using “brown” p-value merge method (109) and corrected using the FDR multiple test correction method (110). Unstable edges were filtered if they weren’t supported by at least two correlation methods, did not have a p-value ≤ 0.05, or were outside of the 95% confidence interval of the bootstrapping distribution (Fig. S3). Global network analysis was conducted using the NetworkAnalysis tool in Cytoscape and network clustering was done with the plugin CytoCluster and clusterMaker using default settings for the OH-PIN, HC-PIN, DCU, ICPA, MCODE, ICP-MCE, ClusterONE, MCL, SPCS and Connected Components algorithms (99, 128). Ten different algorithms were used to identify correlations among the same taxa despite the method. The clustering algorithms maximized the correlations within a cluster and minimized the number of correlations among clusters (99).

3. RESULTS

3.1 Overall Community Dynamics and Composition

Cooling Tower Environmental Factors

The 47 samples were collected from the water tower through March to August 2016. Fluctuations in environmental parameters can be seen in Table 1 and Supplemental Figure 4. The temperature of the water tower went as low as 12.7 °C in April and increased as high as 25.8 °C in July. Based on temperature levels, March and April were arbitrarily assigned as spring (below 19 °C) and May through August were assigned as summer (Above 19 °C). The pH fluctuated from 8.39 to as high as 8.69 throughout the sampling period. Dissolved oxygen (DO) levels were consistent until July when they dropped to 99.7%. Conductivity and turbidity fluctuated as well but the lowest

conductivity occurs in August (0.24 mS/cm) and the lowest turbidity occurs in June (1.4 NTU). The free chlorine and bromine concentrations are high in March (7.5/17.1 ppm) then decrease to 0.00 ppm in June and increase again in August (2.87/6.55 ppm). The tower had a chemical disinfection device that when functioning correctly maintains Chemtreat (a solution containing both chlorine and bromine) at the recommended level of 0.5-1.0ppm. There were several occasions where the levels exceeded the recommended concentration. This occurred in March (7.5/17.1 ppm), April (3.92/8.74 ppm), May (4.01/9.14 ppm), and August (2.87/6.55 ppm). The disinfectant level fell below the recommended concentration range in June (0.00/0.00 ppm) and July (0.18/0.33 ppm).

Community alpha and beta diversity metrics

The total number of sequence counts obtained and sampling depth, or number of sequences subsamples for normalization, for each community can be observed in Table 2. The number of observed OTUs in the bacterial (16S rRNA) community was 537, where four were assigned to Archaea (0.744%), two were only classified to the domain level of Bacteria (2.23%) and the remaining 531 OTUs (98.9%) had further classification levels. In the eukaryote (18S rRNA) community, there were 168 observed OTUs, where only one was assigned just to the domain level of Eukaryote (0.595%) and the remaining had further classifications (99.4%). After filtering of low abundance and low variance OTUs, the number of OTUs present in the bacteria community was 219 and 75 OTUs in the eukaryote community (one sample removed for bacteria and three samples removed for eukaryote when analyzed separately). Overall, there were 123 genera, 108 families and 22

phyla/candidate phyla. Specifically, 103 genera, 82 families and 15 phyla/candidate phyla were from the bacteria community and 20 genera, 25 families and 7 phyla were from the eukaryote community.

Generally, the Shannon alpha diversity values ranged from 1.2 to 6.3 for the bacterial communities, and 2.4 to 6.9 for the eukaryotic communities (Fig. 1). Alpha diversity for the bacterial and eukaryotic communities was correlated with environmental factors (Fig. 1). Temperature and Br level were represented in the figure since they had the strongest correlation coefficients with the respective community's alpha diversity.

The bacterial community's richness significantly increased with increasing temperature (correlation coefficient= 0.7194; $P < 0.05$), month (c.c=0.7053; $P < 0.05$) and days running (c.c.=0.6940; $P < 0.05$). As seen, temperature increased over time from spring (March-April) to summer (May-August; Fig. 1A and B). The alpha diversity was greater with higher pH (c.c.= 0.6360; $P < 0.05$). The richness was negatively correlated with Cl/Br levels as it decreased with increasing Chemtreat levels (c.c.= -0.4846 for both; $P < 0.05$), which again decreased over time. The alpha diversity was highest when Cl/Br levels were zero in June and when the temperature was 25.8C in July (also low Cl/Br levels). In the eukaryotic community, there were no significant correlations with any of the environmental factors (all P values > 0.05) and the richness remains relatively constant over the sampling period despite fluctuations in environmental factors (Fig. 1C and D). All correlations with environmental parameters and alpha diversity were also weaker in the eukaryote community than in the bacteria community (month (c.c.=0.2252), temperature (0.1488), Br (-0.2662), Cl (-0.2662), pH (0.1722), days running (0.6940)). When grouping by alpha

diversity measures by sample type, there was no significant difference among bright sediment, dark sediment, and water samples for the bacterial and eukaryotic communities ($P > 0.05$ for both).

To further investigate the difference among spring and summer samples, the alpha diversity was correlated with season (Spring=March and April; Summer=May-August). Spring was significantly different from the summer months/samples ($P<0.05$; Fig. 2A) in the bacterial community but the seasons were not significantly different in the eukaryote community (Fig. 2B; $P=0.294$).

In Figure 3, the beta diversity differences among samples within each community (bacteria and eukaryote) can be seen. There was clear stratification among each month for each community (Fig. 3A and 3B), however, there was a large separation between the spring months and summer months in the bacterial community. Pairwise ANOSIM results indicated that the spring community was significantly different than the summer community for the bacteria ($P<0.001$), but the eukaryote communities showed no significant difference ($P=0.118$; Supp. Table 2). When grouping the communities by month, the eukaryote community showed no significant differences among months ($P>0.05$), whereas all summer months were significantly different to spring months in the bacterial community ($P<0.05$). Interestingly, May was also significantly different from the remaining summer months as well ($P<0.05$).

To further explore the community differences, the samples were grouped into sample type. In the bacterial community, there was no separation among sediment and water samples

in the spring (ANOSIM; $P>0.05$) but as the summer months progressed, water separated from the sediment samples (ANOSIM; $P<0.05$; Fig. 3C; Supp. Table 2). Nevertheless, in contrast to alpha diversity, beta diversity was significantly different among sediment samples and water samples in the summer (Supplemental Table 2). There was no significant difference among bright and dark sediment samples in either season ($P>0.05$). In the eukaryotic community, eight samples grouped separately from the remaining 39 samples (Fig. 3B and D). There were no distinct features (i.e. sample type, month) about these samples that might explain these differences in beta diversity. In the spring, the bright sediment separated from the water and dark sediment samples, however, this separation was not significantly different (ANOSIM; $P>0.05$; Supp. Table 2). In the summer months, water samples separated from the sediment samples (ANOSIM; $P<0.05$), while the bright sediment and dark sediment were significantly different as well (ANOSIM; $P<0.05$). This may be explained by the separation in bright and dark sediment samples in July and August (Fig. 3D).

Environmental factors correlated with community structure

Environmental parameters were correlated with the beta diversity measure of each community (Table 3). When the samples were separated into sample type for the bacterial and eukaryotic communities, there was significant environmental correlations with the biological data (all $P<0.05$). Temperature was the top correlated environmental factor for all bacteria and eukaryote sample types except for the bacteria water sample type, which was the most correlated with Br level (c.c= 0.458).

Temporal community composition

Temporal changes in the community composition can be seen as the environmental factors fluctuate over time (Fig. 4). Proteobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, and Actinobacteria comprised the most prevalent phyla in the bacterial community (Fig. 4A), while the most prevalent phyla in the eukaryote community were Opisthokonta, SAR (Super clade consisting of Stramenophiles, Alveolates and Rhizaria), Archaeplastida, Amoebozoa and Excavata (Fig. 4C). The most prevalent bacterial genera were bacterial genera observed were *Flavobacterium, Pseudomonas, Methylotenera, Sphingomonas, Novosphingobium* and *Clostridium* (Fig. 4B); while the most prevalent eukaryotic genera were Ascomycota, Streptophyta, Bilateria, CONthreeP, and Cercomonas (Fig. 4D).

Focusing on the most prevalent taxa, general trends were observed in response to the changing environmental parameters. Proteobacteria was dominant each month and slightly fluctuated over time with little response to the environmental factors. Excavata and Actinobacteria remained relatively constant throughout the sampling period despite changing environmental factors. However, other phyla did show responses to the changing environmental parameters. Bacteroidetes was dominant except in March when Cl and Br levels are at the highest level (7.5/17.1 ppm) and temperature was low (13.9 C). Opisthokonta had a rapid increase when Cl/Br levels dropped to zero in June. Cyanobacteria decreased as temperature increased and Cl/Br levels dropped. Firmicutes was relatively constant until August when the Cl/Br levels increased. The supergroup phyla SAR increased each month with the rising temperature and decreasing Cl/Br levels.

Archaeplastida decreased rapidly in May after Cl/Br levels decreased and temperature increased. Amoebozoa had relatively large fluctuations over time. There was a huge spike from March to April and then rapid decrease after April.

The largest shift in community composition (bacteria and eukaryote) can be observed between spring and summer (May through August) (Fig. 4B and D). Streptophyta (Archaeplastida) and *Pseudomonas* (Proteobacteria) are the most prevalent taxa in the spring when Cl/Br levels are the highest and temperature the lowest. There was a rapid decrease in relative abundance of both taxa when the temperature began to increase and the Cl/Br levels decreased. Once those taxa diminished, other taxa became more prevalent, such as CONthreeP and Ascomycota (eukaryote), and *Flavobacterium* and *Methylotenera* (bacteria). Interestingly, *Acanthamoeba* had a spike in April after the Cl/Br levels began to decrease, then rapidly collapsed in the remaining months when temperature was high and Cl/Br levels were low. The relative abundances of each taxa within each sample type is shown in supplemental material (Supplemental Table 3).

