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A MULTI-TAXA METAGENOMIC EVALUATION OF THE EVERGLADES SOIL MICROBIOME AND THE IMPACT OF SALINITY ON COMMUNITY STRUCTURE AND BIOGEOCHEMICAL CYCLES WITH A SOIL FORENSIC APPLICATION

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Deidra Christina Jordan

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To: Dean Michael R. Heithaus
College of Arts, Sciences and Education

This dissertation, written by Deidra Christina Jordan, and entitled A Multi-Taxa Metagenomic Evaluation of the Everglades Soil Microbiome and the Impact of Salinity on Community Structure and Biogeochemical Cycles with a Soil Forensic Application, having been approved in respect to style and intellectual content, is referred to you for judgment.

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Florida International University, 2022

DEDICATION

I dedicate this dissertation to my parents, Leroy and Darlene Jordan. Thank you for always supporting me. I couldn't have done this without you.

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ABSTRACT OF THE DISSERTATION

A MULTI-TAXA METAGENOMIC EVALUATION OF THE EVERGLADES SOIL MICROBIOME AND THE IMPACT OF SALINITY ON COMMUNITY STRUCTURE AND BIOGEOCHEMICAL CYCLES WITH A SOIL FORENSIC APPLICATION

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Coastal wetlands, such as the Everglades, are increasingly being exposed to stressors that have the potential to modify their existing ecological processes because of global climate change. Their soil microbiomes include a population of organisms that are important for biogeochemical cycling, but continual stresses can disturb the community's composition, causing functional changes. The Everglades features wetlands with varied salinity levels, implying that they contain microbial communities with a variety of salt tolerances and microbial functions. Therefore, it's critical to track the effects of stresses on these populations in both freshwater and brackish marshes. The study addressed this by first constructing a baseline microbial community, then analyzing taxonomic alterations that happened after a long-term disturbance like seawater intrusion, and then determining the soil characteristics that most contributed to the difference in soil communities.

To estimate the microbiome’s diversity, two standard genetic study approaches were compared in Chapter 2. Length Heterogeneity-PCR (LH-PCR) was used to detect

the initial diversity, and Next Generation Sequencing (NGS) was used to detect the taxonomic diversity. The Archaea and bacteria populations were examined. With both techniques, it was determined that each location had a distinct bacterial and Archaeal community distribution. Changes in relative abundance of taxonomies before and after experimental saltwater treatment revealed that various taxa react to stresses in different ways, providing insight into how communities are responding.

By analyzing taxonomic alterations, Chapter 3 defined the microbial communities participating in important biogeochemical cycles and evaluated how their functional potential altered following long-term salt inputs. The nitrogen, carbon, sulfur, and phosphorus cycles were studied by sequencing a microbial functional gene involved in each of these processes. The relationships between the observed soil physiochemical properties and microbial composition before and after saltwater treatment were investigated in Chapter 4. Once saltwater was introduced, changes in the properties responsible for alterations in community variation were observed. The microbiome's value for ecological and forensic purposes was also investigated, specifying its enormous potential. These findings add to the understanding of microbiomes by demonstrating how changes in soil qualities impact communities both before and after a disturbance such as saltwater intrusion.

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CHAPTER 1

1. Introduction

1.1. Saltwater intrusion and its influence in the Florida Coastal Everglades

Coastal wetlands, such as the Everglades, provide many valuable ecological and economical functions, including filtering and storing water, buffering the inland from storms, cycling nutrients, and storing carbon (Bodelier & Dedysh, 2013; Chambers et al., 2013; Servais, Kominoski, Davis, et al., 2019). However, global climate change is rapidly introducing stressors into coastal wetlands with the potential to degrade and threaten their current ecosystem functions (Green et al., 2017). As a result, coastal freshwater and brackish wetlands are increasingly being exposed to saltwater intrusion. This saltwater initially acts as a subsidy to plant growth by supplying newly available phosphorus and stimulating soil microbial processes. But long-term exposure can ultimately become a stressor on that environment, causing osmotic stress on both microbes and plants (Servais, Kominoski, Charles, et al., 2019; Wilson et al., 2019). Microbial communities drive the critical biogeochemical cycles, but these communities can differ between marine and freshwater environments. Thus, a perturbation such as saltwater would likely have a more significant impact on freshwater than brackish ecosystems, its overall function and could even change the environment permanently.

Vegetation, microbial communities, and microbially-mediated metabolic reactions can also be immensely affected by saltwater intrusion as microbes are the first responders to changes in the environment, even before plants, because they quickly respond to the availability of nutrients and environmental stressors (Chambers et al., 2016; Jacoby et al., 2017; Servais, Kominoski, Charles, et al., 2019). Saltwater also introduces sulfate

allowing for the shift and predominance of sulfate-reducing bacteria in freshwater sediments. A byproduct from these bacteria is hydrogen sulfide (HS^-) which acts as a phytotoxin when in excess. The saltwater osmotic stress and the accrual of HS^- in soil porewater lead to plant mortality (Chambers et al., 2015). The decreasing diversity or abundance of the vegetation could directly disrupt the feedback mechanisms that occur between the plant and microbes, leading to a holistic decrease in ecosystem composition and function (Morrissey et al., 2014). One study that examined the microbial community changes from sediment in the Everglades along a salinity gradient indicated that the community's diversity was similar. Still, even when sequenced with Sanger sequencing, the identity of the microbes significantly differed, further highlighting the importance of microbial identification, specifically for distinguishing perturbation impacts (Ikenaga et al., 2010).

Several factors have been analyzed in the Everglades, such as enzymatic responses, microbial respiration, porewater chemistry, and biomass carbon changes (Servais, Kominoski, Charles, et al., 2019). However, understanding how microbial communities are shifting and contributing to nutrient cycling after saltwater intrusion is uncertain. Microbial function can be described by microbially-mediated reactions dependent upon the availability of electron acceptors and donors. Salinity alters soil chemistry by increasing the ionic strength and conductivity, causing microbes that are not capable of osmoregulation to disrupt metabolic functions or even experience cell lysis (Oren, 2008). Microbes capable of osmoregulation can often adapt to this new environmental impact by utilizing different biogeochemical metabolic nutrient cycling. The biogeochemistry can be examined by extracellular enzyme measurements or by

studying the intrinsic functional genes present in the microbiome. While enzymatic activity tracks changes in the enzyme concentrations released by the microbes as a response to the environment (Servais, Kominoski, Charles, et al., 2019; Servais et al., 2020), functional gene composition and diversity provides a view into the metabolic potential of the biotic community's ecological function in the environment and its potential to adapt to a rapidly changing environment (Wang et al., 2017). Maintaining an abundant and diverse soil microbiome is essential to maintain the vital functions necessary to cycle nutrients and protect soil health in the Everglades. ***Therefore, the objective of my dissertation is to examine the soil microbiome from Everglades soils to deduce information about the microbial communities and how they are responding to saltwater stressors.***

1.2. Soil biota, their role in biogeochemical cycles, and changes due to saltwater intrusion

Microorganisms such as bacteria, fungi, and Archaea, are known for their critical roles in cycling nutrients and minerals and mediating biogeochemical cycles in all ecosystems. In this dissertation, I explored the intrinsic metabolic potential of microorganisms and their role in the nitrogen, carbon, sulfur, and phosphorus cycles by examining functional genes encoding for the components of the respective enzymes– Nitrite reductase (*nirS*), Methyl-coenzyme M reductase subunit alpha (*mcrA*), Dissimilatory sulfite reductase subunit alpha (*dsrA*), and Alkaline phosphatase (*phoD*).

The nitrogen cycle is one biogeochemical cycle where bacteria have vital roles. Nitrogen is a necessary nutrient for all forms of life to synthesize biomolecules important for growth and can even be used as a source of energy by some microbes (Albright et al.,

2019). Although nitrogen is the most abundant molecule in the atmosphere and very abundant within the pedosphere, plants cannot use it in its common inorganic form, dinitrogen (N_2). Therefore, a process known as nitrogen fixation must occur. Microbial biological nitrogen fixation reduces atmospheric nitrogen (N_2) to ammonia (NH_3), which biota can directly take up for building proteins. Often nitrogen fixation is done in a symbiotic relationship in the rhizosphere between the plant and the bacteria living in its roots (Mus et al., 2016). Nitrification, a two-step process, converts ammonia to nitrites (NO_2^-) and then to nitrates (NO_3^-) which are assimilated by plants, while denitrifying bacteria convert these excess products back to nitrogen gas. Removal of excess nitrogen is imperative in freshwater wetlands, but previous studies have shown that saltwater intrusion decreases rates of nitrification and denitrification, suggesting a shift in microbial community composition, or suppression of functions or both, that significantly affects water quality (Neubauer et al., 2018; Xie et al., 2020). Several functional genes are involved within the denitrification pathway, but *nirS* can be found in over 70% of known denitrifiers, making it an ideal candidate to uncover the diversity and composition of this community as it adapts to perturbations (Gu et al., 2019).

In coastal wetlands, anaerobic soil conditions cause slow decomposition and biogeochemical processing rates, allowing organic matter to build up and soils to store the carbon. Within the first step of the carbon cycle, bacteria and fungi break down organic matter releasing complex carbon compounds into the soil for storage. Some of that carbon is cycled and eventually released as carbon dioxide, CO_2, or methane, CH_4 (Galagan, 2002). Elevated salinity can cause several ecosystem and microbial community modifications stemming from habitat changes in ionic strength, alkalization, and

sulfidation (Tully et al., 2019). Studies have shown saltwater decreases the amount of stored soil carbon, suggesting increased microbial respiration rates (Morrissey et al., 2014; Servais, Kominoski, Charles, et al., 2019; Servais et al., 2020). However, investigating the functions of microbes by measuring extracellular enzyme activity has shown both increases and decreases of enzymatic activity exhibiting the sensitivity of these communities to saltwater intrusion (Morrissey et al., 2014; Servais, Kominoski, Charles, et al., 2019).

Previously, it was thought that the only microorganisms contributing to biogeochemical cycles were bacteria and Eukarya. However, the expanded usage of molecular biology techniques leading to the discovery of Archaea, revealed their critical roles within these cycles (Offre et al., 2013). Archaea contribute to the carbon, sulfur, and nitrogen cycles and are abundant in various ecosystems such as soil and marine waters (Anantharaman et al., 2018; Liu & Whitman, 2008; Treusch et al., 2005). One pathway where Archaea presence dominates is methanogenesis, a pathway within the carbon cycle that produces CH_4. Methane, a well-known greenhouse gas, is produced from the reduction of a one-carbon compound such as CO_2. In soil, the CO_2 along with hydrogen, H, is reduced to CH_4 under anoxic conditions. Mediated by methanogens, this process is critical in decomposing organic matter (Galagan, 2002). However, the addition of saltwater brings high levels of sulfate, SO_4^{2-}, and sulfate-reducing bacteria can outcompete methanogens for essential metabolic substrates, thus suppressing methanogenesis (Ember M. Morrissey, 2015). Furthermore, a byproduct of sulfate reduction is HS, which slows plant growth and inhibits nitrification, disrupting the nitrogen cycle (Chambers et al., 2015; Tully et al., 2019). To detect the effect that

saltwater intrusion has on methanogens and sulfate reducers, the *mcrA* and *dsrA* genes, respectively, were evaluated (Morris et al., 2014; Sela-Adler et al., 2017).

Phosphorus, an essential nutrient for plant growth, is a limiting nutrient in the Everglades. Microbes such as fungi and bacteria are prevalent in its acquisition and cycling by releasing phosphatases when the presence of orthophosphates is low (Megonigal & Neubauer, 2009). But saltwater intrusion can increase orthophosphate levels when the saltwater penetrates limestone bedrock, releasing the nutrient into the ecosystem. An increase in this bioavailability would infer suppression of the microbial activity (Flower et al., 2017). The *phoD* gene is widely found within both terrestrial and aquatic environments. It has even been found to shift microbial taxonomic composition and diversity with long-term additions of phosphorous fertilization, making it an ideal marker to explore the role saltwater intrusion has on microbes and phosphorus cycling (Ragot et al., 2017).

By beginning to uncover the soil's microbiome, we can identify ways to monitor soil health and understand how ecosystems respond to climate change and other perturbations (Bardgett & Caruso, 2020; Fierer, 2017). For example, long-term exposure to saltwater in freshwater coastal wetlands can accelerate decomposition leading to peat collapse and dramatically changing the water quality of a vital ecosystem. Assessing the soil microbiome only strengthens the holistic information about soil health, biotic and abiotic diversity, and even the soil uniqueness that can have a forensic or intelligence application. Therefore, understanding the shifts in diversity and function of these soil microorganisms due to saltwater intrusion is imperative. Hence, the impact of global warming on microbial communities must be recognized.

1.3. Evaluation of microbial communities with genetic analyses

It is well known that microbes are drivers of the biogeochemical cycles, so investigating the functional genes responsible for these processes can give insight into nutrient cycling potential within these communities and the ability to quickly respond to perturbations (Pereyra et al., 2010). Microbes are the "first responders" to disturbances within their environment that disrupt these processes. By monitoring the impact on the organisms and the community, information can be obtained about the condition of that particular environment (Chambers et al., 2016; Jacoby et al., 2017; Servais, Kominoski, Charles, et al., 2019). The utilization of molecular microbial ecology techniques to study how the organismal genomes and genes change can provide a better understanding of their responses to environmental factors is termed ecogenomics (Chapman et al., 2006).

Previously, exploring the microbiome diversity within various environments has relied on either morphological or culturable microbes' characteristics (Allwood et al., 2020). It is estimated that the culturable microorganisms only represent a small portion of the microbial diversity in any environment. As a result, culture-independent molecular methods have been adopted to explore these diverse communities (Saenz et al., 2019). By extracting the DNA from environmental samples and amplifying specific DNA sequences of interest universal to taxa, culturable and non-culturable microorganisms can be identified. For bacteria and Archaea, the 16S ribosomal RNA gene is commonly targeted due to having nine hypervariable domains within genes that can be associated with microbial taxonomic identification (Clarridge, 2004). Amplifying and subsequent sequencing of these regions can provide taxonomic affiliations.

Common genetic analysis methods involve using the polymerase chain reaction (PCR) to create DNA fragments of different sizes and electrophoresis to separate the fragments based on the amplicon's base pair number/length. Previous methodologies utilized in environmental analyses involved gel or capillary electrophoresis such as denaturing gradient gel electrophoresis (DGGE) and length heterogeneity-PCR (LH-PCR) (Damaso et al., 2018; Guevara et al., 2014; Ikenaga et al., 2010; Moreno et al., 2006). These methods have played an integral role in identifying microbial patterns based on their DNA fragment length but cannot be directly linked to specific taxa. For example, when using universal primer sets and analyzing length heterogeneity, one amplicon of a specific nucleotide length in a profile could represent different taxa, masking the true taxonomic diversity within a community. Other methods, single-strand conformational polymorphism (SSCP), or various sequencing analyses can elucidate more detailed genomic information (Damaso et al., 2014; Young et al., 2014). These methods provide inexpensive and rapid ways to monitor gross changes in any environmental sample and are helpful screening tools before moving to more complex and expensive technologies such as Next Generation Sequencing (NGS).

The newest genetic technology developed is Massively Parallel Sequencing (MPS) or more commonly known as Next-Generation Sequencing (NGS). NGS allows clusters of genomic regions to be sequenced in one analysis using PCR, adapters, oligonucleotides, and a flow cell (a glass slide with lanes) (Ansorge, 2009). This technology can sequence thousands of genomic regions simultaneously, allowing for whole-genome or metagenomic sequencing (Gettings et al., 2016). Since NGS is a high throughput technology, it is attractive for soil analyses where sequencing these

communities will provide much better information than conventional methodologies, leading to identifying non-culturable species while simultaneously identifying nondominant groups or species.

1.4. Soil Forensics

Soil is easily transferable therefore making its abiotic and biotic information viable forensic evidence. Historically, geological analyses have been utilized to identify the elemental composition of a soil sample and can be helpful in forensics (Siegal, 2016). However, in some instances where the soil may be similar over a large area, the use of mineralogy may not be very discriminative (Giampaoli et al., 2014). It is known that soil supports thousands of different microbes unique to each habitat, known as the "soil's microbiome" (Jansson & Hofmockel, 2018). Elucidating the inhabitants of the soil and their particular function must be investigated to identify their significance to the soil and establish that most important forensic element--uniqueness. Recent studies have begun to assess the microbial communities within the soil that may provide better or additional discrimination to the elemental and geological aspects of soil (Damaso et al., 2018; Giampaoli et al., 2014; Habtom et al., 2017; Moreno et al., 2006). A study was even able to prove the utility of machine learning algorithms to correlate biotic composition and geographic location, therefore establishing provenance (Damaso et al., 2018). Research involving genetic analyses combined with bioinformatic techniques to assess soil's inhabitants and classify them by soil type or origin will lead to advances in the forensic community resulting in more accurate and discriminatory methods in soil forensics.

1.5. Experimental overview and specific statement of research objectives and hypotheses

Samples will come from archived soils from a previous Everglades study (Servais et al., 2020). Freshwater and brackish marshes within the Everglades National Park, Florida, USA, were chosen for a field saltwater dosing experiment. At each site, 12 polycarbonate chambers, mesocosms, were installed along a boardwalk for a total of 24 chambers. Within each site, six chambers were designated as ambient controls, and six were designated as saltwater treatments. The experiment began in October 2014 and lasted until November 2016. Treatment chambers were dosed with monthly pulses of artificial seawater (Instant Ocean) mixed with water from the study site (Atkinson & Bingman, 1997). Porewater was collected from each chamber 24 h after each dosing, and the physiochemistry was measured. Soil cores collected in 2014 and 2016 were used for DNA extractions in my study (Servais et al., 2020).

A multi taxa approach—Archaea and bacteria-- is analogous to DNA typing methods applied in human identity testing. Concatenation of the alleles at the different loci creates a unique profile with a high power of discrimination for human forensic identification. The multi taxa approach presented here makes a unique metagenomic profile for each soil sample. Microorganisms play a vital role in the biogeochemical cycles that are essential for nutrient cycling and soil health. Investigating the functional genomic diversity is imperative in understanding how the microbes interact with nutrients and perturbations of their environment. Functional gene data, combined with the taxonomic data, can confer even greater uniqueness to a sample that not only provides

ecological knowledge but will also provide provenance of soil that can be used in intelligence or forensic cases.

This dissertation is divided into the following chapters.

Chapter 2 tests the *hypothesis that saltwater intrusion, simulated by saltwater dosage experiments on freshwater and brackish soils, will not impact the microbial community structure at the taxonomic level.* This hypothesis was tested by first screening the diversity of the bacteria and Archaea communities with LH-PCR. Next, these microbial communities were sequenced with NGS to characterize the changes in the composition of microbes within the soil sites after treatment.

Chapter 3 investigates the *hypothesis that the microbial community's functional potential in soil microbiomes adapted to brackish environments will not change with salinity dosage experiments compared to those in freshwater environments.* Microbial functional genes and their diversity were evaluated to differentiate between sites and assess the impact of salinity treatments on critical functional genes and biogeochemical cycles. Functional genes that were assessed are the *nirS*, *mcrA*, *dsrA*, and *phoD* genes.

In chapter 4, two hypotheses are tested. The first *hypothesis states that combining the taxonomic and functional gene information along with measurements from porewater chemistry will determine that there is no correlation between salinity dosage experiments and shifts within the microbiome.* This was examined by combining the biotic and abiotic data to better assess the driving factors and consequences of the environmental impact of sea-level rise on the whole community. Additionally, the potential of adapting this research to increase forensic utility to determine soil provenance was introduced. This *hypothesis states that bioinformatic algorithms of taxonomic and functional gene*

information will not provide discriminatory provenance between brackish and freshwater environments. The combination of these data types provides biogeographical patterns, therefore predicting the provenance of the soil for forensic and intelligence information.

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CHAPTER 2

2. Characterizing microbial community composition after simulated saltwater intrusion experiments in freshwater and brackish marshes

2.1. Introduction

The movement of seawater towards land and into freshwater, saltwater intrusion, is becoming an increasing issue within tidal wetlands like the Everglades. This inward movement can have detrimental effects on a coastal freshwater wetland, such as the loss or inland movement of a marsh, eutrophication, and habitat degradation (Tully et al., 2019). It can also impact the water quality for human consumption as the shallow aquifers located near the coastline are used as freshwater sources for populations within these areas (Kummu et al., 2016). Increased salinization, caused by saltwater intrusion or other factors, is also known to affect soil quality, alter plant composition, and decrease overall species diversity. To measure these increased salinization effects on the environment, biogeochemical factors, i.e., dissolved organic carbon (DOC), nitrogen, and pH, just to name a few, are commonly measured. However, microbial communities responsible for cycling nutrients and mediating biogeochemical cycles are also altered, but these shifts are not as well studied (Chambers et al., 2016; Zhang et al., 2019). Driven by climate change consequences such as increased frequency and intensity of hurricanes and sea levels rising, pushing saltwater inland, there becomes a more urgent need for monitoring microbes in these environments (Herbert et al., 2015). In a consensus statement about climate change, microorganisms were highlighted in their roles in the production and consumption of greenhouse gases, but the importance of monitoring how

they will be affected by climate change was also emphasized (Cavicchioli et al., 2019). Studying the soil microbiome is a highly efficient way of monitoring how microorganisms will be affected and will provide critical data to incorporate into climate models.

The soil microbiome community structure is not only diverse but also dynamic due to the microbes' sensitivity to microscale environmental conditions that trigger immediate microbial responses. Shifts in nutrient availability with disturbances such as saltwater intrusion has been shown to alter the microbial community composition within wetlands (Dang et al., 2019; Neubauer et al., 2018). The slow rate of organic matter breakdown and anaerobic conditions within wetlands allow for large amounts of carbon storage. However, the recent loss of soil elevation within the Everglades, likely a result of saltwater intrusion and sea-level rise, has caused an increase in plant matter input and accelerated microbial breakdown (Chambers et al., 2015; Chambers et al., 2019). As a result, a concern for climate scientists is that the soil microorganisms could mineralize this stored carbon and contribute further to greenhouse gas emissions (Cavicchioli et al., 2019; Jansson & Hofmockel, 2020).

Sulfate reducers and methanogens prefer the common substrates- acetate and hydrogen and are known to compete when these microbial groups co-exist in the same environment. Therefore, measuring sulfate and methane concentrations can lead to inferences about the rate of each process and how these microorganisms compete for substrates in varying environments. The outcompeting microorganism, whether it is a sulfate reducer or a methanogen, can be the difference between the production of carbon dioxide or the more potent greenhouse gas, methane (Sela-Adler et al., 2017). Previous

studies that monitored methane production after salinity manipulation experiments have shown that hydrogenotrophic sulfate reducers outcompete hydrogenotrophic methanogens for the same substrates, so the less competitive methylotrophic methanogens were expected to be favored in sulfate-rich marine environments (Liu & Whitman, 2008; Oremland & Polcin, 1982). However, newer research, supported by next-generation sequencing, has found methanogens within taxonomic groups involved in hydrogenotrophic methanogenesis (*Methanomicrobia, Methanocellales, and Methanomicrobiales*) are favored in sulfate-rich environments, although they are known to directly compete with these sulfate reducers (Dang et al., 2019). In this example, the dominant pathway, evidenced by higher abundance of either hydrogenotrophic or methylotrophic methanogens, can provide insight into the availability of substrates, environmental conditions, and provide knowledge useful for mechanistic climate models (Conrad, 2020). This contrast in microbial taxonomic composition within methanogenesis further highlights the importance of identifying microbes, dependent upon the availability of varying substrates within the soil microbiome, and how saltwater intrusion affects ecosystem function.

The purpose of this chapter was to investigate the taxonomy of the microbes that shifted after increased salinization within freshwater and brackish sites in the Everglades ecosystem to better understand the long-term effects on the microbial community. Previous research indicated that microbial enzymatic activities were not sensitive to the saltwater dosage experiments so similarly, the community structure responsible for the enzymatic activities would be unaffected (Servais et al., 2020). It was then hypothesized that *saltwater intrusion, simulated by saltwater dosage experiments on freshwater and*

brackish soils, will not impact the microbial community structure at the taxonomic level. To test the hypothesis, soil chambers within these two marshes were subjected to elevated salinity levels for two years. Soil cores were collected before experimental manipulation and after the two-year long experiment. DNA was then extracted from these samples, and their initial diversity was screened using length heterogeneity-PCR (LH-PCR), and the taxonomic composition was evaluated using Next Generation Sequencing (NGS).

2.2. Materials and Methods

2.2.1. Sample Selection

Soil samples from a previous Everglades study were collected from freshwater and brackish marshes within the Everglades National Park, Florida, USA (Servais et al., 2020). Within the Servais et al. (2020) study, 12 polycarbonate chambers were installed along an 80 m long boardwalk at each freshwater and brackish site. Each chamber was 1.4 m in diameter and embedded 30 cm deep into the soil. Six chambers were designated as ambient controls at each location, and six were designated for saltwater treatments. The chambers designated for saltwater treatment were dosed monthly from October 2014 to November 2016, with artificial seawater (Instant Ocean, Spectrum Brands, Blacksburg, VA, USA) mixed with water from the study site (Atkinson & Bingman, 1997). It is important to note that the current study began three years after the completion of the initial study, so some samples collected from the chambers were misplaced over time. Therefore, some treatment groups will only include five soil cores instead of six. A total of 33 soil cores from the top layer of soil, 0-10 cm, were used for this study, Table 1 and Table 2. Control samples were collected during the initial year of the study (2014) and were not exposed to saltwater pulses—five samples from the freshwater site (FW_Y0)

and five samples from the brackish water site (BW_Y0). After two years of monthly saltwater pulses, samples were collected again in 2016. Soils from each of the experimentally manipulated sites were selected and from control soils to determine the natural change over time within the microbial community. This consisted of five samples from the freshwater site (FW_Y2), six samples from the freshwater site exposed to saltwater (FW_Y2_Saline), six samples from the brackish site (BW_Y2), and six samples from the brackish site exposed to saltwater (BW_Y2_Saline).

Table 1: Bacteria samples from brackish (BW) and freshwater (FW) sites with corresponding year of collection: initial (Y0) or 2 years later (Y2). Samples with no salt treatment (Control) or treated with saltwater pulses (Salt) are indicated in the treatment column. The chambers indicated as controls were sampled at Y0 and again at Y2 while the treatment chambers were sampled only at Y2. Total Observed OTUs (Operational Taxonomic Units) indicate the total number of reads identified as a taxonomic cluster. Observed Unique OTUs indicate the richness within the sample as explained by the various diversity indices, Chao1, Shannon, Simpson, and Inverse Simpson.

Group	Treatment	Chamber	Total Observed OTUs	Observed Unique OTUs	Chao1	Shannon	Simpson	Inverse Simpson
BW_Y0	Control	3B	6364	4566	62700.573	7.639	0.991	109.085
BW_Y0	Control	5B	7578	4647	37596.308	7.530	0.992	120.082
BW_Y0	Control	8B	10071	7759	362600.537	8.423	0.995	183.641
BW_Y0	Control	1B	3126	3082	3082.000	8.013	1.000	2700.132
BW_Y0	Control	7B	6269	4692	68550.889	7.759	0.991	107.977
BW_Y2	Control	9B	6857	6774	1268464.556	8.812	1.000	6525.541
BW_Y2	Control	5B	4116	4061	8146691.000	8.283	1.000	3521.059
BW_Y2	Control	3B	158333	21	34.000	2.899	0.936	15.622
BW_Y2	Control	7B	552	536	536.000	6.248	0.998	443.133
BW_Y2	Control	8B	2678	2437	2437.000	7.683	0.999	1339.786
BW_Y2	Control	1B	7143	6708	210099.142	8.745	1.000	4231.803
BW_Y2_Saline	Salt	15B	1634	1620	1620.000	7.387	0.999	1604.145
BW_Y2_Saline	Salt	11B	1587	1521	1521.000	7.293	0.999	1316.747
BW_Y2_Saline	Salt	12B	6120	5522	139450.830	8.490	1.000	2742.259
BW_Y2_Saline	Salt	13B	4493	4288	8851609.000	8.278	1.000	2798.522
BW_Y2_Saline	Salt	16B	3555	3283	3283.000	8.012	0.999	1878.299
FW_Y0	Control	3F	5225	5131	725507.556	8.522	1.000	4605.709

FW_Y0	Control	2F	8463	8228	2588393.000	9.001	1.000	7642.557
FW_Y0	Control	6F	9164	7908	332953.161	8.671	0.997	301.075
FW_Y0	Control	9F	3020	3007	3007.000	8.006	1.000	2980.523
FW_Y0	Control	8F	5621	5436	396067.784	8.543	1.000	3069.422
FW_Y2	Control	2F	9190	7985	472149.818	8.865	1.000	3114.015
FW_Y2	Control	8F	8736	8167	1099642.933	8.950	1.000	5214.107
FW_Y2	Control	9F	7767	6944	171512.333	8.711	1.000	2246.194
FW_Y2	Control	10F	1707	1707	1707.000	7.442	0.999	1707.000
FW_Y2	Control	3F	16538	838	3203.069	5.589	0.974	38.627
FW_Y2	Control	6F	790	720	720.000	6.401	0.996	223.109
FW_Y2_Saline	Salt	12F	12270	210	611.538	4.628	0.969	32.778
FW_Y2_Saline	Salt	15F	4757	4244	94735.739	8.156	0.999	1537.137
FW_Y2_Saline	Salt	16F	6786	6582	794657.037	8.756	1.000	4878.411
FW_Y2_Saline	Salt	13F	2294	2068	2068.000	7.532	0.999	1006.166
FW_Y2_Saline	Salt	14F	4415	4193	8456409.000	8.237	1.000	2236.854

Table 2: Archaea samples from brackish (BW) and freshwater (FW) sites with corresponding year of collection: initial (Y0) or 2 years later (Y2). Samples with no salt treatment (Control) or treated with saltwater pulses (Salt) are indicated in the treatment column. The chambers indicated as controls were sampled at Y0 and again at Y2 while the treatment chambers were sampled only at Y2. Total Observed OTUs (Operational Taxonomic Units) indicate the total number of reads identified as a taxonomic cluster. Observed Unique OTUs indicate the richness within the sample as explained by the various diversity indices, Chao1, Shannon, Simpson, and Inverse Simpson.

