ENVIRONMENTAL EFFECTS ON CONSTRUCTED WETLAND MICROBIAL DIVERSITY AND FUNCTION IN THE CONTEXT OF WASTEWATER MANAGEMENT

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ABSTRACT

ENVIRONMENTAL EFFECTS ON CONSTRUCTED WETLAND MICROBIAL DIVERSITY AND FUNCTION IN THE CONTEXT OF WASTEWATER MANAGEMENT

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Microbial communities play a crucial role in ecosystems, yet we know little about how microbial diversity influences ecosystem functioning. An important gap in our understanding is how environmental change affects microbial Biodiversity-Ecosystem Function relationships (BEF). These complex interactions between microbial biodiversity and ecosystem function can influence major biogeochemical processes, such as the nitrogen cycle in wetland ecosystems, which play an important role in managing wastewater. To address the effect of biodiversity on function, my study investigates the BEF relationships between microbial diversity and the function in terms of ammonia removal from wastewater at the Arcata Wastewater Treatment Facility (AWTF) both spatially and temporally. The AWTF uses several natural treatment systems consisting of Oxidation Ponds and constructed wetlands for secondary wastewater treatment. These natural treatment systems provide a unique opportunity to study microbial community BEF relationships because they are interconnected by the flow of nutrients in the wastewater and are exposed to seasonal changes. First, I conducted a field study where I sampled the AWTF natural treatment system from Autumn to Spring. Based on classical BEF studies, I expected a positive relationship between microbial biodiversity and

ecosystem function, but also anticipated a potential effect of seasonal and spatial factors in strengthening or weakening the relationship. Instead, I found a significant negative BEF relationship between microbial community richness and ammonia removal. Ammonia concentration significantly decreased through the wastewater purification system, yet microbial diversity was unrelated to the different locations in the wastewater treatment system. In turn, seasonality significantly affected the microbial community diversity where richness was lower during Spring. Following the field study, I conducted a microcosm experiment to determine the direct effect of an environmental change in terms of dissolved oxygen (DO) concentration on biodiversity and ecosystem function. The DO concentration had a positive relationship with evenness and a negative relationship with richness. In addition to the DO relationships, I observed a negative correlation between evenness and nitrification which reflects the BEF relationship findings from the field study. Because the lower evenness values are associated with more ammonia removal, these results further support that ammonia removal capabilities of the AWTF are most efficient when fewer species dominate the microbial communities in the natural treatment system, regardless of oxygen levels and other environmental factors. By expanding our search for more microbial community BEF relationship scenarios we can further unravel how richness and evenness influence ecosystem processes in natural and humanized systems.

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INTRODUCTION

Biodiversity and ecosystem functioning (BEF) relationships have been an area of inquiry for community and ecosystem ecologists for the past three decades (Wardle et al., 2000). Evidence supports positive BEF relationships, leading to a general acceptance that higher diversity leads to greater ecosystem function (Wardle et al., 2000). For example, extensive research in plant communities supports a positive relationship between plant species biodiversity and ecosystem functions like primary productivity and biomass accumulation (Morin et al. 2011). These patterns have been further applied to understanding the impact of climate change and human activities on biodiversity for terrestrial, marine, and aquatic ecosystems (Tilman et al. 2012). In major contrast to prevailing hypotheses, microbial ecosystems reveal a multitude of different BEF relationships and the general acceptance that higher diversity leads to greater ecosystem function is not strictly supported (Hagan et al., 2021).

Understanding which BEF relationships are involved at both a macro and micro scale is critical to predicting how environmental changes will affect the function of the world's ecosystems. Importantly, we know little of how environmental change at different spatial scales will impact BEF relationships. To understand the effects of environmental factors at different spatial scales, I borrow from two main theories to address complex biodiversity and ecosystem questions: (1) the metacommunity theory and (2) the meta-ecosystem theory. The metacommunity theory describes the interactions between a set of local communities that are linked through the dispersal of interacting

species. The meta-ecosystem theory is defined as a set of habitats connected by the flow of biotic communities, nutrients, and energy across ecosystem boundaries. Considering how heavily microbes impact ecosystem resilience, it is problematic that dispersal and environmental fluctuations on microbial diversity are largely unknown (Townsend et al. 2003).

A major biogeochemical cycle that has been disrupted by global anthropogenic activities is the nitrogen cycle (Erisma et al. 2013, Fields 2004, Fowler et al. 2013, Galloway et al. 2008, Vitousek et al. 1997). Although, the nitrogen cycle is complex, wastewater treatment systems provide an opportunity to focus on specific and concrete functions to better understand how BEF relationships impact cycling of this important nutrient.

Two main types of systems in which wastewater treatment can be studied are conventional wastewater treatment and natural wastewater treatment in constructed wetlands. In both cases, nitrogen enters the wastewater treatment system through two channels: (1) as atmospheric nitrogen gas across air-water boundaries throughout the system and (2) as ammonia via influent. Ammonia is one of the main pollutants found in wastewater, originating from industrial and house-cleaning chemicals, amino acid products and urine in sewage (Minocha et al., 1987). In conventional wastewater treatment systems, the ammonia is released as nitrogen gas after being treated in large concrete basins, where introduced nitrifying and denitrifying bacteria remove a large portion of ammonia. This process is often energetically intensive and produces large amounts of biosolid waste. A cost effective and sustainable alternative is to treat

wastewater in constructed wetlands. The wetlands provide a series of habitats where microbial communities perform nitrification and denitrification to remove ammonia from the untreated wastewater (Dong et al., 2011) (Fig. 1). Nitrifying bacteria metabolize the ammonia into bioavailable nitrates which plants require for growth. Although plants also use ammonia as a nutrient source, it is highly saturated in wastewater and the nitrate-ammonia ratio needs to be balanced for optimal treatment efficiency by plants (Errebhi & Wilcox, 1990). Following nitrification, a large portion of the nitrate is reduced to molecular nitrogen and released into the atmosphere by denitrifying bacteria (Fig. 1). Denitrifying and nitrifying bacteria are crucial in constructed wetland ecosystems because they directly impact algal blooms, effluent quality, and efficiency of pollutant treatment processes (Bodelier et al. 2013).

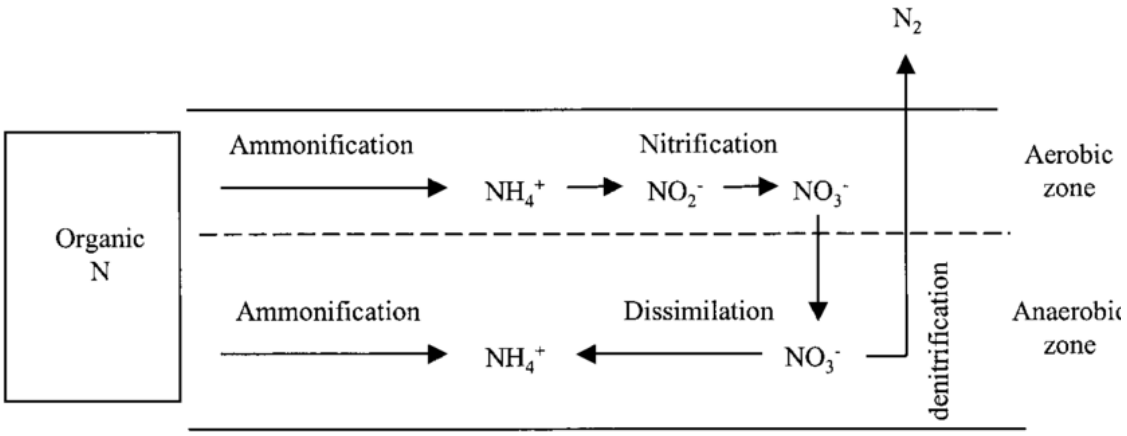

Figure 1. Model of the nitrogen cycle in wetlands. This study focuses on nitrification and denitrification (Figure modified from Pinay et al., 2002)

In addition to offering wastewater treatment, wetlands are vital ecosystems in sustaining the Earth's biodiversity and biogeochemical cycles around the world (Erwin,

2009). Wetlands are valued for their high biological productivity, as pollutant filters, nutrient cycling, and carbon sinks (Hook, 1993). The value of wetlands has proved increasingly significant as urban and agricultural development further reduces the global wetland surface area to a fraction of its size prior to industrialization (Mitsch & Gosselink, 2000). Wetland restoration and creation helps us mitigate the effects of fossil fuel generated global warming (Mitsch et al., 2013). Besides providing nutrient cycling benefits, wetlands also act as coastal buffers between human development and tidally influenced regions, a function that should be taken advantage of in the face of sea level rise (Schuerch et al., 2018).

Wetlands are also an ideal system to study microbial BEF relationships in the context of meta-ecosystem and metacommunity ecology because they create microhabitats interconnected by the flow of nutrients and microbial communities (Brisson et al., 2020). The proven water purification capability of wetlands has encouraged the expansion of constructed wetlands to mimic the purification potential found in their natural counterparts (Truu et al. 2009, Ottovà et al. 1997). The Arcata Wastewater Treatment Facility (AWTF) is suitable for testing metacommunity dynamics of the bacteria because the natural treatment systems are interconnected in a series. Additionally, the microbial communities found throughout the AWTF metaecosystem are the key drivers for the bioremediation processes. The use of naturally occurring microorganisms to metabolize and degrade environmental pollutants, with ammonia being the principal interest in this study.

The central goal of this study is to demonstrate how the microbial communities are impacted by environmental, and how microbial biodiversity in turn affects the concentration of wastewater ammonia. Because ammonia is a crucial toxin in wastewater, ammonia removal serves as a measure of ecosystem function in the BEF relationships. Furthermore, microbial community composition may be as, or more important than, diversity in determining ecosystem function, thus I will also characterize patterns in bacterial relative abundances. A key environmental factor of interest is oxygen concentration, which is proven to increase ammonia removal rates, and thus often added to wastewater systems via aeration pumps (Regmi et al., 2015). Despite its common use, the effect of added oxygen on microbial community diversity via aeration remains unknown in natural wastewater systems. This thesis is the first comprehensive molecular assessment of the microbial communities throughout the wetlands of the AWTF and it will provide useful insights into the ammonia removal functions of bacterial communities in wastewater wetlands. There are two main sections which will address the BEF relationship question using different methods:

1. A field study assessment of the effect of location and season on microbial community BEF relationships and changes in microbial community composiiotns.
2. A microcosm experiment to test the direct effect of an environmental change on community composition observed through a microcosm experiment that tests the role of oxygen addition on microbial community dynamics and nitrification.

By combining environmental surveys and a manipulative microcosm experiment, this research will increase our knowledge of microbial BEF relationships by providing key information about how microbial communities may change over locality and season. These microbial community dynamics can be further applied to expand wastewater ammonia bioremediation strategies.

Study Site

The Arcata Marsh in Arcata, California became one of the world's first wastewater treatment facilities to incorporate constructed wetlands into its natural treatment design in 1984. Management of a conventional system, lacking wetlands, requires precise control of the environment which necessitates expensive energy inputs. In a natural wastewater treatment system where the wastewater is diverted through constructed wetlands, the system uses biotic processes, thereby reducing energetic costs (Ayaz et al. 2001). Working in series, the oxidation ponds, treatment marshes and enhancement wetlands improve wastewater quality in the natural treatment processes (Adrados et al. 2014). Important advantages of a natural wastewater treatment system over a conventional system are its ability to treat wastewater with minimal anthropogenic energy requirements and auxiliary production of key wildlife habitat (Crites, R.W. et al. 2014). The Arcata Marsh Wildlife Sanctuary houses over 70 year-round resident bird species and 330 migratory birds visit the marsh annually, as well as threatened species such as the Red-Legged frog (*Rana aurora*) and the Northern American river otter (*Lontra canadensis*). The large area and depth of landscape covered by the constructed

wetlands is diverse in habitat types, which leads to a broad range of metabolic activity and niches (Kawecki 1995).

