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Development of soil microbial community structure in created and natural wetlands

By Wendy A Owens

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Environmental Science

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July 26, 2022

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Table of Contents

Table of Tables........IV

Table of Figures........V

Acknowledgements........VI

Abstract........1

Chapter 1 - Overview........**2**

Chapter 2 - Microbial community structure across zones in a salt marsh chronosequence..**5**

Introduction........5

Methods........8

Results........13

Discussion........31

Conclusion........33

Chapter 3 Succession and soil management in freshwater depressional wetlands........**35**

Introduction........35

Methods........39

Results........47

Discussion........69

Conclusions........73

Appendices........74

Bibliography........92

Table of Tables

Table 2.1- Plant and environmental characteristics across the four vegetation zones in each Marsh. 18
Table 2.2- Environmental Factor- Statistical Analyses. 19
Table 2.3: Relative abundance (RA) of the top 10 bacterial classes across all zones in each marsh. 23
Table 2.4:Diversity Indices for each Marsh. 24
Table 2.5:Bray-Curtis Distance with PERMANOVA results for each pairwise comparison of marshes and zones. 27
Table 2.6-Metabolic function in each Marsh and within each Marsh 29
Table 2.7-Metabolic function by percent in each of the zones and in each Marsh. 30
Table 3.1-Environmental factors for HANA Emergent. 54
Table 3.2-Environmental Factors for HANA Wooded Wetlands. 55
Table 3.3-Mill Seat Wooded Wetlands. 56
Table 3.4-Relative abundances (RAs) of the top 10 bacterial classes across all treatments in each wetland. 60
Table 3.5-Relative abundances of the top 10 bacterial classes across treatment in each wetland. 61
Table 3.6-Microbial Diversity between wetlands and within each wetland. 63
Table 3.7- Microbial Diversity across the three treatments at each site. 63
Table 3.8-Metabolic function in each wetland and across treatments within each wetland. 67
Table 3.9-Metabolic function by percent in each of the treatments within each wetland. 68
Table A.1-Grain Size portions for each of the vegetation zones. 74
Table A.2-Young Marsh all Metabolic functions. 74
Table A.3-Mature Marsh all Metabolic functions. 78
Table A.4-HANA Emergent Plant Species List. 81
Table A.5-HANA Wooded Plant Species List. 81
Table A.6-Mill Seat Wooded Plant Species List. 82
Table A.7-Soil Grain Size for all treatments in each Wetland. 82
Table A.8-HANA Emergent -All Metabolic functions. 84
Table A.9-HANA Wooded All Metabolic functions. 86
Table A.10-Mill Seat Wooded All Metabolic functions. 89

Table of Figures

Figure 2.1- Map of Hog Island Locations......9
Figure 2.2- Total aboveground biomass for each zone in each marsh.(Mean ± Standard error (SE)). Unique letters above bars indicate statistical differences based on two-way ANOVA using Marsh, Zone and their interaction as fixed factors.......17
Figure 2.3-Relative Abundances of the top 10 Phyla in each Marsh.......20
Figure 2.4:Relative Abundance of the top 10 phyla in each zone for the Young and Mature Marshes.......21
Figure 2.5-Heat Map of Relative Abundances at the taxonomic level of Class in each Marsh divided by Zone.......22
Figure 2.6- Microbial diversity in each of the Marshes separated by vegetation zone.......24
Figure 2.7-Principal coordinates analysis (PCoA) of bacterial community structure.......25
Figure 2.8: Bray Curtis Cluster Plot depicting both Marshes and their vegetation Zones.......26
Figure 2.9-RDA depicting Environmental Factors driving sediment microbial communities.......28
Figure 3.1-Location of Experimental and Reference sites at High Acres and Mill Seat Restoration Areas......41
Figure 3.2-High Acres Nature Area Experimental Design.......41
Figure 3.3-Compost Experimental Design at Mill Seat Wetland Restoration Area.......42
Figure 3.4-Vegetation characteristics in control, compost and reference sites at the three wetland locations.......53
Figure 3.5-Relative Abundances of the top 10 Phyla across treatments in each of the wetlands.......58
Figure 3.6-Heat map of the top 20 microbial classes, including both Bacteria and Archaea, across treatments in each of the wetlands.......59
Figure 3.7- Alpha diversity metrics in control, compost and reference sites at the three wetland locations.......62
Figure 3.8-PCoA of microbial communities across treatments.......64
Figure 3.9-Bray-Curtis Cluster of microbial communities sampled in control, compost, and reference plots.......65
Figure 3.10-RDA of environmental factors correlated with key bacterial classes.......66
Figure A.1-Relative Abundance on replicates of Soil Amendment experiment......83
Figure A.2-Heat Map of Relative Abundances of Class for Soil Amendment replicates......83