Group	Treatment	Chamber	Total Observed OTUs	Observed Unique OTUs	Chao1	Shannon	Simpson	Inverse Simpson
BW_Y0	Control	3B	22	22	253.00	3.09	0.95	22.00
BW_Y0	Control	8B	63	63	2016.00	4.14	0.98	63.00
BW_Y0	Control	1B	3	3	6.00	1.10	0.67	3.00
BW_Y0	Control	7B	1	1	1.00	0.00	0.00	1.00
BW_Y2	Control	9B	46	40	391.50	3.57	0.96	27.13
BW_Y2	Control	5B	38	38	741.00	3.64	0.97	38.00
BW_Y2	Control	3B	4247	4160	1077715.00	8.29	1.00	2874.42
BW_Y2	Control	7B	326	226	7681.33	4.93	0.98	53.30
BW_Y2	Control	8B	67	38	286.00	3.19	0.93	14.25
BW_Y2	Control	1B	13	12	39.50	2.46	0.91	11.27
BW_Y2_Saline	Salt	14B	49	49	1225.00	3.89	0.98	49.00
BW_Y2_Saline	Salt	11B	61	54	691.50	3.90	0.98	41.81
BW_Y2_Saline	Salt	12B	65	61	916.50	4.07	0.98	53.48
BW_Y2_Saline	Salt	13B	10	10	55.00	2.30	0.90	10.00
BW_Y2_Saline	Salt	16B	89	83	853.25	4.38	0.99	74.03
FW_Y0	Control	3F	28	28	406.00	3.33	0.96	28.00
FW_Y0	Control	2F	55	55	1540.00	4.01	0.98	55.00
FW_Y0	Control	6F	35	35	630.00	3.56	0.97	35.00

FW_Y0	Control	9F	42	42	903.00	3.74	0.98	42.00
FW_Y0	Control	8F	46	46	1081.00	3.83	0.98	46.00
FW_Y2	Control	2F	71	59	416.75	3.92	0.97	36.27
FW_Y2	Control	8F	77	77	3003.00	4.34	0.99	77.00
FW_Y2	Control	9F	42	39	372.00	3.63	0.97	35.28
FW_Y2	Control	10F	113	113	6441.00	4.73	0.99	113.00
FW_Y2	Control	3F	22	17	47.33	2.71	0.92	12.74
FW_Y2_Saline	Salt	12F	9	9	45.00	2.20	0.89	9.00
FW_Y2_Saline	Salt	15F	74	55	1381.00	3.64	0.94	18.13
FW_Y2_Saline	Salt	16F	55	55	1540.00	4.01	0.98	55.00
FW_Y2_Saline	Salt	13F	41	39	742.00	3.63	0.97	35.77
FW_Y2_Saline	Salt	14F	16	16	136.00	2.77	0.94	16.00

2.2.2. DNA Extraction

Metagenomic DNA was extracted from 250 mg of each soil sample using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. The DNA was quantified using the Qubit® dsDNA HS Assay Kit on the Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Aliquots were diluted to 5 ng/μL and stored at 4°C while the remaining stocks were stored at -20°C until needed.

2.2.3. Microbial Community Amplification and LH-PCR

To screen the diversity of the bacteria and Archaea communities within each soil sample, the extracted DNA was amplified, and their LH-PCR profiles were analyzed. The bacterial community was assessed by targeting the V1-V3 hypervariable regions of the 16S rRNA gene. The primers used to amplify these regions were 27F (5'-AGAGTTTGATCMTGGCTCAG -3') and 536R (5'-GWATTACCGCGGCKGCTG -3') (Suzuki et al., 1998). To assess the archaeal community, the V1-V3 hypervariable region of the 16S rRNA gene was targeted by utilizing the 21F (5'-TTCCGGTTGATCCYGCCGGA-3') and 518R (5'-ATTACCGCGGCTGCTGG-3') primers (Damaso et al., 2018). The forward primer of each primer set was labeled with FAM fluorophores (Integrated DNA Technologies, Skokie, IL, USA). PCR amplifications were conducted on a ProFlex PCR System (Applied Biosystems, Foster City, CA, USA) in a 20 μL volume consisting of 1X Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific), 0.5 μM of each forward and reverse primer, ten ng of DNA and water added to volume. PCR conditions were as follows: initial

denaturation of 98°C for 10 seconds, 30 cycles of 98°C for 10 seconds, 59.7°C for 5 seconds for bacteria and 65.5°C for 5 seconds for Archaea, extension at 72°C for 15 seconds, and a final extension at 72°C for 1 minute. The PCR products were maintained at 4°C for 5 minutes before removing from the thermal cycler and visualized on a 1% m/v agarose gel before fragment analysis.

LH-PCR amplicons were separated on the SeqStudio Genetic Analyzer (Applied Biosystems) by mixing 11.5 µL of HIDI Formamide™ (Applied Biosystems), 0.5 µL of Genescan™ 600 LIZ™ dye Size Standard v2.0, and one µL of PCR product. Samples were then loaded onto the genetic analyzer, and results were analyzed with the cloud-based Peak Scanner™ Software (Applied Biosystems) with a minimum analytical threshold of 50 relative fluorescent units (RFU). Electropherogram data were then transformed into relative ratios by dividing each individual peak height by the total intensity from each sample. Relative ratios were used for subsequent statistical analyses (Mills et al., 2006).

2.2.4. NGS Library Construction and Sequencing

The NGS libraries were constructed by modifying the 16S rRNA primers to contain Illumina-specific adaptor sequences. An adaptor sequence was added to the front of each forward (5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG + forward primer -3') and reverse primer (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG + reverse primer -3'). The protocol for NGS sequencing was followed from the Illumina 16S Metagenomic Sequencing Library Preparation Guide (Amplicon et al., 2013). PCR amplifications were

conducted with the same conditions as before, except in a 25 μL volume with 12.5 ng of DNA. After PCR, 1 μL of the product was visualized on a bioanalyzer trace to verify size before sequencing. Amplification products were purified using Bulldog Bio CleanNGS SPRI Beads (Bulldog Bio, Inc. Portsmouth, NH, USA) and quantified using the Qubit® 2.0 Fluorometer and the Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific). The Nextera XT v2 Index Kit (Illumina, Inc. San Diego, USA) was utilized to create the sequencing libraries. Normalized DNA libraries were diluted to 6 pM combined with 15% PhiX Control Spike (Illumina). Sequencing was performed with a MiSeq v3 reagent cartridge (Illumina) on a MiSeq 2 x 300 platform (Illumina).

2.2.5. NGS Data processing and Statistical Analysis

Raw sequences were processed using Mothur version 1.45.3 (Schloss et al., 2009) following the MiSeq SOP (Kozich et al., 2013). All sequences were aligned and classified using SILVA v138.1 reference alignment and database within Mothur (Pruesse et al., 2007; Quast et al., 2013; Yilmaz et al., 2014). Sequences were then clustered into operational taxonomic units (OTUs) at 97% similarity (Chappidi et al., 2019). The resulting dataset was analyzed using the R package 'phyloseq' (McMurdie & Holmes, 2013). All subsequent statistical analyses and visualizations were performed using R Statistical Software v4.1.1(Team, 2021).

Alpha diversity for each sample was evaluated with Chao1, Shannon, Simpson, and Inverse Simpson diversity measurements to examine OTU richness and evenness, Table 1 and Table 2. Assessing alpha diversity aids in the initial characterization of a community and multiple index calculations are used because each diversity index measured has their own biases. The observed count of distinct sequences or species present within the

sample is known as richness. Evenness measures how similar each unique species' population size is to that of other species in the community. Chao1considers the observed richness as well as the number of singletons and doubletons to calculate overall richness. The Shannon and Simpson indices are both richness and evenness estimators. While Shannon emphasizes the richness, Simpson emphasizes the evenness (Kim et al., 2017). The higher the Shannon diversity value, the more species with a relatively equal abundance. The Simpson index determines the likelihood that two species picked at random from the community belong to the same species. A high likelihood suggests a low diversity of species, while a low probability indicates a large diversity of species. Simpson's value ranges from 0 to 1, increasing as diversity diminishes. However, because this has little biological relevance, the Inverse Simpson is frequently used to offer context.

2.3. Results

2.3.1. Beta Diversity

The diversity patterns for Archaea and bacteria communities were first screened with LH-PCR. An Analysis of Similarities (ANOSIM) test using Bray-Curtis dissimilarity was used to identify significant differences ($R>0.5$, $p<0.05$), **Table 3,** between communities, beta diversity. Bray-Curtis dissimilarity is a beta diversity measurement of how different the microbial communities are from one another (Bray & Curtis, 1957). This dissimilarity is visualized on a Nonmetric Multidimensional Scaling (NMDS) plot where samples that are more similar are plotted closer together and vice versa. ANOSIM tests are often used to evaluate multivariate ecological data by ranking the results based on a measure of dissimilarity, in this instance Bray-Curtis dissimilarity

(Bray & Curtis, 1957; Clarke, Somerfield, Airoldi, & Warwick, 2006). The rank is calculated as a ratio of the differences between groups to the differences within groups. This ratio can vary from -1 to 1, although it is most commonly between 0 and 1. Higher numbers imply poorer similarity and an uneven distribution, whereas values near to 0 suggest an equal distribution and great similarity. Negative numbers, on the other hand, might imply more dissimilarity within samples than across groups (Chapman & Underwood, 1999). Control samples collected from year 0 and collected after two years documented the baseline shifts that occurred over time. These samples were used to determine normal temporal shifts, if any, that would occur within the microbial community at each site. Samples collected after salinity manipulation were compared to the control samples collected from that same year to investigate if saltwater shifted the communities in either site or taxa.

Overall, the Archaeal community structure showed distinct differences between the freshwater and brackish sites, Figure 1. ANOSIM results showed that there was not a significant difference in Archaea over time in either brackish, Figure 2, or freshwater, Figure 3, control samples. Additionally, ANOSIM results from LH-PCR profiles determined that there was not a significant difference within freshwater or brackish marshes after salinity manipulation. These results are supported in the NMDS plots within Figure 1.

Table 3: Results of Analysis of Similarities (ANOSIM) test using Bray-Curtis dissimilarity from LH-PCR and NGS. Statistically significant differences are bolded.

	Temporal		**Treatment**	
	BW_Y0 vs BW_Y2	**FW_Y0 vs FW_Y2**	**BW_Y2 vs BW_Y2_Saline**	**FW_Y2 vs FW_Y2_Saline**
LH-PCR				
Archaea	R= -0.02, p>0.05	R= 0.02, p>0.05	R= 0.13, p>0.05	R= -0.06, p>0.05
Bacteria	**R= 0.62, p<0.05**	**R= 0.32, p<0.05**	R= -0.11, p>0.05	R= 0.05, p>0.05
NGS				
Archaea	**R= 0.47, p<0.05**	**R= 0.25, p<0.05**	**R= 0.03, p<0.05**	**R= 0.24, p<0.05**
Bacteria	R= 0.061, p>0.05	**R= 0.20, p<0.05**	R= -0.11, p>0.05	R= -0.003, p>0.05

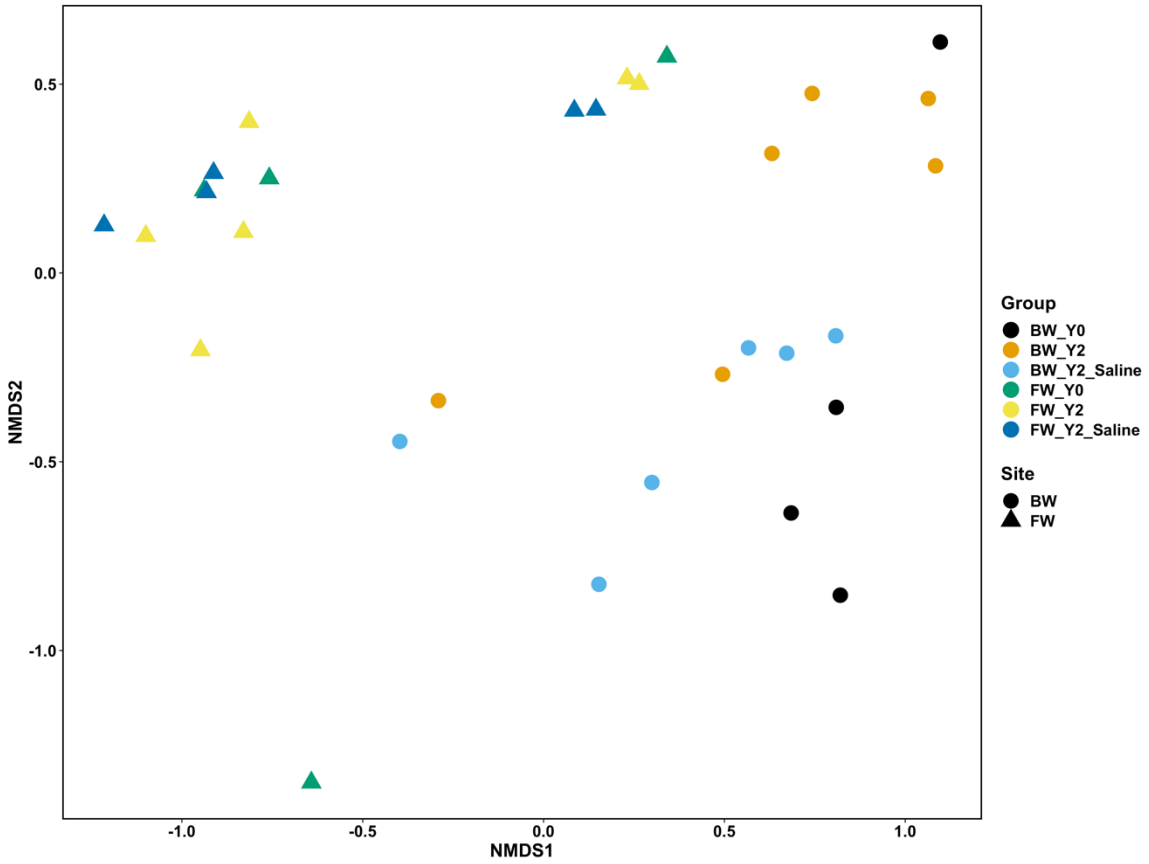

Figure 1: Nonmetric multidimensional scaling (NMDS) plot displaying clear separation between freshwater (triangle) and brackish (circle) sites from Archaea LH-PCR profiles. In the brackish site, control samples were widespread indicating no significant difference in diversity over time ($R = -0.02$, $p > 0.05$). Saltwater also did not have a noticeable effect on the community as there was no clear distinction amongst groups. A similar occurrence was observed in the freshwater site.

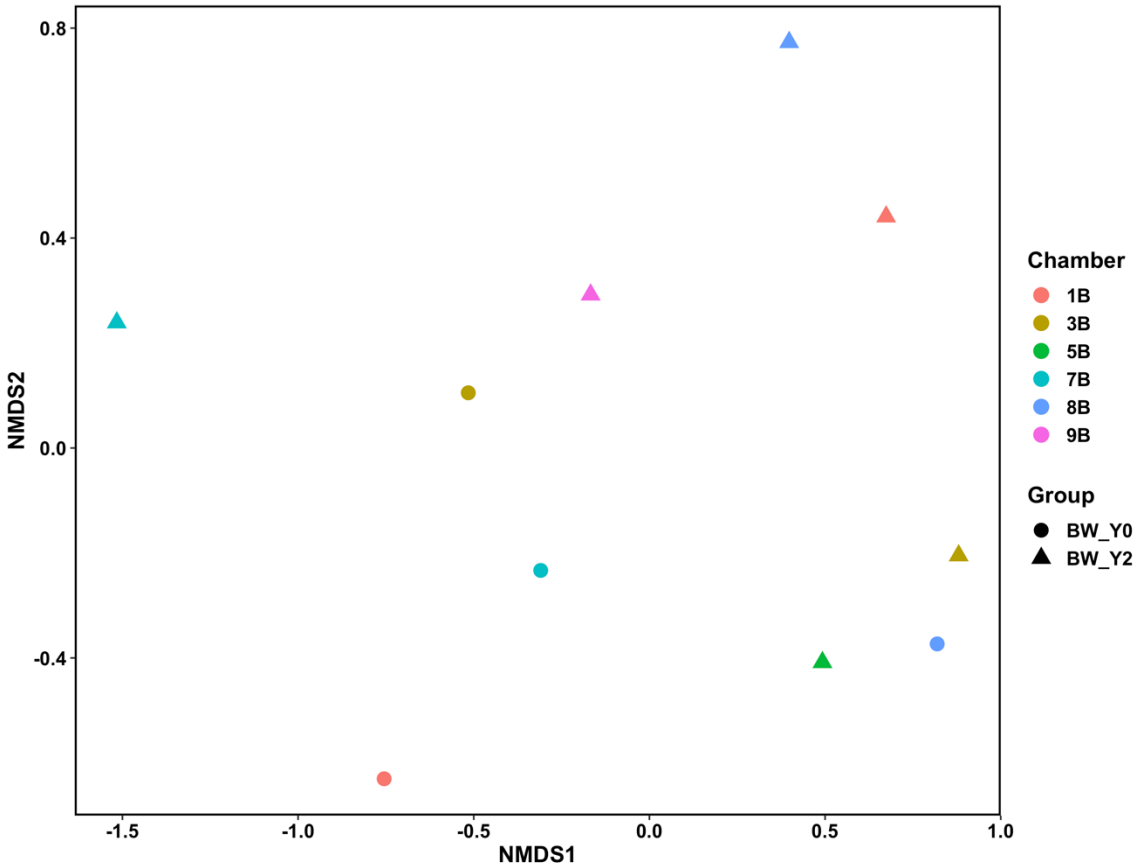

Figure 2: NMDS plot comparing the Archaea community control chambers at the brackish site from the initial year of sample collection (circle) to the sample collection after two years (triangle). No distinct grouping within the collection years occurred indicating changes in each chamber over time. Chambers 5 and 9 did not have a matching sample for the initial collection year.

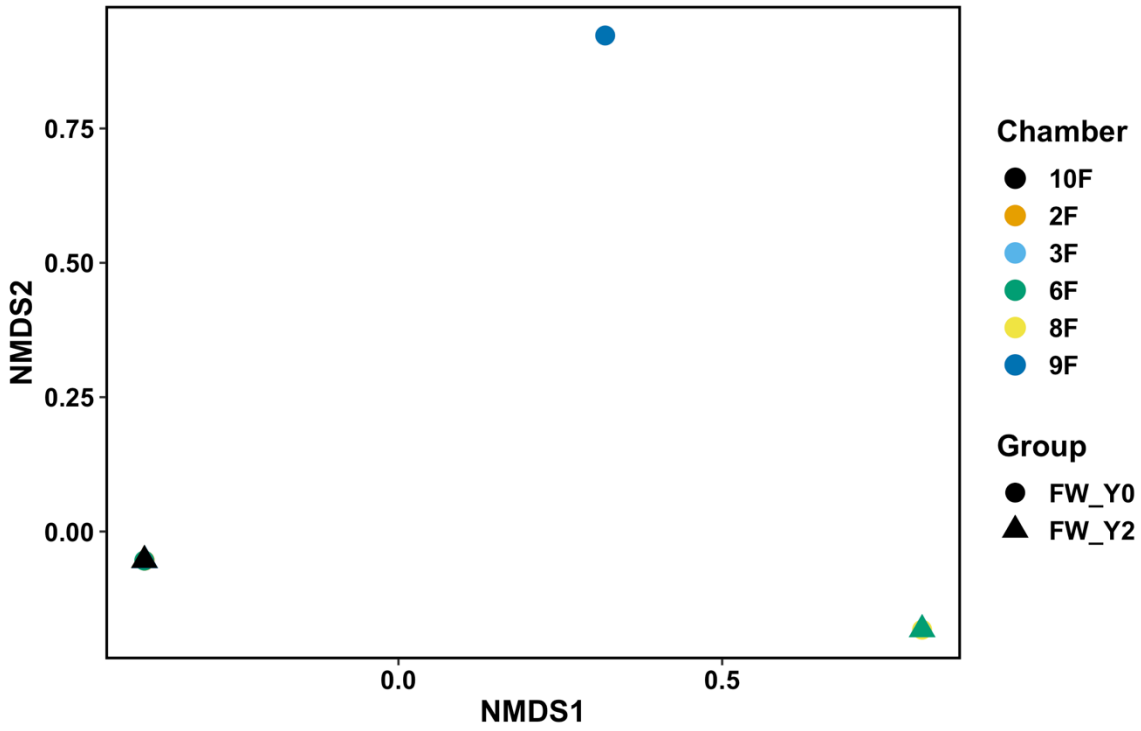

Figure 3: NMDS plot comparing the Archaea community control chambers at the freshwater site from the initial year of sample collection (circle) to the sample collection after two years (triangle). Tight grouping occurred between the chambers from the collection years, indicating that overall, the diversity did not change over time.

Within the bacteria community, there were also distinct differences between the freshwater and brackish sites indicating their unique compositions, but two freshwater samples grouped closer to the brackish site which contributed to the lower dissimilarity index, Figure 4. The control samples for the bacteria communities were also compared to determine shifts over time, and both the brackish site, Figure 5, and freshwater Figure 6, revealed significant differences in their communities. Since these samples were not experimentally manipulated, this indicates that there were some natural disturbances that could have contributed to the shifts in microbial communities. Shifts in individual chambers in Figure 5 and Figure 6 exhibit the temporal dissimilarity. However, salinity

manipulation did not have a significant effect on the bacterial community in freshwater or brackish marshes.

Archaea and Bacteria taxa were examined with LH-PCR to determine if shifts in their communities were revealed. Both taxa revealed significant differences between the brackish and freshwater sites. The Archaea samples did not reveal any significant differences in their control samples at either site. But the bacteria did show temporal shifts that were significant within both sites. Additionally, it was determined that Archaea did not have any statistically significant shifts in their community after saltwater manipulation. This same result was seen in the bacteria community.

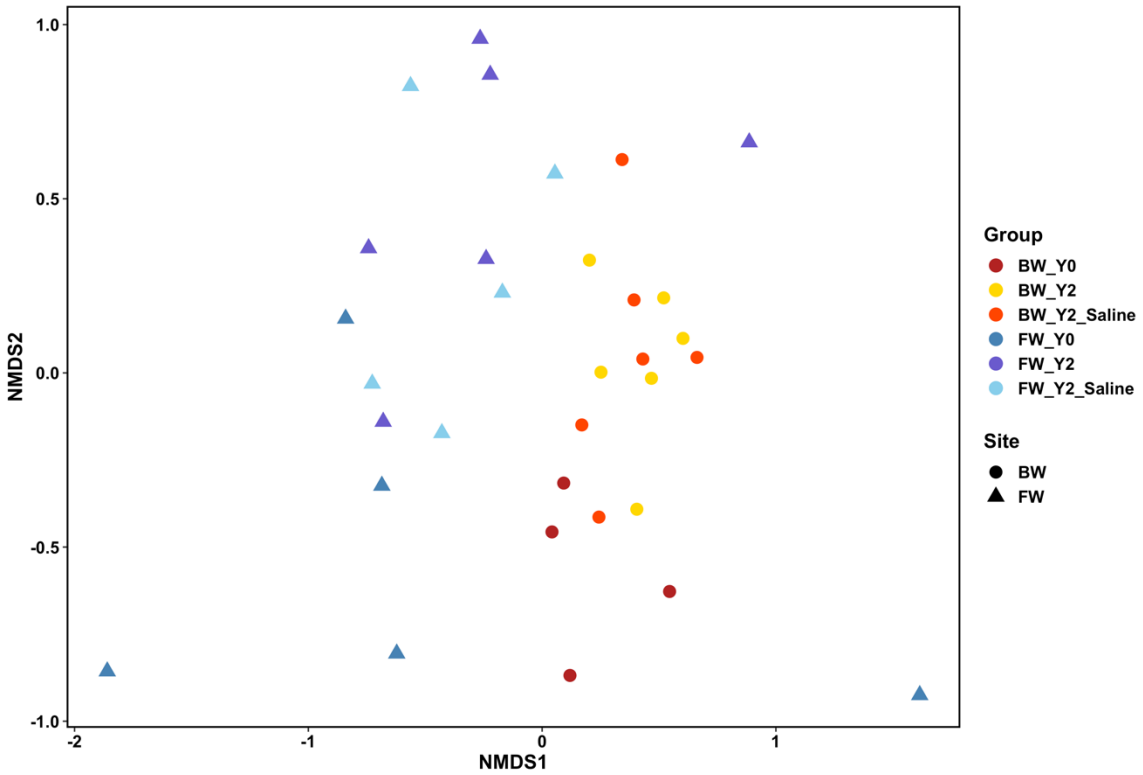

Figure 4: Nonmetric multidimensional scaling (NMDS) plot displaying clear separation between freshwater (triangle) and brackish (circle) sites from Bacteria LH-PCR profiles. In the brackish site, distinct grouping occurred between the control samples from the initial collection year (red circle) and the collection after two years (yellow circle) displaying the temporal microbial diversity shifts. Samples treated with saltwater (orange circle) grouped closely with the control samples from year 2 suggesting that the treatment had little effect on the diversity at that site. A matching occurrence was observed in the freshwater site.

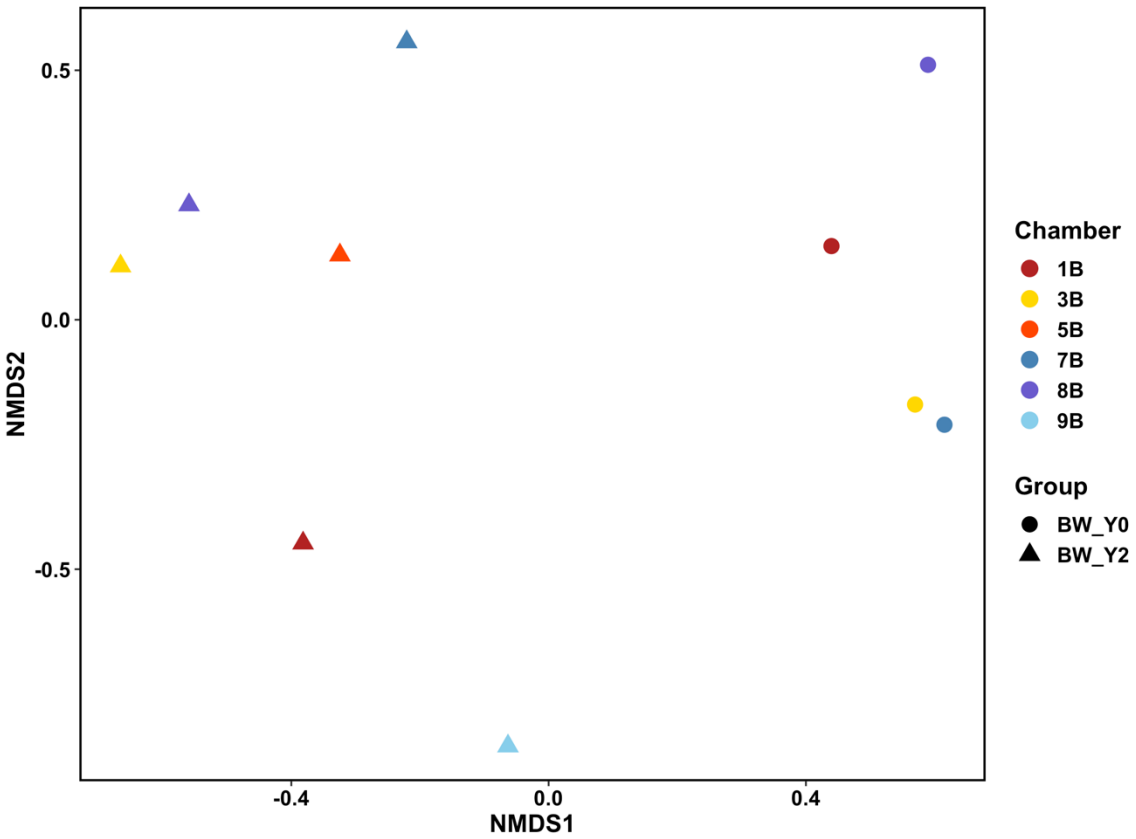

Figure 5: NMDS plot comparing the Bacteria community control chambers at the brackish site from the initial year of sample collection (circle) to the sample collection after two years (triangle). Distinct differences between the collection years occurred indicating significant change in diversity over time. Chambers 5 and 9 did not have a matching sample for the initial collection year.

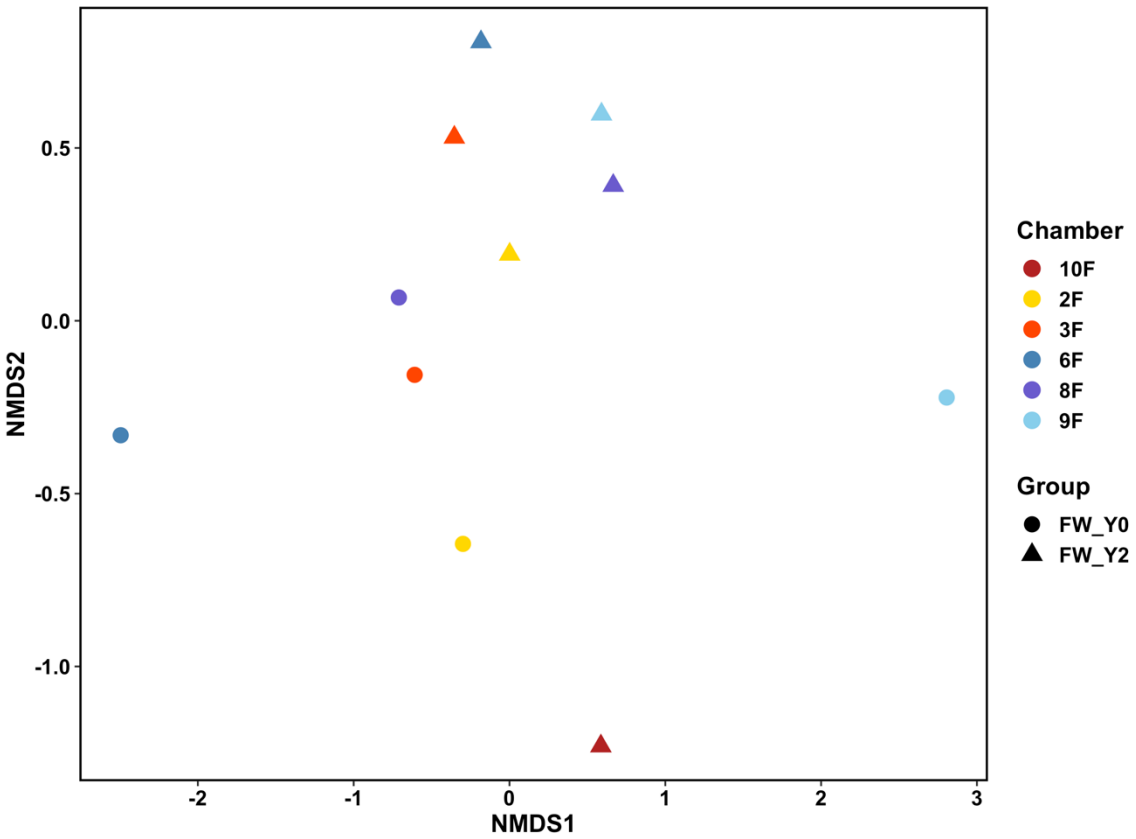

Figure 6: NMDS plot comparing the Bacteria community control chambers at the freshwater site from the initial year of sample collection (circle) to the sample collection after two years (triangle). Distinct difference across the collection years occurred indicating changes in diversity over time. Chamber 10 did not have a matching sample for the initial collection year.

Spatial differences between chambers just a few meters apart at each marsh were shown by analyzing samples from each individual chamber on an NMDS plot, (Figure 2, Figure 3, Figure 5, Figure 6). Their placement on the plots show that each chamber had their own unique microbial community. In Archaea, communities from each chamber at the brackish site, Figure 2, shifted after two years but their differences in diversity, as measured by ANOSIM, were not enough to be significant. In the freshwater site, the Archaea communities from several chambers grouped closely as indicated in Figure 3. Therefore, there were not any large shifts in the Archaea soil microbiome at the

freshwater marsh after two years. When looking at the bacteria, the communities within the control samples shifted after two years at both the freshwater and brackish sites as indicated in Figure 5 and Figure 6**,** indicating the high variability and seasonal dynamics within this community.

2.3.2. Taxonomic Diversity

The taxonomic microbial community composition of the sites was assessed with NGS, and ANOSIM tests were done to evaluate their dissimilarity, Table 3. There were 5,518 unique OTUs within the Archaea community, and the freshwater and brackish sites were considerably different. Sequencing revealed temporal shifts within the Archaea community at the freshwater and brackish sites. Experimental salinity manipulation modified this community within the freshwater site as well as the brackish sites.