Ammonia concentration, biological oxygen demand (BOD) and total dissolved solids (TSS) are the three primary water quality parameters for wastewater management. The BOD is a measurement of the oxygen concentration (*mg/L*) required for the microbial community to breakdown all the organic matter in a specific volume of wastewater sample. The BOD thus serves as an indicator of the carbon and organic material load in the wastewater. TSS is a measure of turbidity measured in milligrams of solids per liter of water (*mg/L*). In this study I focus on ammonia concentration and the change in ammonia between ponds, focusing on the nitrogen cycle to determine ecosystem function.

The AWTF system includes conventional headworks and primary settling followed by a series of three natural treatment steps and disinfection. These stages are referred to as the "treatment train" by wastewater facility operators and can be envisioned as a linear treatment flow path from the plant influent towards the effluent, while progressively reaching further purification levels (Fig. 2). I selected sampling points that encompass the entirety of the treatment train while considering important differences between locations. The first stage of the natural purification process takes place in two oxidation ponds which cover 49 acres combined. In the oxidation ponds photosynthesizing algae provide oxygen to facultative heterotrophic bacteria that break down waste particles in the wastewater. The large area of open water has high oxygen and sunlight exposure in surface waters which leads to algal growth. This can increase

the BOD and TSS in the oxidation ponds, but this stage is critical in reducing initial ammonia concentration.

Following the oxidation ponds, the water is directed to the six parallel Treatment Marshes which are densely vegetated with submergent plants such as cattails (*Typha angustifolia*), bullrush (*Typha latifolia*), marsh pennywort (*Hydrocotyle vulgaris*) and water celery (*Oenanthe javanica*). The roots of the plants and shading helps to reduce the high concentration of algae created in the oxidation ponds. Plants act as physical barriers for the remaining solids and limit sunlight availability to the microalgae. This results in the algae senescing and sinking to the bottom of the treatment marsh, where they degrade via anaerobic microbial pathways. The final stage of the wastewater purification flow path is to pass through the three enhancement wetlands- Allen (point 4), Gearheart (point 5) and Hauser (point 6) in series (Fig. 2). In total these 30 acres of enhancement wetlands provide a similar function as the treatment marshes,

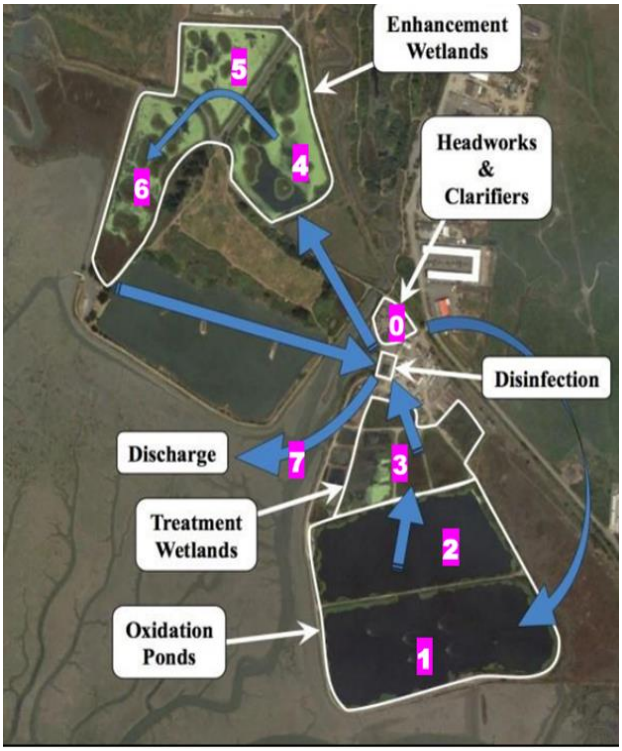

Figure 2. Google Earth image of the AWTF with numerical flow path of wastewater. 130 acres total wetland surface area with blue arrows indicating subterranean pipes. In order in the treatment train the ponds are: (1) Oxidation Pond 1, (2) Oxidation Pond 2, (3) Treatment Marshes, (4) Allen Marsh, (5) Gearheart Marsh, (6) Hauser Marsh and (7) Bay Discharge. (Image courtesy of AMRI)

but a higher habitat heterogeneity with more open water facilitates increased nitrification potential in the enhancement wetlands.

Disinfection is an important part of wastewater treatment and must be considered during bacterial analyses. There are two chlorination/de-chlorination steps within the 130 acres of the treatment train. Chlorination occurs before entering Allen marsh (point 4) and finally after leaving Hauser marsh (point 6) before purified wastewater is discharged into Humboldt Bay (Fig. 2). These chlorination steps are required by the California Water Board to ensure the total coliform load is below permit violation limits.

As part of the wastewater treatment process, the decaying organic molecules sink to the bottom of the wetlands and accumulate over the years into a biosolids layer. The average lifespan of constructed wetlands is approximately 40 years until biosolids accumulation eventually fills the wetland entirely (Bavor et al., 1995). Constructed wetlands require a biosolids removal plan for the long-term preservation of the wetlands (Gerba et al., 2009). This can be accomplished by dredging or pumping the settled solids out of the wetlands and relocating them for further aerobic breakdown until the solids are ready for any additional application such as use for fertilizer and compost (Uggetti et al., 2011). Operators use the term legacy load to describe the accumulation of toxins, bacteria, and organic matter accumulation in the biosolids layer that remains relatively inactive with the rest of the water column until disturbed (Uggetti et al., 2012). There are ongoing scientific questions concerning interactions between the undisturbed biosolids layer and the water column, including the ammonia diffusion back into the water from the biosolids, as well as the bacterial communities migrating back and forth between each

substrate type. However, at this point most research concludes that nitrogen cycling in the biosolids layer remains relatively decoupled from the water column, until biosolids are mechanically disrupted (Wiegman et al., 2020). I collected and sequenced samples from the biosolids layer in the Oxidation Ponds during field work but the analysis of the samples will be part of another project which will solely focus on the biosolids microbial community. I provide the general comparison between the microbial communities of biosolids and aqueous samples solely as evidence of their distinction. This decision allows for a more thorough analysis and understanding of the aqueous samples for this thesis.

METHODS

Sample Collection

Sampling points included all wetlands in the treatment train for a total of 16 sampling points. The specific sampling strategy for the large oxidation ponds at the beginning of the treatment train differed from the smaller and shallower wetlands further down the line due to their different sizes, biotic characteristics, and accessibility. Their accessibility made it feasible to collect both water and biosolids samples from both oxidation ponds. In this study, only aqueous samples were collected from the subsequent wetlands. The two oxidation ponds are 25 acres each and to capture the variance within Ponds, I collected five samples from each Oxidation Pond, tracking their influent to effluent water indicated by yellow arrows (Fig 3). Sampling locations in the oxidation ponds were accessed using a 4.6-meter aluminum flat bottom boat propelled by an electric motor. The subsequent six parallel treatment marshes were not sampled individually. One effluent weir of oxidation pond 2 was selected as a representative treatment marsh influent sample, and the effluent was collected as a composite sample at a pre-existing pipe that served as a common effluent for the treatment marshes. The following three enhancement wetlands were sampled via weirs and pump stations chosen to match sampling points used on previous research conducted by the Arcata Marsh Research Institute (AMRI) and to assess microbial and water quality transformation occurring within each pond. Figure 3 displays a map of sampling points at the AWTF with red crosses indicating the sampling points following the oxidation ponds.

Figure 3. Google Earth map of the study sample points. Yellow arrows are sludge and water samples pointing in the general direction of the wastewater flow within the Oxidation Ponds. The subsequent samples were collected on the marked red crosses from the weirs and faucet of the treatment marshes, enhancement wetlands and bay discharge.

At each location, I obtained dissolved oxygen (DO), ammonia and temperature measurements. The field work portion of this study was conducted from August 2019 to February 2020; hence the analysis is for the period Autumn of 2019 through the Spring of 2020. Coastal Humboldt County, where the study site is located, experiences mild seasonal changes throughout the year which are categorized as a wet and dry season. The

rainy season averages 100 cm in the Winter (December to February) and the dry season (May to September) experiences as low as 12 cm of rain (NOAA, 2020).

Collection and Filtration

For biosolids samples, I used a "sludge sampler" designed with rubber plugs along a transparent plastic PVC pipe to retrieve the biosolids layer from the benthic zone. After collecting, I stored the biosolids in 50 *mL* Falcon tubes. The aqueous samples were directly collected into autoclaved sterile Nalgene one-liter bottles. To preserve the integrity of the microorganisms in the water and biosolids samples, the sample flasks and bottles were immediately stored in an ice cooler and transported to the field laboratory for further processing. For each water sample, 400 *mL* were filtered to concentrate the microbial content for DNA extraction. I filtered water samples through sterile analytical filter funnels with a pore size of 0.45 μm to minimize clogging from the high algae concentration, however, some of the bacteria can be lost due to this pore size being larger than many bacterial species. I captured the flow-through in a sterile filter flask then filtered a second time through the same type of filter funnel but with a pore size of 0.22 μm to capture smaller classes of bacteria that would otherwise be lost if only filtered with a 0.45 μm filter. All processed filters were preserved in 1.5 *mL* microcentrifuge tubes with 1 *mL* of DNase/RNase buffer (Zymo Shield) and stored at -80º C until ready for extraction. The biosolids were extracted using the same method as aqueous samples without prior filtration.

For each sampling site, I recorded the water temperature, dissolved oxygen (DO), GPS coordinates (Garmin Etrex 10) and ammonia concentration. Temperature and DO were determined using a Hannah multiparameter probe at 3 depths (surface, 30 cm, 90 cm deep) for each location. The samples were aseptically brought in autoclaved one liter Nalgene bottles to the field lab where I measured ammonia and nitrate concentration using an Orion meter and the ISE high performance ammonia electrode with substrate specific buffer and 1000 ppm standard solutions (USAbluebook). The ammonia test was done with an ammonia probe and calibrated with 100 ppm, 10 ppm, 1 ppm and 0.1 ppm standards.

DNA Extraction and Sequencing

To capture the fullest extent of microbial diversity in each sample I used ZymoBIOMICS DNA/RNA extraction kits designed for mixed microbial community samples as described by the manufacturer. DNA sequencing followed methods described by Canter et al. (2018). The 16S rRNA genes were amplified from 20 μL of purified DNA extractions using archaeal and bacterial primers 515F and 806R, which target the V4 region of *Escherichia coli* in accordance with the protocol described in similar previous work (Caporaso et al. 2011, 2012) and applicable by the Earth Microbiome Project (http://www.earthmicrobiome.org/emp-standard-protocols/16s/). Raw sequences were demultiplexed and then quality filtered using the DADA2: high resolution sample interference from Illumina amplicon data R package. Demultiplexed data matching Phi-X reads were removed using the SMALT 0.7.6 akutils phix_filtering command (Krohn

2016). Chimeras were removed using VSEARCH 1.1.1 (Rognes et al. 2016). Sequences were clustered into Amplicon sequence variants (ASVs) using Greengenes version 13.5 (McDonald et al. 2012) to determine taxonomy. Using the Phyloseq package, I removed ASV assigned to Archaea, Mitochondria, unassigned taxa and those shorter than 1800 base pairs sequence results via Amplicon sequencing with Illumina MiSeq.