Since there was a shift in the community composition due to the dramatic fluctuations in temperature, chlorine and bromine levels, LefSE was performed to determine which taxa were most impacted by the environmental parameters (Fig. 5; Supplemental Table 4). The LDA scores were the same for each taxa despite which environmental parameter was used to group the samples, hence, only temperature was portrayed in Figure 5. In the bacterial community (Fig. 5A), the top 25 taxa ranked by LDA score had significant distributions for temperature ($P<0.05$), whereas in the eukaryote community (Fig. 5B), only Streptophyta, *Acanthamoeba*, *Gymnophrys, Vannella, Gregarinasina* and *Ptolemeba*

bulliensis were significantly distributed among temperature levels ($P<0.05$). The other eukaryote taxa were not significantly distributed among temperature levels ($P>0.05$). *Pseudomonas* yielded the largest effect size (LDA score= 3.83) and was most prevalent in March when the temperature was 13.9 °C. Streptophyta had the largest effect size (LDA score= 2.72) among the eukaryotes and was also most prevalent in March. *Acanthamoeba* was the next significant taxa with a high effect size (LDA score= 1.78) and was most prevalent in April when the temperature was 12.7 °C.

3.2 Microbial Association Network and Correlations

Microbial association networks were created to further explore co-presence (positive correlations) and mutual exclusion (negative correlations) relationships within the community among taxa and environmental parameters (74). The global combined (bacteria and eukaryote) network (not shown) had 119 nodes (or taxa) and 1,040 edges (links between nodes). The environmental factors were included in the creation of the network to determine correlations among the parameters and taxa, however, the environmental factors did not yield any significant correlations with taxa in the combined network.

The global combined network was explored by separating the positive edges (co-presence) and the negative edges (mutual exclusion) into separate networks and clustered. After clustering of taxa within the networks using the 10 different clustering algorithms, seven of the ten methods yielded overlapping positive correlations (Supplemental Table 5). Overall, 62 taxa were significantly positively correlated with

Legionella spp., where seven taxa were eukaryotes (Fig. 6). *Blastomonas, Halomonas, Candidatus Protochlamydia,* and *Candidatus Xiphenobacter* were bacterial genera that were significantly correlated with *Legionella* in six of those 7 clustering methods. Other bacteria correlated within these clusters include taxa from the phyla Proteobacteria, Verrucomicrobia, Bacteroidetes, and Firmicutes. *Vermamoeba vermiformis,* Streptophyta, Bilateria, ConTHREEp, Incertae sedis, *Gregarinasina* and *Vannella* were eukaryotes shown to have a significant positive correlation with *Legionella* in at least six of the clustering methods (*V. vermiformis* was in 6).

3.3 *Legionella* community dynamics and relationship with eukaryotic taxa.

Despite Proteobacteria being the dominant phylum for each month in the bacterial community, overall, the abundance of *Legionella* was relatively low (Fig. 4B). Nevertheless, the genus increased rapidly in June and July when the Cl/Br levels reached very low levels, then decreased in August once Chemtreat increased again.

Looking more closely at the eukaryotes associated with *Legionella* in the network analysis, major compositional changes were observed with Streptophyta (as described above) and *Acanthamoeba,* which had a large spike in April then crashed as the Cl/Br levels decreased (Fig. 4D). The remaining taxa exhibited patterns that indicated response to the changing temperature and Cl/Br levels. Particularly, ConTHREEp and Bilateria became prevalent in the summer months, while the other taxa increased in summer months as well when Cl/Br levels were low and temperature was high. Throughout the time period, there

was at least one eukaryote present in each month and eukaryotic richness increased as time progressed.

To further explore community differences among the bacteria and eukaryotes, ANCOM and LeFSe tests were performed to detect differentially abundant taxa among sample type. The results of ANCOM showed that there were no differentially abundant taxa when grouped by sample type into spring and summer for both the bacterial and eukaryotic communities (results not shown).

LefSE LDA results also yielded no significant differentially abundant organisms among sample type when grouped by season (Supp. Fig. 5). However, skewed distributions can be observed for the top 25 taxa ranked by LDA score for the spring and summer months. Bacterial taxa were more prevalent in the dark sediment and water during the spring, while in the summer, taxa were more prevalent in the sediment (Supp. Fig. 5A and 5B). *Legionella* was most prevalent in the dark sediment during spring (Supp, Fig. 5A), and transitioned to being most prevalent in water during the summer (Supp. Table 3). As for the eukaryote community, majority of the eukaryotes were more prevalent in the water samples during the spring, including ConTHREEp, Bilateria, Incertae sedis, and *Vannella* (Supp. Fig. 5C). While Streptophyta, *Gregarinasina* and *Vermamoeba vermiformis* were more prevalent in the sediment samples. In the summer, eukaryotes transitioned to being more prevalent in the sediment (Supp. Fig. 5C and 5D). Specifically, ConTHREEp, *Vannella* and Incertae sedis transitioned to being more prevalent in the sediment during spring. Bilateria remained most prevalent in water, while *Gregarinasina* abundance crashed in the summer and was not prevalent in any sample type. Interestingly,

Vermamoeba vermiformis, Korotnevella (amoeba)*, Cryptodifflugia* (amoeba) and *Acanthamoeba* were most prevalent in the dark sediment during spring months when *Legionella* was also most prevalent. While, Bilateria, *Gymnophrys* (amoeba)*, Korotnevella* and *Acanthamoeba* were most prevalent in the water during the summer when *Legionella* was also most prevalent.

4. DISCUSSION

Even if properly maintained, man-made water systems, specifically water cooling towers, can serve as a reservoir for many bacteria (53, 62, 68, 58, 59). These systems depend on numerous factors to properly function and the variability seen in the microbiome of cooling towers from different studies (2, 6, 24, 27), suggests that the system may be very complex. Previous studies have assessed the bacterial community in relationship with environmental factors in cooling towers, but it is important to understand the relationships and associations occurring within the entire microbiome, including eukaryotes. Eukaryotes, specifically amoeba, are known hosts to *Legionella* but also other pathogenic bacteria such as *Pseudomonas* and *Mycobacterium* (101). Understanding these associations will give insight into how the microbial communities are shaped and how they function (111, 112), leading to possible explanations as to how disease outbreaks occur.

Microbial Network Associations

Microbial co-occurrence and co-exclusion networks are useful in predicting the associations among different taxa within an environment and can be used to understand complex community dynamics in microbiomes (111). It is expected that non-random

microbial co-occurrence and significant correlations will occur in such environments (112). It is difficult to completely determine the reason for the interaction among taxa, but possible explanations can include similar niches and ecological relationships such as predator/parasite and commensal associations (111, 112).

Within our combined community networks, positive significant correlations with *Legionella* were observed among five bacterial phyla and seven different eukaryotic taxa. Of interest, *Halomonas* was correlated with *Legionella* in a separate Spearman correlation method (not shown, $P< 0.05$, $FDR< 0.10$) as well as in 6 of the clustering methods. *Halomonas* is a Gammaproteobacteria found in marine or high salinity environments, such as salt lakes and salted foods, and has recently been found in dialysis fluid and considered potentially pathogenic based on blood culture results (88, 129). While salinity was not measured directly in the tower, conductivity was measured and increased as temperature increased (Table 1). Conductivity differs among water systems (i.e. rivers vs. freshwater) and it has been reported that conductivity in industrial waters can be as high as 10,000 umhos/cm (130), or 10 ms/cm. It is possible that the salinity was high enough to produce a saline environment for *Halmonas* to colonize and survive.

Other taxa seen in 6 clustering methods were *Candidatus Xiphinematobacter , Candidatus Protochlamydia,* and *Blastomonas. Candidatus Xiphinematobacter* is a Gram-negative Verrucomicrobia and known symbiont *of Xiphinema* (nematode; 116, 117). *Candidatus Protochlamydia* is a new genus representing a symbiont of *Acanthamoeba* spp. (106). While *Blastomonas* is a Gram-negative alphaproteobacterial found in brackish and freshwater lakes (131). Taxa positively correlated with *Legionella* supported by at least

four clustering algorithms were *Alishewanella, Mycoplana, Pedobacter* and Rickettsiales (order). *Alishewanella* is a widespread Gram-negative Gammaproteobacteria found in natural environments (107). *Mycoplana* is a Gram-negative Alphaproteobacteria common in soils (108). *Pedobacter* is a Gram-negative Bacteroidetes that has been found in drinking water, soil and freshwater environments (118, 119). The Rickettsiales order encompasses a diverse group of Alphaproteobacteria that are obligate, intracellular parasites of eukaryotes (120) and based off of the GreenGenes taxonomy lineage, the correlated Rickettsiales had an association with *Vermamoeba.*

While these networks only suggest associations, it is possible by the significant correlations and known information on the genera, that the bacteria may have a similar host replication mechanism or relationship to FLAs or other protozoa as *Legionella*. The correlations may also just suggest similar ecological niches or response to the changing environmental parameters based on their abundances. The clustering algorithms have been mainly used in protein interaction networks to determine overlapping protein functions (99, 113). However, it could be argued that microbes have overlapping functions within different groups of microbes, which could explain the overlapping correlations observed in the different clustering algorithms. For instance, the OH-PIN algorithm has been used for hierarchical overlapping of function in protein networks (113) and has been successfully used to show ecological niche overlap in a soil microbiome in joint with coculture experiments (100). Overall, based on known information about the taxa, these relationships should be further explored to see if they could play a role in legionellosis disease outbreaks.