The bacterial community composition was highly abundant within the sites and totaled 318,776 unique OTUs. ANOSIM results from NGS revealed considerable differences between the freshwater and brackish site. Temporal shifts were identified at the freshwater site, but not at the brackish site. Additionally, saltwater manipulation did not have a strong effect at the freshwater or the brackish site.

An increase in resolution was observed within both the Archaea and bacteria samples after NGS. Within the Archaea community, temporal differences were observed at both the brackish and freshwater sites, but LH-PCR did not reveal these differences. Within the bacteria community, NGS determined that samples monitoring temporal shifts within the brackish site were not considerably different but, this was the opposite result with LH-PCR analysis.

2.3.3. Microbial Structure

To identify the bacteria that were more frequently present and abundant within specific sites, indicator species analysis was done with the NGS results using the R package 'indicspecies' (De Caceres & Legendre, 2009). Each group had several taxa that were statistically significant and could be identified as indicators. However, only the taxa with the strongest statistical associations are reported here. Alphaproteobacteria, members of the Proteobacteria phylum, were predominantly present in all samples. Rhizobiales and *Methyloligellaceae* were indicated as being the most strongly associated with the brackish site. *Hyphomicrobiaceae* was most prominent within the freshwater site along with *SHA-26*, a member of the Chloroflexi phylum. Each site -controls and treatments – was also tested for indicator species. *Peptostreptococcaceae*, a member of the Firmicutes, was predominately associated with the BW_Y0 samples. The composition shifted after two years, and *GIF3* within the Chloroflexi phylum and *Hyphomicrobiaceae* of the Proteobacteria phylum were significantly associated with the BW_Y2 samples. *Anaerolineaceae* and *Methyloligellaceae* were strongly associated with BW_Y2_Saline samples. *Clostridiaceae* of the Firmicutes phylum and *Defluviicoccaceae* within the Proteobacteria phylum were strongly associated with FW_Y0 samples. Within the FW_Y2 samples, *Rhodomicrobiaceae* of Proteobacteria phylum and *Aminicenantales* of the Acidobacteriota phylum were indicators of this group. FW_Y2_Saline indicators are *Alphaproteobacteria* and *Defluviicoccaceae,* which are both members of the Proteobacteria phylum.

Indicator species analysis was also utilized to identify more frequently present and abundant OTUs within the Archaea. Out of the 5,518 unique OTUs, 2,226 could not

be classified at a lower taxonomic classification than the Archaea Domain. These specific OTUs were classified as unclassified Archaea at the Phylum level and below. Therefore, those Archaea will be identified as their unique OTU number. Sequences that fell under OTU0001 and OTU0002 were strongly associated with the brackish site, but none were specified as indicators for the freshwater site. The BW_Y2 group included three indicators, OTU0007, OTU0016, and OTU0001. The FW_Y2 group included indicators from *Aenigmarchaeales,* a member of the Aenigmarchaeota phylum, and OTU0020 and OTU0012. Groups BW_Y0, BW_Y2_Saline, FW_Y0, and FW_Y2_Saline did not have any OTUs identified as indicators.

2.3.4. Compositional shifts after salinization at each site

The most abundant bacteria phyla for both brackish and freshwater sites were Proteobacteria (48.5% and 62.1%), Firmicutes (31% and 15.6%), Chloroflexi (8.6% and 8.5%) and Actinobacteriota (6.1% and 7.1%). Shifts in relative abundance were determined within these abundant phyla indicating an underlying compositional shift between taxa, as in Figure 7. Within the freshwater site, the relative abundance of each of these phyla decreased except for Proteobacteria which increased from 63.4% to 71.5%. Firmicutes notably decreased after salinization in the freshwater site from 11.8% to 7.5%. Additional phyla that experienced decreases in the freshwater site were Desulfobacterota (0.4% to 0.1%), Planctomycetota (0.7% to 0.5%), Bacteroidota (0.7% to 0.4%), Myxociccota (0.6% to 0.4%), and Gemmatimonadota (0.6% to 0.2%). Within the brackish site, the opposite pattern was seen as Proteobacteria decreased from 74.3% to 61.3% and other abundant phyla increased after salinization. Notably, the Actinobacteriota phylum increased from 4.8% to 12.1% relative abundance. Additionally

slight increases were seen in Desulfobacterota (0.5% to 0.9%), Planctomycetota (1.1% to 1.6%), and Acidobacteriota (0.6% to 1.5%) after saltwater pulses.

Notable shifts at the family level were identified in the Proteobacteria phylum since saltwater pulses caused its relative abundance to increase in the freshwater site and decrease at the brackish site, Figure 8. Additionally, there were unique compositional changes seen at the less abundant phyla like Desulfobacterota, Figure 9. A unique composition was established between the two sites at the Order level and taxonomic abundances shifted displaying the effect of saltwater on this community.

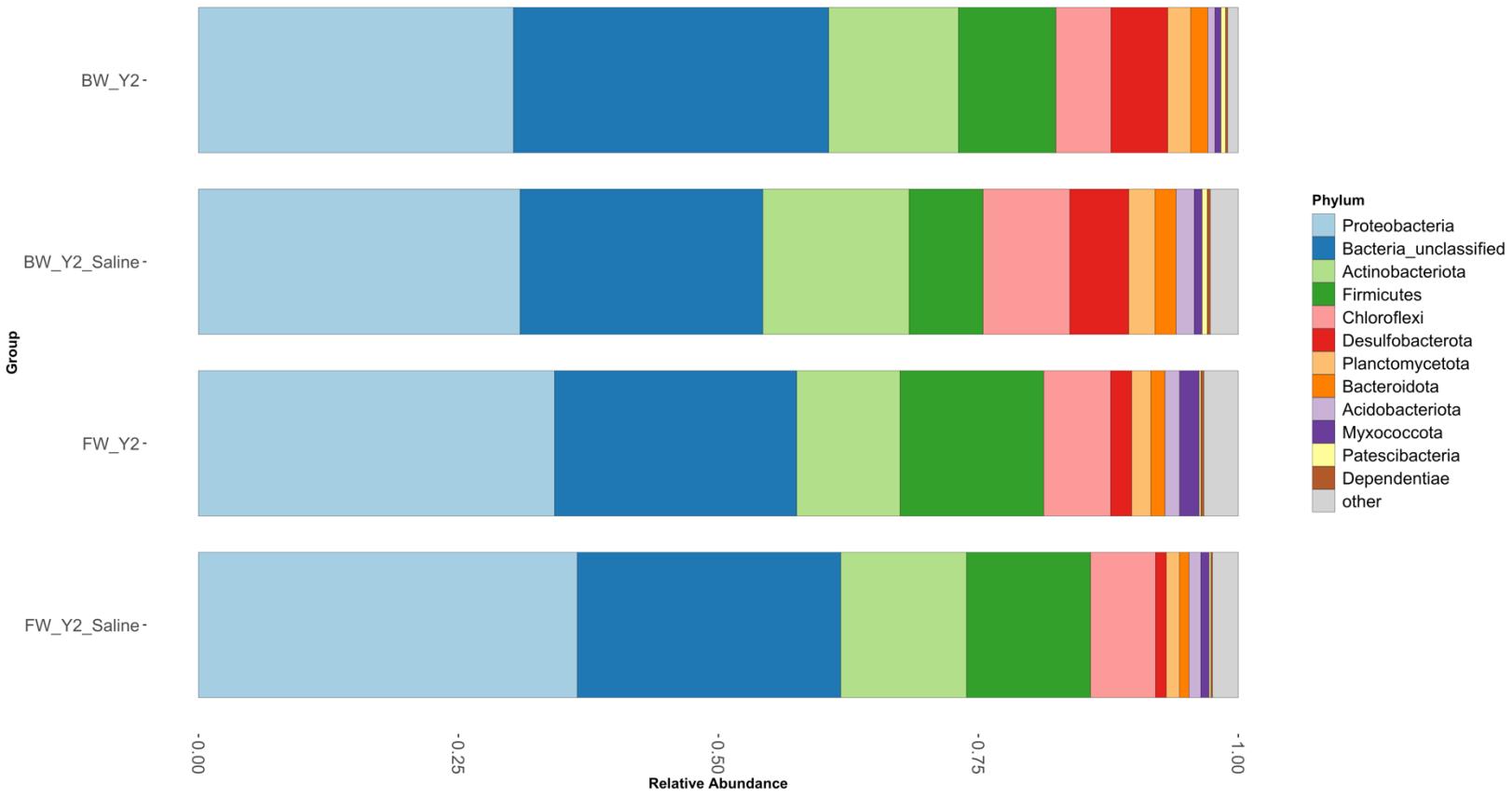

Figure 7: Relative abundance of the 12 most abundant Phyla found within Bacteria samples at year 2. Shifts in relative abundance were observed after the saltwater treatment in the brackish (top two bars) and freshwater sites (bottom two bars). The BW_Y2 group is compared to the BW_Y2_Saline group and the FW_Y2 group is compared to the FW_Y2_Saline group.

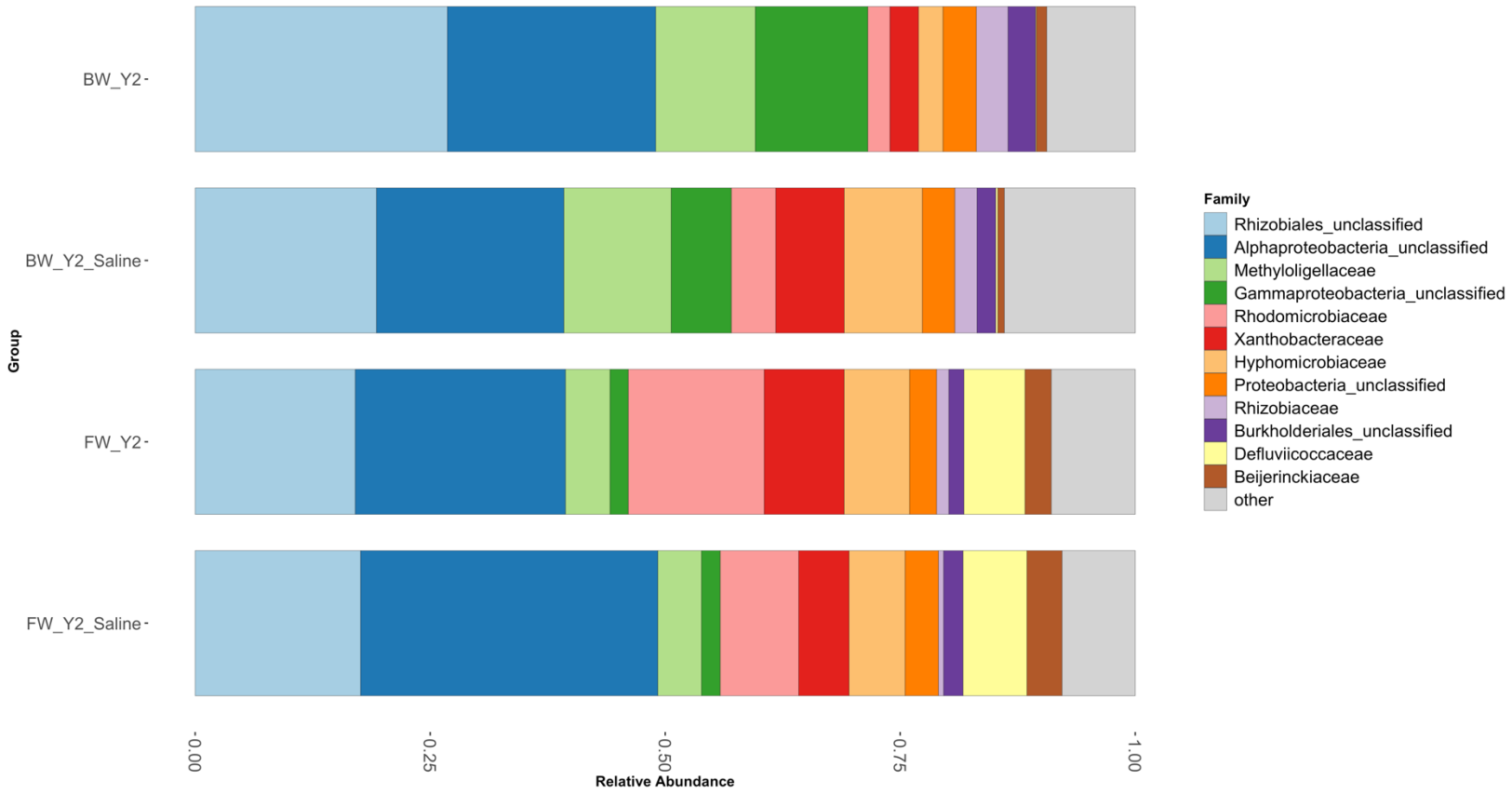

Figure 8: Relative abundance of the most abundant Families within the Proteobacteria phylum at the two sites. A comparison of the BW_Y2 and the BW_Y2_Saline groups showed that the saltwater treatment shifted the relative abundance of certain taxa within the brackish site. A comparison of the FW_Y2 and the FW_Y2_Saline groups revealed that the saltwater treatment shifted the relative abundance of certain taxa within the freshwater site.

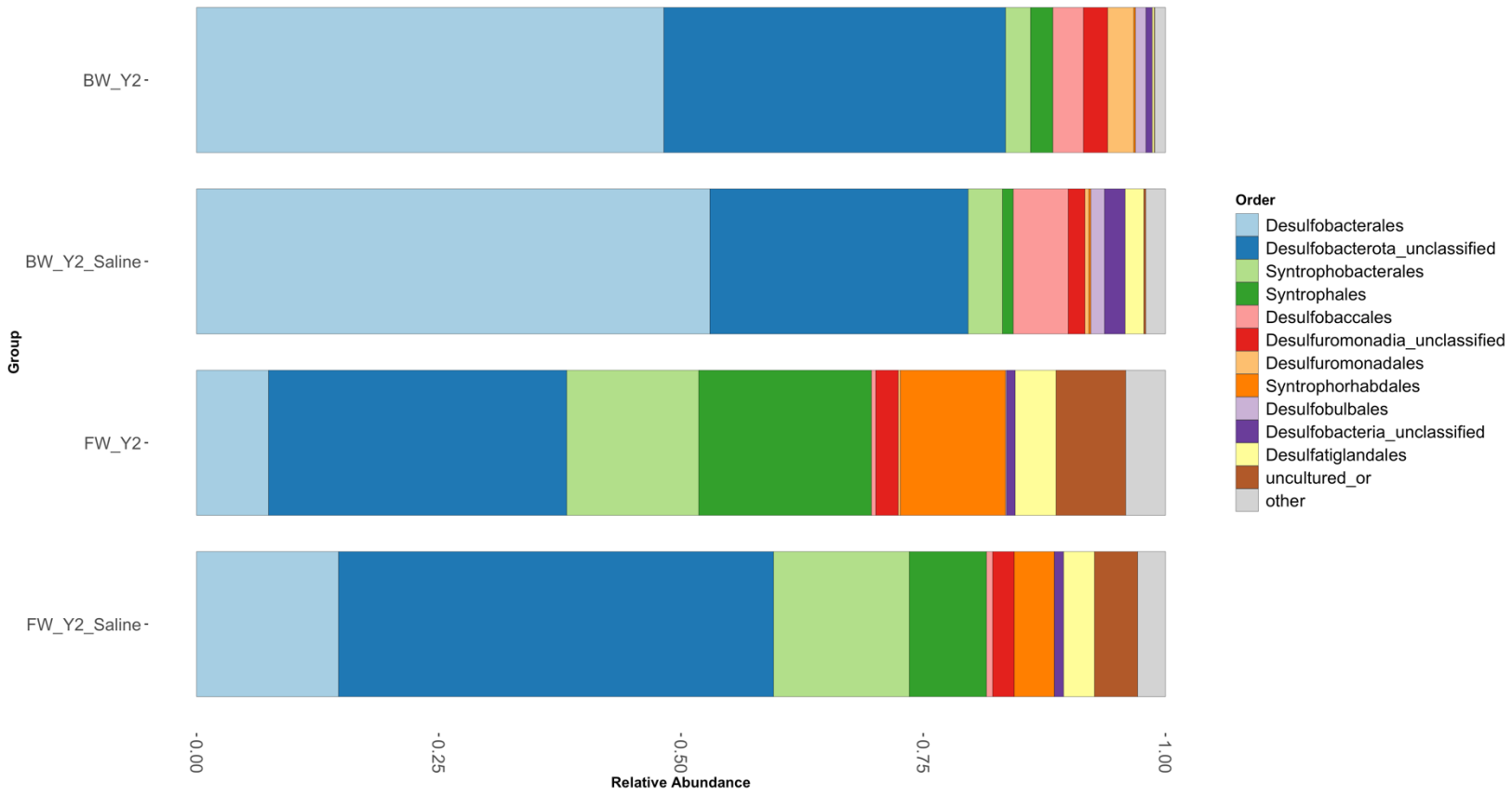

Figure 9: Relative abundance of the most abundant Orders within the Desulfobacterota phylum. The brackish site (top two bars) has a different microbial distribution than the freshwater site (bottom two bars). The saltwater treatment shifted the relative abundance of several different taxa within this phylum at the brackish and freshwater sites.

There were apparent taxonomic differences of Archaea between the freshwater and brackish sites. Overall, approximately 48% of all sequences were unclassified Archaea in the brackish site and 50% in the freshwater site. But specific taxa were found to be more predominant between sites and treatments. The Thermoplasmatota phylum was predominant in the freshwater site, while the Halobacterota phylum was predominant in the brackish site, Figure 10. Halobacterota slightly increased from 0.5% to 1.5% in the freshwater site after salinization and was also seen to greatly increase in the brackish site from 26.6% to 52.8%. Thermoplasmatota, which was prominent in the freshwater site decreased (13.6% to 9.2%) after saltwater in the brackish site and increased in the freshwater site (25.6% to 42.5%). Aenigmarchaeota was found at low levels in the brackish site before (1.8%) and after treatment (0.3%) but at a higher relative abundance in the freshwater site. However, their presence decreased after salinization from 9.0% to 5.9%.

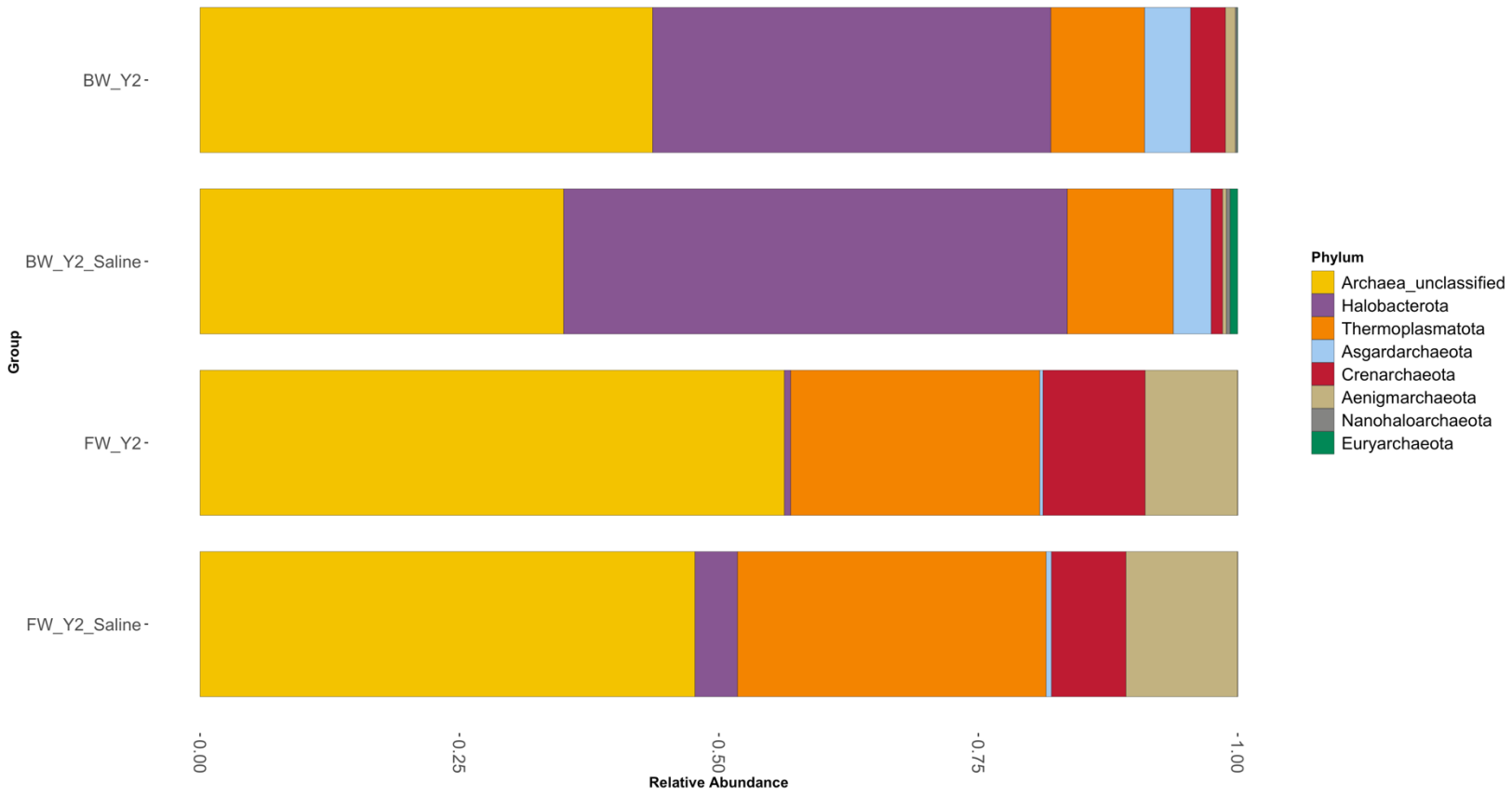

Figure 10: Relative abundance of the eight most abundant Phyla found within Archaea samples at Year 2. Shifts in relative abundance were observed after the saltwater treatment in the brackish (top two bars) and freshwater sites (bottom two bars). Of the classified Archaea, Halobacterota (purple) dominated the brackish site. Thermoplasmatota (orange) dominated the freshwater site. The BW_Y2 group is compared to the BW_Y2_Saline group and the FW_Y2 group is compared to the FW_Y2_Saline group.

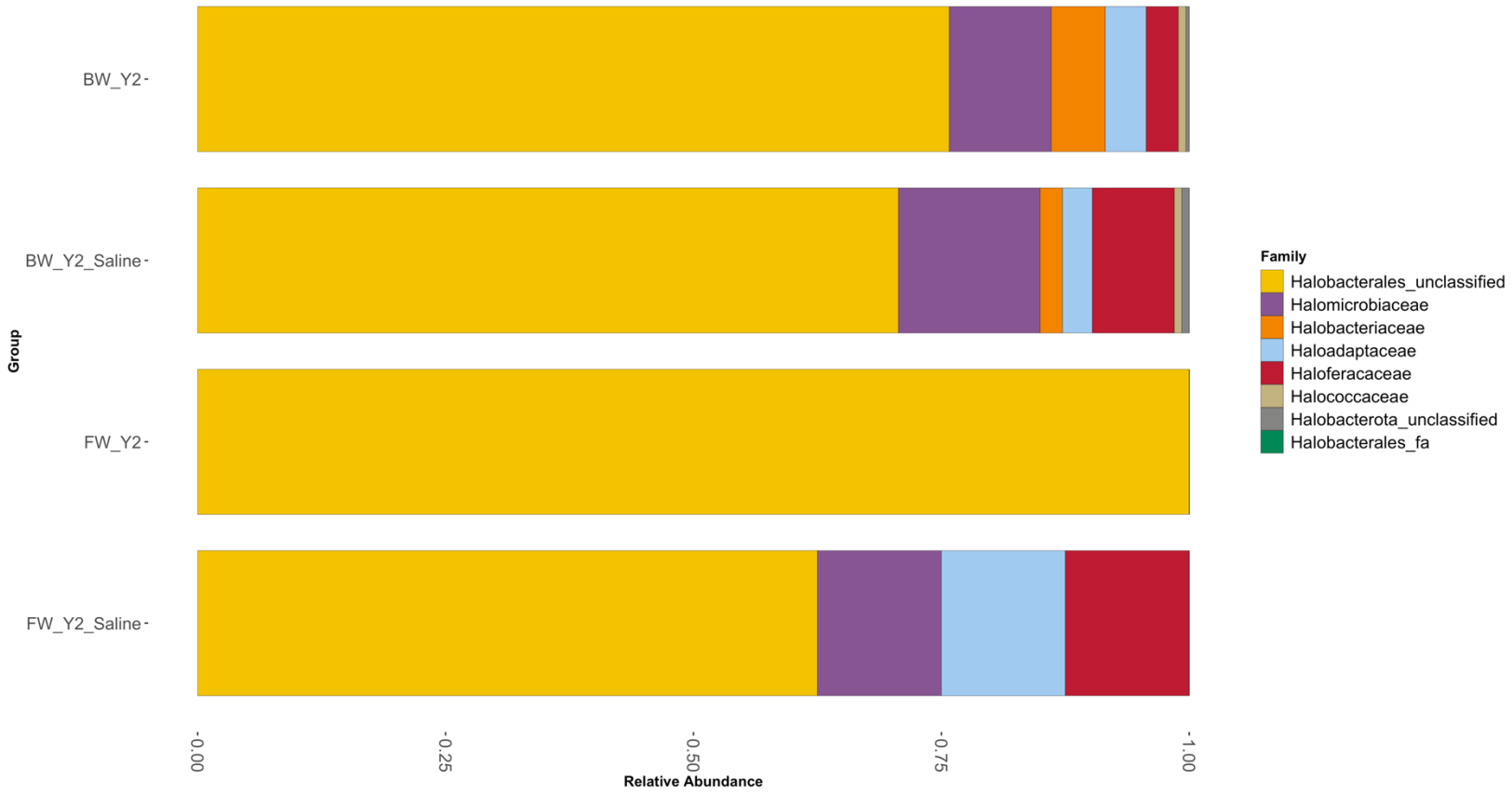

Figure 11: Relative abundance of the most abundant Families within the Halobacterota phylum at the two sites. A comparison of the BW_Y2 and the BW_Y2_Saline groups showed that the saltwater treatment shifted the relative abundance of certain taxa within the brackish site. A comparison of the FW_Y2 and the FW_Y2_Saline groups revealed that the saltwater treatment increased the relative abundance of various taxa within the freshwater site.

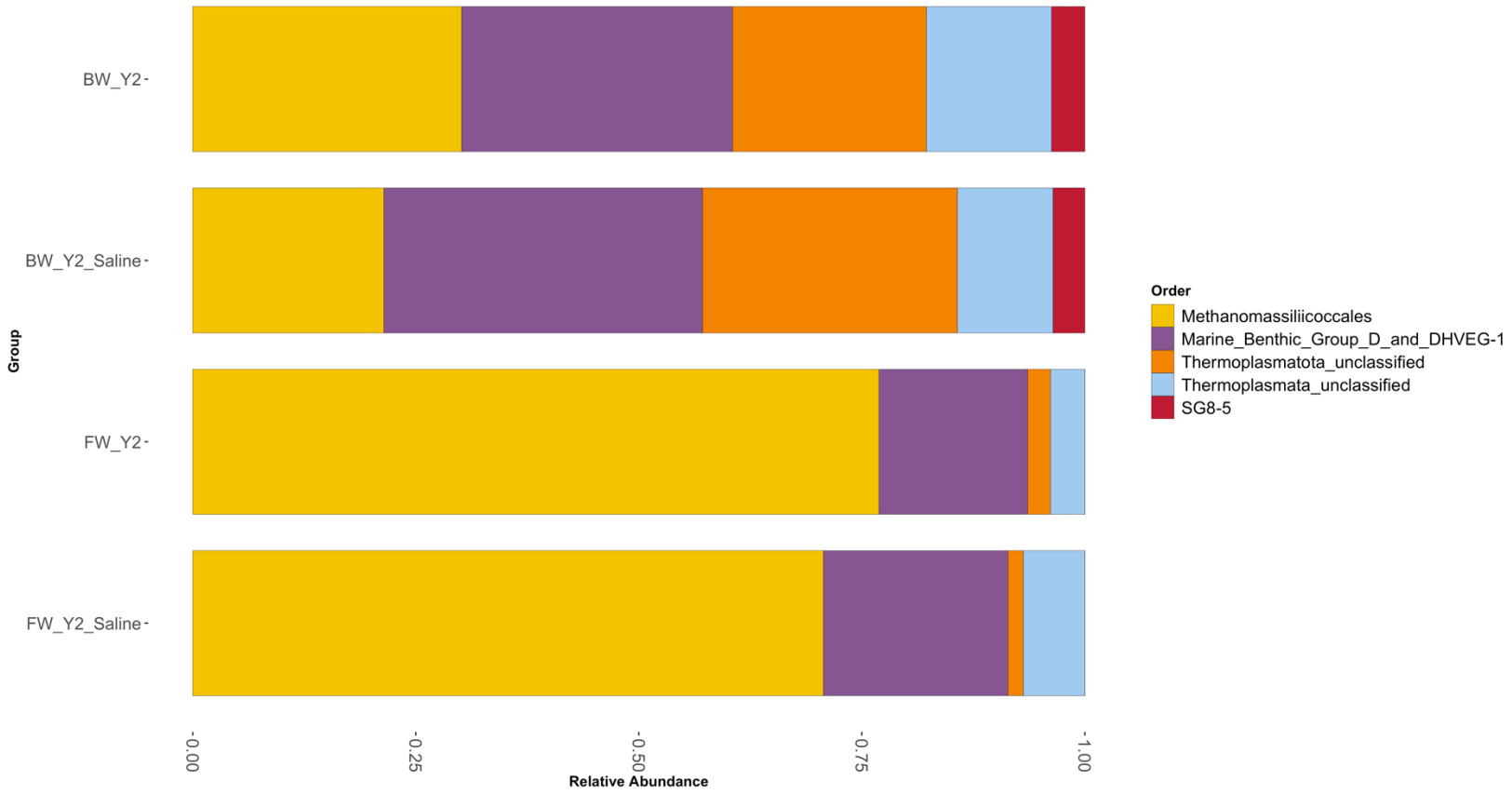

Figure 12: Relative abundance of the most abundant Orders within the Thermoplasmatota phylum. The brackish site (top two bars) has a more even microbial distribution than the freshwater site (bottom two bars). The saltwater treatment shifted the relative abundance of several different taxa within this phylum at the brackish and freshwater sites.

2.4. Discussion

Soil microbial communities play important roles in driving biogeochemical cycles; therefore, one of the first steps to monitoring these communities is establishing baseline community identities and then monitoring their shifts after environmental perturbations. Within this study, the environmental perturbation was simulated saltwater intrusion and LH-PCR and NGS methods were both utilized to observe the communities. LH-PCR provided an initial look into the minimum diversity just based on the abundance and number of fragment lengths of the microbial communities. NGS allowed a deeper dive into the taxonomic diversity and allowed for identification of taxa that contribute to compositional shifts.