Microcosm Experiment Design

I set up a microcosm to test the effect of oxygen addition on microbial biodiversity and function. To build the microcosms, I filled thirty 3.6 L tanks (23x15x15 *cm)* with 3 liters of water from oxidation pond 2. Oxidation pond 2 was chosen because oxidation pond 1 already has large aerators installed, therefore oxidation pond 2 provides wastewater without added aeration. The microcosms were subjected to three treatments, which were replicated three times for a total of 30 tanks. Treatments included (1) added homogeneous aeration, (2) added heterogeneous aeration and (3) a control exposed to ambient oxygen only. I used standard aquarium aeration pumps attached to tubes with bubblers at the end that were submerged in the water tanks. To preserve the aseptic conditions and avoid cross contamination, the bubblers were autoclaved for sterilization, and each tank only received added aeration from a single bubbler. Tanks in the heterogeneous group had a plastic partition that divided the tank in half and reached completely to the top, but the side edges were not sealed against the tank and therefore allowed marginal flow exchange between the partitioned sides. In the heterogeneous group, the bubbler was only on one side of the partition and the homogeneous group did

not have a divider and the bubbler was in the center. Tanks with no added aeration were used as a control group where gas exchange only occurred with ambient air at the water-air interphase. The microcosms were stored on a wooden table in a dark unheated shed at the AMRI field laboratory. The temperatures were between 18º C and 21º C for the duration of the experiment, and the tanks were organized at random so that all treatments were mixed evenly on the table. Weekly, I collected 625 *mL* of a composite sample from each treatment group for filtering, ammonia, and nitrate determinations as described above. For the heterogeneous tanks I collected the samples from the side without the bubblers. The experiment had a total duration of 3 weeks until concentrations of the nitrate stabilized.

Data and Statistical Analysis

All analyses and calculations were completed in RStudio (version1.4.1564) and all graphs and plots were constructed with package ggplot2 (Wickham, 2016) and ggpubr (0.4.0; Kassambara, 2020). To assign taxa to the ASV table I used the function dada() from the *dada2* package (10.1371; Mcmurdie & Holmes, 2013). I calculated diversity metrics from the processed ASV table to obtain richness and evenness (Pielou, 1959) using functions specnumber() and diversity() in the *vegan* package (2.5-7; Oksanen et al., 2020). To establish a metric of microbial function, I calculated the delta ammonia concentration of ponds for each sample date by subtracting the effluent ammonia concentration of each location from its influent concentration (Equation 1).

$$\Delta NH3 = [NH3]_{in} - [NH3]_{out} \quad (1)$$

. To determine if there were differences in variance in microbial community richness and evenness between the aqueous and biosolids samples, I used a Bartlett's homogeneity of variance test. I also performed a Bartlett's test to determine if the variance between the microbial communities in the biosolids between oxidation pond 1 and oxidation pond 2 were different.

I performed an ANOVA on microbial evenness and richness with location and season as factors. Then, I used separate generalized mixed linear models (glmm) with a Gaussian distribution to test the influence of diversity on the delta ammonia. I used richness or evenness as fixed factors, location and season as random factors and ammonia concentration as the response variable. Significance was then tested for each glmm with a type II Wald test using the function Anova() from the *car* package (Fox & Weisberg, 2019). I used ANOVAs to test the effect of locality and season on richness and evenness. To determine if there were significant differences between the locations and the seasons in the diversity metrics and the ammonia concentration, I conducted a Tukey's test on the ANOVA models. I used the Shapiro-Wilk test to test the normality of the sample distribution. Finally, I used ANOVA's to test the effect of temperature on diversity and ammonia concentration.

To consider the influence of community composition on BEF relationships, I used a combination of multivariate and qualitative analysis. I used perMANOVAs to establish the effects of locality and season on microbial composition with the function adonis() from the package *vegan.* To illustrate these patterns, I ran a Non-metric Multidimensional Scaling (NMDS) with three dimensions. I then assessed whether these patterns were

driven by differences in community similarity by calculating the Bray-Curtis dissimilarity index and running a multivariate analog to Levine's homogeneity of variances, using the function betadisper() from the package *vegan.*

To establish patterns in bacterial relative abundance, I used the function heatmap() from base R and normalized the matrix by using the scale argument of the heatmap() function. By constructing heatmaps at the Phylum, Family and Genus level, I described several qualitative patterns in relative abundance across seasons and location.

To establish some potential functional consequences to these compositional changes, I completed a literature review using peer reviewed literature to identify key metabolic traits from the bacteria in the six must abundant Families from the Kingdom Bacteria. For the literature review I used Google Scholar and searched the Family name and key words nitrogen cycle, wastewater, wetlands, and biodegradation.

Lasty, I used data from the microcosm experiment to determine the significance of treatment on DO, evenness, richness and ammonia on data collected on the last day. The effect of DO on the diversity metrics (richness and evenness) was determined with glm models with treatment as a fixed factor. Significance was established as above with type II Wald test on resulting models. To evaluate the difference between treatments in dissolved oxygen concentration, ammonia concentration and evenness value I conducted an ANOVA followed by the post-hoc test with Tukey's Honest Significance Difference test to see which treatments were significantly different from each other.

RESULTS

Field Study

After quality filtering, the total number of taxa in all wastewater samples combined was 17,508 ASVs throughout the treatment plant for the duration of the three seasons of the study: Autumn, Winter, and Spring. The taxa belonged to 61 Phyla and 417 Families from the Kingdom Bacteria. To clarify which samples will be included in my analysis, I will first address differences in bacterial diversity between water samples and biosolids samples. Then, I will investigate more in detail the patterns of bacterial diversity and function as they respond to seasonality and locality.

Biosolids and Water Diversity Patterns

While both aqueous and biosolids samples were collected from oxidation pond 1 and oxidation pond 2, only the aqueous samples are included in the following analysis of this study for two reasons. First, the microbial communities from the biosolids samples were less variable than the communities in the aqueous samples (Bartlett's: $\chi^2 = 5.21$, $df = 16$, p-value < 0.05) (Fig. 4).

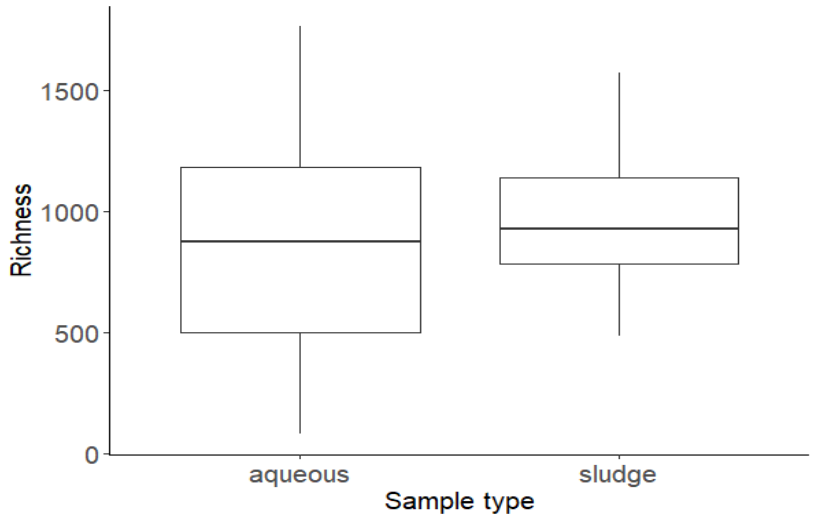

Figure 4. Boxplot of the effect of sample type on species richness.

Additionally, biosolids microbial communities in oxidation pond 1 and oxidation pond 2 did not show significant difference in variance (Bartlett's: $\chi^2 = 0.36$, $df = 2$, p-value = 0.83). Due to the homogenic nature of the microbial communities in the biosolids, I did not include them in any of the results following this sample type analysis.

Aquatic Bacterial Samples

Overall, I found significant effects of seasons and location on microbial diversity and ammonia removal. An ANOVA showed that season significantly affected microbial richness, but location did not significantly affect richness or evenness (Table 1). I also found significant effects of location on changes in ammonia concentration. I further discuss these patterns with three sections: (1) the relationship between microbial diversity and function, (2) the effects of location and (3) changes associated with seasons.

Table 1. Anova of microbial evenness and richness with location and season as factors.

Interaction	*df*	*F*-value	*p*-value
A. Evenness			
Location	7	1.483	0.36
Season	2	6.484	0.12
Residuals	52	NA	NA
B. Richness			
Location	7	0.849	0.48
Season	2	6.166	0.03*
Residuals	52	NA	NA

Diversity and Function

I found a significant negative BEF relationship between richness and function (GLMM: $\chi^2 = 3.86$, $df = 1$, p-value = 0.04). The change in ammonia was the response variable to determine the effect of the species richness on the ecosystem function (Fig. 5). In contrast, there was no significant relationship between delta ammonia and species evenness (GLMM: $\chi^2 = 3.38$, $df = 1$, p-value = 0.07).

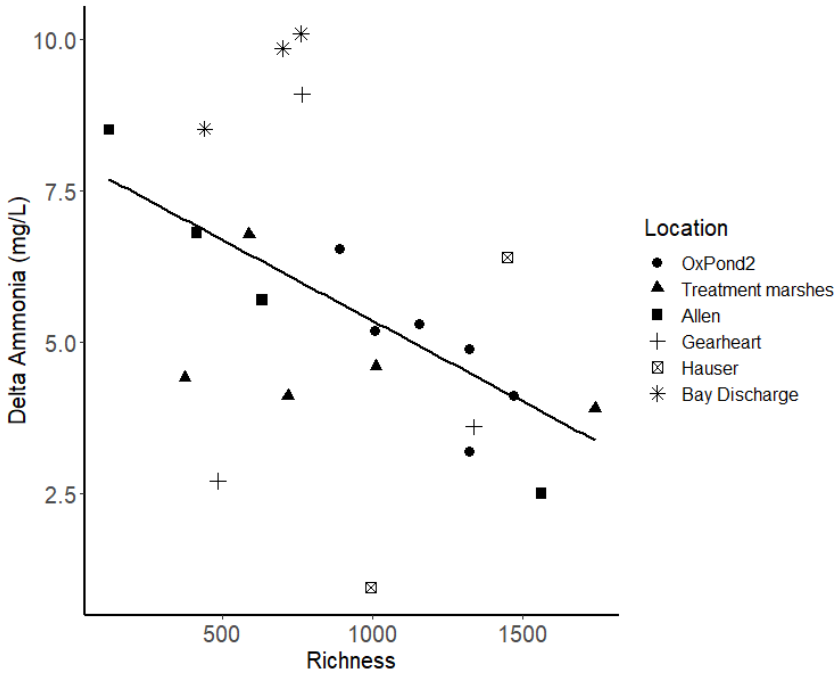

Figure 5. Negative BEF relationship between diversity and the average change in ammonia between each pond.