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Abstract

Wetlands are ecologically and economically important, providing ecosystem services such as biogeochemical cycling, carbon sequestration, flood mitigation, and wildlife habitat. Many of these services are the result of the unique microbial communities found in wetlands. Degradation and destruction of wetlands, from direct human development or indirect stressors caused by climate change, pollution, or invasive species, disrupts community structure and provision of services. Although restoration has been adopted as a mechanism to counteract net loss of function, the success rate in achieving functional equivalence with natural wetlands is low. To improve wetland conservation and outcomes for wetland creation, a better understanding of biotic community structure and biotic-abiotic relationships in developing wetlands is needed. By using the metagenomic approach of 16s rRNA amplicon sequencing, we can better understand the role of microbial communities as drivers of wetland biogeochemical cycling and predict future resilience. I evaluated environmental factors and microbial community structure of young and mature back barrier salt marshes and depressional freshwater wetlands. Salt marshes are particularly vulnerable to climate change. Evaluation of amplicon data suggests an increase in diversity and functional redundancy with marsh age, but also potential for greater resilience in the Young Marsh, where sandier sediments limit waterlogging and anoxia. In created freshwater wetlands, antecedent land use and hydrology may drive soil physico-chemistry and shape microbial community structure, which is distinctly different from mature, reference wetlands. Management of soils by amending with leaf litter compost drives abiotic factors closer to a mature marsh, but at the same time results in a unique microbial community unlike either a young or mature wetland. This suggests a potential shift in function away from the desired trajectory. Evaluation of the microbial community structure provides insight into underlying wetland function and promotes development of management practices to maximize function and overall resilience.

Chapter 2 - Overview

Throughout history wetlands have been drained, dredged, or filled to accommodate for agriculture or development, ultimately resulting in loss of essential ecosystem services (Johnston 1994, Gibbs 2000, Mitsch and Gosselink 2007). Different cultures throughout the world either revered wetlands, used wetlands to their advantage, or destroyed them (Mitsch and Gosselink 2007). In the last century, attitudes slowly changed as the value of wetlands was more fully recognized. This shift in appreciation is reflected in the conservation efforts around waterfowl habitat culminating in the 1971 adoption of a treaty to protect wetlands worldwide (RAMSAR 1971, Mitsch and Gosselink 2007). Since then, countries around the world have implemented different policies to protect wetlands. The current policy in the United States is based on the concept of "no net loss" of wetland function as described in the Clean Water Act ("Clean Water Act: Section 404" 2022). Although this policy requires the creation or restoration of wetlands by the responsible party when wetland destruction has been deemed unavoidable, it is riddled with challenges and there is lack of success in achieving the goal of no net loss of function over time as the wetland ages (Zedler 2004, Moreno-Mateos et al. 2012). Even under excellent management plans, the created wetland may deviate from expectations. A multitude of studies have focus on finding strategies to achieve success in created wetland ecosystems, with a focus on wetland plant community assessments, invasive plant species management, and soil additions, with an general assumption that soil biogeochemistry and hydrology are the underlying factors driving the biotic system (Ballantine and Schneider 2016, Stefanik and Mitsch 2017, Brown and Norris 2018). There are still gaps in knowledge about biogeochemical cycling in wetlands, especially related to the structure and function of microbial communities in developing systems.

Wetland ecosystem function depends on many interacting components, including biogeochemical cycling, hydrology, plant species composition, soil microbial communities, and climate (Mitsch and Gosselink 2007, Schlesinger and Bernhardt 2020). The hydrology of wetlands creates anoxic environments where the soil microbial community becomes the most important driver of the biogeochemical cycles (Schlesinger and Bernhardt 2020). Previous studies on wetlands focus on the soil nutrients and they often refer to the importance of the soil microbial communities (e.g., Tyler et al. 2003, Maietta et al. 2020, McGowan 2020). However, few studies have used microbial detection tools to understand community composition (Maietta

et al. 2020, Abbott et al. 2022). It is clear that in order to more adequately achieve our goals of functionally successful created wetlands, we need to better understand the composition and role of the soil microbial organisms in driving wetland succession.

The successional trajectory of a created wetland is determined based on the type of wetlands as defined by its hydrology and vegetation type (Brinson 1993, Mitsch and Gosselink 2007, Brooks et al. 2011). The hydrogeomorphic classification determines the extent to which hydrology influences the successional trajectory. Depressional wetlands are dependent on autochthonous organic matter, and are therefore slow to accumulate soil organic matter, while the riverine and tidal wetlands accumulate allochthonous materials that may drive succession at a faster rate (NRCS 2008, Stefanik and Mitsch 2017, Maietta et al. 2020, Schlesinger and Bernhardt 2020). Within the depressional wetlands, the plant community type will also determine the rate of succession. Created emergent depressional wetlands may reach the desired outcome more quickly than forested wetlands that take a long time to achieve the climax plant community and as such may more frequently deviate from the intended reference wetland (Matthews and Spyreas 2010, Atkinson et al. 2022).