LH-PCR provides an initial look at the communities and determined that the composition differed between the brackish and freshwater sites. Other studies have found similar results as microbes have variable tolerance for salinity levels displaying unique patterns between salinity gradients (Fei Xi et al., 2014; Morrissey et al., 2014; Nold & Zwart, 1998). Since samples were taken from the control chambers before the salinity manipulation began and again after two years, we were able to identify natural shifts within the soil microbiome over time. Differences in composition within the Archaeal samples were not detected over time at either site. But differences in the bacteria samples were detected at both the brackish and freshwater sites. Control samples were taken from chambers only a few meters apart and differences were detected not only within chamber samples but also over time. These variations in samples over time from each chamber highlight the importance of monitoring microbial communities from a spatial and temporal view (Meyers & Foran, 2008; Zhang et al., 2020). Within the Zhang et. al.

study, study sites were monitored, to explore the spatiotemporal variation that occurred within microbial communities between two different seasons, winter, and summer. The researchers took samples from locations within a large study site and compared the amount of variation that occurred between the locations to the amount of variation that occurred over time. They determined that the space had more of an effect on the soil microbial variation than time. Within the Servais et. al. study, (originator of these soil samples) the various chambers also provided a comparison between time and space (Servais et al., 2020). As depicted in the NMDS plots above, the communities did differ between chambers taken from the same year (spatial) as well as temporal (time). Although more differences were detected within bacteria than Archaea at the sites, these results further highlight the spatial variability of microbial communities. Indicator analysis results were provided to detect common species between the chambers to serve as an indicator for each site and to help explain the variation over time.

By sequencing the 16S rRNA gene of bacteria and Archaea, the community compositions within each site were characterized. Extremely low sequencing reads were detected within the Archaea and was determined to be a limitation of the selected primer set. The primers, 21F and 518F, did not only amplify Archaea but also selectively amplified bacteria (Bai et al., 2012). The comparison to an Archaea only database, helped to identify the sequenced Archaea. Therefore, the initial diversity when screened with LH-PCR is likely attributed to the presence of both Archaea and bacteria.

Nonetheless, sequencing analysis was able to recover and identify Archaea OTUs from the reads and ANOSIM results indicated significant differences between sites after saltwater treatment. Within the Archaea samples, Halobacterota were prominent within

the brackish site and their presence in the freshwater site slightly increased after salinization. This was expected as these phyla are, as their name suggests, halophiles and commonly found within high saltwater environments (Bräuer, 2020). Crenarchaeota were found to be more prominent within the freshwater site and slightly decreased with the input of saltwater. Additionally, Euryarchaeota were present within the brackish site and more prominent with the increased saltwater. These results correspond with earlier research where Euryarchaeota were found to prefer high saline environments and Crenarchaeota preferred lower salinity (Dong et al., 2006; Jiang et al., 2008).

Characterizing the bacteria communities at each site, was also done by sequencing the 16S rRNA genes. Significant differences between sites after saltwater treatment within bacteria samples were not statistically determined with ANOSIM. However, the taxonomic composition and abundance shifted between sites after saltwater treatment at various phyla. The decrease in the Proteobacteria phylum at the brackish site and its increase at the freshwater site is indicative that the naturally occurring taxa are physiology diverse between sites. For example, Defluviicoccaceae were much more abundant at the freshwater site (7.3%) than the brackish site (0.3%), regardless of treatment. These microbes are responsible for nitrogen removal and corresponds with an earlier study where porewater measurements from these two sites determined that the freshwater site had much lower levels of ammonia, and total dissolved nitrogen (Servais et al., 2020; Zhang et al., 2022). The relative abundance of the Desulfobacterota phylum decreased in the freshwater site after saltwater and increased in the brackish site. Members of this phylum are known for their sulfate reducing capabilities and their increased presence within this brackish site could mean that higher levels of sulfate

reduction are occurring (Langwig et al., 2022). Looking at taxa that had relatively variable abundances between sites provides more insight. After saltwater treatment, an increase of the sulfate reducing bacteria, Desulfobaccales, occurred in the brackish site but low levels were detected in the freshwater site and only slightly increased after the pulses. This is likely a result of the competition between methanogens and sulfate reducers -- since they compete for the same substrates, hydrogen and acetate, an inverse relationship between the two functional groups is often observed (Muyzer & Stams, 2008; Oremland & Polcin, 1982). In the freshwater site, the dominant phyla are Thermoplasmatota which are composed of methanogens and many studies have shown that methanogenesis is inhibited as sulfate concentrations increase (Bra¨uer, 2020; Michas et al., 2020; Siegert et al., 2011). Porewater measurements showed that the sulfate levels were much higher in the brackish site than in the freshwater site and increased as saltwater was added (Servais et al., 2020). The taxonomic changes in the microbial community seemed to be directly responding to this input as sulfate reducing bacteria are likely outcompeting methanogens at the brackish site, while the opposite is occurring at the freshwater site.

In conclusion, these results simulate how the microbial structure of a community can change when elevated levels of saltwater are introduced into a community. It was hypothesized that since the enzymatic activities were not very sensitive to the saltwater treatment, then the community structure was likely to be unaffected. However, from sequencing, we saw that the taxonomic distribution was affected by the treatment at both sites. Archaea seemed to be less sensitive to the saltwater than bacteria but is likely an effect of the low observed OTU abundance. Overall, the relative abundance of taxa

changing two years after the saltwater treatment depicts how the community evolves and adapts to a perturbation over time. This information is useful for environmental monitoring strategies because if the microbial community shifts too much from its original composition, then the overall community function can be permanently altered. Here, changes after a 2-year perturbation did not drastically alter the microbial composition but shifts in certain taxa were large enough to suggest that continuous saltwater over a longer period can lead to further changes in relative abundance and potential function.

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CHAPTER 3

3. The Impact of Salinity on Microbial Composition on Various Functional Genes Involved in Biogeochemical Cycles

3.1 Introduction

Microorganisms drive various biogeochemical cycles, making them essential in influencing how an ecosystem functions (Ren et al., 2017). These microorganisms harbor large sets of functional genes within their genomes that encode for enzymes that catalyze redox reactions in soil processes involved in cycling nutrients like carbon, nitrogen, and phosphorous. Identification of microorganisms with functional genes involved in these processes gives insight into the functional potential of the community and potential outcomes of biogeochemical cycles. Despite the vital role these microorganisms play, there is insufficient information on how the microbial community composition relates to biogeochemical cycles (Ma et al., 2021; Zhalnina et al., 2018). Additionally, little is known about how the functional potential of the microbial community shifts, after a perturbation such as saltwater intrusion.

The microbial-mediated carbon, nitrogen, sulfur, and phosphorus cycles are essential processes in wetland ecosystem functioning (Bodelier & Dedysh, 2013). One way to study these cycles from a microbial standpoint is through metagenomic sequencing. Metagenomic sequencing, also known as Next Generation Sequencing (NGS), is a culture independent technology allowing for rapid identification of whole microbial communities at once which will help us understand the coupling of these communities to biogeochemical cycles. Majority of targeted sequencing technologies utilize highly conserved 16S rRNA genes to elucidate the identities of cultivated and

uncultivated microorganisms within communities. These genes are not very practical for identifying microbes that are members of various phylogenetic groups involved in specific enzymatic pathways (Singh et al., 2019). However, regions of DNA characterized by similar traits and encode the same enzymes--functional genes/markers--(Andersen & Lubberstedt, 2003) are better suited for these purposes.

Several studies have shown that saltwater intrusion can alter the biogeochemical functioning of wetlands (Chambers et al., 2013; Neubauer et al., 2018; Tully et al., 2019; Xie et al., 2020). With the effects of climate change, perturbations are increasing and understanding how ecosystem functions change, along with the responses of microbial communities within biogeochemical pathways after these perturbations, must be examined. Monitoring the responses of microbial communities across various environments allows for a more holistic picture of the effects disturbances have on them (Cavicchioli et al., 2019; Hutchins et al., 2019).

The Everglades is one coastal wetland increasingly threatened by climate change and damaging water management practices. Having a salinity gradient that increases from north to south, the various salinity levels produce distinctively diverse biotic communities within freshwater and brackish marshes (Steinman & Kindervater, 2020). Moreover, it can be expected that the biogeochemistry differs as well, leading to disparities in microbial metabolism indicating that the microbial communities will not react similarly to certain perturbations. For example, microorganisms within freshwater environments are more likely to perform methanogenesis while those within more saltwater environments are prone to reducing sulfate. However, when saltwater is introduced into a freshwater system, the increase in ionic concentration results in microorganisms capable

of sulfate reduction to outcompete those capable of methanogenesis because it is now a more energetically favorable process (Dang et al., 2019). Consequently, the carbon and sulfur biogeochemical cycles have been disrupted.

This chapter aims to characterize the microbial functional potential of a freshwater and brackish marsh within the Everglades through metagenomic sequencing and assess the changes in diversity after salinity manipulation experiments. The functional potential is estimated by the changes in abundance. We hypothesized that *the microbial community's functional potential in soil microbiomes adapted to brackish environments will not change with salinity dosage experiments compared to those in freshwater environments.* This hypothesis was tested by sequencing one functional gene encoding for an enzyme that catalyzes a reaction from each biogeochemical cycle-nitrogen, carbon, sulfur, and phosphorus. The Nitrite reductase (*nirS*), Methyl-coenzyme M reductase subunit alpha (*mcrA*), Dissimilatory sulfite reductase subunit alpha (*dsrA*), and Alkaline phosphatase (*phoD*) genes were sequenced as a representative for each respective cycle.

3.2 Materials and Methods

3.2.1 Sample preparation for Metagenomic Sequencing

The same soil samples from Chapter 2 were utilized for these analyses. The extracted DNA was amplified with primers that targeted functional genes instead of 16S rRNA genes. Libraries were prepared by first modifying each functional gene primer set to contain Illumina specific adaptor sequences. The adaptor sequence at the front of each forward primer was (5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG + forward primer -3') and each reverse primer contained (5'-

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG + reverse primer -3'). The primers used for each gene and their respective annealing temperatures are listed in Table 4. PCR amplifications were performed on a ProFlex PCR System (Applied Biosystems, Foster City, CA, USA) in a 25 μL volume consisting of 1X Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific), 0.3 μM of each forward and reverse primer, 12.5 ng of DNA and water added to volume. PCR conditions were as follows: initial denaturation of 98°C for 10 seconds, 30 cycles of 98°C for 10 seconds, an individual annealing temperature for each gene at 5 seconds, extension at 72°C for 15 seconds, and a final extension at 72°C for 1 minute. The PCR products were maintained at 4°C for 5 minutes before removal from the thermal cycler and 1 μL of the product was visualized on a bioanalyzer trace to verify size before sequencing. Amplification products were purified using Bulldog Bio CleanNGS SPRI Beads (Bulldog Bio, Inc. Portsmouth, NH, USA) and quantified using the Qubit® 2.0 Fluorometer and the Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific). The Nextera XT v2 Index Kit (Illumina, Inc. San Diego, USA) was utilized to create the sequencing libraries. Normalized DNA libraries were diluted to 6 pM combined with 15% PhiX Control Spike (Illumina). Sequencing was performed on a MiSeq 2 x 300 platform (Illumina) with a MiSeq v3 reagent cartridge (Illumina).

Table 4: Primer sequences used to amplify the functional gene sequences. Within the nitrogen cycle, the Nitrite reductase gene (*nirS)* was amplified. The Dissimilarity sulfite reductase gene (*dsrA*) was amplified within the sulfur cycle. Within the carbon cycle, the Methyl Coenzyme M reductase (*mcrA)* gene was amplified. The Alkaline Phosphatase D (*phoD)* gene was amplified within the phosphorus cycle.

Gene	Primer	Sequence 5' – 3'	Annealing Temp (°C)	Reference
nirS	cd3aF	GTSAACGTSAAGG-ARACSGG	61°C	(Gu et al., 2019)
	R3cd	GASTTCGGRTGSGTCTTGA		
dsrA	dsrAF	ACSCACTGGAAGCACG	58°C	(Sela-Adler et al., 2017)
	dsrAR	CGGTGMAGYTCRTCCTG		
mcrA	mcrF	GGTGGTGTMGGATTCACACARTAYGCWACAGC	57.7°C	(Morris et al., 2014)
	mcrR	TTCATTGCRTAGTTWGGRTAGTT		
phoD	phoD_F733	TGGGAYGATCAYGARGT	55.8°C	(Ragot et al., 2015)
	phoD_R1083	CTGSGCSAKSACRTTCCA		

3.2.2 Functional Gene Amplicon Data Processing and Statistical Analysis

Demultiplexed paired end amplicons reads were first joined together with PANDAseq, the Ribosomal Database Project (RDP) modified version (Masella et al., 2012). The assembled sequences were then processed with the RDP Sequence Initial Processor to further remove low quality sequences. High quality assembled sequences were then further processed with the FunGene pipeline (Cole et al., 2014; Fish et al., 2013). To create a taxonomic database for each functional gene, reference sequences were downloaded from the FunGene Database (http://fungene.cme.msu.edu/). After processing, sequences were clustered at 80% similarity to the reference sequence (Wang et al., 2013). Output from the pipeline was imported into the statistical software R Statistical Software v4.1.1 and analyzed with 'RDPutils' and 'Phyloseq' R packages (McMurdie & Holmes, 2013; Quensen, 2018; Team, 2021).

Cluster richness and evenness were evaluated with Chao1, Shannon, Simpson, and Inverse Simpson alpha diversity measurements as seen in Table 5, Table 6, Table 7, and Table 8. Alpha diversity measurements summarize the mean diversity within a site, or sample which helps to provide a better initial community characterization. Each diversity index measured has different biases which is why multiple index calculations are considered. Richness is the observed count of different sequences or species, present within the sample. Evenness looks to compare how similar the population size of each individual species is to others present within the community. Chao1 is estimates total richness, by considering the observed richness and the number of singletons and doubletons. Shannon and Simpson indices are both estimators of richness and evenness. Shannon places more weight on the richness while Simpson places more weight on the

evenness (Kim et al., 2017). The larger the value of Shannon diversity, the more species that have a relatively even abundance. The Simpson index calculates the probability that two species randomly drawn from the community are from the same species. A high probability indicates low species diversity, and a low probability indicates a high diversity. The value for Simpson is between 0 and 1 and increases as diversity decreases. However, this does not provide much biological value, so the Inverse Simpson is often given to provide context.

Table 5: *nirS* gene samples from brackish (BW) and freshwater (FW) sites with corresponding year of collection: initial (Y0) or 2 years later (Y2). Samples with no salt treatment (Control) or treated with saltwater pulses (Salt) are indicated in the treatment column. The Chambers indicated as controls were sampled at Y0 and again at Y2 while the treatment chambers were sampled only at Y2. Total Observed OTUs (Operational Taxonomic Units) indicate the total number of reads identified as a taxonomic cluster. Observed Unique OTUs indicate the richness within the sample as explained by the various diversity indices, Chao1, Shannon, Simpson, and Inverse Simpson.

Group	Treatment	Chamber	Total Observed OTUs	Unique Observed OTUs	Chao1	Shannon	Simpson	Inverse Simpson
BW_Y0	Control	3B	8590	574	574.00	4.52	0.95	19.99
BW_Y0	Control	5B	8600	151	151.00	3.87	0.94	16.21
BW_Y0	Control	1B	8570	1113	1113.00	5.43	0.98	43.65
BW_Y0	Control	7B	8623	1465	4540.40	4.47	0.93	14.48
BW_Y2	Control	9B	8635	1899	5716.62	5.07	0.95	21.45
BW_Y2	Control	5B	8672	2199	4380.67	5.76	0.97	29.86
BW_Y2	Control	3B	8622	232	232.00	4.72	0.97	30.70
BW_Y2	Control	7B	8542	1342	2822.17	5.04	0.97	34.11
BW_Y2	Control	8B	8551	1363	3397.14	4.85	0.96	26.84
BW_Y2	Control	1B	8533	1279	2516.28	5.08	0.97	29.85
BW_Y2_Saline	Salt	14B	8655	2079	3310.86	5.61	0.96	25.01
BW_Y2_Saline	Salt	15B	8554	1727	3455.40	5.25	0.94	17.11
BW_Y2_Saline	Salt	11B	8562	1912	5576.08	5.33	0.95	20.61
BW_Y2_Saline	Salt	12B	8531	1438	3333.55	4.99	0.96	27.18
BW_Y2_Saline	Salt	13B	8546	1748	4047.95	5.27	0.95	22.20
BW_Y2_Saline	Salt	16B	8525	1527	3405.12	5.16	0.97	28.79
FW_Y0	Control	3F	8554	1425	2925.94	5.61	0.98	45.03
FW_Y0	Control	2F	8562	337	337.00	5.43	0.99	119.43
FW_Y0	Control	6F	8593	771	771.00	5.27	0.97	39.72

FW_Y0	Control	9F	8607	441	441.00	5.40	0.98	58.91
FW_Y0	Control	8F	8620	219	219.00	4.91	0.98	45.28
FW_Y2	Control	2F	8529	1313	2230.56	5.52	0.98	52.42
FW_Y2	Control	8F	8687	2263	8012.75	6.33	0.99	126.37
FW_Y2	Control	9F	8654	1762	3015.69	5.92	0.99	77.12
FW_Y2	Control	10F	8581	560	560.00	4.80	0.96	24.78
FW_Y2	Control	3F	8661	2036	5043.99	5.95	0.99	67.25
FW_Y2	Control	6F	8541	1221	195597.00	5.52	0.98	50.35
FW_Y2_Saline	Salt	12F	8556	136	136.00	4.53	0.98	46.62
FW_Y2_Saline	Salt	15F	8586	57	57.00	3.63	0.96	22.26
FW_Y2_Saline	Salt	16F	8699	1506	1506.00	6.26	0.99	123.29
FW_Y2_Saline	Salt	13F	8557	1236	2915.58	5.32	0.98	49.88
FW_Y2_Saline	Salt	14F	8660	1426	1426.00	6.04	0.99	68.56

Table 6: *mcrA* gene samples from brackish (BW) and freshwater (FW) sites with corresponding year of collection: initial (Y0) or 2 years later (Y2). Samples with no salt treatment (Control) or treated with saltwater pulses (Salt) are indicated in the treatment column. The Chambers indicated as controls were sampled at Y0 and again at Y2 while the treatment chambers were sampled only at Y2. Total Observed OTUs (Operational Taxonomic Units) indicate the total number of reads identified as a taxonomic cluster. Observed Unique OTUs indicate the richness within the sample as explained by the various diversity indices, Chao1, Shannon, Simpson, and Inverse Simpson.

Group	Treatment	Chamber	Total Observed OTUs	Unique Observed OTUs	Chao1	Shannon	Simpson	Inverse Simpson
BW_Y0	Control	3B	2740	466	1054.09	3.88	0.91	10.63
BW_Y0	Control	8B	2943	321	321.00	4.67	0.95	20.67
BW_Y2	Control	5B	2744	567	938.13	4.79	0.95	21.91
BW_Y2	Control	3B	2918	593	1303.03	4.31	0.92	12.26
BW_Y2	Control	7B	2831	488	730.31	4.43	0.94	17.29
BW_Y2	Control	8B	2616	709	1241.05	5.47	0.99	72.84
BW_Y2	Control	1B	2741	571	995.23	4.46	0.94	16.82
BW_Y2_Saline	Salt	11B	2810	369	369.00	4.52	0.94	16.48
BW_Y2_Saline	Salt	12B	2747	447	920.61	3.82	0.90	9.84
BW_Y2_Saline	Salt	13B	2780	548	968.00	4.86	0.96	27.12
BW_Y2_Saline	Salt	16B	2766	637	1248.85	4.63	0.95	19.41
FW_Y0	Control	2F	2879	26	26.00	3.19	0.96	22.39
FW_Y0	Control	6F	2770	534	44194.00	5.02	0.98	42.28
FW_Y0	Control	9F	2791	296	296.00	5.02	0.98	56.51
FW_Y0	Control	8F	2909	65	65.00	4.09	0.98	53.40
FW_Y2	Control	2F	2639	644	1039.00	5.46	0.99	92.54
FW_Y2	Control	8F	2880	736	91687.00	5.61	0.99	78.04
FW_Y2	Control	9F	2784	463	742.43	4.46	0.96	22.26
FW_Y2	Control	10F	2784	171	171.00	4.68	0.98	46.02

FW_Y2	Control	3F	2795	821	1461.06	5.57	0.98	53.38
FW_Y2	Control	6F	2923	458	458.00	5.58	0.99	111.63
FW_Y2_Saline	Salt	12F	2813	308	308.00	4.97	0.98	44.41
FW_Y2_Saline	Salt	15F	2916	693	1254.16	5.30	0.98	47.17
FW_Y2_Saline	Salt	16F	2760	546	761.47	4.52	0.94	16.46
FW_Y2_Saline	Salt	13F	2707	723	1392.77	5.40	0.99	69.07
FW_Y2_Saline	Salt	14F	2872	767	1434.26	5.64	0.99	77.37

Table 7: *dsrA* gene samples from brackish (BW) and freshwater (FW) sites with corresponding year of collection: initial (Y0) or 2 years later (Y2). Samples with no salt treatment (Control) or treated with saltwater pulses (Salt) are indicated in the treatment column. The Chambers indicated as controls were sampled at Y0 and again at Y2 while the treatment chambers were sampled only at Y2. Total Observed OTUs (Operational Taxonomic Units) indicate the total number of reads identified as a taxonomic cluster. Observed Unique OTUs indicate the richness within the sample as explained by the various diversity indices, Chao1, Shannon, Simpson, and Inverse Simpson.

Group	Treatment	Chamber	Total Observed OTUs	Observed Unique OTUs	Chao1	Shannon	Simpson	Inverse Simpson
BW_Y0	Control	3B	7734	2130	5402.49	5.95	0.98	52.90
BW_Y0	Control	5B	7591	1312	2521.48	5.15	0.95	18.43
BW_Y0	Control	8B	7462	1071	1071.00	5.80	0.99	88.41
BW_Y0	Control	1B	7530	651	651.00	5.38	0.98	41.88
BW_Y0	Control	7B	7610	1530	2927.29	5.58	0.98	40.11
BW_Y2	Control	9B	7651	1897	3312.70	5.86	0.98	48.64
BW_Y2	Control	5B	7372	1113	1113.00	6.28	0.99	170.20
BW_Y2	Control	3B	7461	682	682.00	5.87	0.99	121.60
BW_Y2	Control	7B	7828	2991	9887.94	6.80	1.00	208.82
BW_Y2	Control	8B	7687	2394	4847.61	6.59	0.99	130.49
BW_Y2	Control	1B	7394	1814	602970.00	6.32	0.99	99.53
BW_Y2_Saline	Salt	14B	7602	1144	1144.00	6.33	0.99	179.84
BW_Y2_Saline	Salt	11B	7379	1252	1252.00	6.19	0.99	110.37
BW_Y2_Saline	Salt	12B	7389	1449	2351.21	5.55	0.97	37.78
BW_Y2_Saline	Salt	13B	7654	1177	1177.00	6.27	0.99	120.91
BW_Y2_Saline	Salt	16B	7682	2856	12800.67	6.85	1.00	204.65
FW_Y0	Control	3F	7545	1721	3053.44	6.04	0.98	63.78
FW_Y0	Control	2F	7476	1488	2414.88	5.87	0.99	71.32
FW_Y0	Control	6F	7664	1738	2831.79	6.20	0.99	118.47

FW_Y0	Control	9F	7673	1328	1328.00	5.95	0.98	58.45
FW_Y0	Control	8F	7503	1578	3033.00	5.68	0.97	32.65
FW_Y2	Control	2F	7434	1079	1445.34	4.97	0.95	20.33
FW_Y2	Control	8F	7407	1151	1151.00	5.94	0.98	53.50
FW_Y2	Control	9F	7709	1939	4389.58	5.58	0.95	19.04
FW_Y2	Control	10F	7686	1806	4191.54	5.89	0.98	54.47
FW_Y2	Control	3F	7472	615	615.00	5.41	0.98	41.01
FW_Y2	Control	6F	7637	807	807.00	5.95	0.99	124.16
FW_Y2_Saline	Salt	12F	7415	1535	367475.00	6.09	0.99	94.87
FW_Y2_Saline	Salt	15F	7663	2161	3288.05	6.44	0.99	105.01
FW_Y2_Saline	Salt	16F	7526	1705	3209.98	5.82	0.97	37.30
FW_Y2_Saline	Salt	13F	7441	1576	4073.50	5.62	0.98	44.34
FW_Y2_Saline	Salt	14F	7453	1260	1260.00	6.07	0.98	58.25

Table 8: *phoD* gene samples from brackish (BW) and freshwater (FW) sites with corresponding year of collection: initial (Y0) or 2 years later (Y2). Samples with no salt treatment (Control) or treated with saltwater pulses (Salt) are indicated in the treatment column. The Chambers indicated as controls were sampled at Y0 and again at Y2 while the treatment chambers were sampled only at Y2. Total Observed OTUs (Operational Taxonomic Units) indicate the total number of reads identified as a taxonomic cluster. Observed Unique OTUs indicate the richness within the sample as explained by the various diversity indices, Chao1, Shannon, Simpson, and Inverse Simpson.

Group	Treatment	Chamber	Total Observed OTUs	Observed Unique OTUs	Chao1	Shannon	Simpson	Inverse Simpson
BW_Y0	Control	3B	52728	2030	3011.34	5.72	0.98	64.91
BW_Y0	Control	5B	30314	2030	3238.32	5.25	0.97	35.11
BW_Y0	Control	8B	26518	2274	3342.92	5.55	0.98	44.43
BW_Y0	Control	7B	21473	1866	2878.99	5.51	0.98	54.52
BW_Y2	Control	9B	120	77	77.00	3.85	0.95	19.12
BW_Y2	Control	5B	40047	3659	8922.92	6.10	0.98	60.77
BW_Y2	Control	3B	15084	2176	2176.00	6.12	0.99	110.79
BW_Y2	Control	7B	53926	2380	3482.41	5.99	0.99	93.20
BW_Y2	Control	8B	69216	2239	3013.03	5.64	0.98	44.37
BW_Y2	Control	1B	7535	1561	1561.00	6.18	0.99	146.61
BW_Y2_Saline	Salt	14B	8	8	8.00	2.08	0.88	8.00
BW_Y2_Saline	Salt	11B	53608	2449	3765.25	6.19	0.99	121.88
BW_Y2_Saline	Salt	12B	69406	2351	3212.06	5.84	0.98	61.75
BW_Y2_Saline	Salt	13B	33095	2995	4027.02	6.00	0.98	55.07
BW_Y2_Saline	Salt	16B	45124	3573	7425.83	6.32	0.99	113.96
FW_Y0	Control	3F	11022	2258	2258.00	6.48	0.99	195.86
FW_Y0	Control	2F	32950	3349	4407.27	6.28	0.99	77.76
FW_Y0	Control	6F	32849	3107	4122.22	6.22	0.99	106.19
FW_Y0	Control	9F	32635	3525	4664.10	6.56	0.99	164.77

FW_Y0	Control	8F	19818	2403	623458.00	5.36	0.94	17.89
FW_Y2	Control	2F	21813	2133	3408.92	5.83	0.98	58.28
FW_Y2	Control	8F	13512	2196	2196.00	6.30	0.99	120.75
FW_Y2	Control	9F	26863	2714	4181.54	6.28	0.99	136.03
FW_Y2	Control	10F	9379	1318	1318.00	5.88	0.99	133.74
FW_Y2	Control	3F	27703	2423	3773.81	5.73	0.97	35.81
FW_Y2	Control	6F	23099	2186	3253.55	5.33	0.97	31.95
FW_Y2_Saline	Salt	12F	1762	600	600.00	5.29	0.97	37.48
FW_Y2_Saline	Salt	15F	5169	1367	1367.00	6.26	0.99	189.75
FW_Y2_Saline	Salt	16F	18256	2650	708916.00	6.40	0.99	166.23
FW_Y2_Saline	Salt	13F	26360	2746	4274.37	6.06	0.99	74.43
FW_Y2_Saline	Salt	14F	20649	2981	855146.00	6.40	0.99	122.74

3.3 Results

3.3.1 Beta Diversity Patterns of Functional Genes

An ANOSIM test using Bray-Curtis dissimilarity was utilized to estimate the dissimilarity in functional potential of each gene between the two sites, Table 9. Sample dissimilarity was visualized using Principal Coordinates Analysis (PcoA) ordination plots. ANOSIM tests are frequently used to assess multivariate ecological data using a measure of dissimilarity, in this case Bray-Curtis dissimilarity (Bray & Curtis, 1957; Clarke et al., 2006). The dissimilarity is determined by dividing the differences between groups by the differences between individuals. The ANOSIM R value can range from -1 to 1, although it is usually between 0 and 1. Higher numbers indicate a weaker resemblance and an uneven distribution, whereas values close to 0 indicate a balanced distribution and high similarity. Negative values, on the other hand, may indicate that there is greater variation within samples than across groups (Chapman & Underwood, 1999). The test was measured at $p<0.05$ level of significance.

The *nirS* gene showed distinct community differences between the freshwater and brackish sites, Figure 13. Samples from the brackish site seemed to have very similar microbial compositions and plotted close together. However, more variation was seen in the microbial communities in the freshwater site as displayed in the plot. After two years of monthly salinity pulses, the communities did not significantly shift at the brackish or freshwater site. Samples were collected during Year 0 and Year 2 from each site to determine if the community naturally shifted over time. Within the brackish site, temporal shifts were not detected, indicating a possibly more stable community**.** The freshwater site seemed to have a significant shift in composition over time.

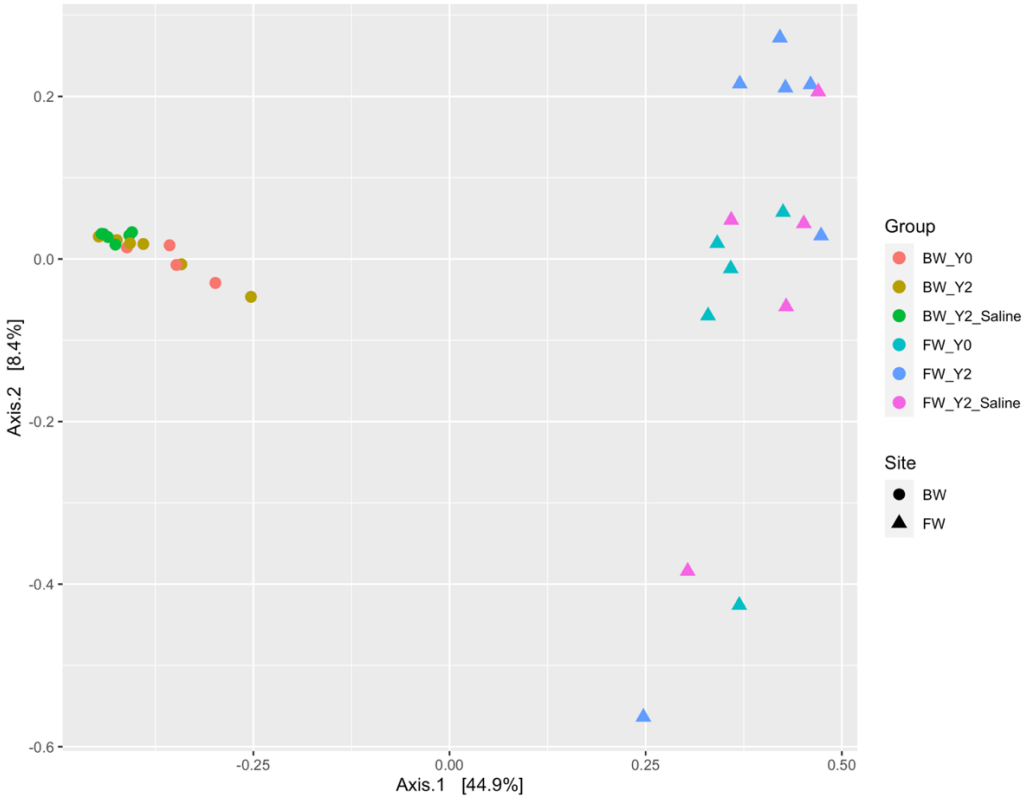

Figure 13: Principal Coordinates Analysis (PcoA) plot displaying clear separation between brackish (circle) and freshwater (triangle) sites from *nirS* microbial community profiles. There was more variation between chambers at the freshwater site than the brackish site. Tight grouping of samples within the brackish site indicates no change in diversity over time or after treatment. Distinct grouping among the FW_Y0 and FW_Y2 samples suggest temporal changes in the freshwater site. The scattered plot of the FW_Y2_Saline samples suggest that saline did shift the diversity for the *nirS* microbial community in the freshwater site for some of the chambers.