Effects of Locality

The operators of AWTF and other natural wastewater treatment plants rely on the fact that ammonia concentration decreases throughout the purification ponds as a function of treatment time and location. Likewise, I observed the same pattern when I measured the concentration of ammonia for each sample site during my study (Fig. 6). The location had a significant effect on ammonia concentration (F-value = 6.82, df = 7, p-value < 0.0006). The ammonia concentration in oxidation pond 1, oxidation 2, and the treatment marshes was significantly higher than the ammonia concentration in the bay discharge (oxidation pond 1: p-value < 0.002; oxidation pond 2: p-value < 0.002 and treatment marshes: p-value < 0.006).

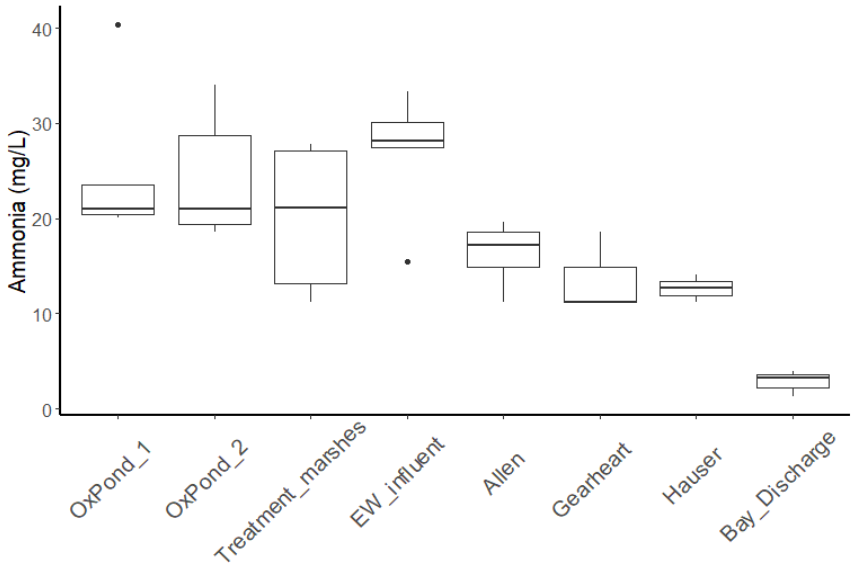

Figure 6. Ammonia concentration from each location in the series of wetlands in order from the facility influent through the discharge into Humboldt Bay.

In contrast, location did not have a significant effect on richness (F-value = 1.25, df = 7, p-value > 0.3). The oxidation ponds showed the highest average microbial richness followed by Gearheart marsh. These low points corresponded with the chlorination steps in the treatment train. Allen marsh had the widest distribution in richness but was not significantly different from the other locations (p-value > 0.8)(Fig. 7).

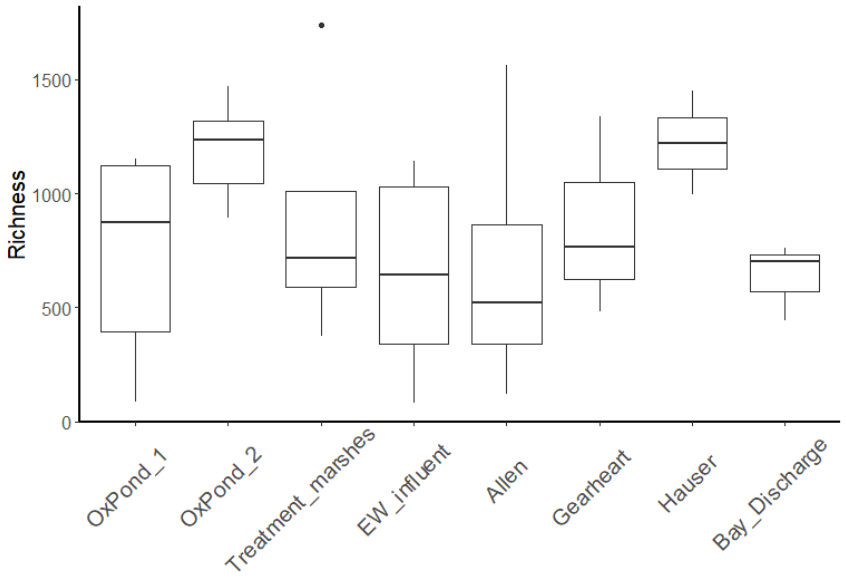

Figure 7. The change in richness throughout locality in the series of wetlands in order from the plant influent to the discharge point into Humboldt Bay.

Effects of Seasons

Species richness was significantly affected by the seasons (df = 2, F value = 3.81, p-value < 0.01). Winter had the most species diversity and the highest median richness values, while Spring had the lowest richness median value (Fig. 8). Evenness on the other hand was not significantly different between seasons (df = 2, F value = 2.33, p-value > 0.1).

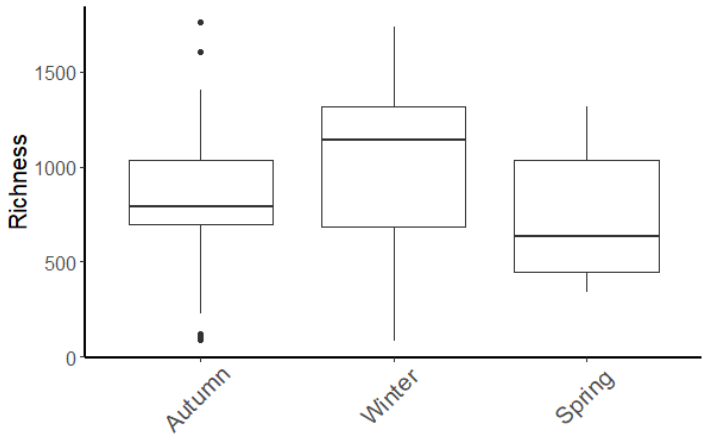

Figure 8. Boxplot of the seasonal effects on species richness for all the locations in study

Similarly, seasons did not have a significant effect on ammonia concentration (GLM: χ^2= 1.12, *df* = 2, *p*-value = 0.09)(Fig.9). Seasons had a significant effect on temperature (GLM: χ^2 = 228, *df* = 2, *p*-value < 0.001), however temperature did not have significant effect on diversity (*F*-value = 0.02, *df* = 1, *p*-value > 0.1) or ammonia concentration (*F*-value = 2.1, *df* = 1, *p*-value > 0.5)(Fig. A5 & A6).

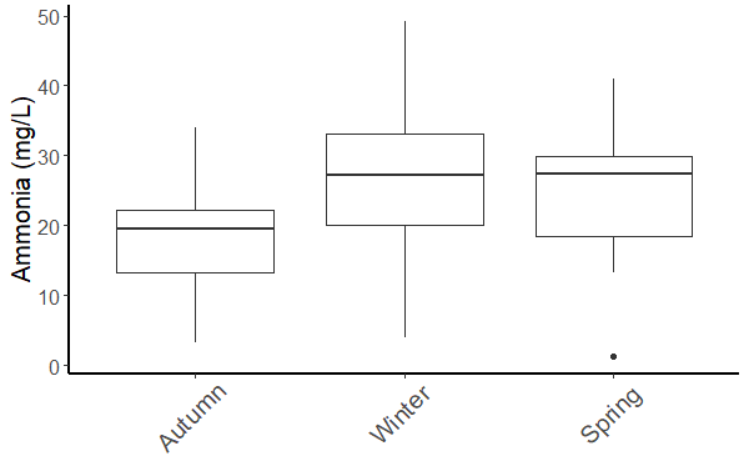

Figure 9. Effect of seasons on ammonia concentration in all locations combined.

With the purpose of considering the influence of community composition on BEF relationships, this chapter investigates patterns in relative abundance of the bacterial taxa in wastewater bioremediation at the AWTF. The current analysis is qualitative and is intended to serve as a general overview of the microbial community compositional patterns to understand the potential effects of location and season on the community composition throughout the treatment systems. Overall, I found that compositional patterns suggested to community similarity differences, instead of compositional shifts in species membership (Fig. 10). When assessing localities, microbial communities within the Oxidation Ponds were more like one another than other sites (Fig. 10a). Similarly, bacterial communities were more similar during Spring than other seasons. (Fig. 10b). However, Bray-Curtis index and betadisper results revealed that community similarity did not change significantly between sites or seasons (PERMANOVA: *df* 6, *F*-value = 0.7202, *p*-value = 0.634)(Fig. A7). Seasonal change in the composition of the microbial Families was particularly striking in Allen marsh which is the first of the enhancement wetlands (Fig. 11a). Similarly, Allen marsh exhibited the highest variability in relative abundances at the level of genera (Fig. 11b). These results reflect the distinctive variability in evenness and richness within Allen marsh (Fig. 7).

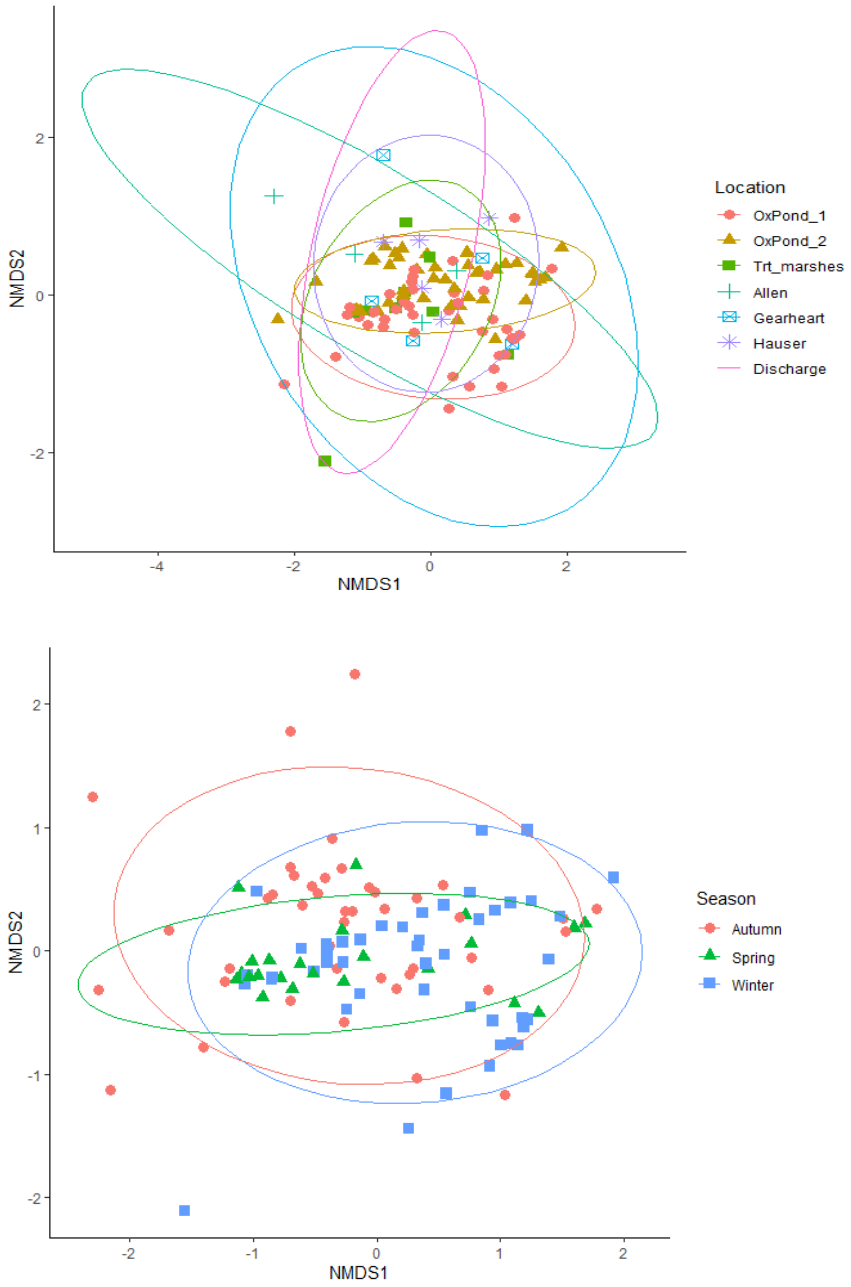

Figure 10. The NMDS shows microbial species composition changes by location and b) by seasons. Color coded orbitals indicate samples from the same site(a) and season (b).