Managing the plant community and soil characteristics during creation is important, but this management must be maintained past a mandatory monitoring period to prevent invasion by non-native species, or shifts in hydrology, that may in turn disrupt the successional trajectory (Moreno-Mateos et al. 2012, van den Bosch and Matthews 2017). At the same time, reference wetlands are subject to exogenous drivers of change, such as invasion by non-native species in unmanaged systems, and may no longer represent the desired restoration outcome (Tillman et al. 2022, Atkinson et al. 2022). The added complication of climate change creates uncertainty in the successional trajectory for created wetlands (Zedler 2010), and suggests the need for more holistic approaches that encompass more than monitoring of hydrology and plant communities.

The underlying soil microbial community structure may give us indications of the direction of the wetland based on the combination of soil physicochemical properties and plant communities. In this study, we analyzed the soil microbial community using 16s rRNA gene to identify key soil microbial organisms driving soil physicochemical characteristics and plant community dynamics (biomass, diversity, and floristic quality) in two distinct wetland types. By identifying soil microbial community structure, microbial taxonomic groups, and microbial functional groups in both created and natural, and young and mature, wetlands we can begin to

tease out function of the natural wetland, and evaluate management techniques that will perhaps aid in the successional trajectory of the created wetlands.

In chapter 2, I focus on a naturally occurring back barrier salt marsh chronosequence along the East Coast of the United States to evaluate how young and mature marshes respond to stressors from climate change such as sea level rise (SLR), higher temperatures and drought. This study gives a unique opportunity to understand a naturally occurring salt marsh with little outside influence from anthropogenic sources. Previous studies at this site (Hog Island, VA) focused on various aspects of the salt marsh ecosystem, including *Spartina alterniflora* biomass (aboveground and belowground), nutrients and soil physicochemical properties (Osgood and Zieman 1993, Tyler and Zieman 1999, Tyler et al. 2003, Goldsmith et al. 2020). With this work, we aim to verify key ecosystem functions by identifying sediment microbial community structure and function in the different age marshes, and as such can support years of prior research. We identify key organisms linked to biogeochemical cycles of sulfur and nitrogen and Metabolic function important to the breakdown of organic matter, and demonstrate variability across both age and zone in the marsh. Much of the heterogeneity within a marsh can be tied to zone-dependent stressors and suggests variability in age-related resilience to climate induce changes.

Chapter 3 focuses on three depressional wetlands groups, encompassing both created and mature sites, in Western New York State. Wooded wetlands were created in 2018 and 2012, and the emergent wetland was created in 2009. Long term studies have been occurring at all sites, including the addition of leaf litter compost to augment soil organic matter and influence plants community structure and soil biogeochemistry (McGowan 2020, Huang 2021, Williams 2021). The corresponding reference sites included a naturally occurring emergent wetland left to fallow roughly 75 yr ago, and a vernal pool and forested swamp that were greater than 100 yr old. Based on previous research we hypothesized that compost additions will drive the trajectory of the created wetland toward that of the nearby reference wetland, with an increase in microbial diversity and function. However, our results suggest that each wetland may follow a site-specific trajectory, and that compost addition may drive wetland physico-chemistry toward a more mature state, but also shift the microbial community. Ultimately, these results are useful to inform management of young wetlands and promote more successful restoration and conservation outcomes.

Chapter 3 - Microbial community structure across zones in a salt marsh chronosequence

Introduction

Coastal wetlands are among the most productive ecosystems in the world, providing a multitude of ecosystem services including carbon sequestration and storm protection (Barbier et al. 2011). Along sedimentary temperate coastlines, salt marshes dominated by emergent herbaceous plants are the typical wetland ecosystem. These valuable land-margin systems are threatened by both direct and indirect drivers related to climate change, including higher temperatures, sea level rise, and increased frequency of storms. These drivers may create significant stressors, including waves, heat, salt, and waterlogging, that in turn cause changes in vegetation zonation, migration of low marsh vegetation into the upland, erosion of marsh edge, and marsh die-off e.g -(Gedan et al. 2009, McLoughlin et al. 2015, Priestas et al. 2015, Valiela et al. 2018, Veldhuis et al. 2019, Silliman et al. 2019). Understanding the impacts of and resilience to these stressors can lead to better management of critical coastal ecosystems.