Associations among eukaryotes and bacteria are key to gaining insight into the complexities of the cooling tower's ecosystem. Bacterial abundances can change due to protozoan predation or replication within the protozoa in both natural and man-made environments (52). In particular, *Legionella* is a key species associated with protozoa, such as FLAs. Within the bacterial and eukaryotic communities, *Legionella* and the eukaryotes were relatively low in abundance (besides Streptophyta and Ascomycota). It has been noted that in water environments, the relative abundance of *Legionella* is usually very low and the microbial community is typically dominated with only a few amoeba species, which makes it difficult to accurately assess the interactions between *Legionella* and potential eukaryotic hosts (52). However, associations among different bacteria and eukaryotic taxa were observed in the combined community clusters. *Legionella* was positively correlated with *Vermamoeba vermiformis* and *Vannella*, known amoebae hosts of *Legionella* (23, 52, 64, 65); as well as Streptophyta, Bilateria, ConTHREEp, Incertae sedis and *Gregarinasina*. *Gregarinasina* is an alveolate that can infect marine, freshwater and terrestrial invertebrates (121), while the Bilateria group contained annelids, arthropods, nematodes, Platyhelminthes, Rotifera, and gastrotrichs. ConTHREEp consists of a group of ciliates (132), while Streptophyta is a unranked clade of plants that can include green algae and land plants (92, 103). Bilateria, ConTHREEp, *Vannella* and *Vermamoeba* were prevalent in the community when *Legionella* was higher in abundance due to the decrease in Chemtreat, whereas Streptophyta and *Gregarinasina* were lower in abundance. Since Streptophtya is an unranked clade of plants, the association with *Legionella* spp. is most likely niche related, while the associations among the other eukaryotes could represent a mutualistic or predator-prey relationship (52, 53). These ecological associations are dependent on numerous factors such as the genetic makeup

of the organisms, the feeding preferences of the protozoa, the relative abundance and the environmental conditions (52, 53).

It has already been stated that amoeba are not the only possible host for *Legionella.* This in part is due to *Legionella* being highly adaptive and the *Legionella* type IV secretion system, Icm/Dot (Intracellular multiplication/Defective organelle transport). This Icm/Dot system allows translocation of more than 300 effector proteins across the host's cellular membrane, which changes the host's cellular processes and allows intracellular survival and replication of *Legionella* (122). In a recent study, copepods, Rotifera and nematodes were identified within a biofilm associated with *L. pneumophila* and the nematodes were shown to have intracellular *Legionella* (122, 123), similar to *Candidatus Xiphinematobacter.* Thus, *Legionella* is capable of using different eukaryotic hosts for replication, transmission and survival and emphasis should be continued to be put on understanding the role of eukaryotes in Legionnaires disease.

While a number of factors may be affecting the prevalence of the bacteria, *L. pneumophila* is capable of surviving in environments with both permissive and restrictive hosts (52). For example, protozoa within the Cercozoa phylum, i.e. *Cercomonas,* are restrictive to *L. pneumophila* growth (52). As observed in our results, *Cercomonas* was consistently present and *Legionella* did not show any significant correlations with it. Also, permissive amoeba may be diminished by the bacteria (52), allowing more prevalence of restrictive hosts so it cannot be ruled out that the permissive hosts played a role in *Legionella* persistence and growth (52). For example, *Acanthamoeba* crashed after April, so portrayal

of any relationship with *Legionella* and other taxa may be missed based on the respective relative abundances of each taxa.

Sediment vs water dynamics

Man-made water systems should typically be free of sedimentation (or biofilms) since sediments can create a suitable environment for bacterial growth and provide nutrients, such as iron (68). The presence of sediment within the tower could contribute to the number of taxa present in the environment and add to the complexity of the cooling tower's community dynamics. Significant differences among the sediment and water samples in the summer suggest different community dynamics occurring between sample type as well as season. Seasonal changes, specifically temperature, can play a role in the community shift seen over time (72). A previous study showed that the community structure within sand and sediment was very different from the overlaying water samples (95). The different coastal water sites were more similar to each other than sediments from the same site (95). When compared to cooling towers, the coastal beach sites have many more external factors affecting them, such as tides and human activity. However, this idea does not disregard the seasonal and disinfection factors that affect water cooling towers, which could result in differences between the water and sediment samples. Sediment could contain up to 1,000 times more bacteria than the water within a water system (77). The greater diversity of taxa within sediment could cause different associations among bacteria and eukaryotes when compared to water environments since sediment can act as a reservoir for biofilms, nutrients and protection from grazing predators.

There were, however, no differentially abundant species when grouped by season among sample type for either community. LefSE results show a difference in bacterial and eukaryotic prevalence among sample type in the spring and summer months. During the spring, there was an even distribution among the top 25 taxa within sample types, however, in the summer, bacteria were more prevalent in the sediment. Specifically, *Legionella* was most prevalent in the dark sediment in spring and switched to being most prevalent in the water. As for the eukaryotes, majority of the top 25 eukaryotes were more prevalent in the water during spring, and switched to being more prevalent in the bright sediment during the summer. This shift in community composition among sample type and season suggest that the microbial community is responding to the change in environmental parameters and may highlight the preference of sample type when temperature or disinfectant are at a certain level. For instance, low Cl/Br levels in the summer may have allowed biofilm formation and could explain why majority of the bacteria and eukaryotes were seen in the sediment. While the high levels of Cl/Br in the spring may have disrupted biofilm formation, causing organisms to be free living within the water. European guidelines for control and prevention of *Legionella* outbreaks state that water towers should be cleaned and free of sediment, which can create a suitable environment for bacterial growth, biofilm formation and provide nutrients, such as iron (68). The presence of sediment within the tower could contribute to the number of taxa present in the and affect the how the community functions.

Furthermore, the eukaryotes associated with *Legionella* in the network analysis show a transition in prevalence among sample type between season as well. In the spring, four amoeba, including *Vermamoeba,* are most prevalent in the dark sediment, where

Legionella spp. is most prevalent as well. Streptophyta and *Gregarinasina* were most prevalent in the bright sediment, while the remaining associated eukaryotes (ConTHREEp*,* Bilateria, Incertae sedis and *Vannella)* were most prevalent in the water. In the summer, only Bilateria is most prevalent in water along with *Legionella* spp. This difference could affect possible host interactions due to proximity in the water system. FLAs are common in man-made water systems where they feed on the biofilms (20), with *Legionella* becoming prey to these amoeba (98). Planktonic *Legionella* may account for the prevalence in the water samples during the summer since sessile cells, typically found in the biofilms or sediment, are more likely to be grazed upon by the FLAs and other eukaryotes. Even so, *Legionella* abundance could be increased with eukaryotic presence due to intracellular replication and expulsion into the water (33). Amoebas are known to release vesicles that contain high copies of replicated *Legionella* that are considered more resistant to biocide and more virulent than naturally replicated *Legionella* (20, 22). This could be a factor contributing to the increase in *Legionella* in water during the summer months since the eukaryotes were more prevalent in the sediment, or biofilm and there was an overall higher abundance of eukaryotes when the Cl/Br levels were low. *L. pneumophila* cell counts also continued to rise with increasing Chemtreat in August, which could be due to replication within eukaryotes present in the summer. It is uncertain where and which eukaryotes are playing a role in *Legionella* replication and persistence, but *Legionella* are highly adaptive and have been suggested to move from host to host (52). This in combination with the distribution of the eukaryotes in sample type could suggest specific niches, i.e. sample types, that are playing a role in the development of Legionnaires' disease. More studies should explore the community dynamics between the

biofilm/sediment and water communities, especially since other studies have shown that the diversity is higher in sediment than water (124, 125).

Temporal and Seasonal Changes affecting microbial and eukaryotic profiles

The environmental parameters fluctuated from month to month with a large difference between spring and summer months. The largest fluctuations were seen in temperature and Cl/Br levels, with these factors having the largest influence on the taxonomic composition of the community. Water systems that are in the range of 20-50 °C pose a greater risk for *Legionella* spp. colonization and growth (54, 68). The tower was above 20 °C in June, July and August, where the Cl/Br levels were low, creating a suitable environment for bacterial proliferation. While *Legionella* relative abundance was relatively low in comparison to the rest of the community, an increase in *Legionella* relative abundance and *L. pneumophila* cell counts in the months when temperature was above 20 °C and Cl/Br levels were low was observed. It cannot be determined based on the relative abundance of *Legionella* if the bacteria reached a level that is considered dangerous, but the *L. pneumophila* cell counts showed that the bacteria never exceeded the alert level of $1.00X10^7$ cells/L, which would require immediate disinfection of the tower as stated by SSRL. Low levels of chemical disinfection promote bacterial proliferation since most disinfection methods are successful at reducing cell counts (62). For instance, a Legionnaires' disease outbreak in Australia was associated with cooling towers that had no detectable disinfectant and thus, allowed aerosolized *Legionella* particles to be released into the environment (96). However, the changing environmental parameters did not lead to alert levels of *L. pneumophila,* as indicated by the DFA

method. The exposure threshold of *Legionella* should be further clarified to assure that the bacteria is not being aerosolized at lower levels.