Apart from the striking microbial community compositional differences of Allen marsh, I found a general seasonal pattern across the entire AWTF showing that some

species increased while some decreased, with the strongest change occurring from Autumn to Winter. Many abundant species in Autumn, such as those in the Family *Acrobacteraceae*, drastically decreased in abundance until they were completely absent in the Spring, which was the season with the lowest richness (Fig. 8 & Fig. 11a). Genera that decreased in relative abundance, included Zoogloeceae (*Thauera)* and Crenotrichaceae (*Crenothrix)*. In contrast, taxa in the Family *Mycobacteriaceae* increased from Autumn to Winter and then slightly more in Spring (Fig 11a). *Mycobacteriaceae* was also the lowest in the Bay Discharge during Autumn and Winter. Other genera that increased with seasonal change were Spirosomaceae (*Runella)* and Arcobacteraceae (*Arcobacte)*. Note that these seasonal patterns were not always homogeneous through space. For example, *Burkholderiaceae* progressively increased from Autumn to Spring except for in Allen marsh.

The genus *Flavobacterium* and genus *Polynucleobacter* decreased drastically in Winter only in Allen marsh. The genus *Methyloparacoccus* was more abundant and consistent throughout the other locations when compared to Allen marsh. Allen and the Bay Discharge were both immediately after a disinfection point and had less *Crenothrix* and *Mycobacterium* than the other locations in the Autumn and Spring. The relative abundance variability between locations was lowest in Winter and highest in Autumn (Fig 11). I also evaluated the relative abundance at the phylum taxanomic level but there was no change between locations (Fig. A3)

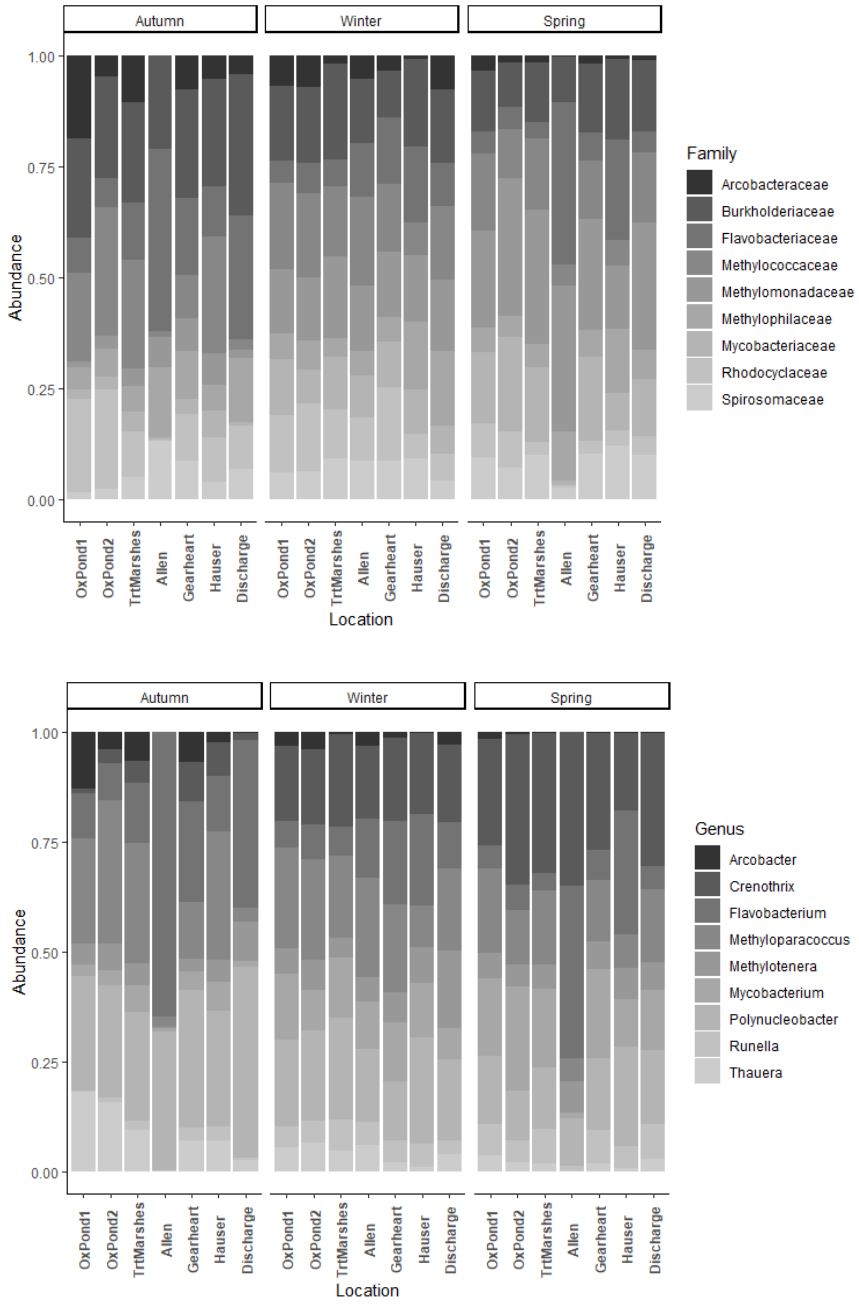

Figure 11. Relative abundance of microbial taxa over seasons and locations at the (a) Family and the (b) Genus taxonomic level.

When focusing on compositional changes across localities, I found that some bacterial taxa were common throughout the entire treatment train while others were

concentrated in a single pond. The heat map qualitative analysis was then done at the family and genus taxonomic level based on the relative microbial community composition. The most abundant bacterial family across all sample points was *Burkholderiaceae* followed by *Methylococcaceae* (Fig. 12a). The most abundant genera were *Polynucleobacter*, *Methyloparacoccus* and *Crenothrix* (Fig. 12b). Of all the locations, Allen marsh had the most bacterial species from the genus *Flavobacterium*.

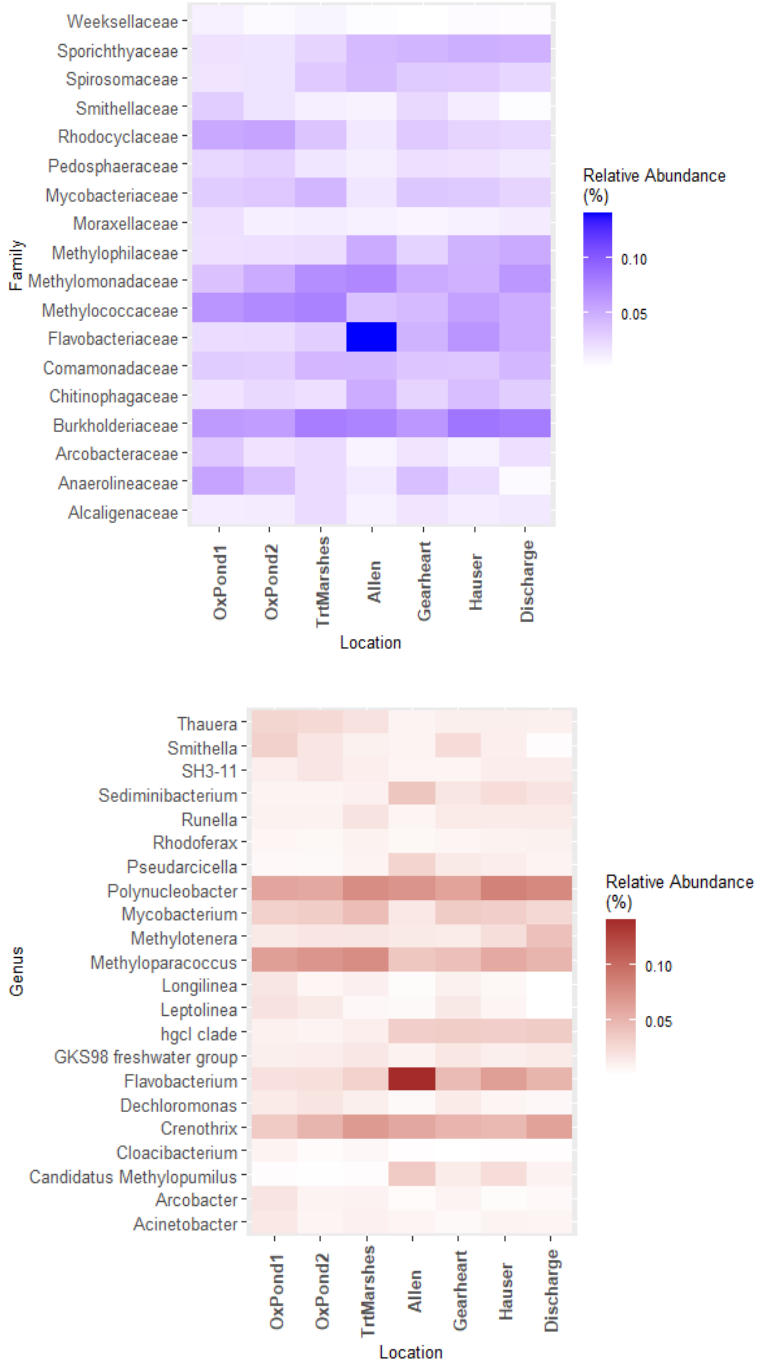

Figure 12. Heatmap displaying cluster analysis of the most abundant bacterial taxa in the microbial communities of the wastewater treatment wetland samples along the treatment train at (a) Family and (b) Genus taxonomic level. The color intensity in each panel shows the percentage in a sample, referring to the color key on the side.

Throughout this study, the most abundant taxa from the AWTF had distinct patterns in the wetlands and changed throughout the seasons, and I was interested in whether these findings could have implications for community function. I found that the most abundant taxa were associated in the literature with ecological functions that were either relevant to a specific portion of the nitrogen cycle or a wastewater bioremediation process (Table 2).

Table 2. Nitrogen cycle and bioremediation associated traits for the top six most abundant Bacterial Families.