Along the mid-Atlantic region of the United States, the seaward edge of salt marshes is mostly dominated by different ecotypes of *Spartina alterniflora* (Mitsch and Gosselink 2007, Barbier et al. 2011). *S. alterniflora* grows in two distinct ecotypes based on the distance from the coast and nutrient availability (Valiela et al. 1978, Valiela 2015). Despite low plant species diversity, because of the high primary and secondary production, salt marshes support commercially important species which includes providing nurseries for fish (Roman et al. 2000). Scientific research in salt marshes historically has been focused on the physical processes (tides, elevation, nutrient availability, and salinity) to understand and predict structure and function (Tyler et al. 2003, Mitsch and Gosselink 2007, Barbier et al. 2011). Long term studies on salt marshes show changes in nutrient availability, elevated salinity in the high marsh, increased submergence and changes in the vegetation composition as low marsh vegetation migrates into the high marsh (Miller et al. 2001, Sallenger et al. 2012, Smith 2015, Valiela et al. 2018, Goldsmith 2019). More recently, studies have focused on tandem shifts in abiotic-biotic structure in order to better predict ecosystem function, services, and resilience, and achieve restoration goals (Silliman et al. 2019, Cahoon et al. 2020, Goldsmith et al. 2020). Understanding abiotic

and biotic factors in concert, and prediction of vulnerability and resilience, is imperative for coastal conservation and to mitigate the impacts of climate change and erosion of critical ecosystem services (Silliman et al. 2019, Cahoon et al. 2020).

Most studies of marsh stress and resilience, however, have taken place in mature salt marshes and have looked at broad patterns of sea-level rise and salt marsh transgression (Leonardi et al. 2016, Miller et al. 2021). Younger marshes, both natural and created, have a different set of biophysical characteristics, and thus may experience and respond to climate-induced stressors in different ways (Davis et al. 2015, Craft 2016, Alldred et al. 2020, Abbott et al. 2022). Young marshes are characterized by sandy sediment, lower nutrient and organic matter availability, and lower levels of hydrogen sulfide (higher oxidation-reduction potential)(Osgood and Zieman 1993, Tyler and Zieman 1999, Tyler et al. 2003, Abbott et al. 2022). Despite these differences, the vegetation community and plant biomass may be largely the same between young and old marshes (Tyler and Zieman 1999, Moseman-Valtierra et al. 2016, Goldsmith 2019). However, differences in function suggest there may be broader distinctions within the microbial communities, and portend potentially divergent responses to external stressors (Tyler et al. 2003, Moseman-Valtierra et al. 2016).

Though many studies have looked at stress-induced changes by studying the vegetation, the fauna, or the biophysical characteristics, newer tools such as high resolution hyperspectral imaging, stable isotope probing and high throughput sequencing (amplicon sequencing) may give us clues to better understand underlying structure-function relationships and responses to stressors (Currin et al. 1995, Veldhuis et al. 2019, Jones et al. 2019, Goldsmith et al. 2020, Stagg et al. 2021, Wilson et al. 2022, Crotty et al. 2022). The use of amplicon sequencing via 16s rRNA is becoming cheaper, easier to use, and is constantly evolving to better analyze sequence data and identify key drivers of salt marsh biogeochemistry (Barreto et al. 2018, Lynum et al. 2020, Abbott et al. 2022). These metagenomic tools illustrate community composition and potentially identify organisms important to ecosystem function (Barreto et al. 2018, Zhang et al. 2020).

It has been long thought that the sediment microbial community in salt marshes drives nutrient transformations crucial to the biogeochemical cycles(Schlesinger and Bernhardt 2020). Studies have looked at the importance of individual bacterial groups such as *Cyanobacteria,* which are known nitrogen fixers (Kaplan et al. 1979, Currin et al. 1996). Salt marshes in general

are nitrogen limited and these organisms contribute to the nitrogen budget(Tyler et al. 2003, Kearns et al. 2016). The process of denitrification is another important process in salt marshes and is determined to access to organic matter (Kaplan et al. 1979, Giblin et al. 2013). Nitrogen cycling species shift as a marsh develops, with different bacterial groups following the development of different biophysical traits characteristic of marsh age (Salles et al. 2017) but may not change with alteration of nutrient loading (Bowen et al. 2020, Hanley et al. 2021). Sulfur is another nutrient in salt marshes where sulfate is the dominate source of sulfur. This process is important in the fate of organic matter (Howarth and Teal 1979, Howarth 1984, Schlesinger and Bernhardt 2020). In highly reducing environments where H_2S is produced by sulfate reducing bacteria the dominant phylum involved in the sulfur cycle is *Desulfobacterota* (Larsen et al. 2015) Newer metagenomic tools such as FAPROTAX aid in facile determination of the dominant Metabolic function in the salt marsh (Louca et al. 2016) and can be used to assess changes in functional capacity across sites.