Table 9: Results of Analysis of Similarities (ANOSIM) test using Bray-Curtis dissimilarity. Statistically significant differences are bolded.

	Temporal		**Treatment**	
	BW_Y0 vs BW_Y2	**FW_Y0 vs FW_Y2**	**BW_Y2 vs BW_Y2_Saline**	**FW_Y2 vs FW_Y2_Saline**
Gene				
nirS	R= 0.071, p>0.05	**R= 0.384, p<0.05**	R= 0.094, p>0.05	R= 0.072, p>0.05
mcrA	R= 0.291, p>0.05	R= 0.151, p>0.05	R= 0.319, p=0.051	R= 0.093, p>0.05
dsrA	**R= 0.307, p<0.05**	**R= 0.608, p<0.05**	R= -0.045, p>0.05	**R= 0.227, p<0.05**
phoD	R= 0.083, p>0.05	**R= 0.504, p<0.05**	R= 0.034, p>0.05	**R= 0.261, p<0.05**

The *mcrA* gene also showed a high dissimilarity between the freshwater and brackish sites, Figure 14. A similar pattern is seen again, with the high similarity between the brackish site and the high variation between the freshwater sites. This suggests that the brackish water did not vary over time and that the saltwater did not influence the overall community composition. However, the spread within the freshwater site suggests that the compositions varied both between chambers and after treatment. The Year 2 samples did plot closer together than the samples from the initial collection year suggesting that the chamber compositions were more similar. Most of the variation within this site occurred between the Year 1 control samples as indicated in the figure below.

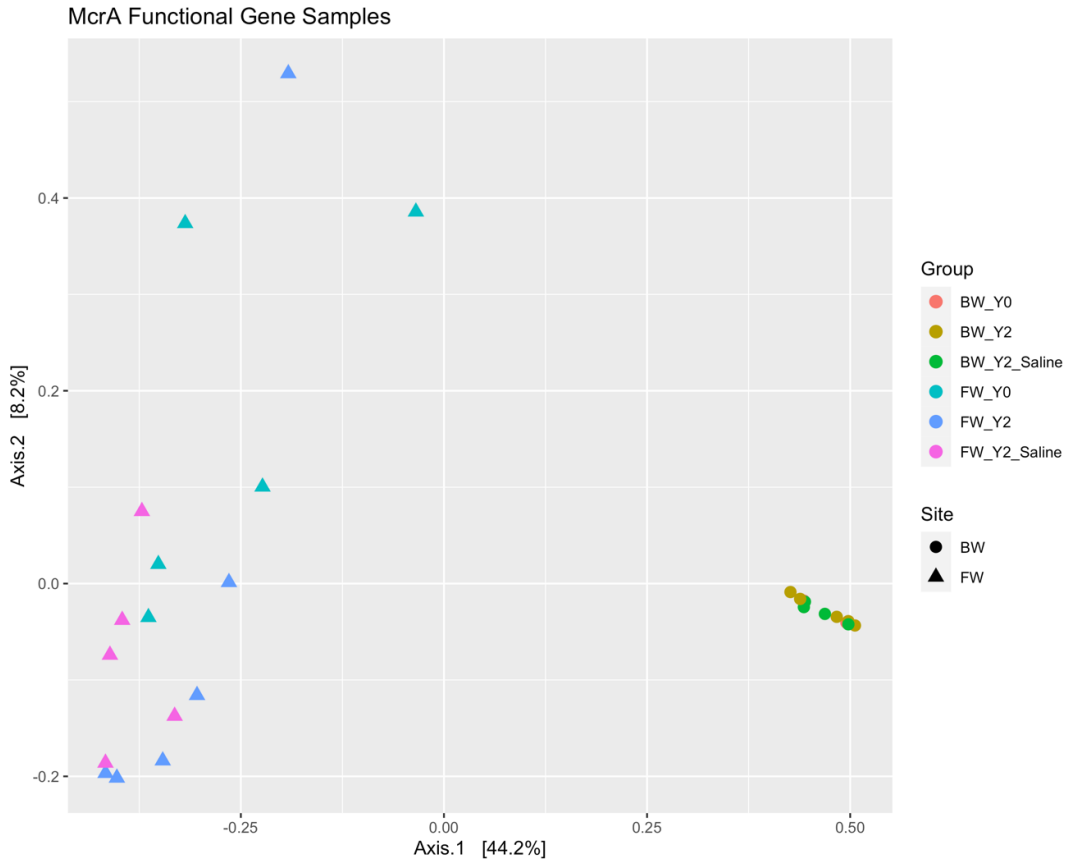

Figure 14: Principal Coordinates Analysis (PcoA) plot displaying clear separation between brackish (circle) and freshwater (triangle) sites from *mcrA* microbial community profiles. High variation between samples at the freshwater site but no distinct grouping suggests changes over time or after treatment, but ANOSIM showed no statistical difference. Close grouping of samples within the brackish site indicates little change in diversity over time or after treatment in the *mcrA* microbial community.

The *dsrA* gene also differed a great deal between the freshwater and brackish sites

Figure 15. Salinity pulses significantly affected the *dsrA* gene microbial community

composition at the freshwater site as displayed by the distinct groupings in the plot.

Salinity had no effect on community within the brackish site. Additionally, significant

temporal shifts were detected at both the freshwater and brackish sites.

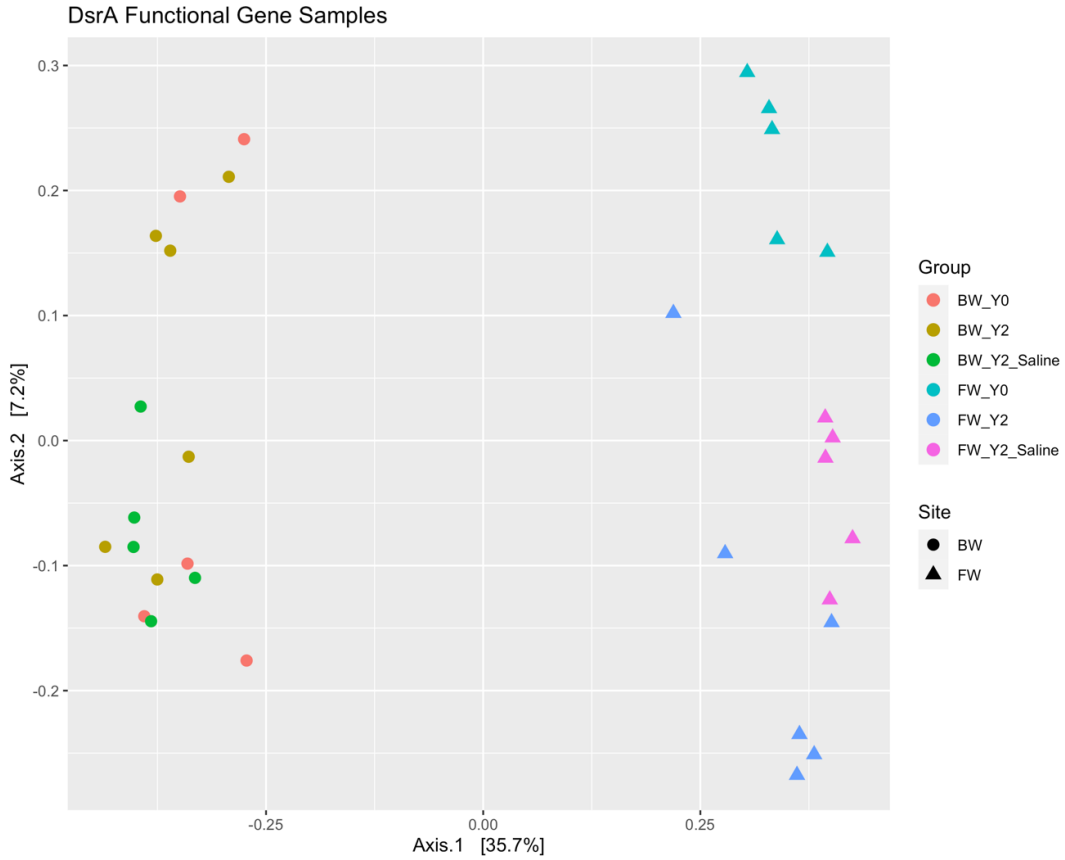

Figure 15: Principal Coordinates Analysis (PcoA) plot displaying clear separation between brackish (circle) and freshwater (triangle) sites from *dsrA* microbial community profiles. Distinct grouping within the samples from the freshwater site indicate changes

in diversity temporally and after saltwater treatment. Close grouping with the BW_Y2 and BW_Y2_Saline samples suggest that saltwater treatment did not significantly shift the *dsrA* microbial community in some chambers. Distinct grouping among the BW_Y0 and BW_Y2 samples suggest temporal changes in the brackish site within this site.

When the *phoD* gene was evaluated, the freshwater and brackish sites had significantly different compositions, Figure 16. Communities harboring this gene were shifted at the FW site after two years of monthly salinity pulses. Communities in the brackish site remained unchanged after salinity manipulation. Samples collected to measure the temporal shifts in the freshwater site significantly differed in composition, but temporal shifts were not detected at the brackish site.

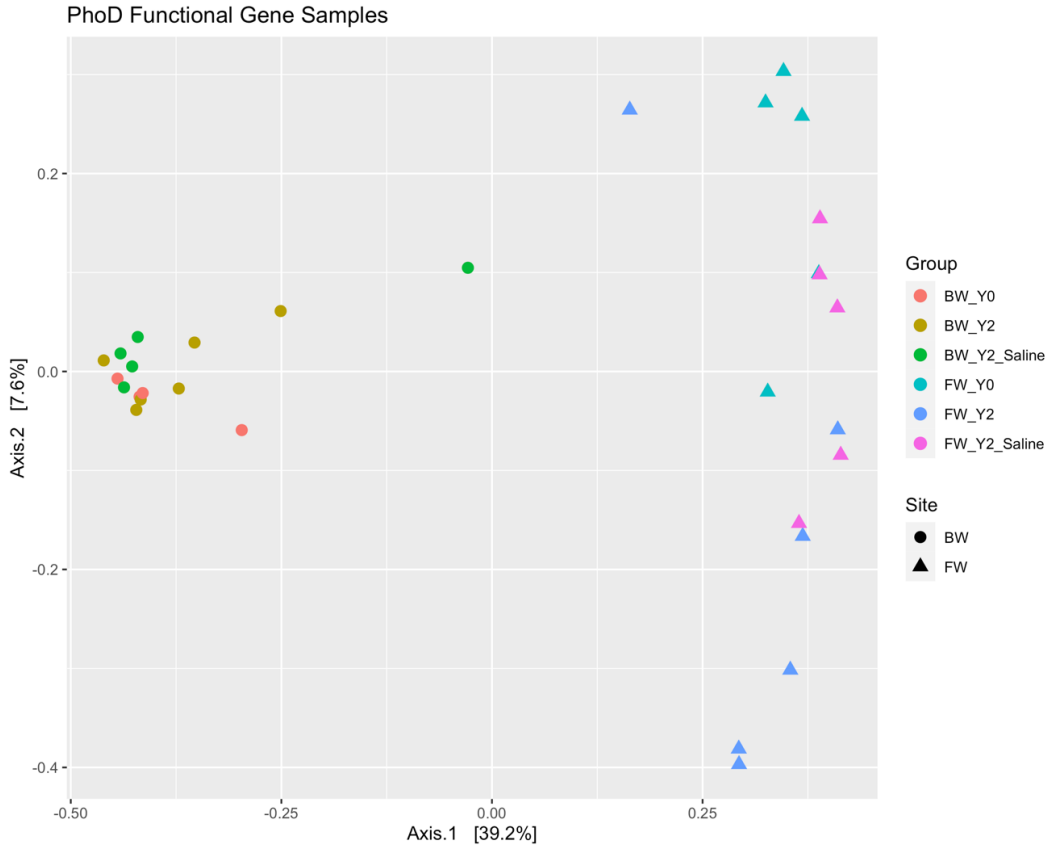

Figure 16: Principal Coordinates Analysis (PcoA) plot displaying clear separation between brackish (circle) and freshwater (triangle) sites from *phoD* microbial community profiles. Close grouping of samples within the brackish site indicates little change in

diversity over time or after treatment. Distinct changes within the samples from the freshwater site indicate changes in diversity temporally and after saltwater treatment.

Beta diversity measurements determined significant differences between the freshwater and brackish sites in each of the functional genes sequenced, indicating unique microbial compositions between the two sites. Temporal shifts in the freshwater site were detected in microorganisms harboring the *nirS*, *dsrA*, and *phoD* functional genes. Within the brackish site, temporal shifts were only detected in communities with the *dsrA* functional gene. These differences show the natural variation of the communities over time and show that many of these communities in the brackish site are stable. The *mcrA* gene did not display any significant changes over time so members of its community were stable in both brackish and freshwater sites. The saltwater manipulation experiments caused significant differences within the *dsrA* and *phoD* communities at the freshwater site. The only community that significantly differed after saltwater manipulation in the brackish site was the *mcrA* community. These differences indicate a change in composition, possibility leading to a change in function due to the increase of saltwater.

3.3.2 Indicator species to determine Functional Gene Microbial Structure

The R package 'indicspecies' was utilized to determine specific clusters within each gene that were found to be more frequently present and abundant with the various sites (De Caceres & Legendre, 2009). OTUs identified here were found at higher rates than in other sites and resulted in them having strong or more robust associations with their specified site or group. Traditionally, an indicator species serves as a measure of the environmental conditions and gives insight into how well other species might do in the same environment. Identification of microorganisms as indicator species can further

characterize the microbial community present within a site and provide information about the status of the soil's sustainability. Additionally, indicator species have the potential to serve as useful predictors of soil provenance. Within the soil samples from this study, numerous clusters were identified as being statistically significant to the sites and groups but only those with the strongest associations within each are reported.

3.3.2.1 *nirS*

The *nirS* gene had a total of 16,479 unique sequence clusters. All identified sequences were of bacteria and consisted of 6 Phyla, 9 Classes, 20 Orders, 28 Families, and 33 Genera including uncultured and unclassified bacteria. Betaproteobacteria, members of the Proteobacteria phylum, were prominent at both freshwater and brackish sites associated with this gene. However, the genera with the strongest association to the brackish site were *Ruegeria* and *Hahella*, which are members of the Alphaproteobacteria and Gammaproteobacteria classes, respectively. At the freshwater site, *Bordetella*, and *Aromatoleum*, both members of the Betaproteobacteria class, were strongly associated with this site. Each group also had clusters that were identified as possible indicator species. Indicator species analysis determined that the *Thiobacillus* genus was the most strongly associated with the BW_Y0, BW_Y2, and BW_Y2_Saline groups. More unique indicator species were identified at the freshwater site. The *Bordetella* and *Cupriavidus* genera, both members of Betaproteobacteria, were most strongly associated with the FW_Y0 group. The *Pseudomonas* and *Magnetospirillum* genera, members of the Gammaproteobacteria and Alphaproteobacteria classes, respectively, were most strongly associated with the FW_Y2 group. *Aromatoleum* of the Betaproteobacteria class was mostly associated with the FW_Y2_Saline group.

3.3.2.2 *mcrA*

The *mcrA* gene had 6,235 unique sequence clusters. All identified sequences were of the Archaea domain and included 1 Phylum, 4 Classes, 7 Orders, 12 Families, and 19 Genera. Members of the Methanobacteria class dominated the composition at each site. When comparing the compositions of each site, the *Methanobacterium* genus was strongly associated with the brackish site, while the *Methanothermobacter* genus was strongly associated with the freshwater site. Both are members of the Methanobacteria class. However, when comparing each group, only the BW_Y0, BW_Y2, FW_Y2, and FW_Y2_Saline groups had clusters that could be significantly associated with their individual groups. BW_Y0 group was strongly associated with the *Methanoregula* genus, a member of the Methanomicrobia class. In comparison, the BW_Y2 group had the strongest association with the *Methanothermobacter* genus. FW_Y2 had the strongest association with *Methanothrix* from the Methanomicrobia class but also had strong associations with *Methanobacterium*. The cluster with the strongest association with the FW_Y2_Saline group was from the *Methanobacterium* genus.

3.3.2.3 *dsrA*

The *dsrA* gene had 18,440 unique sequence clusters. All identified sequences were classified as either Bacteria or uncultured prokaryote. The reads consisted of 7 Phyla, 8 Classes, 13 Orders, 22 Families and 40 Genera, including uncultured and unclassified sequences. Over half of the sequences from each site were identified as uncultured sulfate-reducing bacteria and therefore make up most of the clusters identified as being the strongest associations as indicator species for each site and each respective group. However, to differentiate each sequence read, they will be identified as their

individual cluster number. The brackish site had strong associations with cluster_1303 and cluster_734. The freshwater site had strong associations with cluster_59 and cluster_185. Each individual group also contained strong associations with unique clusters. Clusters_3125 and 5235 were strongly associated with the BW_Y0 group. The BW_Y2 group had strong associations with cluster_7730, cluster_4326. The BW_Y2_Saline group had strong associations with cluster_3419 and cluster_1749. The FW_Y0 group had strong significant associations with cluster_10173 and cluster_769. The FW_Y2 group had strong associations with cluster_5956 and cluster_4169 while the FW_Y2_Saline group was most strongly associated with cluster_8064 and cluster_14109.

3.3.2.4 *phoD*

The *phoD* gene had 22,150 unique sequence clusters. Clusters were distributed into sequences from bacteria, Archaea, or were unclassified sequences, 14 Phyla, 26 Classes, 65 Orders, 126 Families, and 265 Genera. The Proteobacteria phylum dominated all sites, but the Planctomycetes and Actinobacteria phyla followed closely in total composition in brackish and freshwater sites, respectively. Within the brackish site, strong associations were identified with *Streptomyces* and *Pirellula* genera which are members of Actinomycetia and Planctomycetia phyla, respectively. *Anatilimnocola* and *Noviherbaspirillum* from the Planctomycetia and Betaproteobacteria phyla, respectively, were identified as having the strongest associations with the freshwater site. *Pseudomonas* and *Microthrix* were strongly associated with the BW_Y0 group while *Pirellula* and *Haliscomenobacter* were strongly associated with the BW_Y2 group. Within the BW_Y2_Saline group, *Bosea* and *Gemmatimona*s had the strongest associations to this group. In the FW_Y0 group, *Gimesia* and unclassified Nostocales had

the strongest associations. *Nostoc* and *Gimesia* had the strongest associations at the FW_Y2 group and unclassified Betaproteobacteria and *Streptosporangium* were strongly associated with the FW_Y2_Saline group.

3.3.3 Shifts in microbial functional potential after salinization

Differential abundance was analyzed to determine where significant ($p<0.05$) taxonomic shifts in OTUs occurred after saltwater manipulation. This analysis method identifies individual OTUs that have significantly different levels of abundances between samples, groups, or treatments. OTUs that were indicated as having higher abundances at the control or treatment site were plotted in the figures below and provide insight into the effect that saltwater had on individual taxa. The R package "DESeq2" was utilized for differential abundance analysis (Love et al., 2014). Shifts in composition were experienced within all functional genes at freshwater sites and some brackish sites. Changes in composition indicate their affinity and survivability, or lack thereof, for saltwater and the possible subsequent effects on soil conditions, suggesting alterations in functional potential.

In the *nirS* gene, the taxonomic composition varied the most between the freshwater and brackish sites. There were significantly more members of Nitrosomonadales in the brackish site (62.4%) than the freshwater site (4.4%), Figure 17. In comparison, there were considerably more Burkholderales and Pseudomonadales in the freshwater site (41.0% and 23.4%, respectively) than the brackish site (14.5% and 3.5%, respectively). Oceanospirillales were a unique taxon in the brackish site and their relative abundance was increased from 1.01% to 1.65% after saltwater. Rhodospirillales were more abundant in the freshwater site but increased from 3.91% to 4.49% after

saltwater pulses. Differential abundance analysis did not identify any OTUs that were significantly more abundant in the control group or the treatment group in the brackish site. This suggests that the community did not have any significant shifts. However, at the freshwater site, a total of 67 OTUs were identified as being more abundant at either treatment group, with 16 OTUs from the control and 51 OTUs from the treatment. These results suggest that the saltwater pulses caused some taxa to increase at that site. Low level taxa like *Leptothrix*, unclassified Gammaproteobacteria and *Hahella* genera contained OTUs that were more abundant within control samples indicating decreases after saltwater pulses. The relative abundance of *Leptothrix* went from 0.5% to 0%, unclassified Gammaproteobacteria went from 0.5% to 0.2% and *Hahella* went from (0.1% to 0%). These taxa were already present at relatively low levels and saltwater being introduced into the site reduced their abundance more. *Roseibium* increased from 1.9% to 3.9% contributing to the 2% increase observed at the Hyphomicrobiales order. The Burkholderiales order increased from 33% to 48% after saltwater and this increase can be attributed to increases in the *Bordetella, Cupriavidus*, and *Rubrivivax* genera, Figure 18.

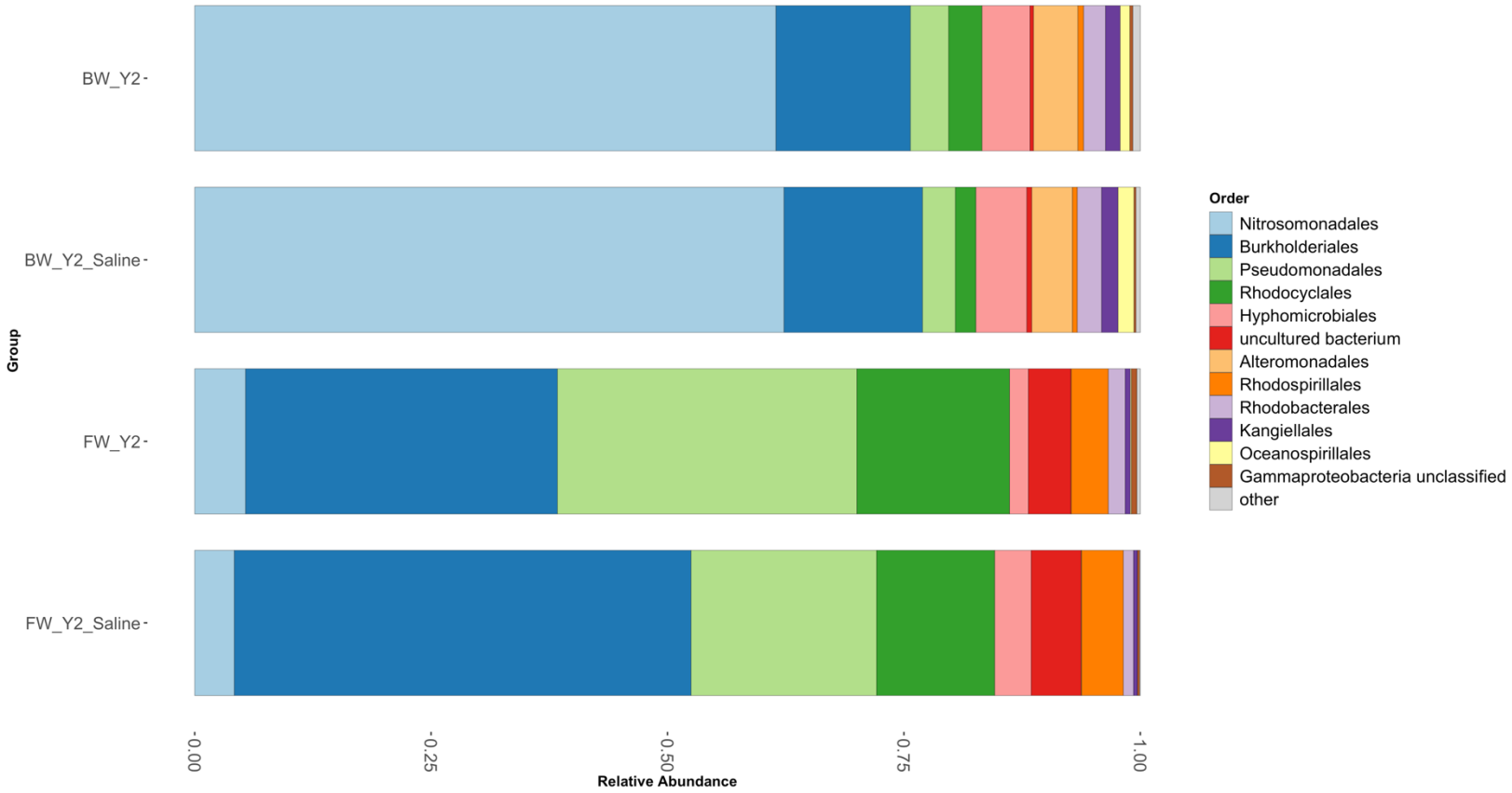

Figure 17: Relative abundance of the most abundant Orders found within the *nirS* gene microbial community. The brackish site (top two bars) and the freshwater site (bottom two bars) have unique microbial distributions. Saltwater did not shift the relative abundance of taxa in the brackish site but shifts in taxa were observed in the freshwater site.

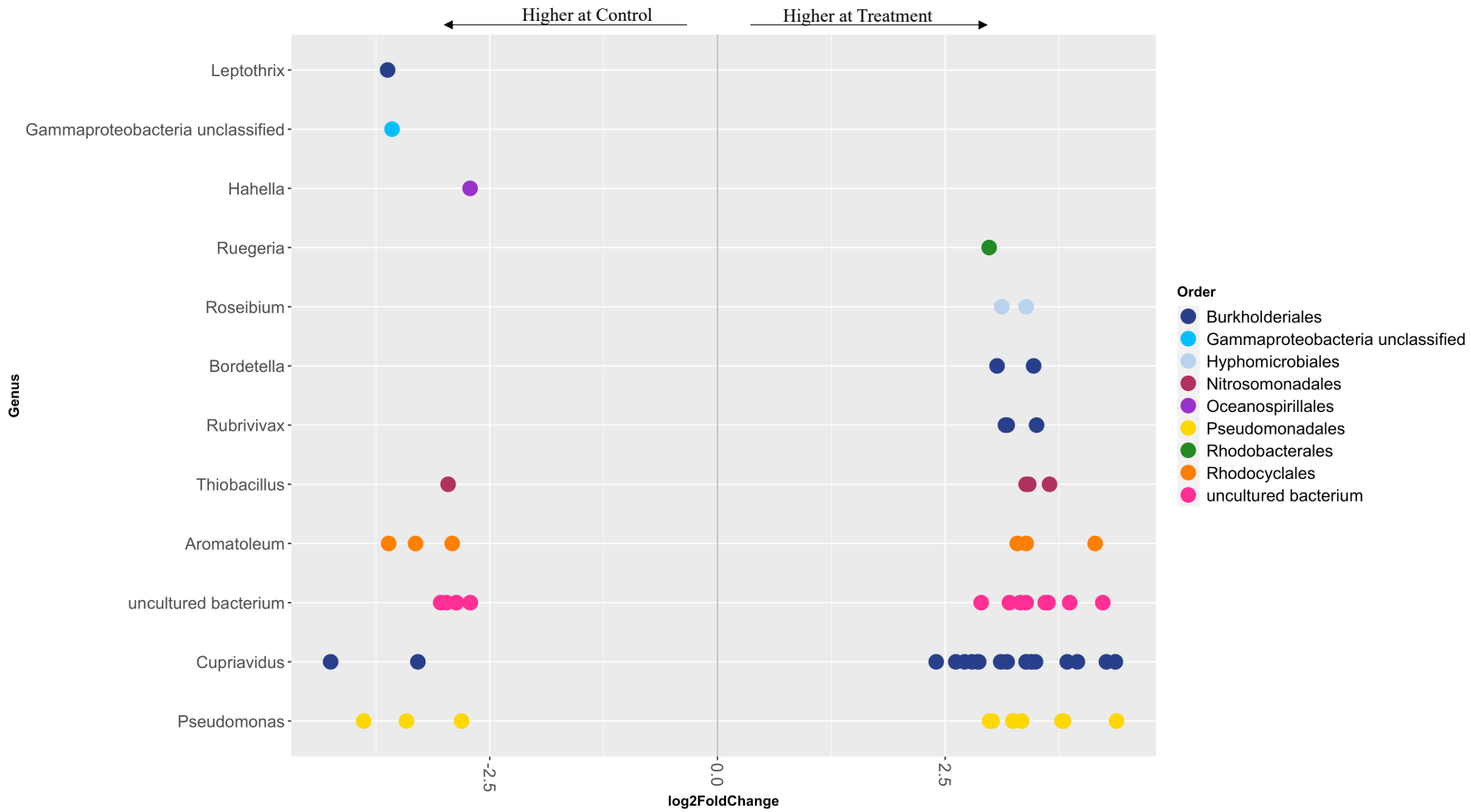

Figure 18: Differential abundance plot between Year 2 samples at the Freshwater site- control and saltwater treatment. Negative log fold changes represent more abundance at the control samples and positive represent the treatment samples and 51 OTUs increased after the saltwater treatment while 16 decreased.

In the *mcrA* gene, the taxonomic composition differed greatly between the two sites. The freshwater site (63.58 $\pm$ 32.77) had an overall higher diversity than the brackish site (25.5 $\pm$ 7.5) as indicated in Figure 19, and by the mean of their Inverse Shannon diversity metric. The brackish site had a relative abundance of 89.8% of *Methanobacterium*, 7.7% of *Methanothermobacter*, and 1.1% of *Methanocella*, the remaining genera were present at less than 1%. The top genera and their corresponding relative abundances at the freshwater site were *Methanobacterium* (32.9%), *Methanothermobacter* (28.3%), *Methanocella* (7.9%), *Methanothrix* (5%), and *Methanoregula* (4.9%). Remaining taxa were present at less than 4.9%. Methanoregula was not present at the brackish site and decreased from 8.8% to 5.1% after the saltwater treatment, suggesting this microbe has an intolerance for saltwater. *Methnobacterium* also decreased at this site after the treatment, from 31.2% to 26.14% relative abundance. Differential abundance analysis identified a total of four OTUs within the brackish site and two within the freshwater site. All were observed within the control samples indicating that their relative abundances decrease after the treatment. Differential abundance analysis revealed that OTUs from *Methanobacterium* and *Methanothermobacter* were contributing more to the control samples at the freshwater site. At the brackish site, OTUs from *Methanobrevibacter* were not present at all in the freshwater site and were more abundant in the control than the treatment. OTUs from *Methanobacterium* were found to be more abundant in the control samples.