Family	**Relevant Genus**	**Metabolic Traits**	**Nitrogen cycle**	**Wastewater Bioremediation**	**Reference**
Acrobacteraceae	*Salmonella* *Shigella* *Escherichia* *Enterobacter*	Facultative anaerobes	Nitrogen fixation	Fecal indicator	(Nordmann et al., 2011; Baylis et al., 2011)
Burkholderiaceae	*Cupriavidus* *Limnobacter* *Polynucleo-bacter*	Saprophytic Phyto-pathogen Opportunistic pathogens	Denitrification Competitive acetate assimilator	Ammonia removal	(Hetz & Horn, 2021)
Methylococcaceae	*Methyl-onomas* *Methylo-paracoccus*	Type-1 methano-trophs	NA	Methane oxidation	(Bowman et al., 2014)
Methylomonadaceae	*Methylospira*	Type-2 methanotrophs Anaerobic methane oxidation	NA	Methane oxidation	(Cabrol et al., 2020; Oshkin et al., 2019)
Rhodocyclaceae	*Azonexus* *Propion-ividbrio*	Wide-ranging	Denitrification Plant associated nitrogen fixers	Fermentation Biodegradation of organic compounds	(Oren et al., 2014)
Flavobacteriaceae	*Flavobacterium*	Diverse metabolic pathways and habitats	NA	Chemotrophic breakdown of organic molecules	(Bernardet et al., 2006)

Microcosm Experiment

In this laboratory-controlled microcosm experiment, I was interested in the effect of aeration on microbial diversity and its ammonia removal functionality, ultimately influencing the BEF relationship. In this section, I first evaluated the effect of aeration treatments on dissolved oxygen levels. Second, to account for the direct effect of oxygen on microbial community diversity and function, I assessed the effects of experimental treatments on diversity metrics and ammonia removal independently. Third, I established the indirect effect of aeration on the BEF relationship.

Below are the sections of this chapter that describe the relationships analyzed from the microcosms to test the effect of the different aeration regimes. Five microcosm tanks had to be removed from the analysis due to contamination of the tanks by insects and wildlife.

Treatment and Dissolved Oxygen

I found significant effects of aeration treatments type on DO concentration (df= 2, F-value = 22.176, p-value < 0.001). The homogeneous aeration treatment and the control group were the most different in DO concentration (p-value < 0.0002). The homogeneous and heterogenous aeration treatments were most similar in DO concentration but still significantly different (p-value < 0.04)(Fig. 13).

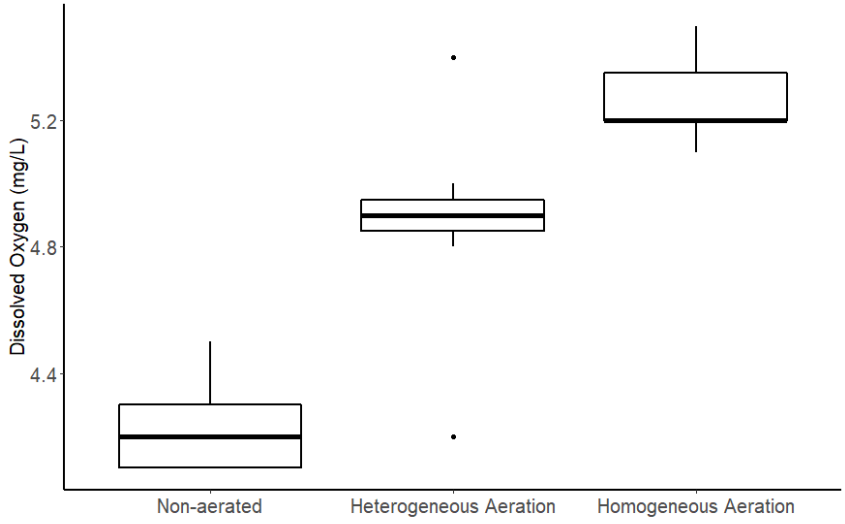

Figure 13. Boxplot showing the significant relationship between treatment and the dissolved oxygen concentration in each group.

Treatment and Diversity

Microbial species evenness was marginally significantly affected by the aeration treatment ($df = 2$, F-value = 3.54, p-value = 0.06) whereas richness was not impacted by the aeration treatment ($df = 2$, F-value = 2.48, p-value = 0.12) (Fig. 14).

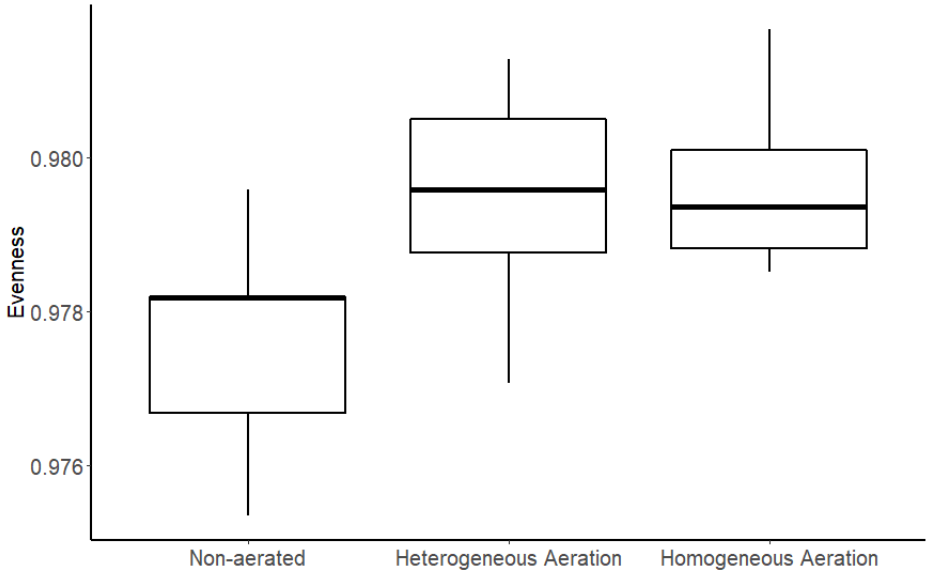

Figure 14. Boxplot showing the significant relationship between treatment and the microbial community evenness for each experimental group

Treatment and Nitrogen

The ammonia and nitrate ratio responded strongly to the treatment type (ammonia; $df = 2$, F-value = 22.17, p-value < 0.0001 and nitrate; $df = 2$, F-value = 67.38, p-value < 0.0001)(Fig. 15). The heterogeneous treatment had the higher change in ammonia concentration (p-value < 0.0001) (Fig. 15b).

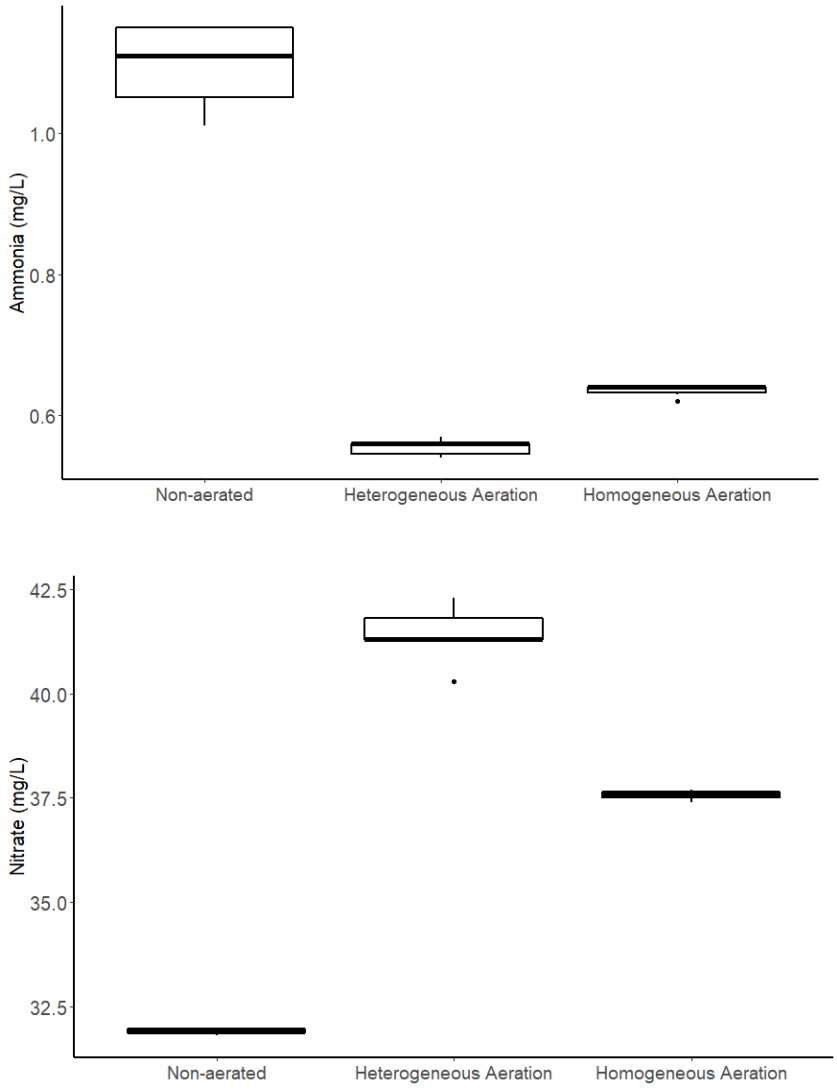

Figure 15. Boxplot showing the significant relationship between treatment and A) the ammonia concentration and B) the nitrate concentration of each experimental group

Effects of DO on Evenness, Ammonia and BEF

The DO concentration had a significant positive effect on microbial species evenness (GLM: $\chi^2 = 18.7$, $df = 1$, p-value < 0.001). Microbial community evenness peaked in the tanks from the homogeneous aeration treatment and was lowest in the

control group (Fig 16a). Comparatively, the relationship between dissolved oxygen and richness was slightly negative ($\chi^2 = 5.12$, $df = 1$, p-value = 0.02). This agreed with richness having the lowest value in the homogeneous treatment which had the highest DO concentration (Fig 16b).