An important note to make is that the DFA method identified *L. pneumophila* SGs 1, 2, and 4 within the cooling tower, whereas the 16S rRNA gene amplicon data only identified *L. pneumophila* in one dark sediment sample in April. Whereas the remaining *Legionella* 16S rRNA amplicon sequences were assigned to the genus *Legionella.* The DFA method has been successfully used to detect serogroup 1 with about 70% sensitivity and ~99% specificity to the organism (85, 86), while there still is the a lack of sensitivity to other *Legionella* species and *Legionella*-like species that could contribute to an outbreak. Thus, we have evidence that *L. pneumophila* is within the cooling tower over the entire time period and that the *Legionella* genus OTU may be representative of other *Legionella* species present in the community. The lack of sensitivity to pick up other *Legionella* species may be due to the sequence information available in the databases utilized for taxonomic assignment. *Legionella* species specific primers could be used in the future in combination with the universal 16S rRNA V4 region primers to compensate for this lack of sensitivity.

When observing the communities as a whole in relation to the environmental and seasonal changes, the bacterial community showed greater sensitivity to fluctuating environmental parameters than the eukaryotic community. Significant differences in the community in May from the remaining months when there was the first transition of increasing temperatures and decreasing Chemtreat levels was observed for the bacterial community, but not for the eukaryotic community. Thus, bacteria showed a greater

response to the transition in environmental parameters and season. It is possible that the eukaryotic species have a greater resistance to environmental factors, such as Cl/Br levels and temperature, than bacteria due to the chemical composition of their cellular membranes, uptake mechanism of chemicals into the cell, cellular response to stress, especially if the taxa are present in biofilms (79). For example, amoebae residing in water systems tend to be resistant to disinfection and unaffected by temperature since they can form dormant cysts that contain layers of cellulose, polysaccharides and proteins (6, 7, 12, 20, 126), supporting the idea that eukaryotes exhibited less sensitivity to the environmental factors as observed in the alpha diversity correlation plots.

While temperature was the main driving force responsible for the community shifts (results from BioENV), bromine (and indirectly chlorine via Chemtreat) was also responsible for changes in the bacterial water samples. Seasonal changes, specifically temperature, can play a major role in the community shift seen over time (72). Another study found residual chlorine levels were a key driving factor responsible for bacterial community shifts (76). Microbes in the sediment or in biofilms tend to have stronger resistance to chlorination or disinfection methods (53, 75). It is possible that Chemtreat had a greater effect on the bacterial water samples because taxa were not protected in a biofilm, which would explain the strongest correlation with the Br levels. There was no significant difference among community richness when grouped by sample type in the bacterial or eukaryotic, but it's possible that Cl/Br levels are not affecting the sediment samples as much as temperature because multiple species are within the biofilm/sediment and there is an increased resistance to the disinfectant. When comparing multispecies and single species biofilms, the multispecies tend to be more resistant to chloramine treatment due to the community

diversity and interactions among microbes (53). This is most likely because nitrifying bacteria are present which lead to the depletion of chloramine.

The two major taxa that were highly prevalent in the spring months (when Cl/Br levels were high) were Streptophyta and *Pseudomonas* and as the temperature increased and the Cl/Br levels decreased, the abundance of these two taxa crashed. Free chlorine has been shown to be ineffective towards *Pseudomonas aeruginosa* (58) which is a player in biofilm formation (44, 45) and known denitrifying bacteria, which again could lead to depletion in chlorine levels (53, 72). The high prevalence of *Pseudomonas* could indicate resistance against disinfection or a niche for high chlorine levels. The crash in Streptophyta could explain the separation of the 8 samples that were separated from the rest of the samples in the PCoA plots of beta diversity. Given the high abundance of Streptophyta in the spring and the high prevalence in the bright sediment during the spring, the separation of bright sediment samples from water and dark sediment samples during spring to the separation of water samples from sediment samples during the summer is supported by the crash in Streptophyta. Streptophyta, which could include algae and land plants, had OTUs assigned to taxonomic lineages of Tracheophyta and Spermtophyta, which are groups of vascular plants. *Posidonia oceanica,* a tracheophyta sea grass, was successfully used for biosorption of heavy metals in waste water samples (103). Chlorine releases different toxic compounds into an environment when used as a bleaching agent and one study showed that aerobic biomass performs biosorption of the harmful compounds in a wastewater environment (104). The high prevalence of Streptophyta in March and April could have been a contributing factor to the rapid decline in Cl/Br levels in the cooling tower from May-July, as the taxa could

have acted as a biosorbent. This mechanism could be further explored to see if the presence of plant material in man-made water systems leads to changes in the levels of disinfectant.

5. CONCLUSION

To our knowledge, this is the first study that has analyzed the community structure with respect to bacteria and eukaryotes in a water cooling tower with naturally occurring *Legionella* spp. It is important to advance our understanding of the association and relationship *Legionella* has with potential hosts and other microbes present in a community. These relationships could create favorable conditions that may provide a suitable environment for *Legionella* to proliferate and cause an outbreak. Positive significant correlations with bacteria such as *Halomonas* and *Candidatus Xiphinematobacter*, may suggest a similar host relationship or similar niches as *Legionella.* Future studies should include a wider scope of the community that explores other bacteria that have similar niches or mechanisms as *Legionella* to identify possible taxa that could contribute to legionellosis. Furthermore, emphasis should be put on better characterization of the protozoa found in natural and man-made water systems since they likely play a role in the proliferation and evolution of *Legionella* (52). Specifically, the eukaryotes Bilateria, ConTHREEp, Streptophyta, Incertae sedis and *Gregarinasina* since they had significant correlations with *Legionella.*

While previous studies have shown that temperature and bromine levels play a role in community dynamics, this is the first time these parameters were measured in relation to the entire community (bacteria and eukaryotes) of a water cooling tower. Our results indicate that there is major stratification between spring and summer with respect to

fluctuating environmental parameters. Bacteria show greater sensitivity to these environmental parameters than eukaryotes, which highlights the need to better understand the community since eukaryotes likely play a role in Legionnaires' disease. Also an important aspect to further explore is the difference in community dynamics between the sediment and water since the distribution of *Legionella* and common hosts of the bacteria were different in each sample type among season. Overall, this study advances our understanding of community dynamics among bacteria and eukaryotes associated with *Legionella* and provides insight into how Legionnaires' disease may develop by identifying possible hosts for *Legionella.* The remaining water cooling towers will be sequenced and analyzed as tower HTF-2 to further investigate the community dynamics and ecology of cooling tower systems.

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APPENDICES

APPENDIX A: TABLES

Table 1. Average and range of cooling tower's environmental parameters from March to August 2016

Parameter	Mar	April	May	June	July	Aug	Mean ± SD
Temp (C)	13.9	12.7	19.7	24.2	25.8	23.4	18.6±5.35
DO (%)	100	100	100	100	99.7	98.6	99.8±0.459
Conduct (mS/cm)	0.55	0.598	0.73	0.496	0.82	0.24	0.562±0.162
pH	8.48	8.39	8.64	8.52	8.69	8.39	8.52±0.112
Cl (ppm)	7.5	3.92	4.01	0	0.18	2.87	2.81±2.43
Br (ppm)	17.1	8.74	9.14	0	0.33	6.55	6.32±5.52
Turbidity (NTU)	3.3	4	3.2	1.4	2.2	5.3	4.75±3.56

DO=Dissolved Oxygen, Conduct=Conductivity, Cl=Chlorine, and Br=Bromine

Table 2. Summary of QIIME2 sequence counts, sampling depth and number of OTUs for each community.

Community	Total Sequences	Sequences Retained	Sampling Depth	Observed OTUs	Used OTUs
Bacteria	2,078,951	631,488	13,738	537	219
Eukaryote	718,071	91,828	2,087	168	75

Table 3. BioENV and two-Sided Mantel test results for the environmental factor correlation with beta diversity

Sample Type	Top Correlated Factor	Correlation Coefficient	P-value
Bacteria-Bright Sediment	Temperature	0.419909	<0.05
Bacteria-Dark Sediment	Temperature	0.573109	<0.05
Bacteria-Water	Br Level	0.458158	<0.05
Eukaryote-Bright Sediment	Temperature	0.447569	<0.05
Eukaryote-Dark Sediment	Temperature	0.369378	<0.05
Eukaryote-Water	Temperature	0.377899	<0.05