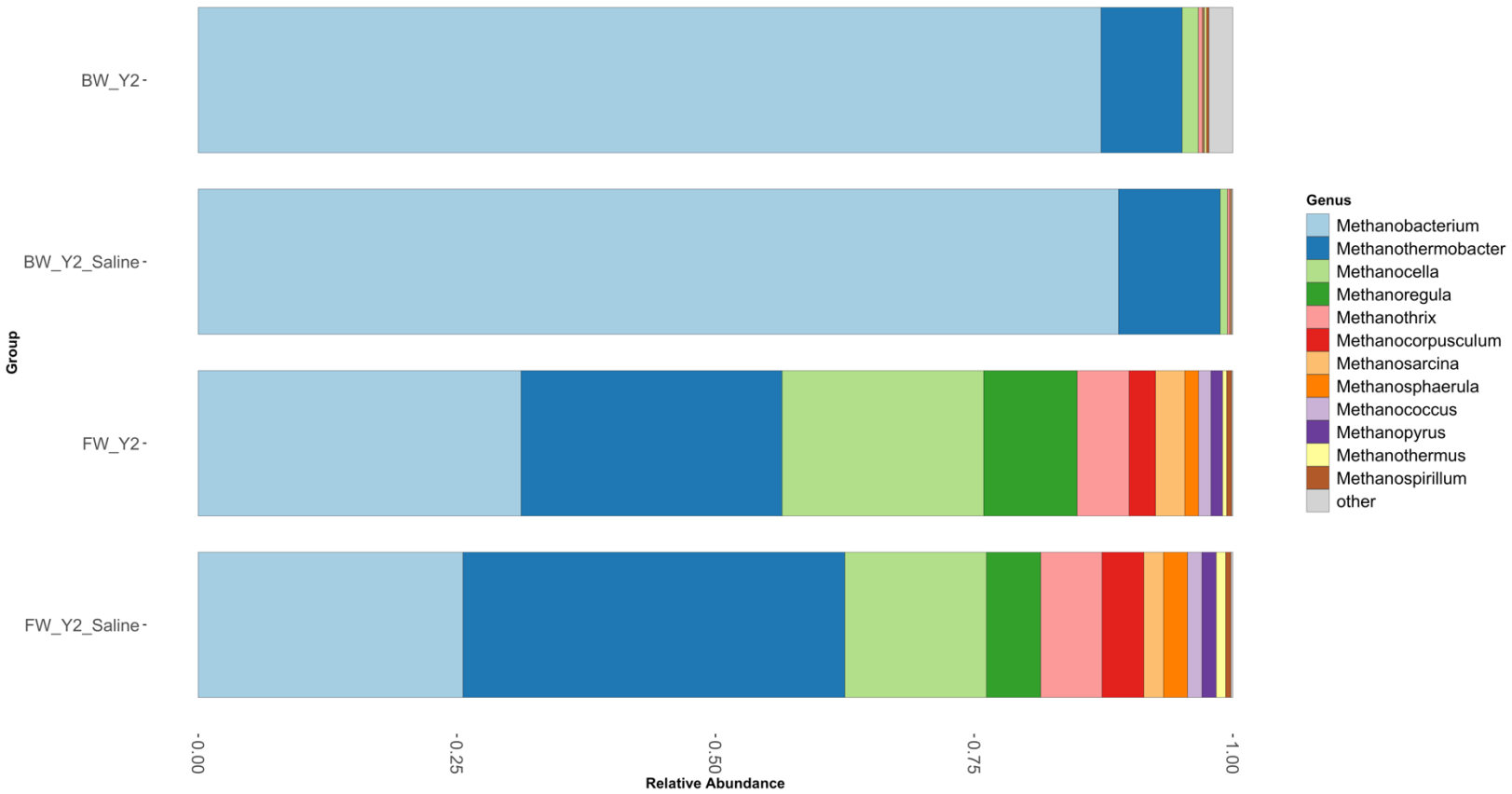

Figure 19: Relative abundance of the most abundant genera found within the Methanobacteria phylum of the *mcrA* microbial community. The brackish (top two bars) and freshwater site (bottom two bars) had differences in observed diversity as exhibited by the taxonomic affiliations.

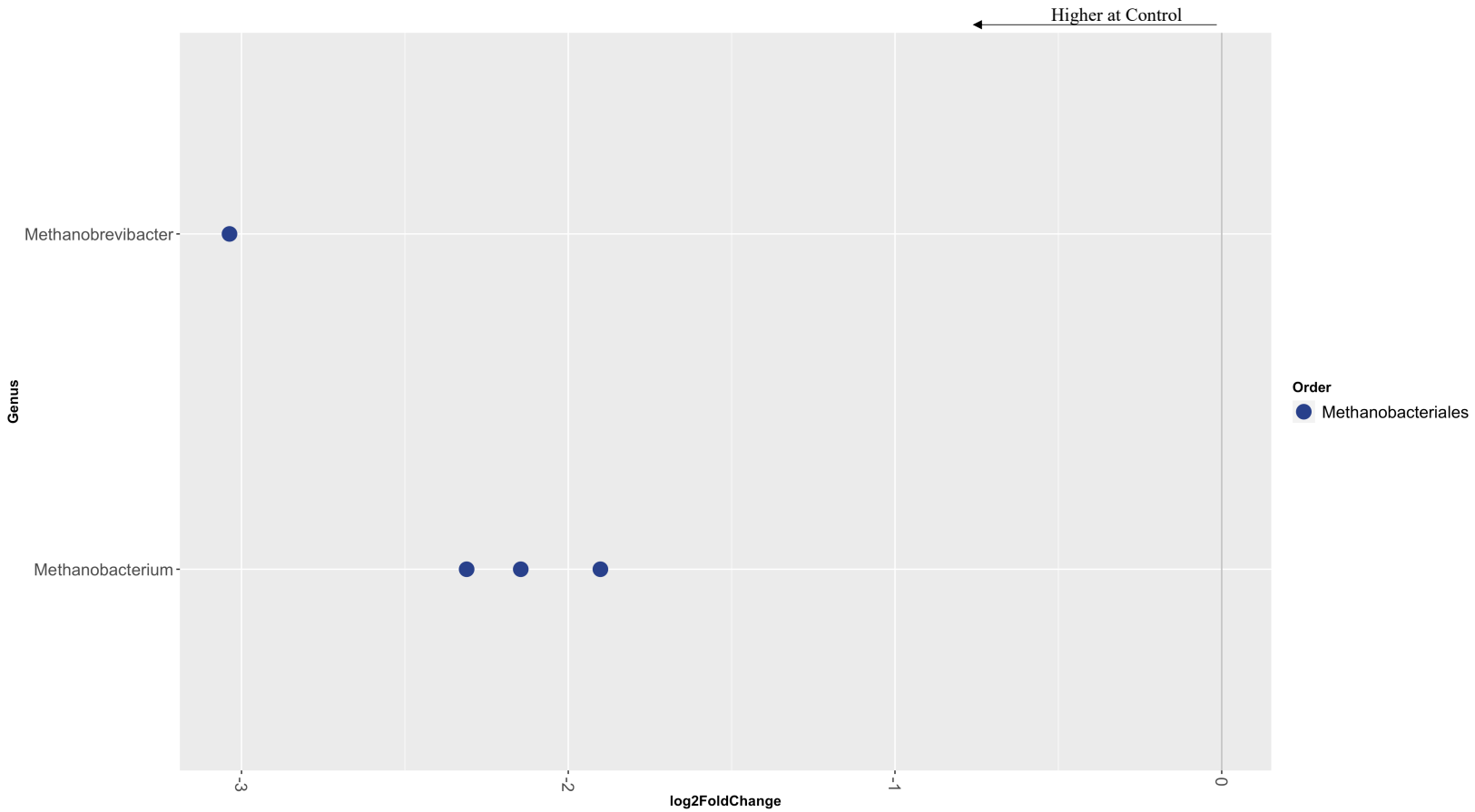

Figure 20: Differentially abundant genera found at the brackish site. Differential abundance plots between Year 2 samples - control and saltwater treatment. Negative log fold changes represent significant differential abundance at the control samples. Decreases in abundance were observed in the Methanobacteriales Order after treatment.

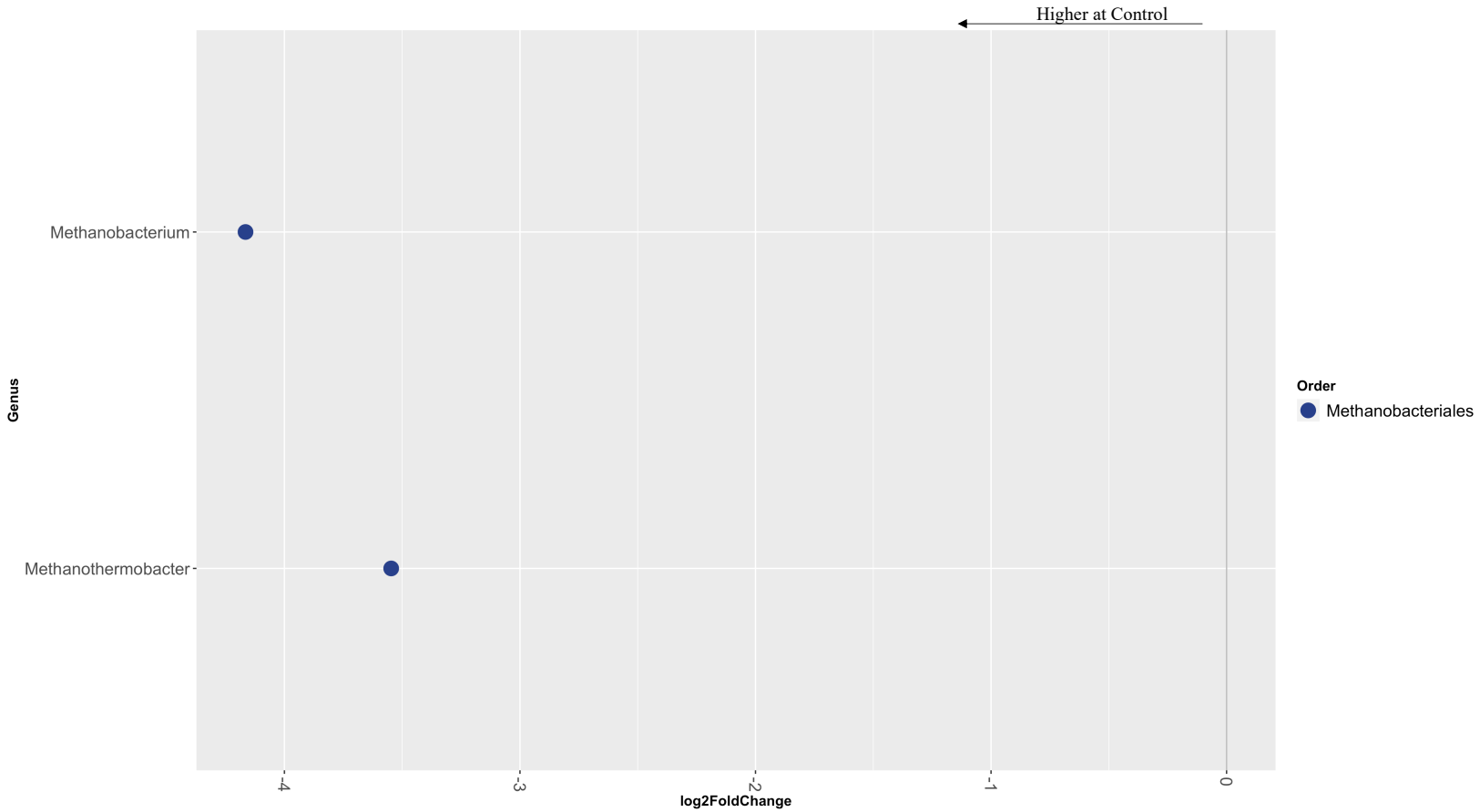

Figure 21: Differentially abundant genera found at the freshwater site. Differential abundance plots between Year 2 samples - control and saltwater treatment. Negative log fold changes represent significant differential abundance at the control samples. Decreases in abundance were observed in the Methanobacteriales Order after treatment.

In the *dsrA* gene, over 75% of clusters were classified as uncultured sulfate-reducing bacteria, even at the genus level, Figure 22. Notable differences between the brackish and freshwater sites included the presence of *Desulfatiglans* in the brackish soil and its absence in the freshwater soil. Additionally, higher levels of *Carboxydothermus* and *Desulfosudis* were present in the freshwater soil and not in the brackish soil. Differential abundance analysis did not identify any OTUs at the brackish site, but 33 unique OTUs were found at the freshwater site with 14 found in the control samples and 19 in the treatment samples, Figure 23**.** These OTUs were observed in the control samples at the *Desofundulus* and *Carboxydothermus* genera. All taxonomically identified taxa were present at less than 2% relative abundance. However, some shifts in the freshwater site were identified as decreases in *Desulfosudis* (1.09%) and *Desulfofundulus* (0.43%) after saltwater treatment. At the brackish site, the presence of *Desulfohalovibrio*, and *Desulfosarcina* increased by 0.48% and 0.3% respectively, after saltwater. While *Desulfovibrio* (0.15%), *Desulfoglaeba* (0.54%) and *Desufofundulus* (0.17%) all decreased after saltwater in the brackish site.

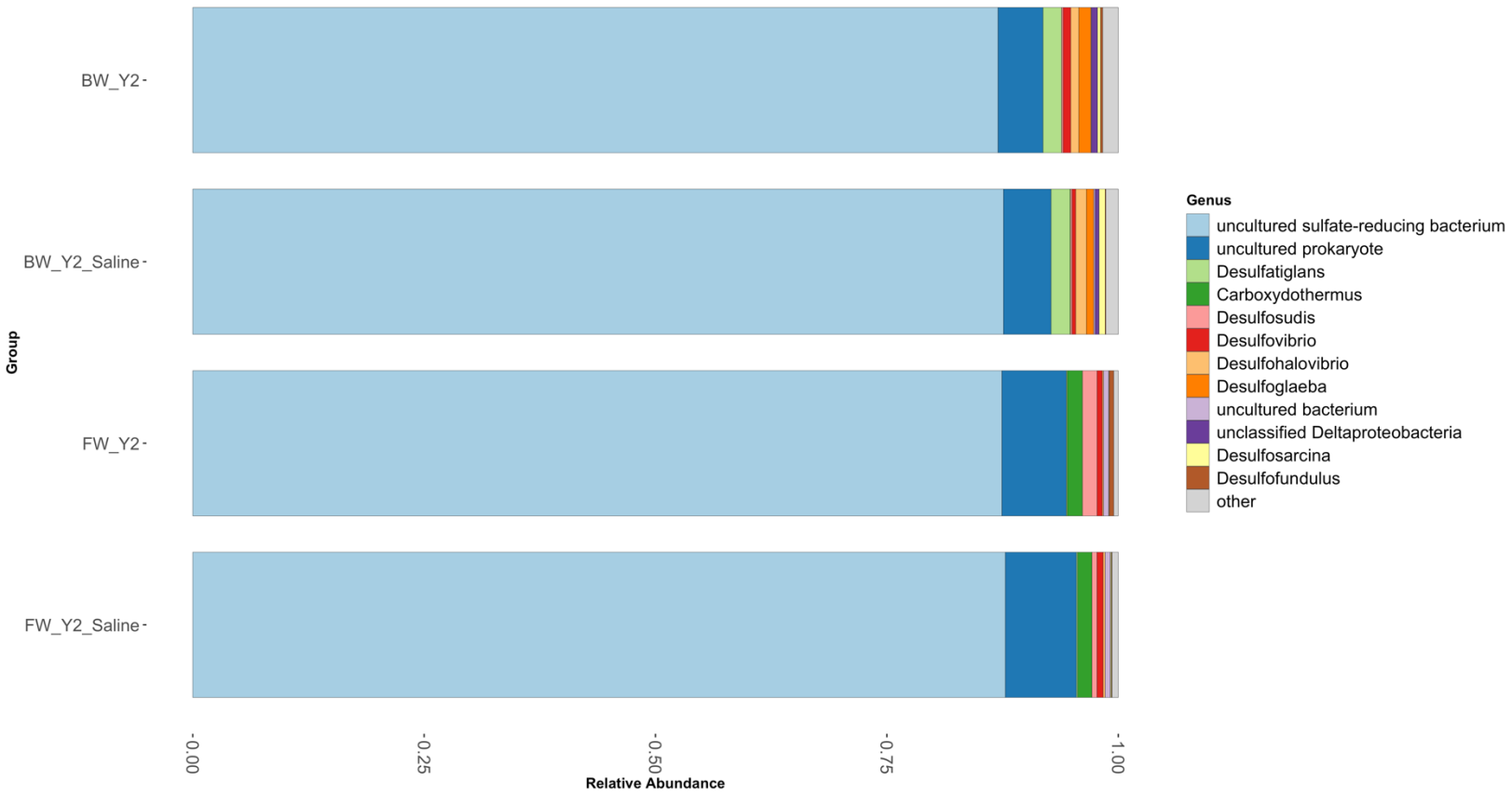

Figure 22: Relative abundance of the most abundant genera found within the *dsrA* microbial community. The brackish (top two bars) and freshwater site (bottom two bars) both had a unique observed composition.

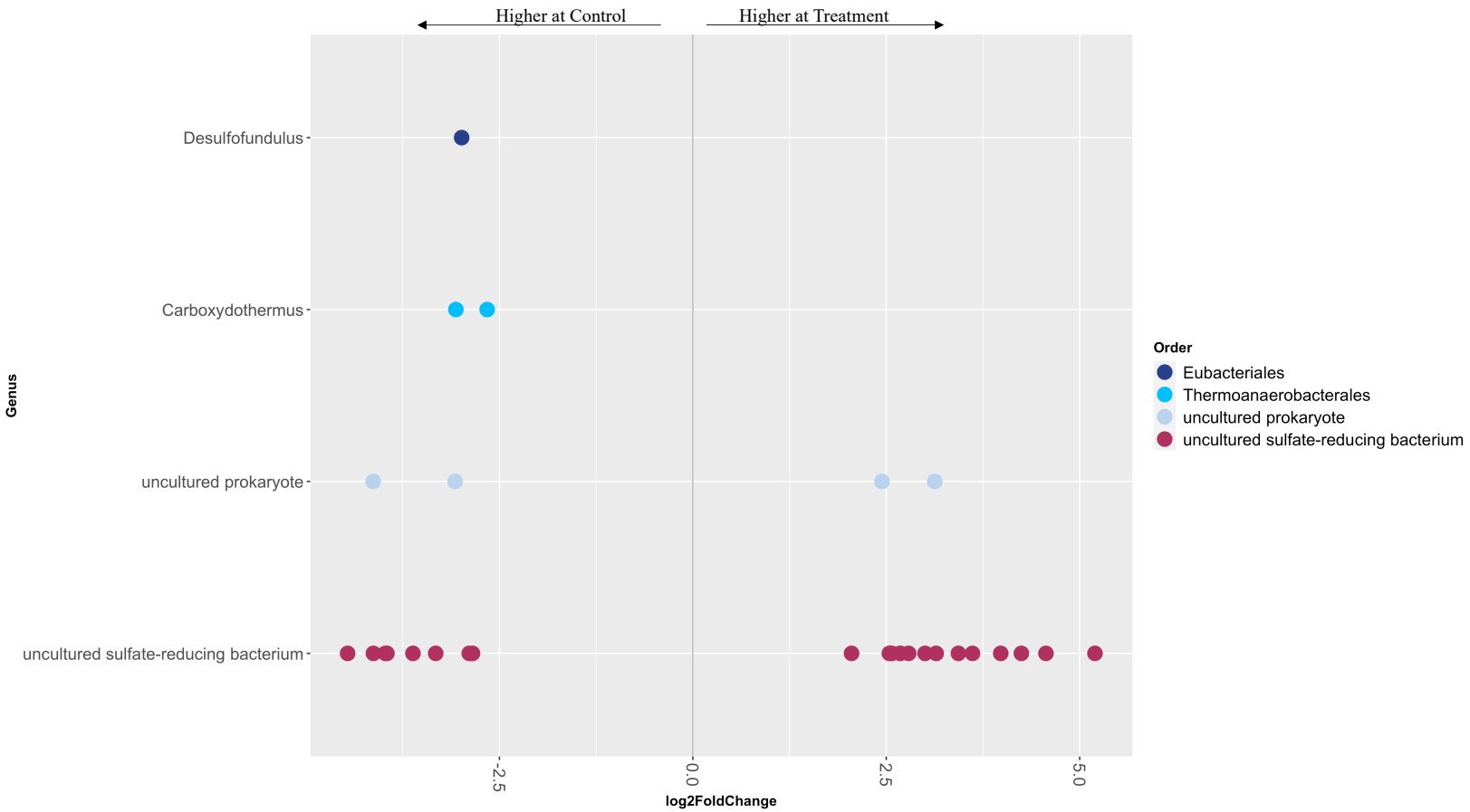

Figure 23: Differential abundance plot between Year 2 samples at the freshwater site- control and saltwater treatment. Negative log fold changes represent significant differential abundance at the control samples and positive represent the treatment samples.

The *phoD* gene contained high diversity in all soil sample groups. At the class level, Planctomycetia (25%) was seen in the highest abundance in the brackish site, while Actinomycetia (23.2%) was found in the highest abundance at the freshwater site, as indicated in Figure 24. At this site, a decrease from 23.2 to 17.6% abundance occurred within the Betaproteobacteria group after saltwater treatment. However, in the brackish site, this group was found at relatively low levels (7.3 to 9.9%) before and after saltwater treatment. The addition of the saltwater treatment caused a decrease in the Gammaproteobacteria group from 32 % to 19.8% in the brackish site while its abundance went from 9% to 10.9% in the freshwater site. Additionally, the Actinobacteria were only found at the BW_Y2_Saline group, indicating that members of this group may have the ability to survive in higher levels of saltwater. Members of the Deinococci class were found in extremely low levels (0.3%) at the brackish site and more prominent (5.2%) in the freshwater. However, a slight decrease to 3.5% in abundance was identified after the addition of saltwater possibly indicating the inability to survive saltwater environments. At the brackish site differential abundance analysis found 43 OTUs, of which 25 OTUs were found in the control and 18 OTUs from the treatment. Within the freshwater site, 38 OTUs were found to be significant at the $p<0.01$ level, 15 OTUs were attributed to the control and 23 OTUs were attributed to the treatment. OTUs from the Bacillales, Pseudomonadales, Nostocales, and unclassified Betaproteobacteria were found to be more abundant in the control samples at the brackish site. Members of the Hyphomicrobiales, Planctomycetales, Corynebacteriales, unclassified Actinobacteria, Xanthomonadales, and Rubrobacterales were found to be more abundant after treatment, Figure 25. Within the freshwater site, OTU from the Burkholderiales,

Solirubrobacterales, Gemmatales, Micrococcales, Caulobacterales, and Pseudomonadales were more abundant in the control samples. The OTUS that were more abundant after treatment at this site consisted of OTUs from the Gemmtimonadales, Streptosporangiales, Bacillales, Hyphomicrobiales, Isosphaerales, Sphingomonadales Gloeobacterales, Chromatiales, and Pirellulales orders, Figure 26.

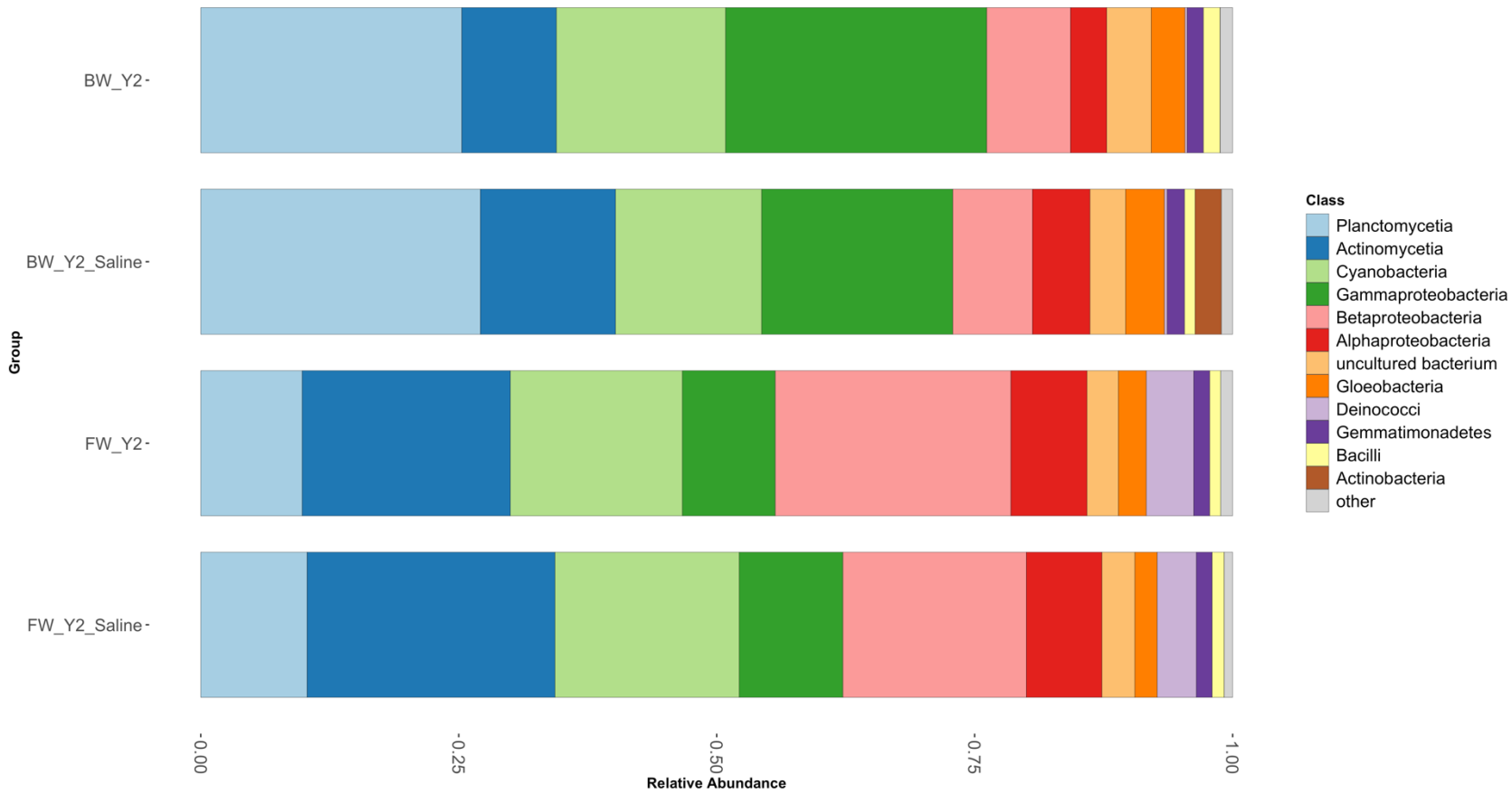

Figure 24: Relative abundance of the most abundant Classes found within the the *phoD* gene microbial community. The brackish site (top two bars) and the freshwater site (bottom two bars) had similar taxonomic affiliations but different microbial distributions. Saltwater shifted the relative abundance of various taxa in both sites.

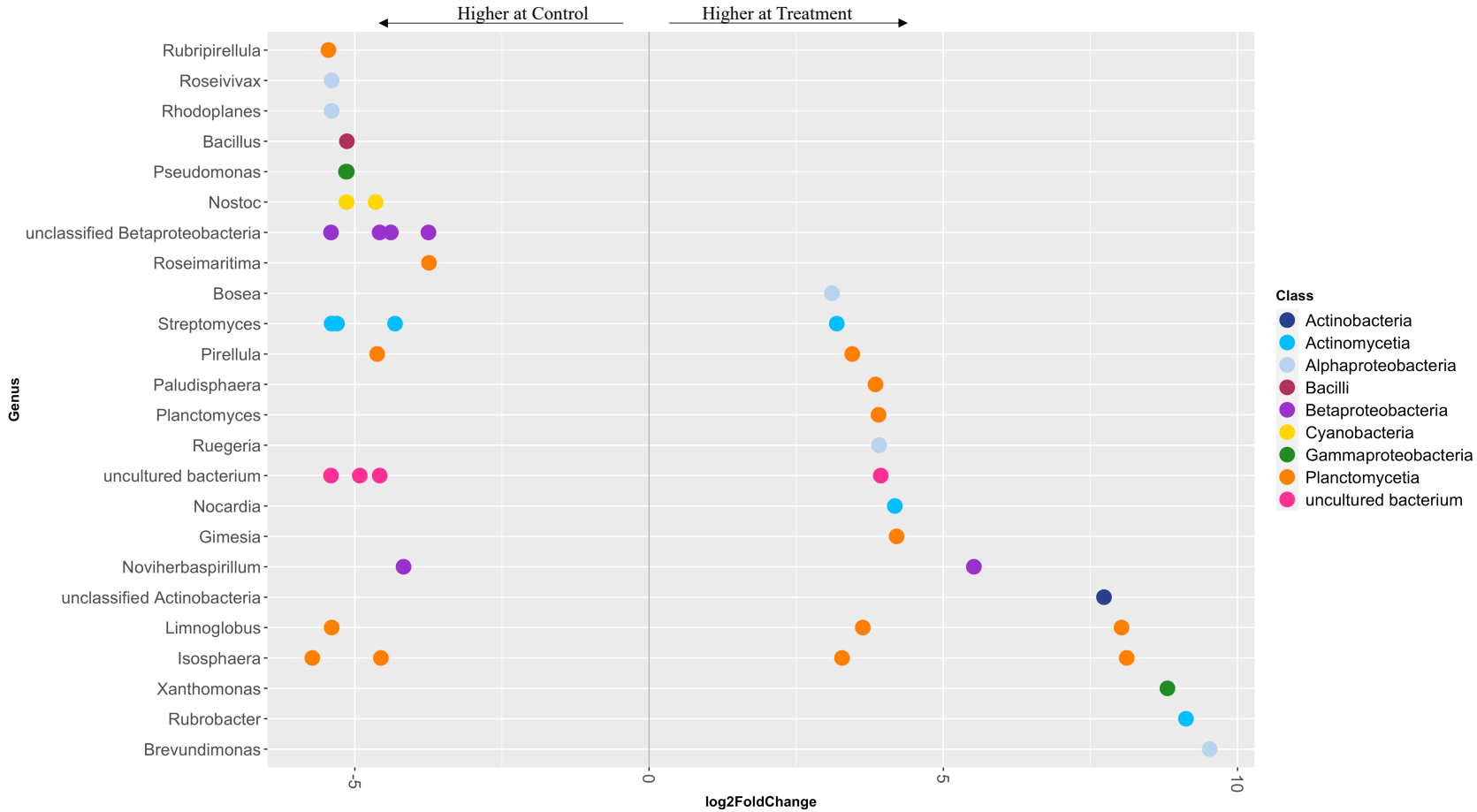

Figure 25: Differentially abundant genera found within the *phoD* gene at the brackish site. Differential abundance plots between Year 2 samples - control and saltwater treatment. Negative log fold changes represent significant differential abundance at the control samples and positive represent the treatment samples. More taxa had significantly higher abundances after the saltwater treatment.