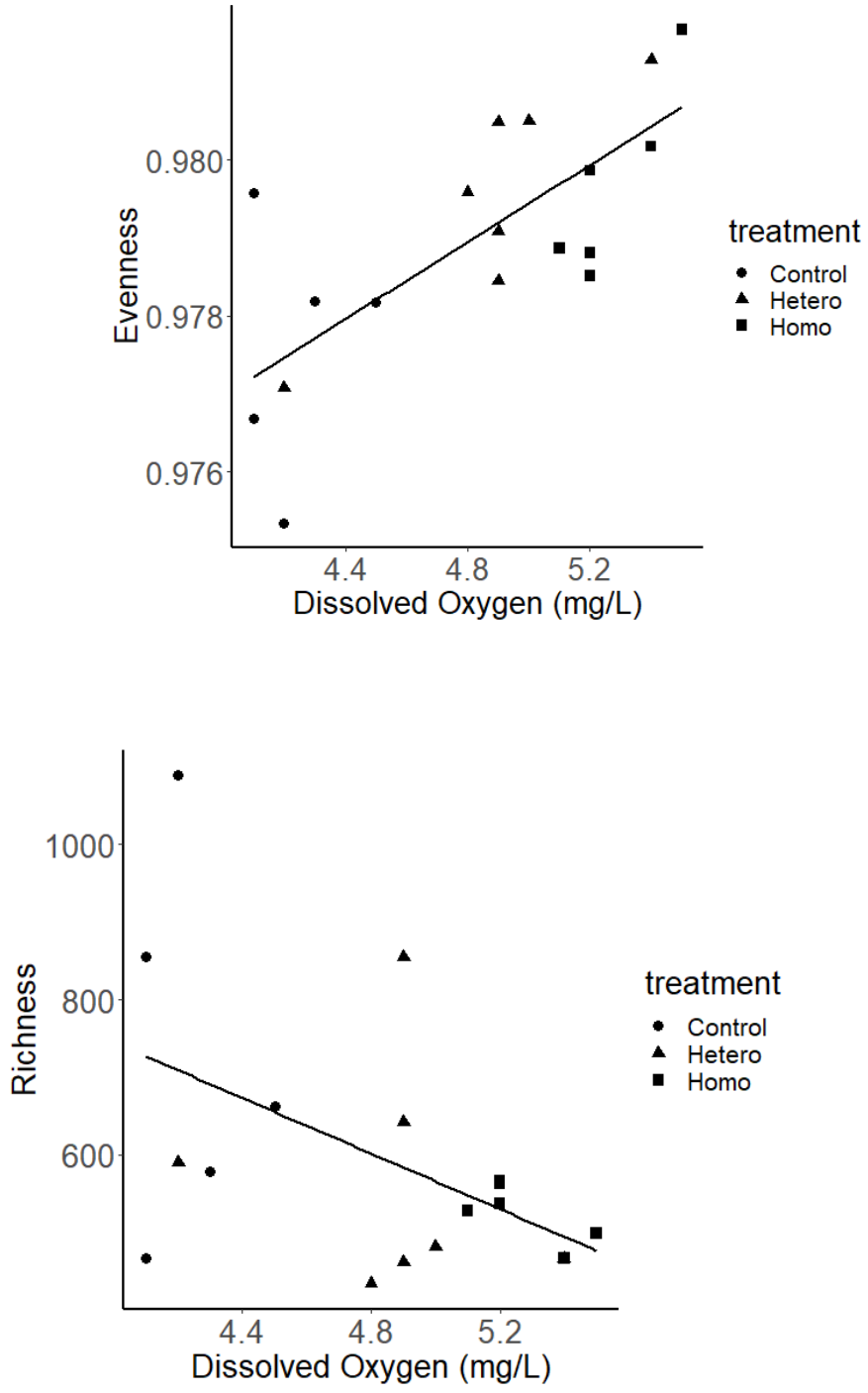

Figure 16. Linear model showing the direct relationship between dissolved oxygen concentration and A)the microbial community evenness and B) species richness

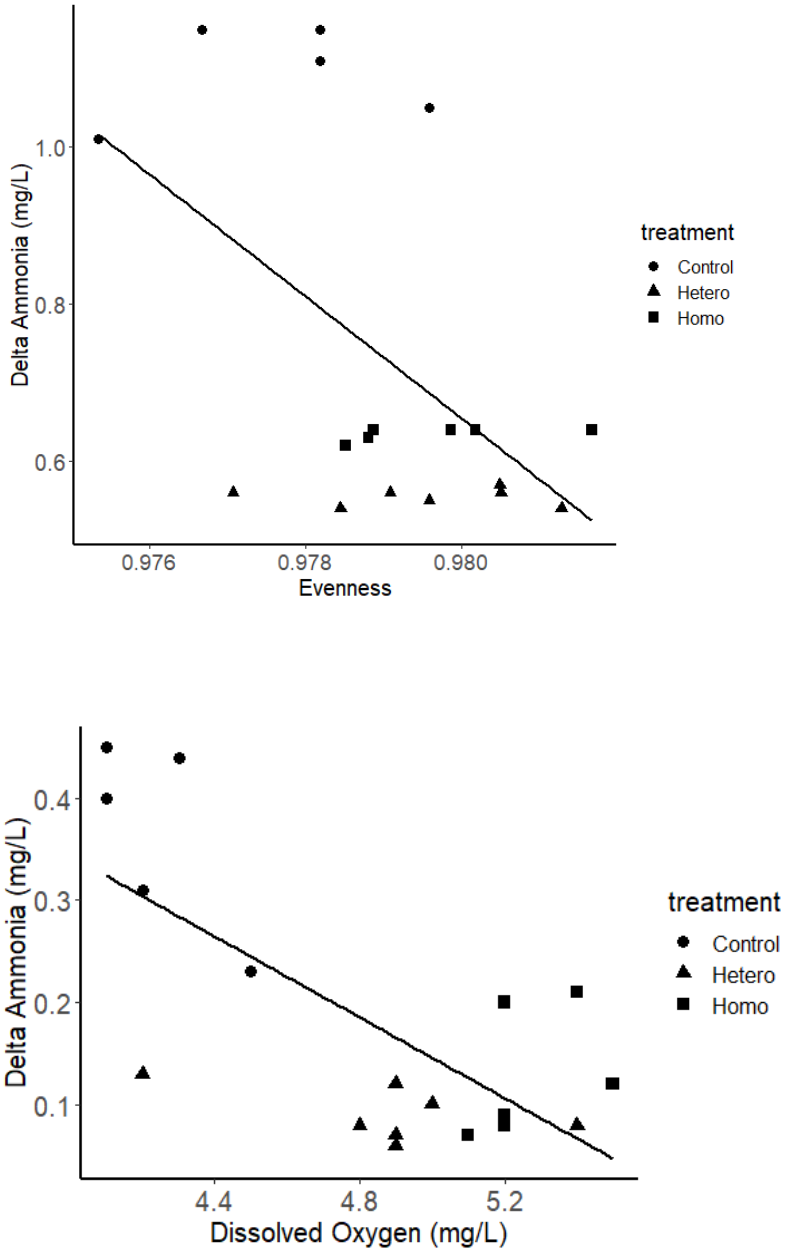

Figure 17. Linear model showing the significantly negative relationships between (a) species evenness and the ammonia and (b) DO and ammonia.

In the microcosm experiment, evenness had a negative effect on delta ammonia (GLM: $df = 1$, $\chi^2 = 6.21$, p-value < 0.01)(Fig. 17a). However, when DO was included in

the glm model the evenness did not have a significant effect on delta ammonia (GLM: χ^2 = 0.02, *df* = 1, *p*-value = 0.88). DO had a significant negative effect on delta ammonia (GLM: χ^2 = 7.38, *df* = 1, *p*-value = 0.006)(Fig. 17b). The negative BEF correlation indicated that a lower microbial community evenness was associated with a higher delta ammonia, yet this relationship may be mediated by oxygen levels (Fig. 17).

DISCUSSION

Throughout this study, I found significant negative BEF relationships between species richness and ecosystem function. This bears a striking contrast with previous studies suggesting positive BEF relationships, mostly in plant diversity and biomass studies (Marquard et al. 2009) and adds to studies demonstrating diverse alternative BEF relationships. For example, a recent study in Switzerland researching the effect of wastewater on stream microbial communities and ecosystem functioning showed a similar negative BEF relationship between richness and ecosystem function (Burdon et al., 2020). Integrating functional trait-based approaches to microbial community BEF research can increase our ability to analyze the connections between microbial diversity and ecosystem function more accurately (Krause et al.,2014). However, even as new research begins to include microbial communities in BEF studies, little has been done for microbial BEF relationships in wastewater wetlands. In the following paragraphs, I describe how location and season influenced diversity and ecological functions in the AWTF and how changes in community composition provide a glimpse into trait-based influences in microbial BEF relationships.

Location determined the ammonia concentration, but the location did not have a significant relationship with the microbial community diversity or composition. As the wastewater moves along the AWTF treatment train, we expected diversity and compositional trends to positively reflect the progressive reduction of ammonia concentration typically observed by AWTF operators. Contrary to these expectations, the lowest points of biodiversity were located after the disinfection steps in the treatment

train, indicating the strong influence of chlorination on microbial communities (Murray et al., 1984). The lack of relationship between location and diversity suggests that other factors besides microbial community richness and evenness are reducing the ammonia along the treatment train. For example, the proven water purification capability of wetlands is often attributed to a complex mechanism of plants and microbes (Brisson et al., 2020). Therefore, there are variables that change between the sampling points which were not measured in this project. A comprehensive analysis of the microbe-plant interaction would likely yield more complex results.

Seasons had a slight effect on microbial diversity throughout the wetlands. This study captures the shift from dry to wet season and provides the environmental change needed to assess seasonal impacts on the microbial community diversity and function at the AWTF. The findings support the hypothesis that the microbial diversity and composition changes based on seasonality which alters the growth of certain microorganisms by providing favorable conditions for niches to be filled by specific taxa. The most abundant Family throughout the seasons at the AWTF was *Burkholderiaceae* which decreases slightly from Autumn to Spring. The bacteria from this Family are competitive acetate assimilators during complete denitrification (Hetz & Horn, 2021). Following *Burkholderiaceae*, the Family *Methylococcaceae* was most abundant in Spring and functions as a methanotroph in aquatic systems. *Methylococcaceae* along with other methanotrophic taxa can reduce the methane flux to the atmosphere by 90 percent via methane oxidation in the surface of wetlands (Dedysh et al., 1998). Finding a consistently

high abundance of both Families at the AWTF across seasons indicates these wetlands are harboring the bacterial capacity to perform effective nutrient cycling.

The seasons significantly affected the ASV relative abundance, but across locations the only shift in relative abundance for microbial communities occurred in Allen Marsh. In Allen Marsh there was a significant decrease of bacteria from the Genus *Flavobacterium* and Genus *Mycobacterium* from Autumn to Winter and these taxa increased again in Spring. The Genus *Flavobacterium* is metabolically diverse with some species categorized as chemoheterotrophs that participate in mineralizing various types of organic matter such as carbohydrates, proteins and amino acids in aquatic ecosystems (Bernardet et al., 2006). The Genus *Mycobacterium* is a common human pathogen found in wastewater and requires chlorination treatment procedures to be removed from the wastewater (Le Dantec et al., 2002 & Radomski et al., 2011). Fluctuations in taxa that are metabolically relevant suggests that understanding the effectiveness of the AWTF at removing toxins may require careful monitoring of microbial dynamics in Allen Marsh.

While the broad goals of the current study preclude a clear explanation behind these unique microbial community dynamics within Allen Marsh, here I propose a hypothesis that may guide future studies disentangling this issue. Because Allen Marsh is the first of the three Enhancement Wetlands which immediately follows a disinfection step, the effect of chlorination on the microbial communities in the wastewater entering Allen Marsh may be causing the lower richness in this location. This effect could be weaker in Winter when the AWTF experiences the highest flow rates due to more precipitation and can dilute the impact of the chlorination. Whether these dynamics can

have future consequences for the whole system seems unlikely, due to the stability of other Enhancement Wetlands, yet should be not fully discarded as a monitoring goal.

In the microcosm experiment, species evenness had a negative correlation with ammonia removal indicating that the most functionally efficient communities were dominated by few species. Interestingly, a similar wastewater microcosm study found the opposite trend between microbial species evenness and denitrification: a positive BEF relationship. They concluded that microbial communities with high evenness were more resilient to stress, and when there was dominance by a few species there was lower denitrification efficiency by the microbes in the wastewater (Wittebolle at al., 2009). This discrepancy may result from differences in nitrifying and denitrifying gene abundances in the microbial communities studied.

The results from my microcosm experiment showed a negative DO-richness relationship. As DO increases the number of bacterial species decreases and the microcosm is regulated by a few species. This concurs with how additional energy availability in high oxygen environments leads to the competitive advantage of a few species (Yadav et al. 2014). Since I directly manipulated aeration, the only direct relationships are the effects of DO concentration on nitrification and evenness. An experimental manipulation of both initial richness and evenness in the microcosms would lead to clearer cause-effect results for the BEF relationships in the context of oxygen availability. Alternatively, quantitative polymerase chain reaction (qPCR) could be used to quantify the nitrifying and denitrifying gene abundance in the microbial populations of the microcosms to yield direct BEF relationships between diversity and delta ammonia

(Zhang et al., 2014). Although the BEF results are only correlational, this experiment indicates that BEF relationships are affected by DO and supports the findings of the negative BEF relationship in the field study.