Salt marsh microbial community composition and structure provides new understanding for how to restore degraded salt marshes (Summers Engel et al. 2017, Lynum et al. 2020, Abbott et al. 2022). However, research is needed to determine microbial community composition across the range of natural "healthy" salt marshes – including the range of ages - to establish baseline information for use in restoration (Summers Engel et al. 2017, Salles et al. 2017, Abbott et al. 2022). Combining this information with biophysical properties of soils and vegetation characteristics can translate to the broader restoration questions in more human dominated systems along our coast and assist with understanding marsh development trajectories in restored salt marshes. Addressing the question of how the sediment microbial community composition of both young and old natural marshes responds to stressors such as SLR, higher temperature, and drought may provide insight into resilience of both natural and created or restored salt marshes.

In this study, we look at a well-studied chronosequence on Hog Island, Virginia (e.g., Osgood and Zieman 1993, Tyler and Zieman 1999, Tyler et al. 2003, Goldsmith et al. 2020). Intense coastal storms have caused overwash events and eroded or buried mature marsh, creating a mosaic of marshes of different ages as *S. alterniflora* recovers on the new sediment platform. Previous work has focused on nutrient availability, biophysical characteristics, and identification of stressors of *S. alterniflora* (e.g., Osgood and Zieman 1993, Tyler and Zieman 1999, Tyler et al. 2003, Goldsmith et al. 2020). These characteristics change in a predictable manner with the

age of the marsh. For example, silt, clay, carbon, hydrogen sulfide, and nutrients build up in sediments over time. Nitrogen is more abundant in the older marshes, and limiting to plant growth in the younger marshes where enhanced microbial nitrogen fixation may supply nutrients to plants (Tyler et al. 2003). However, an updated comparison of chronosequence marshes from the mid 1990s to 2017 showed loss of mature marsh on the lagoon edge, prograding of marsh into the lagoon in the younger marshes, and increasing heterogeneity within the interior of the marsh across ages, represented by die-off, patches of higher salinity, and invasion of *S. alterniflora* into the high marsh (Goldsmith 2019).

With these differences in mind, we sought to evaluate whether the sediment microbial community composition reflects these differences in the biophysical sediment characteristics. We hypothesized that there would be distinct community composition between young and mature marshes, but also among the different zones within a marsh, reflecting the vegetation zonation, the gradient in stressors along the intertidal prism, and age-related variability in sediment physicochemical properties.

Methods

Site Description and Sampling Design

Hog Island is located within the Nature Conservancy's Virginia Coast Reserve and is part of the Virginia Coast Reserve Long Term Ecological Research (VCR LTER) site on the Delmarva Peninsula on the Atlantic seaboard (Figure 2.1) (Day et al. 2001). The island was once populated, but no longer sustains human dwellings due to extreme weather events, lack of freshwater, and eroding coastline (Hayden et al. 1991). Two marshes on the southern end of the island were sampled in July 2019. The "Mature Marsh" site is at least 170-yr old based on its presence on maps dating back to 1853 and sits adjacent to the location of the former town of Broadwater (Tyler and Zieman 1999). The "Young Marsh" site (<30-year) was formed by an overwash event during the Ash Wednesday nor'easter of 1962 that deposited >1 m of sand across the southern end of the island (Stewart 1962). Age is defined as the date when *Spartina alterniflora* first recolonized, based on inspection of aerial images (Osgood and Zieman 1993, Tyler and Zieman 1999). Since that time, the Young Marsh edge has continued to expand toward the lagoon, with a section of very new marsh (<10 yr; Goldsmith 2020).

Within each marsh, four zones were established based on location within the tidal prism and *S. alterniflora* biomass. The “Edge” sites are along the lagoon edge or tidal creek, “Meadow” consists of continuous moderate *S. alterniflora* biomass, and “Upper” lies along the upland edge of the low marsh, fringing the high marsh. In the Young Marsh, this zone represents an area of high marsh invaded by *S. alterniflora*. The fourth zone is “Die-Off”, where *S. alterniflora* is no longer present. In the Mature Marsh, “Edge” sites reflect both the eroding edge of the lagoon and the drowning edge of a well-developed tidal creek. In most analyses below these are combined, but where appropriate the differences are illustrated.