APPENDIX B: FIGURES

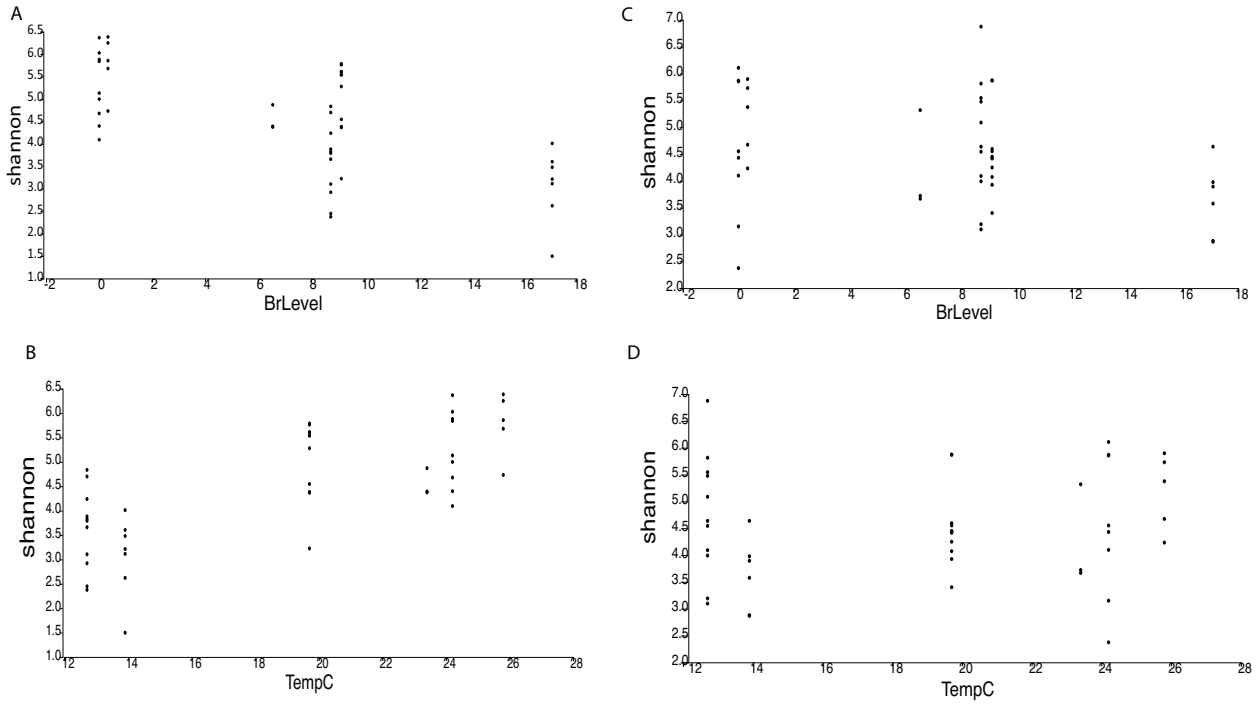


Fig. 1. Alpha diversity correlation plots for temperature and bromine level. The Spearman correlation method was used to correlate the Shannon vector with the environmental factors. Black dots represent each sample within each environmental parameter. A represents the 16S community's alpha diversity correlated with bromine levels (Correlation Coefficient= -0.4846; $P < 0.05$). B represents the 16S alpha diversity correlated with temperature (C.C.= 0.7194; $P< 0.05$). C represents the 18S community's alpha diversity correlated with bromine level (C.C.= -0.2662; $P > 0.05$). D represents the 18S alpha diversity correlated with temperature (C.C.= 0.1488; $P > 0.05$).

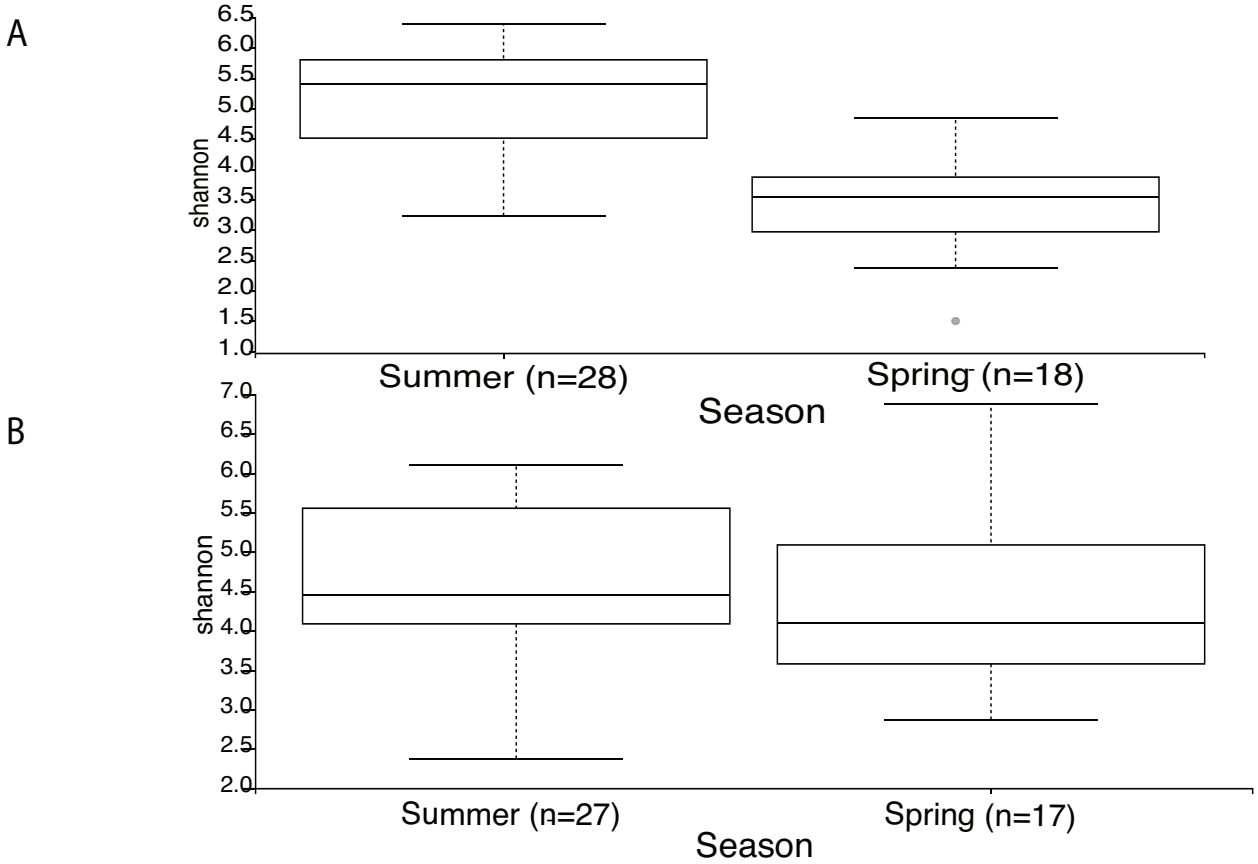


Fig. 2. Alpha diversity grouped into season using the Kruskal-Wallis test among spring and summer samples for the bacteria and eukaryote communities. A represent the bacterial community ($P<0.05$) and B represents the eukaryotic community ($P=0.294$). Spring includes March to April when temperatures were below 19C and Summer includes May to August when temperatures were above 19C.

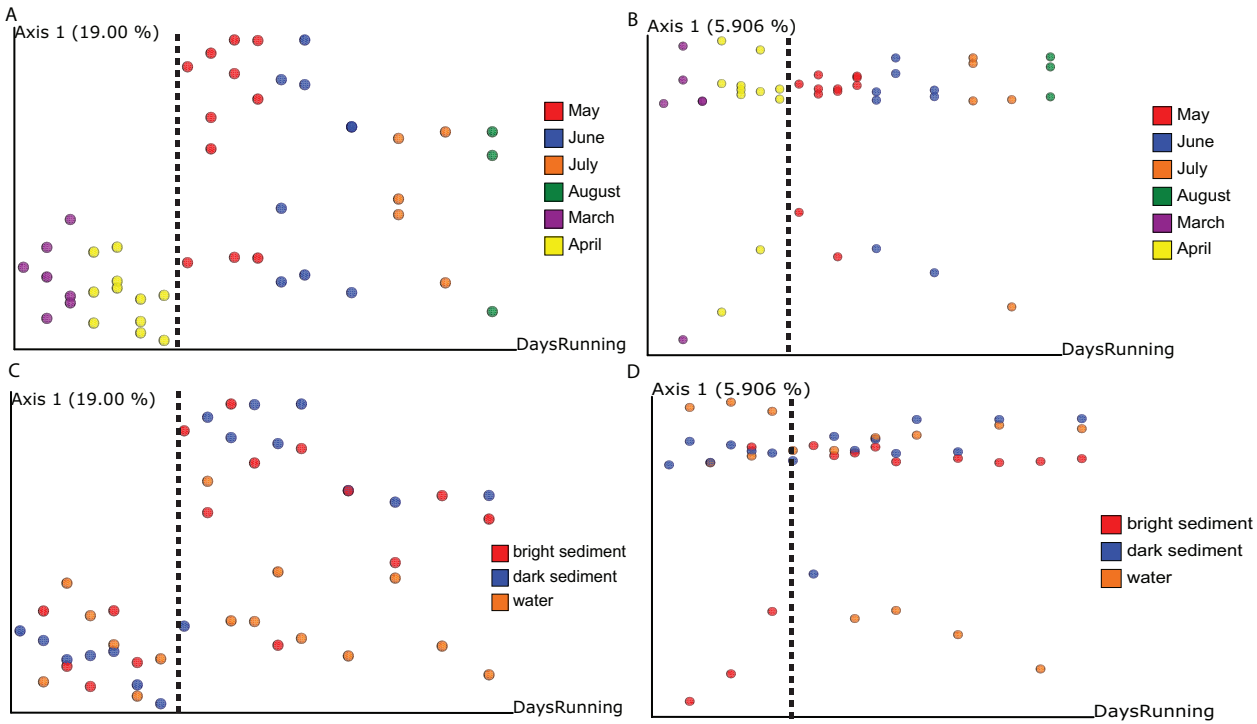


Fig. 3. PCoA plots of the beta diversity for the bacterial and eukaryotic communities with Days Running as the X-axis. A and C represent the 16S community when grouped by month and sample type. B and D represent the 18S community when grouped by month and sample type. The dashed line indicates the switch from spring to summer months.