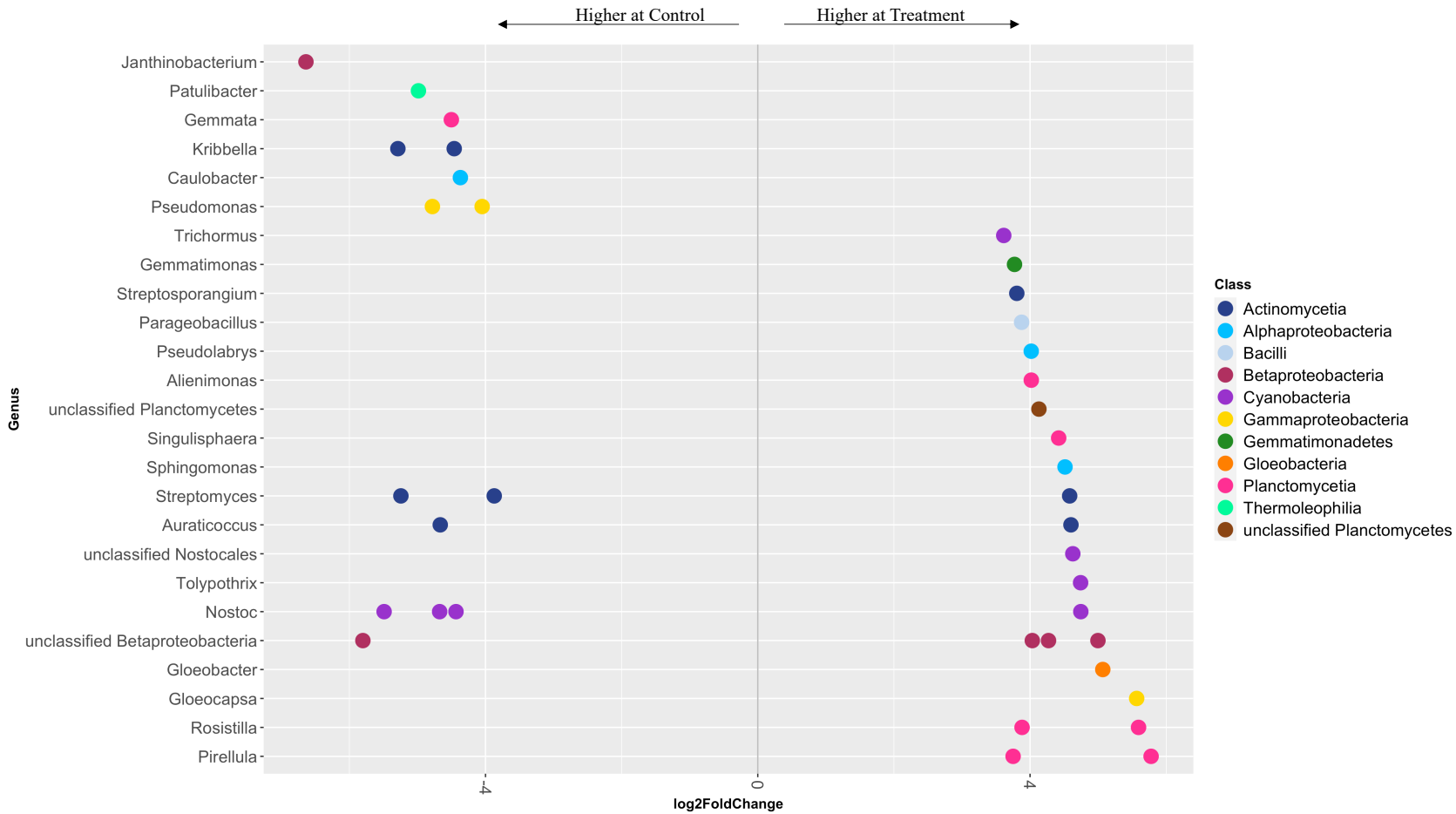

Figure 26: Differential abundance plots between Year 2 samples - control and saltwater treatment. Negative log fold changes represent significant differential abundance at the control samples and positive represent the treatment samples. Differentially abundant genera found at the freshwater site from the *phoD* gene.

3.4 Discussion

Microorganisms carry various functional genes which are directly linked to maintaining healthy ecosystem functioning. Although sequencing the functional genes does not provide information on gene expression, it can provide insight that cannot be obtained with just 16S rRNA genes, its functional potential -the capacity of a microbial community to perform critical environmental functions. Microbial communities are suggested to have high levels of functional redundancy so disruptions in biogeochemical cycling are likely to be unaffected by functional gene species composition (Louca et al., 2018), but perturbations on the environment have been known to disrupt the communities' structure resulting in the loss of microbes that perform some of these functions ultimately disrupting the nutrient cycling within the community (Dang et al., 2019; Xie et al., 2020). Results of this study shed light on the differences in composition of the microbial communities involved in the cycling of these nutrients and how they are affected after a perturbation such as saltwater intrusion in two different marshes.

Comparison of the resulting overall microbial community structures between the freshwater and brackish sites support previous findings where salinity gradients displayed unique compositional patterns (Fei Xi et al., 2014; Morrissey et al., 2014; Nold & Zwart, 1998). This could be explained by the capability of a microorganism's osmoregulatory functions. Many microorganisms do not contain the metabolism to support the additional osmotic stress of saltwater which leads to significant decreases in diversity or a shift in the taxonomic composition of a community as many microbes reach their own cellular limitations (Ruhl et al., 2018; Yang et al., 2016). In this study, the Inverse Simpson index revealed that individual samples from the brackish site seemed less diverse, than those

from the freshwater site in *the nirS* and *mcrA* functional genes, supporting this claim. Within the *dsrA* functional gene, the brackish site (104.66 $\pm$ 62.7) seemed to have an overall higher mean alpha diversity than the freshwater (62.31 $\pm$ 32.69). These results can be explained by the increase of sulfate reduction that is required by the larger amount of sulfate present in saltwater environments (Dang et al., 2019). However, alpha diversity measurements between sites with the *phoD* functional gene did not significantly differ, supporting the findings of Ragot et. al. (Ragot et al., 2017) where microbes harboring this gene were found to be stable across various environments.

Over time microbial compositions shifted within the freshwater site at the *nirS*, *dsrA*, and *phoD* genes. Temporal changes were identified within the brackish site only at the *dsrA* gene. This corresponds with earlier research that showed temporal changes in these samples for total percentage of nitrogen and extracellular enzymes, arylsulfatase and phosphatase (Servais et al., 2020). In that study, total percentage of bulk soil nitrogen increased after two years (from 0.8% to 1.2%), and temporal variation occurred in arylsulfatase and phosphatase extracellular enzymes. Indicator species revealed uniquely abundant taxa within each group that are likely contributing to shifts in the environment and production of extracellular enzymes.

Saltwater manipulation significantly affected the composition of the *dsrA* ($R = 0.227$, $p<0.05$) and *phoD* ($R=0.261$, $p<0.05$) genes at the freshwater site. A possible increase in the functional *dsrA* gene could be suggested by the significant increase in porewater concentrations of sulfate (from 0.01 to 150.03 mg L^{-1}) and sulfide (from 0 to 0.08 μmol L^{-}) after saltwater treatment. The current study found more diversity within this gene after saltwater manipulation. This is the opposite of what has been found in a

separate study where abundance decreased with saltwater manipulation (Zhang et al., 2021). Within that study, functional genes were measured with GeoChip and a notable decrease occurred along the salinity gradient at the *dsrA* gene. The perturbation seemed to lead to a shift in composition which likely shifted the function. Significant increases, as determined by differential abundance analysis, in various OTUs were identified after saltwater manipulation but limitations in the database hindered taxonomic classification. Sequences are assigned a taxonomic affiliation by comparing them to the reference sequences present within a database. Therefore, sequences can only be assigned if they reach a specified percentage similarity to a reference within this database. Over 75% of the sequences were assigned an unclassified taxonomic affiliation so many of these sequences have yet to be classified.

Taxonomic shifts in diversity within the *phoD* community seem to have been affected by the increase in salinity as several taxa were found to be more abundant after treatment. Similar results were seen in a previous study that discovered that some *phoD* harboring species select for more oligohaline environments (Hu et al., 2020). We found that Planctomycetia favored the oligohaline environments, since it was found at 25% relative abundance in the brackish site and only 11.2% within the freshwater site and increased at both sites after saltwater. Betaproteobacteria, like in the Hu et. al. study, preferred freshwater sites. This class was identified at 18% relative abundance at the freshwater site and only 8% in the brackish and decreased after the saltwater inputs. Measured levels of phosphorous from the Servais et. al. study (the origin of these soil samples) included total phosphorus, soluble reactive phosphorus, and total dissolved phosphorus. However, neither measurement significantly differed after saltwater in the

freshwater site (Servais et al., 2020). In an earlier study, by Wei et. al., several phosphorous types were measured and organic phosphorus was found to shape the *phoD* community and it may be shaping the community here (Wei et al., 2021). Members of the *phoD* community utilize organic phosphorus as one of their substrates. In the Michaelis-Menten equation the rate of an enzymatic reaction steady increases as more substate is introduced and eventually will reach a steady state (German et al., 2012). This phenomenon was observed in terms of the observed community and availability of the substrate, organic phosphorus, in the Wei et. al. study. OTU richness and diversity increased and eventually leveled off as the availability of organic phosphorus increased. They also found that, low phosphatases and low diversity were seen in areas with low organic phosphorus. Measurements of the extracellular enzyme, alkaline phosphatase, in the Servais et. al. study, slightly increased but did not significantly differ after saltwater treatment. In the current study, alpha diversity measurements showed a similar occurrence backing up what was observed in Wei et. al. Results suggest that although the function of the community stayed the same, the taxonomic community shifted likely due to the nutrient availability, and therefore has the potential to shift in function as more organic phosphorous becomes available.

The only community that significantly differed after saltwater manipulation in the brackish site was the *mcrA* community. Methanogenesis is already a limited pathway within more oligohaline environments because of the increased presence of sulfate. Sulfate reducers often outcompete methanogens for the same substrate, hydrogen, or acetate, which may be happening here as the *dsrA* community did not significantly shift at this brackish site, but the *mcrA* community did. Previous studies have suggested that

methylotrophic methanogens are likely to be favored in these sulfate-rich environments since they would be less competitive with sulfate reducers (Liu & Whitman, 2008; Oremland & Polcin, 1982). The brackish site is still dominated by hydrogenotrophic methanogens before and after saltwater, namely the *Methanobacterium* genus.

A notable decrease in dissolved organic carbon (DOC) was seen after saltwater manipulation indicating the loss of carbon by some biogeochemical function. DOC has been shown to have a correlation with specific methanogenic archaea suggesting methanogenesis rates vary dependent upon the level of DOC and composition of taxa (Liu et al., 2012). The Liu et al study found that measurements of methane production were significantly correlated with DOC and that Methanobacteriales (hydrogenotrophic methanogens) were found to be predominantly associated with the DOC-rich marsh. In the current study, OTUs from Methanobacteriales were found to be more abundant at the control site, which had significantly higher levels of DOC. These results suggest that methanogenesis may be occurring at a higher rate after the saltwater treatment than before. Findings from this study further support the findings of (Dang et al., 2019) where hydrogenotrophic sulfate reducers and hydrogenotrophic methanogens, which are known competitors, were found to coexist in sulfate rich environments.

In conclusion, taxonomic increases identified within each of each these functional genes after saltwater treatment suggests an increase in functional potential. Therefore, with more microbes to participate in a specific function, increased rates of activity can be expected. The opposite can be expected if decreases in taxa occurred. Results from this study provided insight into which taxa are more sensitive to changes in saltwater concentrations within each marsh. It was hypothesized that the taxa in the brackish site

would not be as affected by the saltwater treatment since the microbes were already accustomed to saline conditions. This was the case for each of the functional genes except *mcrA*, whose community structure shifted after saltwater. This change in taxonomic composition suggests that rates of methanogenesis are expected to shift as saltwater is introduced into the brackish site. These results provide insight for climate scientists since an area of concern is the possibility of increased carbon degradation with saltwater intrusion resulting in higher levels of methane being released.

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CHAPTER 4

4 Salinity Alters Potential Contributing Factors of Microbial Communities and the Utility of Random Forest to Determine Soil Provenance in Freshwater and Brackish Marshes

4.1 Introduction

Environmental factors play a significant role in the complex and dynamic nature of soil microbiomes and the responses of these microbiomes are almost entirely reliant upon them. Because of this, it is important to not only characterize the structure of soil microbiomes but also identify abiotic drivers of these communities to explain what shapes them (Cheung et al., 2018; Zhang et al., 2021). Researchers have suggested that the factors that drive the microbial communities can vary dependent upon the environment or soil type (Li et al., 2021; Qi et al., 2018). As disturbances are introduced into their environment, the community may shift in response, resulting in a change in the factors that drive them. Knowledge in this area can then assist in the mitigation or prediction of fluctuations in microbial communities that can alter the ecological function.

In addition to monitoring the effects of disturbances on their communities, microbes also have the potential to serve as a valuable resource for environmental forensic analyses (Demaneche et al., 2017; Giampaoli et al., 2014). One of the main principles of forensic science is the ability to exclude one sample from another by identifying uniquely distinct features. Human DNA fingerprinting has become the standard of forensic genetics because of the ability to identify multiple short tandem repeats (STRs) that are unique to an individual. One STR region may not be distinct by itself but concatenating a minimum

of 20 core loci greatly increases the power of discrimination (Arenas et al., 2017; Jordan & Mills, 2021). A similar principle can be applied in soil microbial communities to determine provenance by concatenating several different genes or taxa to create a unique signature or fingerprint for differentiation.

However, microbes are highly variable and abiotic factors can affect their response to an environment thus shifting the community composition. Monitoring these factors and predicting the outcome that a variable would have on a microbial community could be highly useful. This can be achieved by utilizing machine learning models which can provide a quick and low-cost way to monitor an environment or predict provenance. Recently, various fields such as the human microbiome, soil health and forensic science have all begun to acknowledge the utility of applying machine learning algorithms to microbiome data (Ghannam & Techtmann, 2021; Marcos-Zambrano et al., 2021; Roguet et al., 2018). Some studies have shown that analyzing high dimensional data, like microbial communities, with random forest provides high accuracy and is less prone to overfitting when compared to other supervised machine learning algorithms (Damaso et al., 2018; Flojgaard et al., 2019; Wilhelm et al., 2022). Within these studies, random forest and microbial communities were utilized to determine soil provenance and monitor soil health exhibiting its robustness. The main distinction between traditional statistical microbial methods and machine learning algorithms is that instead of inferring the connection between the dataset and the variables, an optimized model is used to predict the outcome from a dataset.

This chapter aims to identify the main contributing factors of microbial community structure in two wetlands after saltwater dosing experiments which mimicked

saltwater intrusion. Additionally, we wanted to utilize random forest, a machine learning algorithm, to produce a model that would predict provenance of a soil sample and determine if the soil had suffered from a stressor like saltwater intrusion. Two hypotheses were tested. The first hypothesis was that *combining the taxonomic and functional gene information along with measurements from porewater chemistry will determine that there is not a correlation between salinity dosage experiments and shifts within the microbiome.* The second hypothesis was that *bioinformatic algorithms of taxonomic and functional gene information will not provide discriminatory provenance between brackish and freshwater environments.*

4.2 Methods

4.2.1 Physiochemical properties of the soil microbiomes

Soil samples from the Year 2 timepoint were utilized to reduce confounding effects that may occur from samples collected at a separate time point. These samples consist of control samples from the brackish and freshwater site as well as the saltwater treated soils from both sites. Here we wanted to determine the abiotic factors of the communities without treatment and to see how they change after saltwater pulses. Measurements of soil physiochemical properties in terms of porewater biogeochemistry, bulk soil and extracellular enzyme activities were obtained from an earlier study (Servais et al., 2020). Porewater measurements were collected from 15 cm depth at each site. The top 0-10 cm of each soil core was selected for microbial community analysis. Therefore, corresponding measurements of extracellular enzymes and bulk soil were analyzed from only the top 0-10 cm of the soil cores. A summary of the measurements from the brackish and freshwater sites are in Table 10. A summary comparison of the measurements

between the two sites is in Table 11. Extracellular enzyme activities consisted of alkaline phosphatase, extracellular acid phosphatase, arylsulfatase, Beta-1-4-glucosidase, Beta-1-4-cellobiosidase, and leucine amino peptidase. Bulk soil measurements are from phosphorus, nitrogen, and carbon. Measurements from porewater biogeochemistry consisted of temperature, salinity, alkalinity, Chlorine, Dissolved Organic Carbon (DOC), pH, ammonium (NH_4^+), sulfate (SO_4^{2-}), total dissolved nitrogen (TDN), soluble reactive phosphorus (SRP), total dissolved phosphorus (TDP), and sulfide (HS^-). Microbiome data from chapters 2 and 3 will also be utilized for subsequent analyses. This consists of datasets from the Bacteria and Archaea 16S rRNA genes and the *nirS*, *mcrA*, *dsrA,* and *phoD* functional genes.

Table 10: Summary of soil physiochemical properties of brackish and freshwater sites collected from porewater, and top 10 cm of soil cores at Year 2 timepoint of study. Welch's T-Test using alpha = 0.05. Significant differences are bolded. Adapted from (Servais et al., 2020).

	Brackish Samples			**Freshwater Samples**		
	Mean		**Significance**	**Mean**		**Significance**
Extracellular Enzyme Activity	**Control**	**Treatment**	**p value =**	**Control**	**Treatment**	**p value =**
Alkaline phosphatase	0.41	6.99E-05	0.36	3.82E-03	0.01	0.34
Acid phosphatase	2.80	2.61	0.87	3.89	3.51	0.83
Arylsulfatase	2.07	2.16	0.88	3.26	3.35	0.93
Beta-1-4-glucosidase	1.68	1.87	0.67	5.91	3.46	0.11
Beta-1-4-cellobiosidase	0.23	0.24	0.75	2.66	0.62	0.28
Leucine amino peptidase	0.01	0.00	0.36	0.01	0.04	0.10
Bulk soil						
Phosphorus	522.28	442.37	0.19	500.04	569.71	0.16
Nitrogen	2.47	2.17	0.10	3.25	3.29	0.74
Carbon	36.66	30.70	0.34	35.18	25.16	**< 0.05**
Porewater Biogeochemistry						
Temperature	29.10	29.26	0.70	27.49	27.84	0.46
Conductivity	19.32	26.23	**< 0.05**	0.51	3.80	**< 0.05**
Salinity	11.49	16.00	**< 0.05**	0.24	2.03	**< 0.05**
Alkalinity	597.17	322.02	**< 0.05**	155.00	165.67	0.17
Chlorine	6259.02	8937.19	**< 0.05**	56.79	1230.81	**< 0.05**
DOC	135.37	89.04	**< 0.05**	22.83	22.62	0.94
pH	7.56	7.51	0.29	7.30	7.60	**< 0.05**

NH_4^+	5.37	2.47	**< 0.05**	0.45	0.93	0.07
SO_4^{2-}	362.81	964.63	**< 0.05**	0.10	150.03	**< 0.05**
TDN	8.84	4.58	**< 0.05**	1.09	1.57	**< 0.05**
SRP	5.47	2.76	**< 0.05**	0.04	0.04	0.72
TDP	8.90	3.95	**< 0.05**	0.32	0.34	0.54
HS^-	3.37	1.45	**< 0.05**	0	0.08	**< 0.05**

Table 11: Summary of soil physiochemical properties comparing brackish and freshwater sites collected from porewater, and top 10 cm of soil cores at Year 2 timepoint of study. Welch's T-Test using alpha = 0.05. Significant differences are bolded. Adapted from (Servais et al., 2020).

	Site Samples		
	Mean		Significance
Extracellular Enzyme Activity	**BW**	**FW**	**p value =**
Alkaline phosphatase	0.21	0.01	0.35
Acid phosphatase	2.70	3.66	0.39
Arylsulfatase	2.12	3.32	0.07
Beta-1-4-glucosidase	1.78	4.44	**< 0.05**
Beta-1-4-cellobiosidase	0.23	1.43	0.10
Leucine amino peptidase	4.37E-03	0.03	0.06
Bulk soil			
Phosphorus	482.32	541.84	0.12
Nitrogen	2.32	3.27	**< 0.05**
Carbon	33.68	29.17	0.21
Porewater Biogeochemistry			
Temperature	29.18	27.70	**< 0.05**
Conductivity	22.78	2.49	**< 0.05**
Salinity	13.74	1.32	**< 0.05**
Alkalinity	459.59	161.40	**< 0.05**
Chlorine	7598.10	761.20	**< 0.05**
DOC	112.20	22.70	**< 0.05**
pH	7.54	7.48	0.44
NH_4^+	3.92	0.74	**< 0.05**
SO_4^{2-}	663.72	90.06	**< 0.05**
TDN	6.71	1.38	**< 0.05**
SRP	4.11	0.04	**< 0.05**
TDP	6.43	0.34	**< 0.05**
HS^-	2.41	0.05	**< 0.05**

4.2.2 Statistical analyses

Relationships between the soil physiochemical properties and the microbiome datasets were analyzed with distance-based redundancy analysis (dbRDA) and Mantel tests. dbRDA is a form of redundancy analysis (RDA) but it is not constrained by the linearity that is assumed with Euclidean measures. Instead, a dissimilarity matrix is utilized, which is useful for microbiome data. The method is an extension of multiple linear regression in which it can analyze multiple predictor variables to explain the variance within several response variables at once (Jupke & Schafer, 2020). In this case, the predictor variables are soil properties, and the response variables are the OTUs. Dissimilarity matrices for dbRDA were constructed using Bray-Curtis Dissimilarity. Biplots were produced from dbRDA and their results were evaluated using Analysis of Variances (ANOVA) to determine significant soil properties that are the main contributing factors of the variation within each of the communities (Legendre & Fortin, 2010). Mantel tests were done to calculate linear correlations between the OTUs and the soil properties. Each function was performed in R statistical software v4.1.1 with the vegan package (Oksanen et al., 2020; Team, 2021). Statistical tests were calculated for each analyzed gene, Bacteria, Archaea, *nirS*, *mcrA*, dsrA, and *phoD*.

4.2.3 Machine learning using random forest

Classification of the samples was performed by creating random forest models. Random forest is a supervised machine learning approach that builds several forests of decision trees from a dataset to predict an outcome (Breiman, 2001). The abundance of OTUs from each gene was utilized as features for input. Each node is created by splitting the samples into groups with similar features or values. Bootstrapping is performed on

the dataset and the predictions from the trees averaged to create an ensemble of decision trees. Samples that are not utilized in the bootstrapping method are known as “out of bag” and can be used to estimate error (Ghannam & Techtmann, 2021; Zhou & Gallins, 2019). The ‘randomForest’ package in R statistical software was utilized to create models that would determine provenance of each soil sample (Liaw & Wiener, 2002). The performance of each model was validated with a leave one out cross validation (LOOCV) scheme and compared to the out of bag error rate. These values were similar so only the values from the cross validation were reported,

4.3 Results

4.3.1 Environmental factors of the microbial communities

The influence each soil parameter had on the communities was first evaluated with the Mantel test. Samples were separated by site within each group to determine significant factors influencing the communities before and after saltwater dosing. Mantel tests were performed, and significant factors were identified at a $p<0.05$ significant level. The Archaea and *nirS* communities did not have any factors that identified as having a significant correlation at either site. Bacteria did not have any significant correlations with any soil properties before saltwater treatment. After treatment, a significant correlation was identified in the freshwater site with Bacteria and DOC ($r = 0.71$, $p<0.05$) and in the brackish site a significant correlation with sulfide ($r=0.53$, $p<0.05$). The *mcrA* community correlated with pH ($r =0.71$, $p<0.05$) in the brackish site before saltwater treatment but no significant correlations were identified after the treatment. Additionally, no correlations were found in the *mcrA* community at the freshwater site. Total dissolved

nitrogen ($r = 0.70$, $p < 0.05$) and ammonium ($r = 0.78$, $p < 0.001$) were significantly correlated to the *dsrA* community at the freshwater site after saltwater treatment. A correlation between total dissolved phosphorus ($r = 0.6$, $p < 0.05$) and the *phoD* community was identified in the freshwater site after the saltwater treatment. Correlations with the *phoD* community at the brackish site before treatment were identified as phosphorus ($r = 0.65$, $p < 0.05$), salinity ($r = 0.67$, $p < 0.05$), ammonium ($r = 0.58$, $p < 0.05$), total dissolved phosphorus ($r = 0.75$, $p < 0.01$) and pH which had the highest correlation ($r = 0.82$, $p < 0.01$). After treatment, no correlations could be identified in the *phoD* gene community.

The factors most contributing to the variation within these communities before and after saltwater were then assessed with dbRDA. The results were plotted on a principle coordinates analysis (PCoA) plot with the amount of variation explained by each axis -CAP1 and CAP2. All soil properties that were identified as having significantly ($p < 0.05$) contributed to the amount of variance within the samples were plotted as vectors (arrows) in each plot. The direction and length of the arrow represent the direction and strength of the relationship to each axis, CAP1 and CAP2. In the *dsrA* community sulfide influenced the variation in the freshwater site, Figure 27, and alkalinity and ammonium in the brackish site, Figure 28. Sulfide also was found to influence the variation within the freshwater site at the *nirS* community Figure 29. No correlations were found by Mantel at the freshwater site in *mcrA* and pH, alkalinity, ammonium, and total dissolved nitrogen were identified as influencing this variation Figure 30. At the brackish site of the *mcrA* community, DOC and alkalinity shaped the variation before treatment and Sulfate, Chlorine, and salinity and conductivity shaped the

variation after treatment Figure 31. In the *phoD* community, properties influencing the variation in the freshwater site were DOC and sulfide, Figure 32. In bacteria, soluble reactive phosphorus and DOC were influential at the freshwater site, Figure 33.

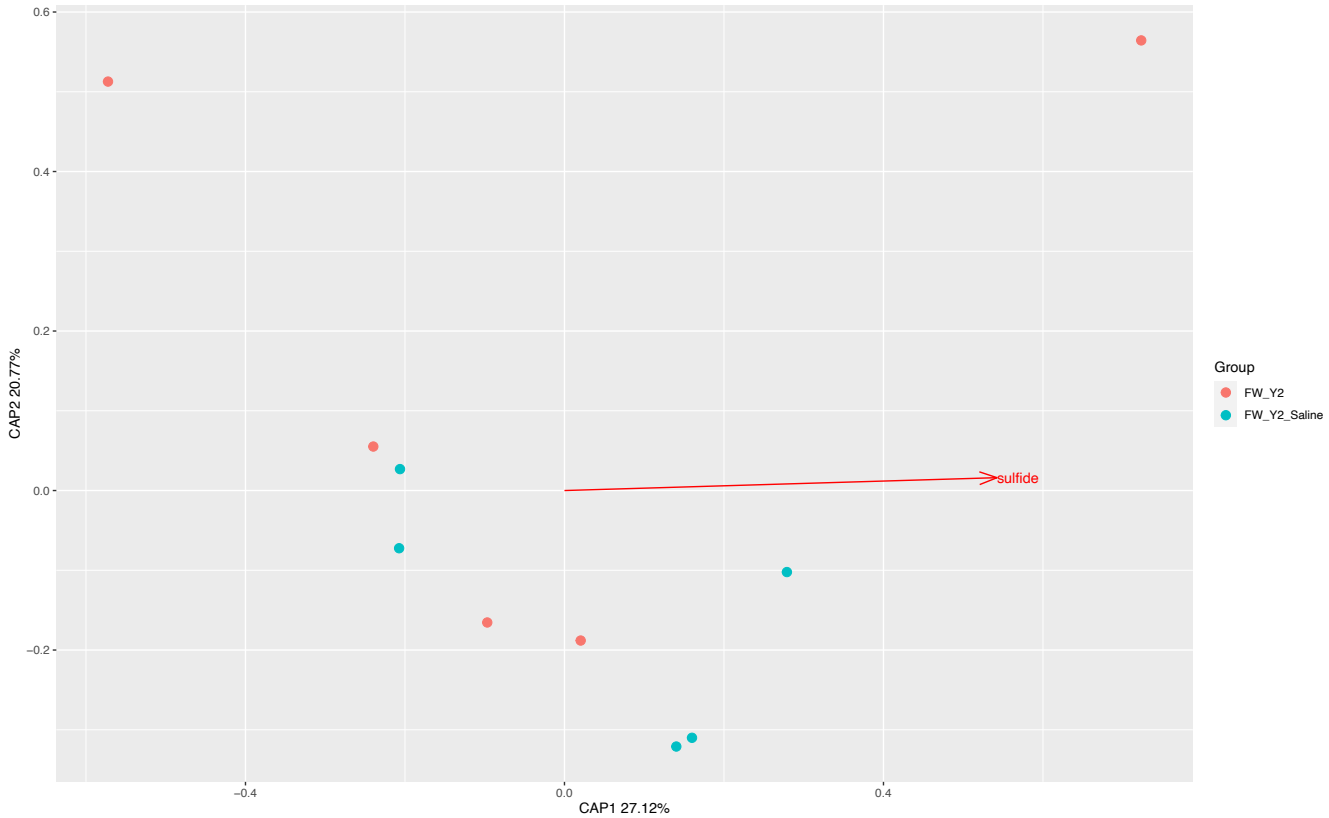

Figure 27: The distance-based redundancy analysis (db-RDA) diagram shows the distribution of soil *dsrA* communities before and after treatment at the freshwater sites. Soil property depicted as red arrow points to positive associations with communities.