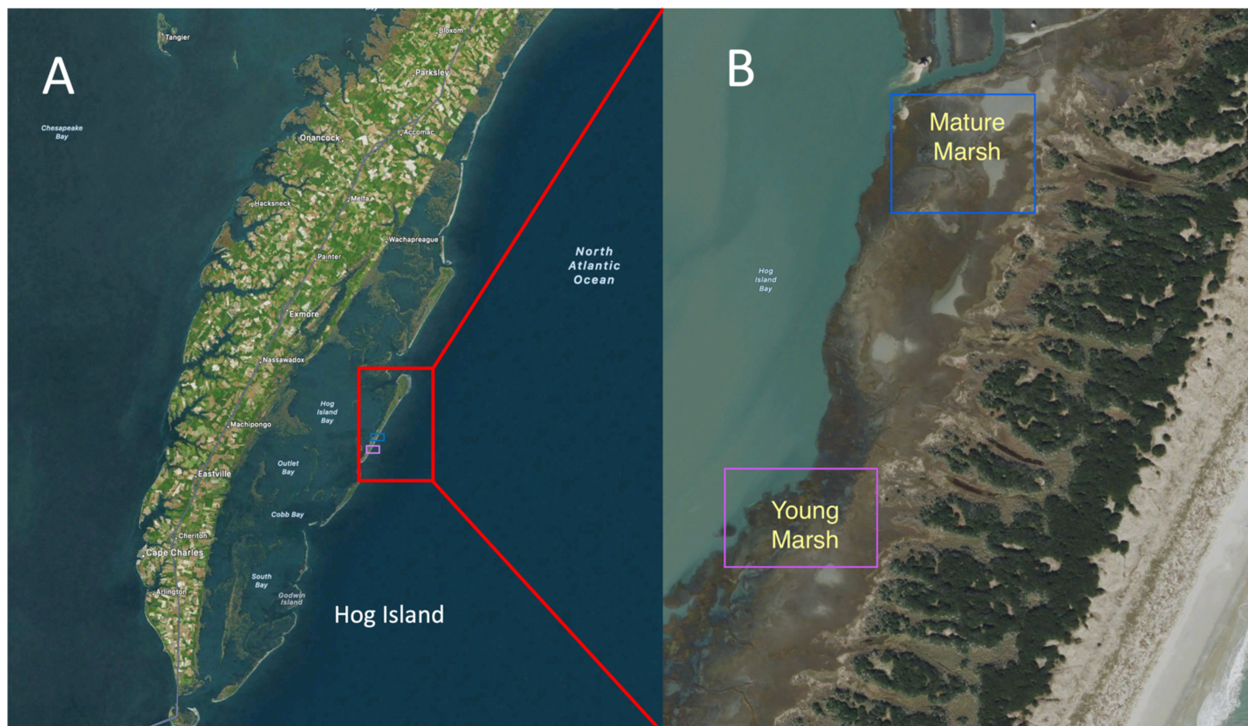

Figure 3.1- Map of Hog Island Locations Eastern Shore of Virginia. B) Research sites on Hog Island Field Measurements, Sample Collection and Analysis

Within each zone, four to seven 0.25 m^2 plots were randomly chosen for collection of plant biomass, sediment samples for physico-chemical parameters, and microbial community characterization, for a total of 20 plots in the Mature Marsh and 17 plots in the Young Marsh (Figure 2). To determine aboveground biomass, at each 0.25 m^2 plot, the total number of *S. alterniflora* culms was recorded. Height was measured for the first 10 *S. alterniflora* culms along a diagonal transect between opposite corners of the quadrat. An empirical relationship was developed between height and culm biomass by collecting 201 culms outside the plots. The

culms were clipped at the surface and brought back to the laboratory where they were washed of debris, measured for height, and weighed after drying at 60 °C for 48 hr (Miller unpub. data). *Salicornia virginica* was evaluated by estimating the percent cover within each plot where it was present. An empirical relationship between cover and biomass was developed by collecting, drying, and weighing all culms from five additional plots (Miller unpub. data).

In each plot, two sets of three replicate cores were taken from the top 1 cm of sediment using a modified 5 cc syringe corer. One set of three cores was pooled and placed in a sterile 15 ml centrifuge tube for microbial analysis and the other three were pooled and placed into a 20 ml acid-washed scintillation vial for physico-chemical characterization. Tubes and vials were placed on ice, transported in a cooler, and stored at -80°C until processing. Within each zone, one additional 6 cm diameter by 10 cm deep core was extracted from a representative plot using a tulip bulb corer and used for grain size analysis. At each plot, porewater salinity was measured one time by extracting approximately 5 ml porewater using a perforated stainless-steel probe inserted to a depth of 10 cm (Berg and McGlathery 2001). Salinity was determined using a temperature compensating refractometer. Position was measured for each plot using a Trimble Real Time Kinematic GPS (X, Y accuracy <1 cm; Z accuracy = 1.5 cm).