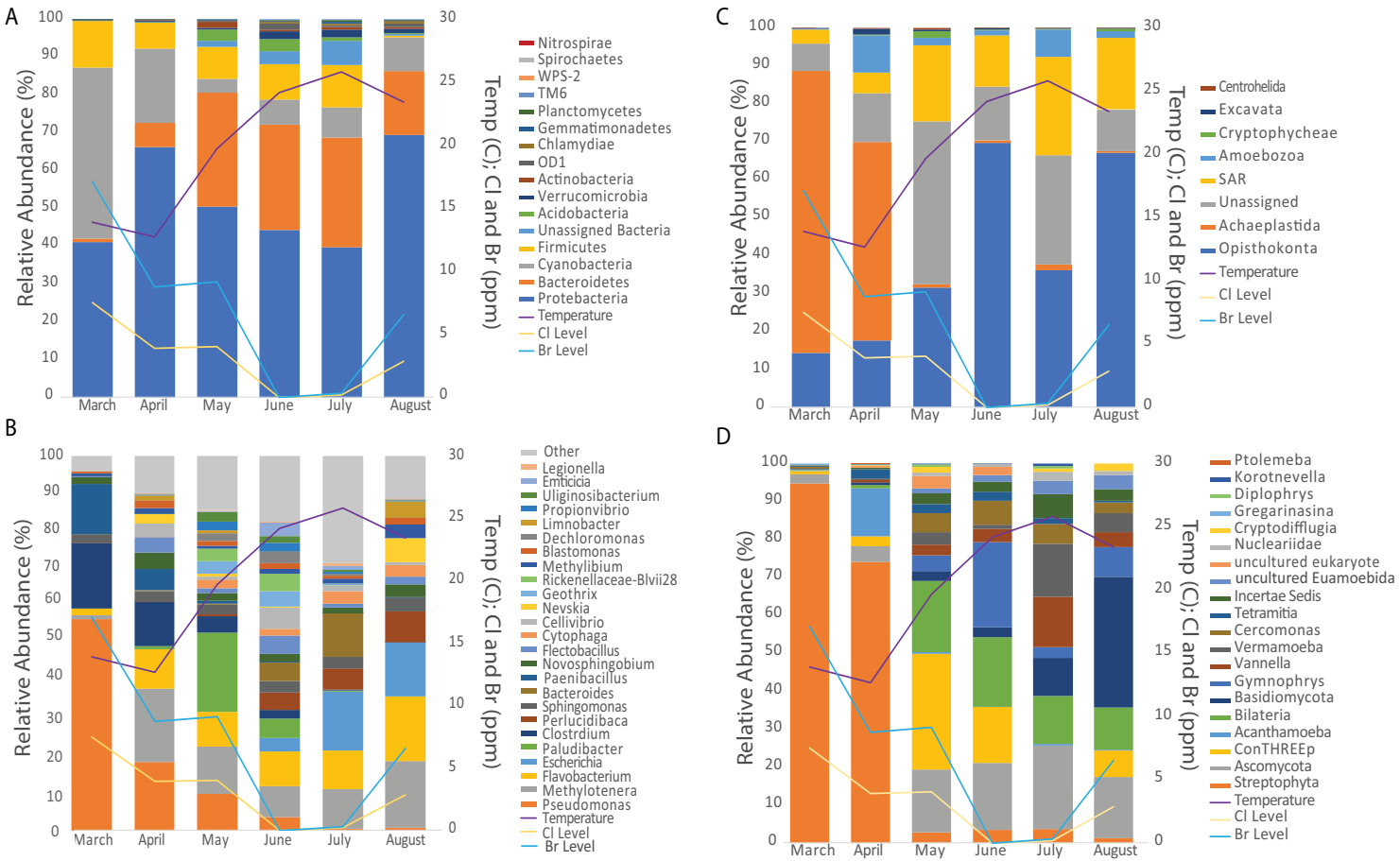


Fig. 4. Community composition of the bacteria and eukaryote community relative abundances by Phyla and Genera grouped into months with environmental parameters overlaid. A represents the bacteria phyla community composition. B represents the bacteria genera community composition. C represent the eukaryote phyla community composition and D represents the eukaryote genera (or similar phylogenetic level) community composition.

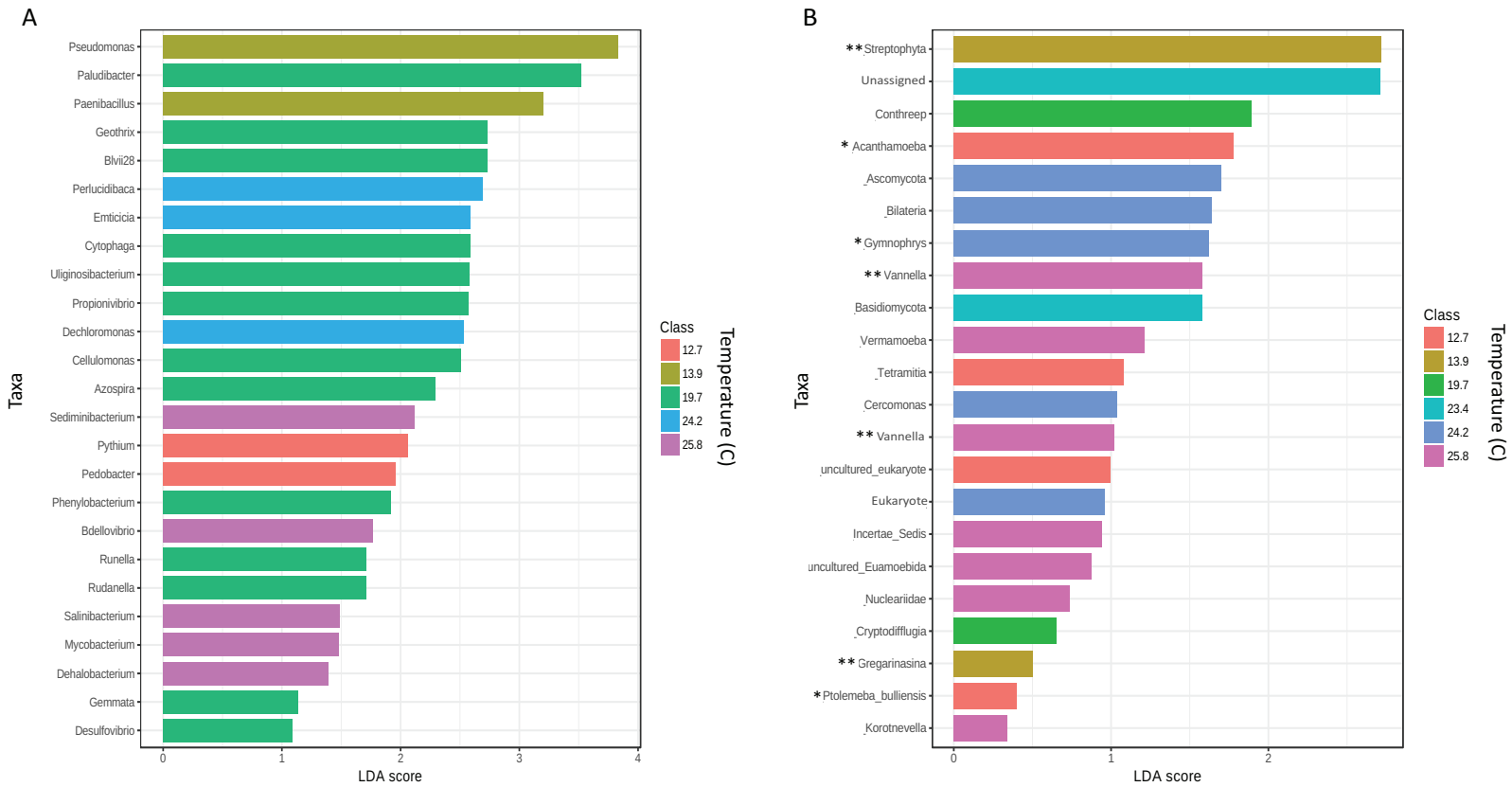


Fig. 5. LefSE LDA results of the top 25 ranked taxa by LDA score for bacterial and eukaryotic communities when grouped by temperature. A represents the bacterial community while B represents the eukaryotic community. Colored bars represent the temperature level where the taxa was most prevalent. All bacterial taxa were significant (adjusted $P<0.05$), while an asterisk next to eukaryote taxa indicates significance (adjusted $P<0.05$). A double asterisk next to eukaryote taxa indicates a significant adjusted p-value as well as the taxa was observed in network analysis.