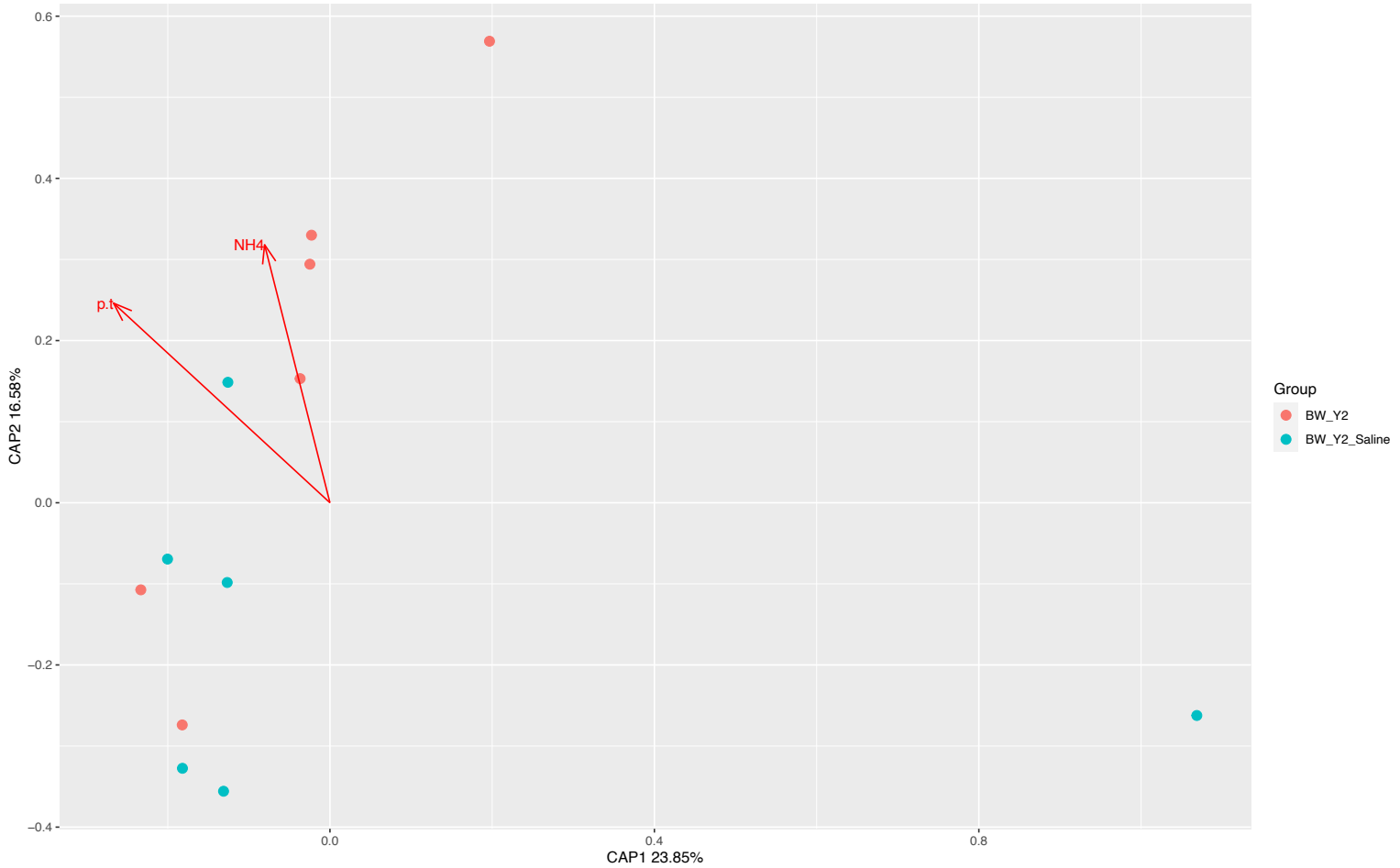

Figure 28: The distance-based redundancy analysis (db-RDA) diagram shows the distribution of soil *dsrA* communities before and after treatment at the brackish sites. Soil properties depicted as red arrows point to positive associations with communities. Phosphorus (p.t), Ammonium (NH_4)

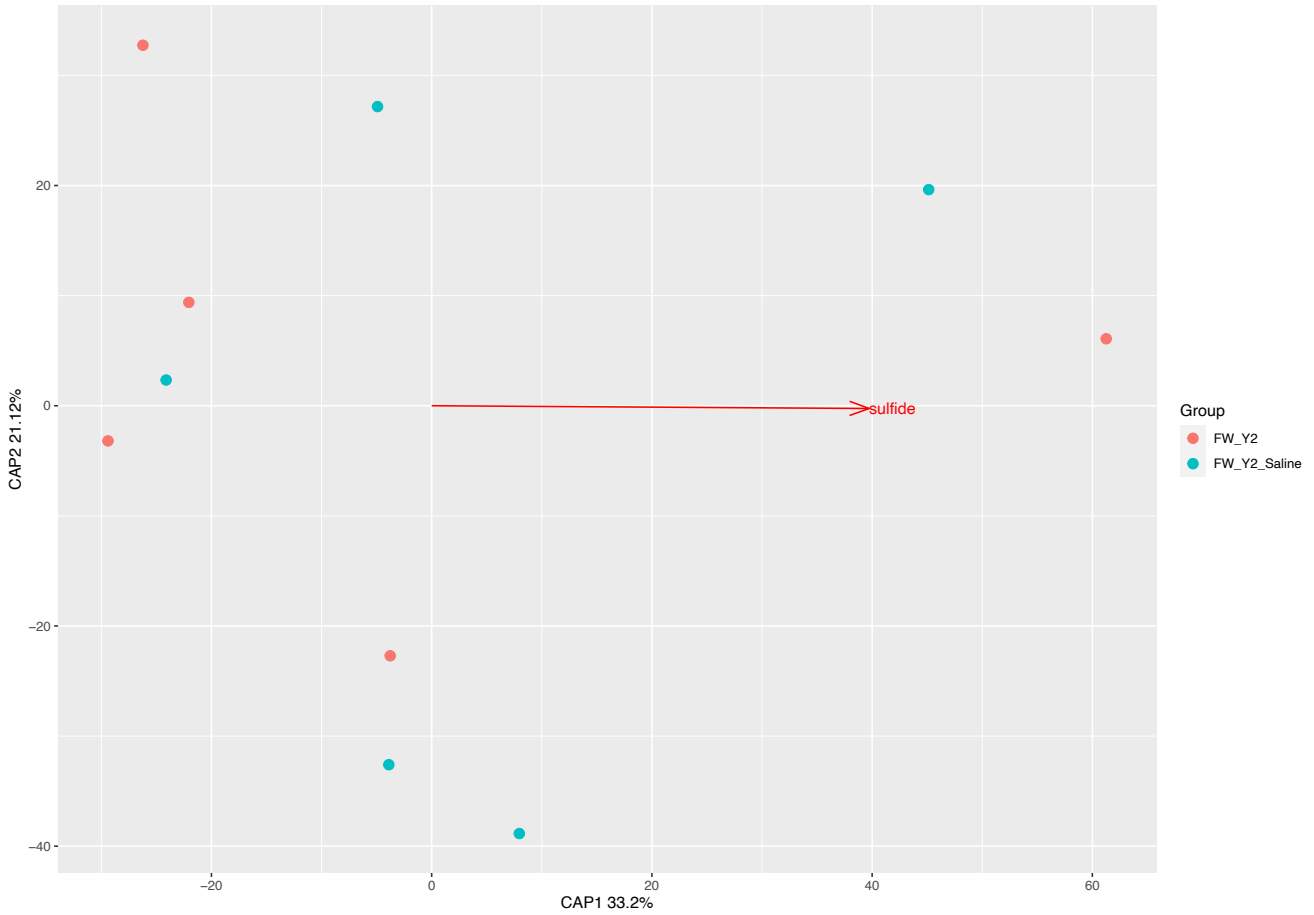

Figure 29: The distance-based redundancy analysis (db-RDA) diagram shows the distribution of soil *nirS* communities before and after treatment at the freshwater sites. Soil property depicted as red arrow points to positive associations with communities.

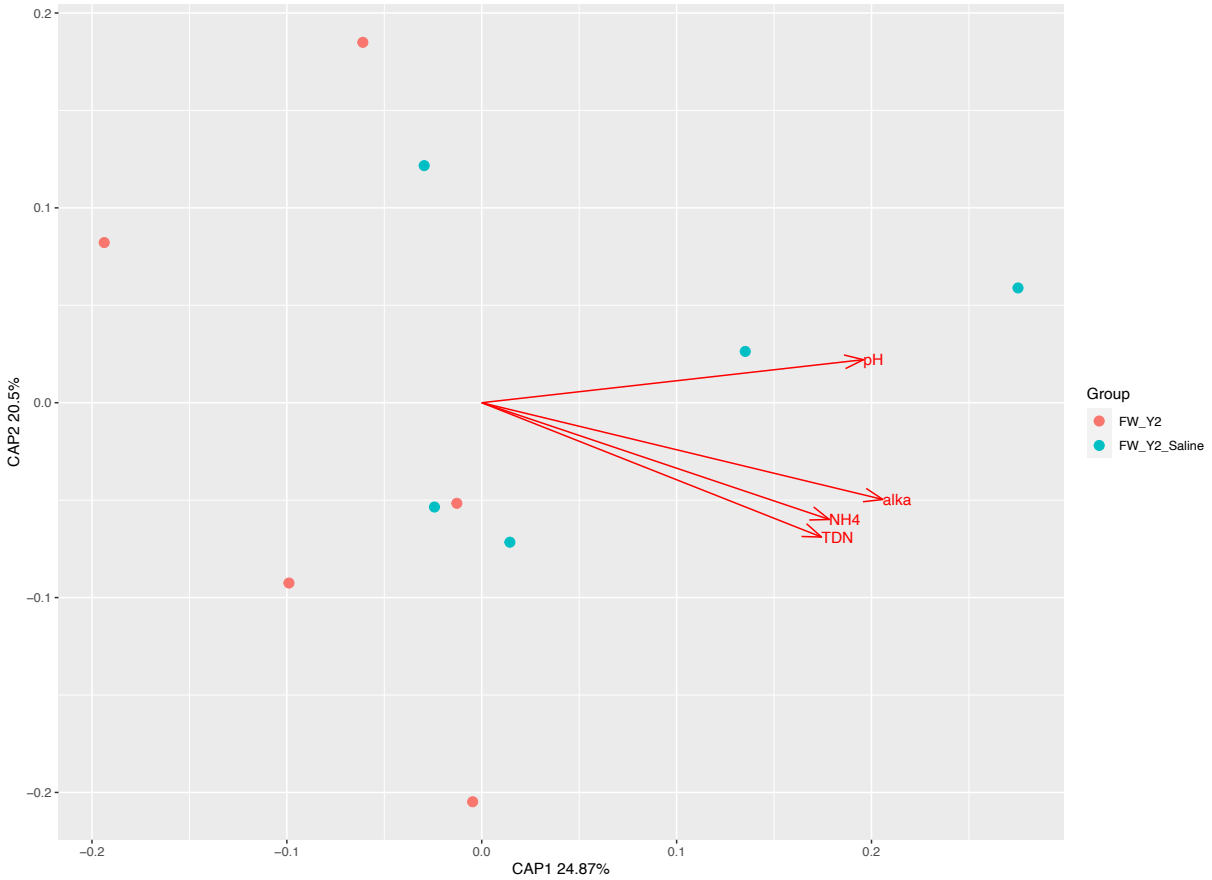

Figure 30: The distance-based redundancy analysis (db-RDA) diagram shows the distribution of soil *mcrA* communities before and after treatment at the freshwater sites. Soil properties depicted as red arrows point to positive associations with communities. Alkalinity (alka), Ammonium (NH_4), Total Dissolved Nitrogen (TDN)

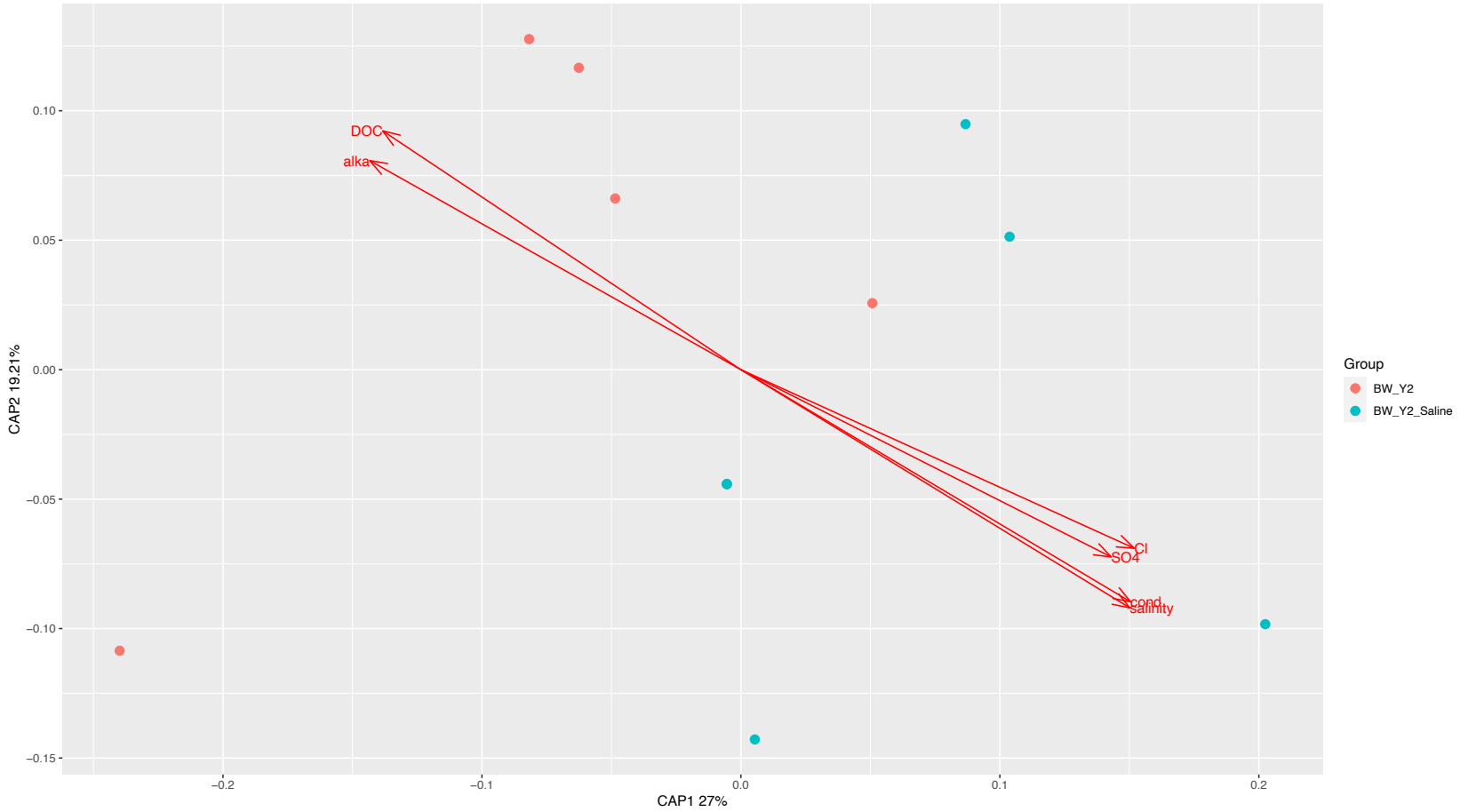

Figure 31: The distance-based redundancy analysis (db-RDA) diagram shows the distribution of soil *mcrA* communities before and after treatment at the brackish sites. Soil properties depicted as red arrows point to positive associations with communities. Dissolved organic carbon (DOC), Alkalinity (alka), Sulfate (SO_4), Chlorine (Cl), Conductivity (cond)

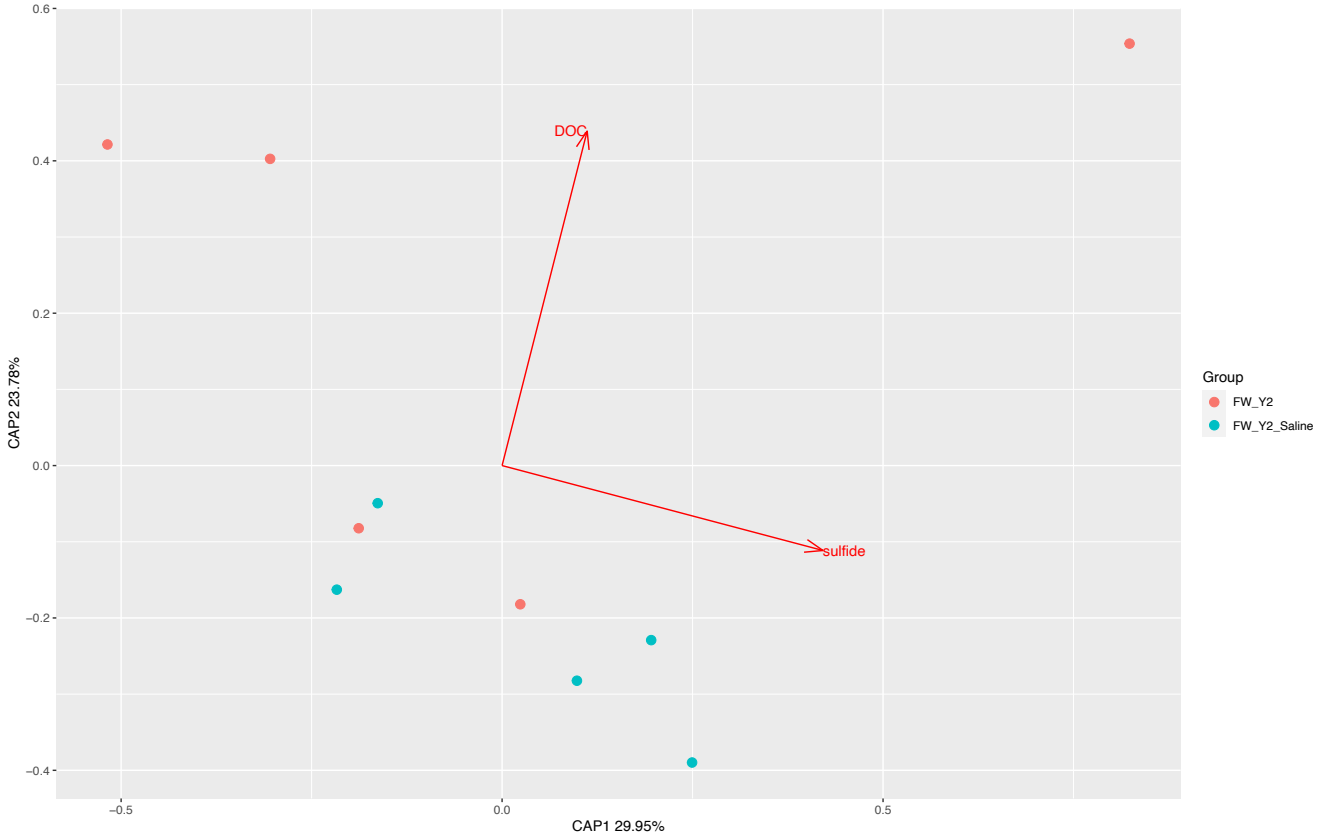

Figure 32: The distance-based redundancy analysis (db-RDA) diagram shows the distribution of soil *phoD* communities before and after treatment at the freshwater sites. Soil properties depicted as red arrows point to positive associations with communities.

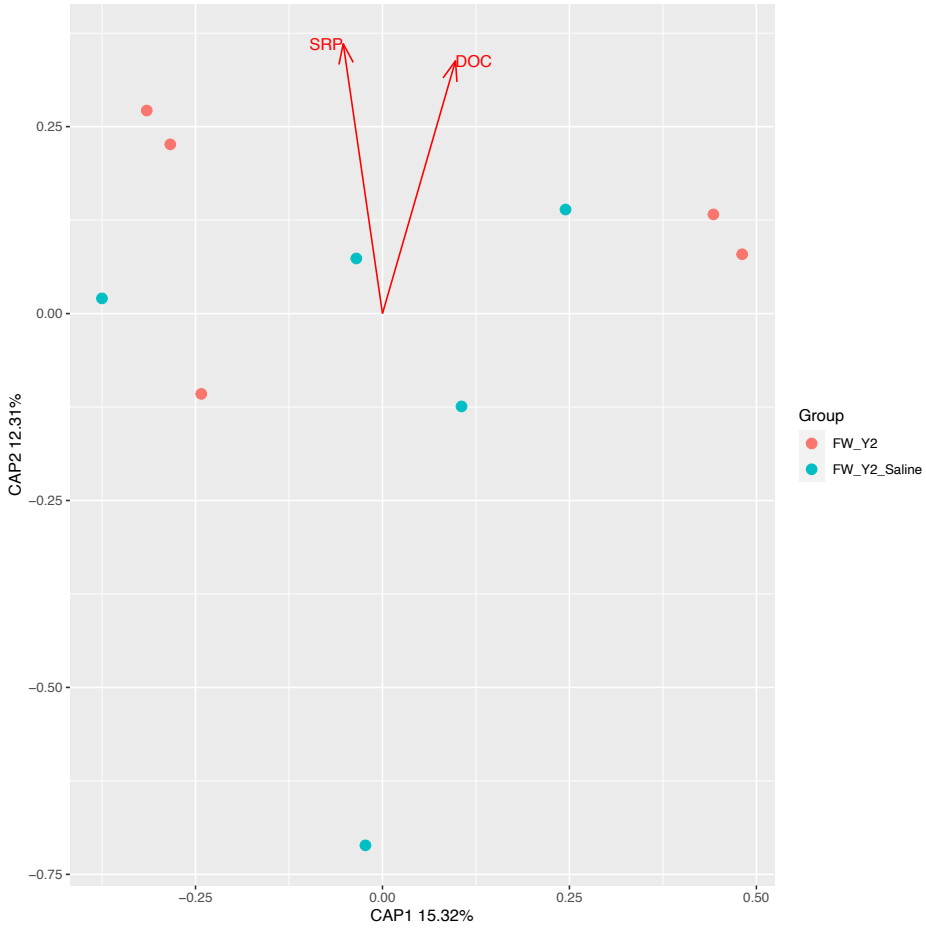

Figure 33: The distance-based redundancy analysis (db-RDA) diagram shows the distribution of soil bacteria communities before and after treatment at the freshwater sites. Soil properties depicted as red arrows point to positive associations with communities.

4.3.2 Random forest modeling

Random forest models were produced for each of the analyzed genes and their accuracy assessed. Using the 16S rRNA genes, bacteria performed the best at predicting the site and to determine groups, but the Archaea model performed better at predicting treatment Table 12.Two of the functional genes, *mcrA* and *dsrA* achieved 100% accuracy when determining the site. However, when a model was created to predict groups, the accuracy dropped. Overall, the individual functional genes performed the best when determining between treatment. Here, the samples were separated by site and their accuracy at determining whether a sample had been exposed to saltwater pulses was assessed. The *mcrA* gene performed had the highest accuracy when predicting treatment in the brackish site. The *nirS* gene had the highest accuracy at the freshwater site. Concatenating the 16S genes created a model that performed slightly better than their individual genes models to predict site. Group accuracy using this approach dropped to 18%, which was lower than the accuracy of the individual Archaea and the Bacteria models. The concatenation of the functional genes performed with 100% accuracy to predict between the sites. The model's accuracy was even lower than each of the individual functional genes when determining groups and treatment within the brackish site. The same effect was seen when the 16S genes were concatenated with the functional genes. However, *dsrA* performed worse than the concatenation of all the genes at the brackish site with an accuracy of 18% compared to its 25% accuracy. Overall, the highest mean accuracy between levels was seen when using the *nirS* gene with an average of 70%. Archaea performed the worst with an overall mean accuracy of only 22%.

Table 12: Accuracy percentage of random forest models validated with leave one out cross validation (LOOCV). Assessments of models with individual genes and then various combinations of genes. Site = brackish and freshwater, Group = BW_Y2, BW_Y2_Saline, FW_Y2, FW_Y2_Saline, Treatment = Control and Saltwater

LOOC Accuracy				
Gene	**Site (n =2)**	**Group (n=4)**	**Treatment (n=2)**	
			BW	**FW**
Archaea	50%	20%	9%	9%
Bacteria	90%	29%	18%	45%
nirS	95%	55%	50%	80%
mcrA	100%	53%	67%	40%
dsrA	100%	43%	18%	50%
phoD	95%	48%	36%	70%
16s Genes	91%	18%	9%	55%
Functional Genes	100%	39%	25%	64%
All Genes	100%	39%	25%	55%

4.4 Discussion

The aim of this chapter was to first determine the main factors that were influencing the variation within the soil microbiome. The second aim was to produce random forest models that could distinguish between sites and determine if concatenating the genes provided any additional accuracy similar to the effect of the increased power of discrimination that is observed with human DNA analysis. Assessing factors that shape the community after saltwater manipulation provide insight that could potentially predict how the microbiome will react in the future, especially with the expectation of climate change bringing increased salinity levels into coastal wetlands. Additionally, bioinformatic machine learning techniques have the potential to aid in determining whether a soil community has been affected by an outside perturbation utilizing its genetic material. Forensic science can also benefit from the increased utility of soil

provenance analyses by targeting specific functional genes instead of broad taxonomic genes like the 16s rRNA of bacteria and Archaea.

4.4.1 Factors contributing to microbial community variation change after saltwater manipulation

Various relationships were determined between the microbial communities and each soil parameter before and after saltwater manipulation. In the freshwater site, the addition of saltwater created significant positive correlations in the bacteria, *dsrA* and *phoD* communities. A relationship could not be established in the bacteria community before the saltwater treatment, but several other studies have found relationships between pH and conductivity with their bacterial communities (An et al., 2019; Fierer & Jackson, 2006; Morrissey et al., 2014). Another study concluded that bacteria community structure is shaped by salinity gradients (Zhang et al., 2021). Results from this study only established relationships in bacteria with DOC and SRP after saltwater manipulation at both the brackish and freshwater site. These factors directly affected the community variation as DOC is the main substrate for heterotrophic bacteria which were found to be the most abundant phyla observed within this site, Chapter 2. Shifts within the Proteobacteria, Firmicutes, and Actinobacteria, all known heterotrophs, were found to be shaped by these substrates (Gawas et al., 2019). A correlation between salinity was not directly established as a factor of the community variation.

A significant correlation with pH was found in the *mcrA* brackish site, similar to what was observed in (Tong et al., 2017). They evaluated the structure of methanogens within a freshwater-brackish marsh and concluded that salinity, along with pH, greatly controlled the community structure. In the current study, soil properties influencing the

variation within this community differed before and after treatment further displaying the effect of salinity on the *mcrA* community in a brackish environment. Before experimental saltwater manipulation occurred at the brackish site, significant correlations were identified with several soil properties to the *phoD* gene, but after treatment no correlations could be established. The opposite effect occurred at the freshwater site with a significant correlation to total dissolved phosphorus and sulfide influencing the community variation. Other studies have documented soil properties such as pH, vegetation, and total phosphorous levels as influential in the *phoD* microbial community in a saltwater gradient and in different soil types (Hu et al., 2020; Lang et al., 2020; Ragot et al., 2015). To our knowledge no other study has evaluated the changes in the properties that shaped the *phoD* community composition in wetlands after long term saltwater inputs.

Sulfide was seen to shape the microbial community within the *dsrA* gene after long term saltwater inputs in the freshwater community. This was expected as saltwater brings in sulfate and the sulfate reducers in turn produce sulfide (Ember M. Morrissey, 2015). Levels of total dissolved nitrogen and ammonium were significantly correlated to this community. This is important because increased levels of sulfide have been shown to inhibit nitrification (Chambers et al., 2015; Tully et al., 2019). Additionally, the *nirS* gene community was seen to be influenced by sulfide here as well. At the brackish site, the properties shaping the *dsrA* community were ammonium and phosphorus. Here, ammonium had a close relationship with the community before saltwater and after saltwater, phosphorous was the main influence on the community variation. This reveals

the effect that saltwater has on interactions between the nitrogen, phosphorus, and sulfur cycles (Flower et al., 2017; Gu et al., 2012).

4.4.2 Gene choice can affect accuracy of random forest modeling

Overall, the random forest models performed best with the functional genes. Recent studies have focused on utilizing 16S rRNA genes microbial communities as input for random forest classification (Ghannam et al., 2020; Roguet et al., 2018). However, focusing on analyzing the taxa within specialized functions can provide additional accuracy that is not achieved by 16S rRNA. When determining soil provenance between the two sites, accuracy was lowest with the Archaea samples and highest with the *mcrA* and *dsrA* functional genes. Bacteria are often utilized for random forest classification and performed better than Archaea but at a lower accuracy than any of the functional genes. This suggests that the wide net of microbes that are found within 16S rRNA genes are hindering the accuracy of the random forest models for classification. Concatenating the genes did not provide much of an increase in accuracy for group and between treatment models. This is likely because of the lack of OTU uniqueness identified within some of the other genes in the concatenation. Some taxa have been found to be more informative for random forest classification (Belk et al., 2018). When comparing between treatments, models performed best on the freshwater site because those communities experienced more variation after the saltwater manipulation. Brackish communities were already adapted to the saltwater, so communities did not experience much of a shift, contributing to the lower accuracy. However, the *mcrA* model likely did better in brackish because methanogenesis is less prominent in oligohaline environments, and their taxonomic

diversity is often low (Dang et al., 2019). This provides for a unique community, that is further reduced after an additional stressor like saltwater.

In conclusion, these results show that overall different nutrients can shape the taxonomic composition of the microbial communities before and after saltwater. The variation in the relationship with the soil properties and the community within different microbial groups suggest a potential for shifts in the community structure and ultimately the ecological function of that community. Knowing that saltwater affects these relationships provides information useful for climate models and predicting how the community will utilize these nutrients. Random forest has proven to be a useful machine learning algorithm for predictions using 16S rRNA genes, but here it was found that functional genes provided higher accuracy. This increased accuracy from targeting microbes that are more focused on a specific function may be useful for ecologists when determining whether a marsh has experienced a perturbation like saltwater intrusion, especially since a perturbation such as this causes changes in the environment that influence their specific functioning. Soil forensics can also benefit from this knowledge by increasing the model accuracy to determine soil provenance by targeting these functional genes. Although outside the scope of this research, targeting specific soil microbial groups that are affected by the presence of a decaying body and utilizing random forest can also assist forensic scientists in time since death estimation.

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CHAPTER 5

5. Conclusion

With the constant threat of global climate change, coastal wetlands like the Everglades, are increasingly being introduced to stressors with the potential to alter their current ecosystem processes. Their soil microbiomes include a community of organisms responsible for biogeochemical cycling, but constant stressors can disrupt the community composition leading to alterations in functioning (Green et al., 2017). The Everglades has marshes with varying salinity levels which infers that they comprise microbial communities with not only different salt tolerances but also different microbial functions. Therefore, it is important to monitor the effects of stressors on these communities in diverse marsh types - freshwater and brackish marshes. This study addressed this need by first establishing a baseline microbial community and then characterizing taxonomic shifts that occurred after a long-term disturbance such as saltwater intrusion and then identifying the soil parameters that most contributed to the soil communities' variation.

In Chapter 2, two common genetic analysis methods were compared to determine the diversity of the community. The initial diversity was observed with LH-PCR and then the taxonomic diversity observed by NGS. Here, the Archaea and bacteria communities were analyzed. With both analysis methods, it was established that each site had a unique distribution of bacterial and Archaeal communities. Moreover, it was found that the choice of analysis method matters, as an additional level of resolution with NGS determined more dissimilar communities in both taxa. Spatial and temporal variations were also observed indicating that the bacteria and Archaea communities differed

between chambers and naturally changed over time. Shifts in relative abundance of taxonomies before and after experimental saltwater manipulation showed that certain taxa react differently to stressors, thus giving insight into how the communities are responding.

Chapter 3 characterized the microbial communities involved in essential biogeochemical cycles and estimated how their functional potential changed after long-term saltwater inputs by investigating the taxonomic shifts. Processes involved in the nitrogen, carbon, sulfur, and phosphorus cycles were examined. Once again, the taxonomic composition differed between the two sites indicating the variation in functional potential between marshes with different salinity levels. Results determined that more dissimilarity was observed over time, than between samples treated with saltwater and those that were not. Diversity indexes revealed that freshwater sites had higher diversity than brackish sites in microbial functional groups that are often suppressed with increasing salinity, such as nitrification and methanogenesis (Chambers et al., 2015; Galagan, 2002; Tully et al., 2019). These functional groups also experienced a decrease in diversity after saltwater, disrupting their functional potential. However, microbial functions that can be accelerated with the increase of saltwater, such as sulfite reduction and the release of phosphates, experienced higher taxonomic diversity, and an increase in diversity after the experimental saltwater manipulation (Flower et al., 2017). Microbes responsible for the potential shift in functioning were identified.

Chapter 4 examined the relationships between the measured soil properties and the microbial composition before and after saltwater. Positive correlations were established but often changed after the input of salinity. Properties responsible for

influencing the shifts in community variation were also altered after the input of saltwater. These results further characterize the microbiomes by identifying how the change in soil properties affect the communities before and after a disturbance like saltwater intrusion. In addition, random forest was utilized to determine if concatenating the various genes provided a higher level of discrimination to establish provenance or monitor soil health. Results showed that concatenation of genes did improve accuracy between sites, but functional genes contributed more to the important features. The individual functional genes provided a higher level of accuracy overall and may be better suited to monitor soil health or in forensic use to determine soil provenance or even time since death.

This study provided a comprehensive overview of the soil microbiome in two Everglades marshes with differing salinity levels. Their communities were evaluated over time and taxonomic differences observed after experimental saltwater manipulation. In addition, changes in the functional potential were estimated and the main contributing factors of the community variation detected. The utility of the microbiome for ecological and forensic purposes was also examined demonstrating the immense potential of the community below the soil.

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