The second set of sediment cores was dried at 60°C for 48 hr and weighed to determine bulk density (BD). The dried sediment was homogenized with a mortar and pestle and soil organic matter (%OM) was assessed by combusting a weighed subsample in a muffle furnace at 550°C for 4 hr and calculating %OM based on the mass loss on combustion (Heiri et al. 2001). Total phosphorus (%TP) was determined by adding 50% w/v magnesium nitrate to 0.1 g oven-dried soil and ashing for 2 hr at 550°C in a muffle furnace. Once cool, 10 mL of 1 M HCl was added, samples were then shaken for 16 hours and allow to settle overnight. Samples were diluted 10-fold and the phosphate content measured at 880 nm using a Shimadzu UV 1900 Spectrophotometer (Aspila et al. 1976). Carbon (%TC) and nitrogen (%TN) content were measured by using the Perkin Elmer 2400 Elemental Analyzer. All analyses were conducted in duplicate or triplicate. Molar ratios of C:N and N:P were calculated from these values. Carbon Density (CD) was obtained by multiplying the bulk density (g/cm^3) by total carbon.

Microbial Analyses

DNA was extracted from sediment using DNeasy PowerSoil Kit (QIAGEN 2021) per manufacturer’s instructions (n=37). The DNA purity and quantity was confirmed by using a

Nanodrop 2000 spectrophotometer. DNA sequence data was generated using Illumina paired-end sequencing at the Environmental Sample Preparation and Sequencing Facility (ESPSF) at Argonne National Laboratory. The V4 region of the 16S rRNA gene (515F-806R) was PCR amplified with region-specific primers that include sequencer adapter sequences used in the Illumina flowcell (Caporaso et al. 2011, 2012). For the PCR reaction, each 1 µL DNA sample was combined with 9.5 µL of MO BIO PCR Water (Certified DNA-Free), 12.5 µL of QuantaBio's AccuStart II PCR ToughMix (2x concentration, 1x final), 1 µL Golay barcode tagged Forward Primer (5 µM concentration, 200 pM final), 1 µL Reverse Primer (5 µM concentration, 200 pM final). The reaction conditions were: 94 °C for 3 minutes to denature the DNA, with 35 cycles at 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s; with a final extension of 10 min at 72 °C to ensure complete amplification. Amplicons were then quantified using PicoGreen (Invitrogen) and a plate reader (Infinite® 200 PRO, Tecan) and then pooled into a single tube so that each amplicon was in equimolar amounts. This pool was cleaned up using AMPure XP Beads (Beckman Coulter), and quantified using a fluorometer (Qubit, Invitrogen). After quantification, the molarity of the pool was determined and diluted down to 2 nM, denatured, and then diluted to a final concentration of 6.75 pM with a 10% PhiX spike for sequencing on the Illumina MiSeq. Amplicons were then sequenced on a 151 bp x 12 bp x151 bp MiSeq run (Caporaso et al. 2011, 2012).

The resulting 16s rRNA sequences were demultiplexed in QIIME2 (Bolyen et al. 2019) chimeras removed and the final 2,323,741 sequences were denoised with the DADA2 plugin for QIIME2 (Callahan et al. 2016). Taxonomy was assigned using SILVA database (Quast et al. 2013). R-Studio and R were used to convert the artifacts into a *phyloseq* object (McMurdie and Holmes 2013, R Core Team 2022, RStudio Team 2022) and then transformed into a 'microtable' for Microeco. All ASVs not assigned to Archaea or Bacteria, and those assigned to "mitochondria" or "chloroplast" were removed. The *tidy_dataset* function was used to trim the dataset to eliminate samples with 0 artifacts and the microtable was rarified to a sample size of 10,000 sequences per sample (only one sample was removed). From this final data, we calculated relative abundance at the Kingdom, Phylum, Class, Order, Family, Genus, and Species level. All data visualizations were performed using the ggplot2 package in Microeco for R (Wickham 2016, Liu et al. 2021). Alpha diversity metrics for each marsh as a whole, and for each zone within each marsh were calculated using Microeco, including species richness,

evenness, Total Observed Features, and Shannon, Chao1, and Faith's Phylogenetic Diversity Indices. Beta diversity analyses were performed in Microeco to evaluate community structure among the two marshes and their respective zones. Dissimilarity was visualized using Principal Coordinate Analyses using pair-wise Bray-Curtis dissimilarity estimates, and relationships were visualized using a cluster graph. Using the Bray-Curtis distance a RDA was performed show correlations among the microbial taxa level of class and the associated environmental factors: BD, total aboveground biomass, CD, %TP, %TC, C:N and N:P. Highly autocorrelated variables assessing similar parameters (e.g., %TC and %OM) were reduced to a single variable (%TC). A PERMANOVA using Bray-Curtis dissimilarity was used to assess Beta Diversity using the *vegan* package within the Microeco package for R (Liu et al. 2021, Oksanen et al. 2022). The top metabolic functions (by percent) were determining by using the FAPROTAX package within the Microeco package for R (Louca et al. 2016, Liu et al. 2021).