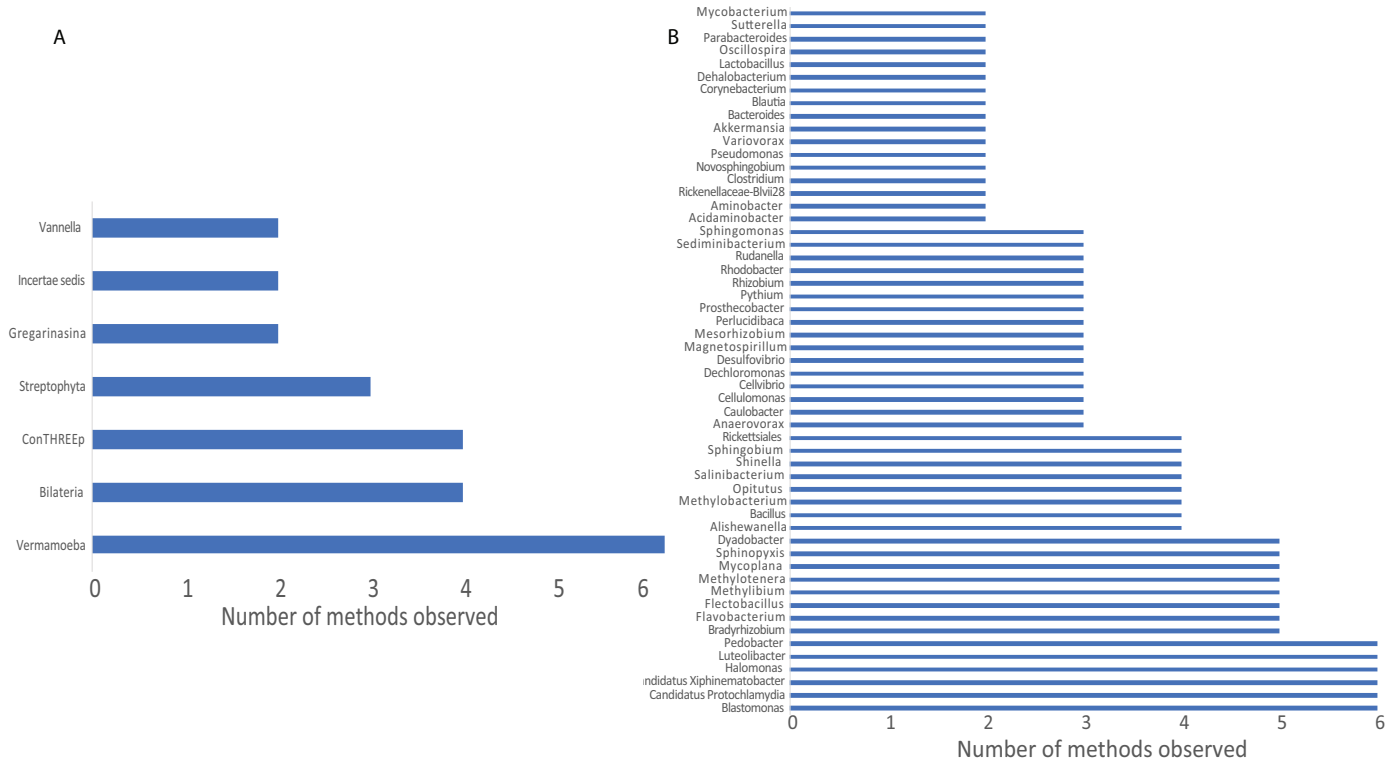
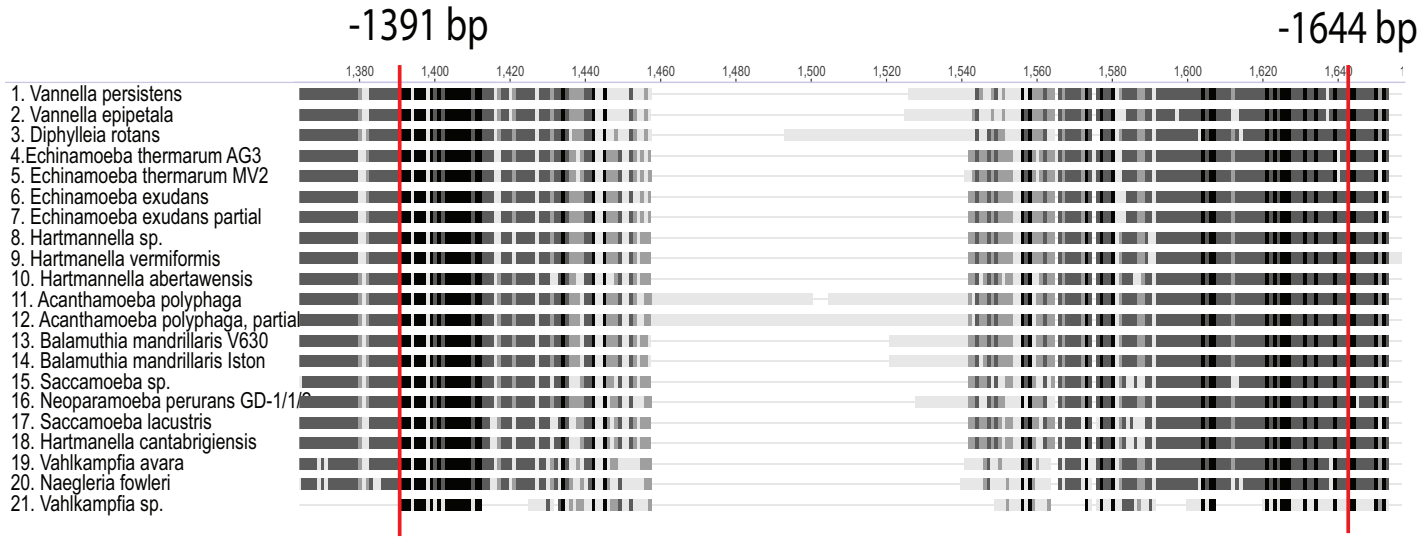
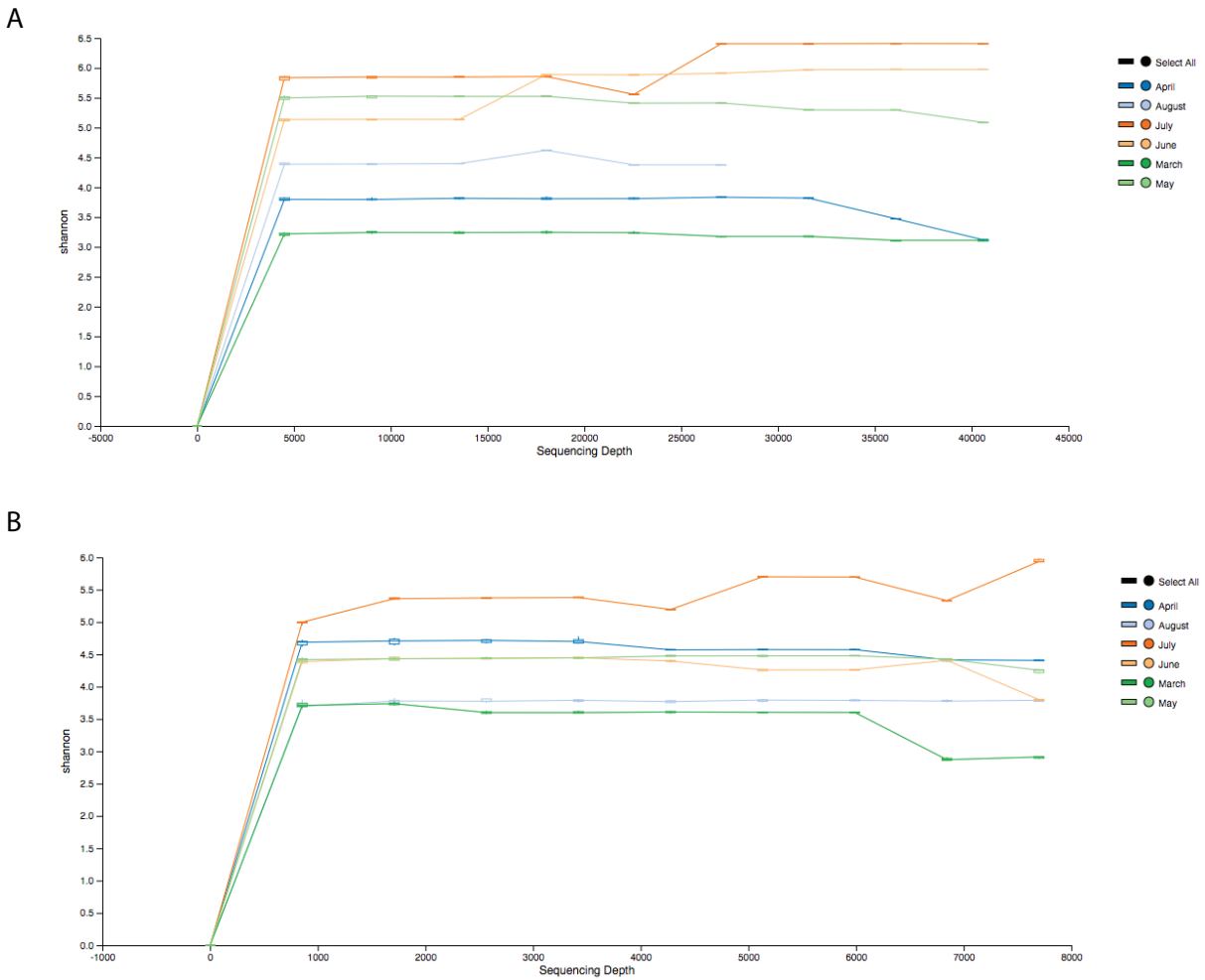


Fig. 6. Summary of overlapping taxa supported by six of ten clustering algorithms. A represents the eukaryotes and B represents the bacteria.