This research was a foundational step towards understanding the bacterial interactions throughout the AWTF and identifying the general patterns between location, seasonality, ammonia removal and microbial diversity. The negative BEF relationship further supports the need for expanding the scope of field-based studies to fully capture the ways in which microbial community diversity interacts with ecosystem functioning. Furthermore, my findings suggest that BEF relationships can dynamically change in space and time, since locality influence function and seasons influence diversity. Moving forward, BEF studies should aim to generalize which ecological processes can best promote or disrupt diversity and function relationships.

The results of this study inform the AWTF that the dominance of a few bacterial taxa may increase the ammonia removal capabilities of the natural treatment system. My project generated new questions that open the door for future studies that address issues like (1) identifying the specific taxa driving nitrogen cycling and the mechanism behind functional decrease in diverse communities (via co-occurrence network analyses or competitive assays), (2) diversity relationships with nitrification and denitrification gene abundance (via qPCR assays), or (3) the role of biosolid microbial communities in transforming and storing waste (DNA extractions are available for further analyses). Additionally, my findings highlight that Allen Marsh should be further investigated to explain its significantly lower species richness and distinct responses to seasonal changes

and its consequences for the efficiency of the AWTF in the long term. Importantly, this research can be applied to other sections of the nitrogen cycle in aquatic systems, as well as other biogeochemical cycles and terrestrial systems, with the objective to understand the role of microbial communities in driving the Earth's ecosystems. Overall, I hope to contribute to our understanding of local and global systems by strengthening our framework to predict and manage microbial functions for ecosystem resiliency.

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APPENDIX

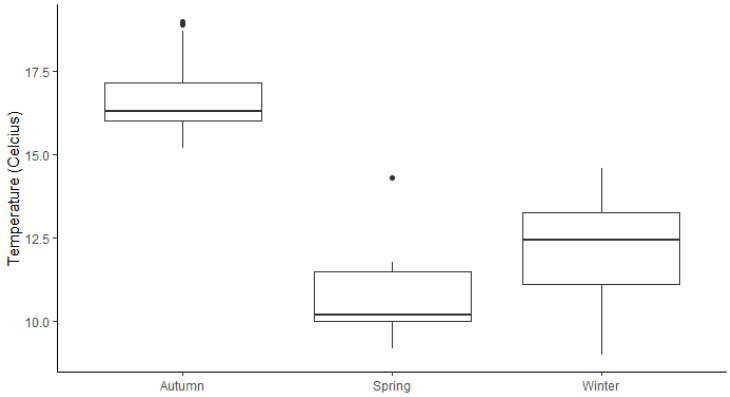

Figure A-1. Water temperature changes across seasons in degrees Celsius (GLM; $X^2 = 228$, DF = 2, p-value < 0.001).

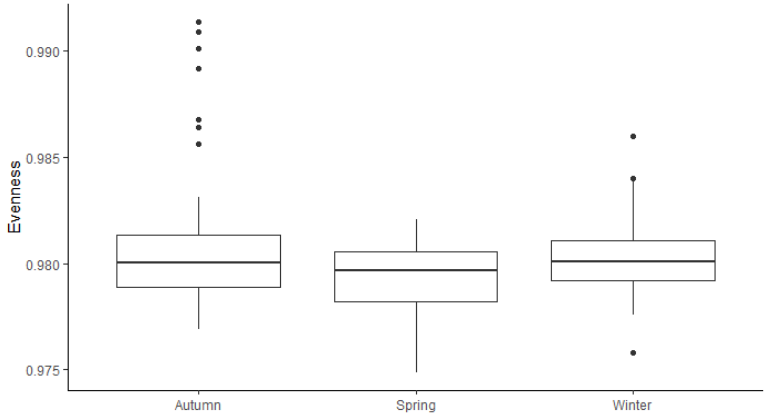

Figure A-2. Boxplot of the effects of seasonality on the species Evenness. (ANOVA; DF 2, F value= 2.32, p value> 0.1)

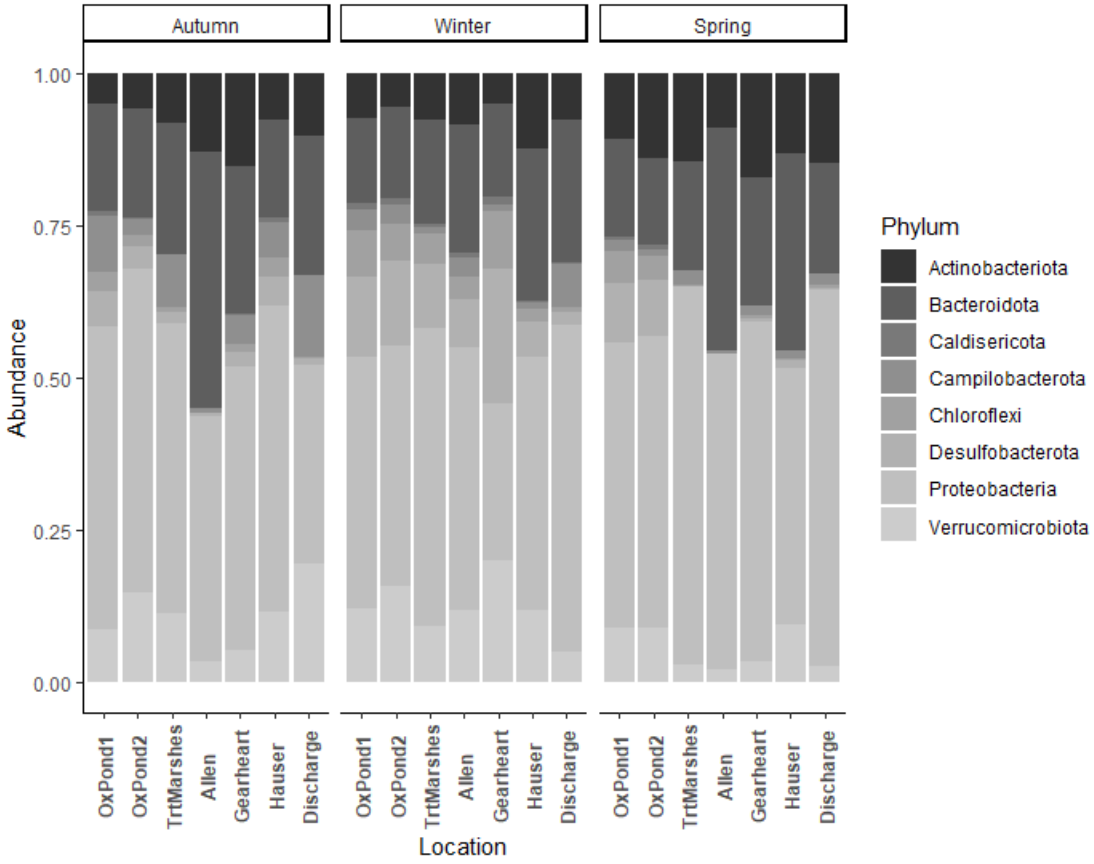

Figure A-3. Relative abundance of bacteria at Phylum level in aqueous samples.

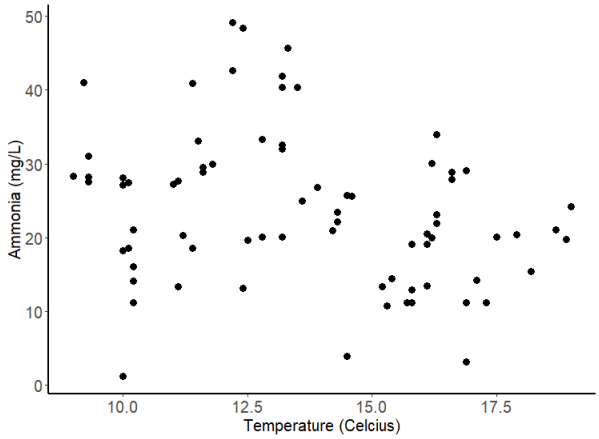

Figure A-4. Scatter plot of temperature and ammonia concentration.

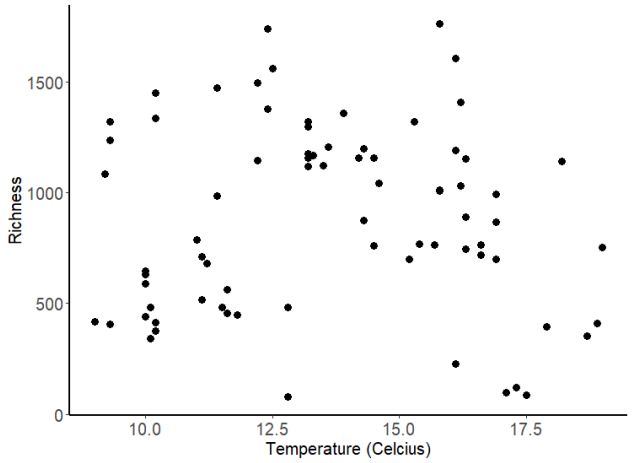

Figure A-5. Scatter plot of temperature and species richness.

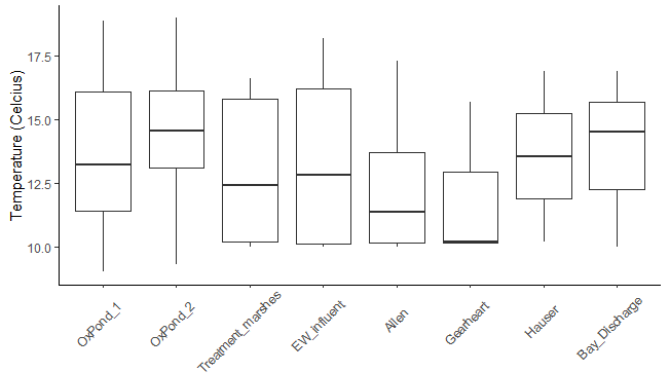

Figure A-6. Distribution of temperature based on locality (ANOVA; F-value = 4.3, DF = 7, p-value > 0.8).

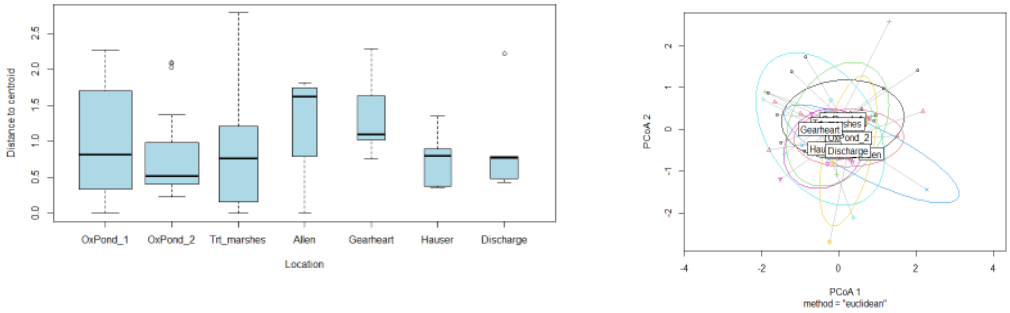

Figure A-7. Bray-Curtis dissimilarity plot shows the microbial community composition does not differ significantly between each site. (PERMANOVA; DF 6, F-value = 0.7202, p-value = 0.634)