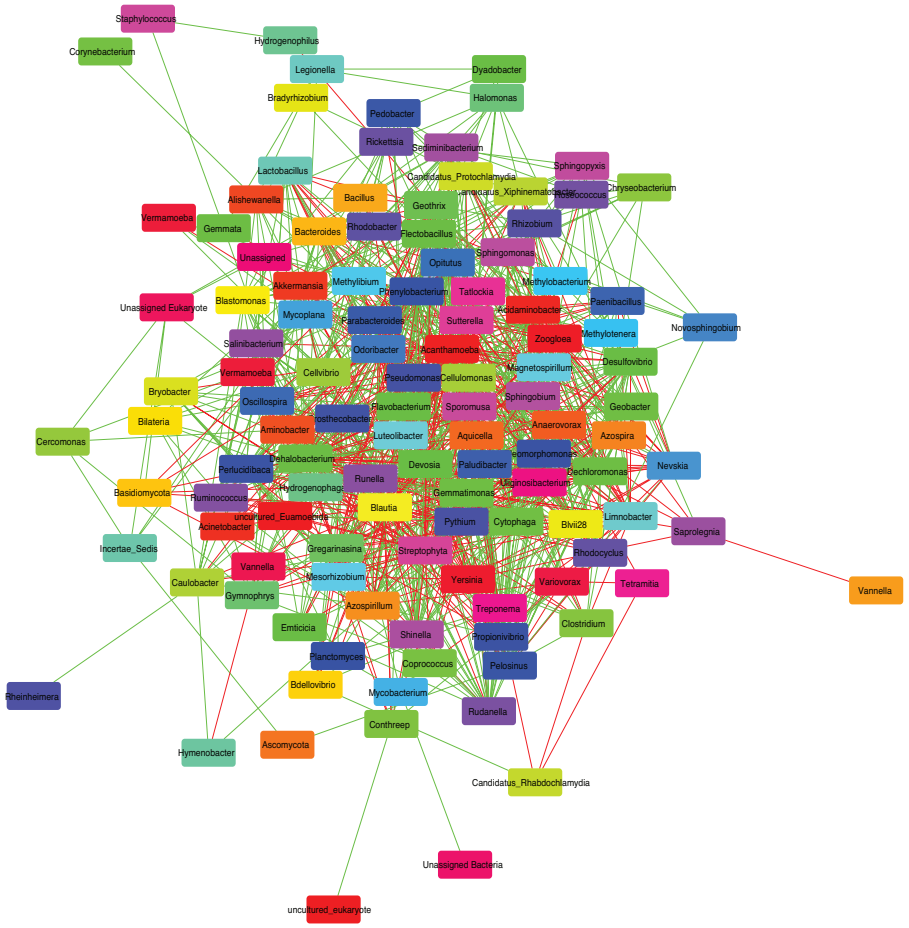
APPENDIX C: SUPPLEMENTAL FIGURES



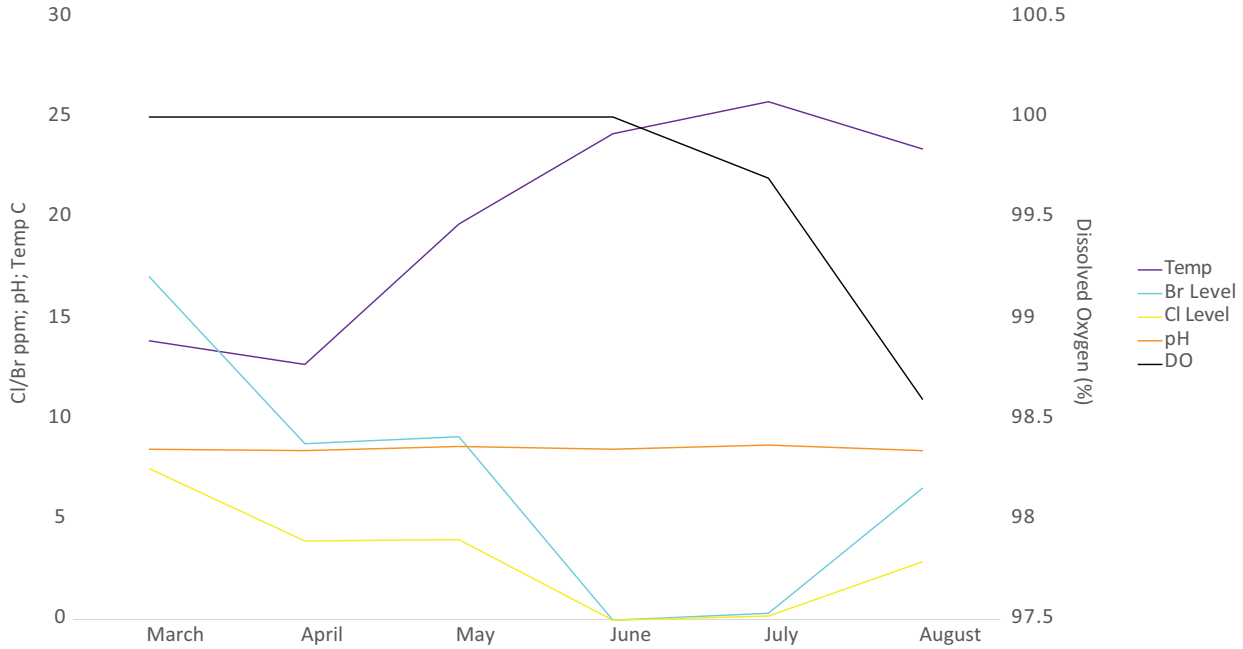
Supp. Fig. 1. The MAAFT alignment of amoeba sequences used to construct the 18S rRNA amplicon primers. The amplicon spans the -1391 to -1644 base pair region.



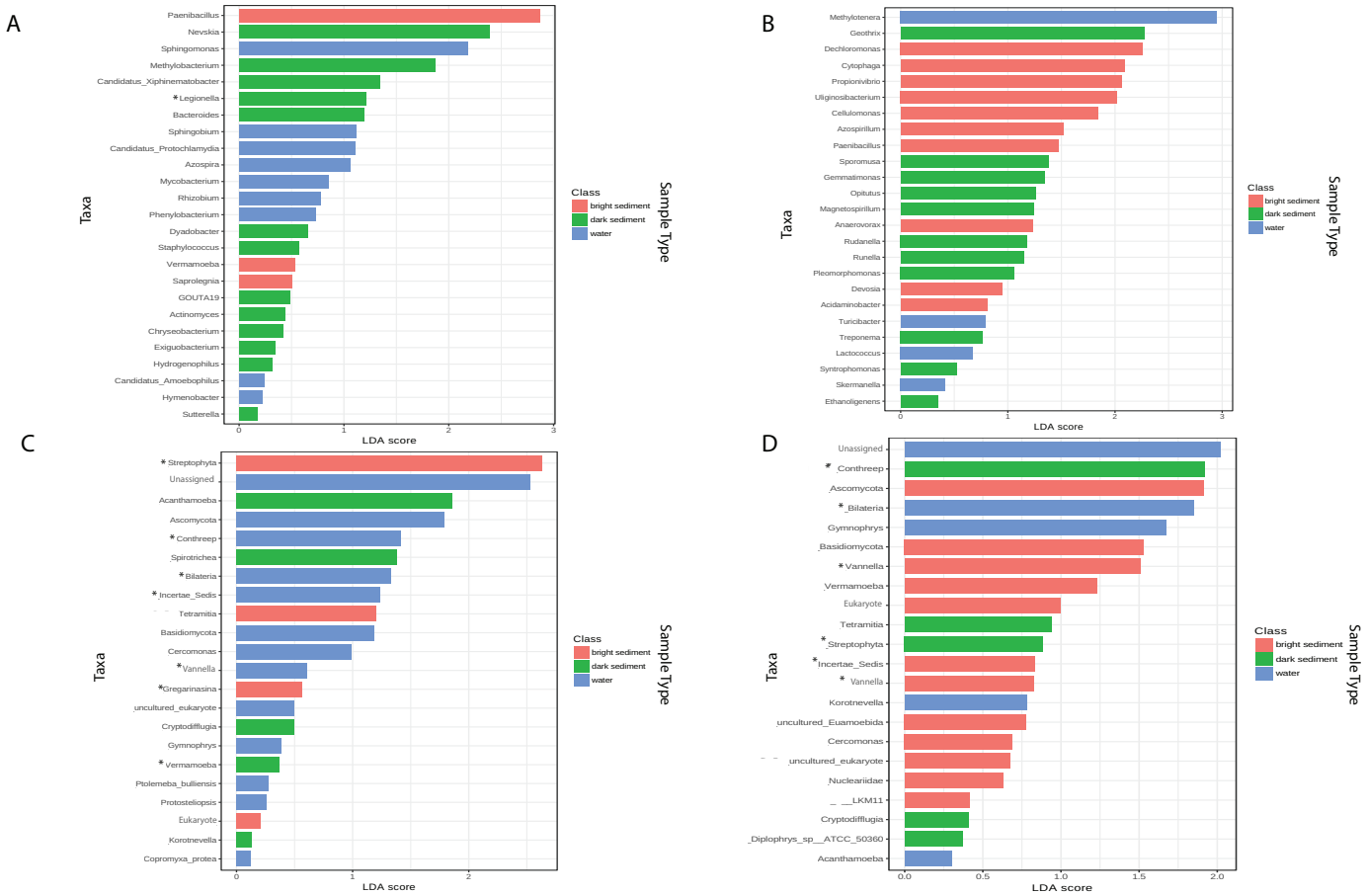
Supp. Fig. 2. Alpha rarefaction curves of the bacteria and eukaryote communities using Shannon diversity metrics. Samples are grouped by month.



Supp. Fig. 3. Initial un-clustered “consensus” network of combined bacterial and eukaryotic communities. Colored squares represent each taxa, or node. Lines connecting the taxa, or edges, are colored by their correlation type. Green lines represent positive correlations, red lines represent negative correlations and black lines represent unknown correlations.



Supp. Fig. 4. Environmental parameters over time. The primary axis contains units for temperature, bromine level, chlorine level and pH. The secondary axis contains units for dissolved oxygen (DO).



Supp. Fig. 5. LefSE LDA results of top 25 ranked taxa by LDA score for bacterial and eukaryotic communities when grouped by sample type and season. A represents the bacterial community during spring months, while B represents the bacterial community during sample months. C represents the eukaryotic community during the spring and D represents the eukaryotic community during summer. Colored bars indicate the sample type where the organisms was most prevalent. Asterisks next to eukaryote taxa represent the taxa observed in network analysis. All adjusted p-values were greater than 0.05.