Statistical Analyses

Differences in biomass, salinity, elevation, and sediment characteristics were evaluated using a two-way analysis of variance (ANOVA) in JMP 16 with Marsh, Zone, and their interaction as fixed factors. All data were assessed for normality (Anderson-Darling) and homogeneity of variance (Levene's test) prior to analysis to ensure adherence with the assumptions of the test. Where assumptions were violated, data were transformed. If transformation failed, the non-parametric Kruskal-Wallis test was applied to evaluate differences between marshes or among zones within an individual marsh. The Kruskall-Wallis test was also used on the 10 most abundant microbial taxa at the level of class, the diversity indices, and metabolic function to separately evaluate age-related differences between marshes and within marsh differences among zones. Where significant zone effects were found, a Dunn's pairwise comparison was used to evaluate differences among zones within each marsh.

Results

Age related differences separate the two sites, with distinct differences in vascular plant characteristics, biophysical variables, and sediment microbial communities. There were also unique characteristics across vegetation zones within each marsh that suggest the potential for unique structure-function relationships.

Environmental characteristics

Total aboveground biomass (as *S. alterniflora* plus *S. virginica*) for Meadow zones was similar across ages at approximately 600 g m^{-2}, but a significant interaction between Marsh and Zone ($p = 0.0005$) suggests site specific differences in stressor zones. Edge biomass was roughly 4-fold greater in the Mature marsh than the Young Marsh, but the Upper and Die-Off zones are similar between sites and with each other. The Mature Marsh exhibits a significant gradient in biomass from Edge to Upper zones, which is absent in the Young Marsh where biomass is statistically similar across all zones. *Salicornia virginica* was present only in the Young Marsh at the edge (trace amounts) and in the Upper zone, where it was mixed with *S. alterniflora* but comprised 56% of the total biomass (Figure 2.2)

Similar interactions were observed between Marsh and Zone for elevation, salinity, %OM, %TC and %TN ($p < 0.01$ for all interactions; Table 2.1 and Table 2.2). The elevation of the Upper marsh was roughly 0.4 m higher than all other zones, which was reflected in a similar difference of approximately 10 ppt greater salinity in the Young Upper zone ($p<0.01$ for Zone x Marsh interaction for both). Bulk Density was more than two-fold greater in the Young Marsh than the Mature marsh (1.12±0.05, 0.46±0.04; $p<0.0001$), with higher values closer to the marsh edge in both marshes (1.32±0.01, 0.58±0.04). Percent OM was higher in the Mature marsh overall (8.5±0.4, 2.9±0.3, $p<0.0001$). Within each marsh, all zones were similar in %OM. Percent TC was also greater in the Mature marsh (3% vs 1%, $p<0.0001$), with the greatest difference between sites at the Edge, where the Mature Marsh had 10-fold greater carbon than the Young Marsh, reflecting the very new nature of the prograding edge. All remaining Zones within the Young Marsh, and all Zones within the Mature Marsh, were similar to one another. The Young Die-Off was slightly higher (1.33±0.6%), and similar to the old Marsh (Table 2.1 &

2.2). Percent TN followed a similar pattern, with the lowest value at the Young Edge, moderate values in the young Meadow and Upper zones, and slightly higher values in the Young Die-Off that were similar to the Mature zones. Percent TP was only different between marshes ($p < 0.0001$), with two-fold greater values in the Mature Marsh. Carbon Density was similar in all zones but between the two marshes, CD was significantly higher in the Mature Marsh (1253±97 mg m^{-2} vs 816±105 mg m^{-2}, $p<0.01$). C:N generally increased with distance from the Edge in the Young Marsh, and decreased in the Mature Marsh. The Die-Off zones were similar to all other Zones. N:P was similar within each marsh, but substantially greater in the Mature Marsh (7.7±0.4, 4.4±0.5, $p<0.001$), suggesting a buildup of N over time.

Soil Microbial Analyses

Bacteria Community Composition

The overall bacterial communities across the two marshes and their vegetation zones are comprised of similar Phyla, but there are distinct differences in the relative abundance between marshes, and within each marsh among the zones (Figure 2.3 & Figure 2.4). Overall, the top two phyla in both marshes are *Proteobacteria* and *Desulfobacterota*, but with higher relative abundance in the Young Marsh (32% and 20%, respectively) than the Mature Marsh (25% and 19%, respectively). *Chloroflexi* is substantially more abundant in the Mature Marsh (14%) versus the Young Marsh (6%) and *Bacteroidota* is higher (15%) in the Young Marsh than the Mature Marsh (9%). (Figure 2.3 & 2.4).

Differences between the two different-aged marshes become more apparent at the class level. The Mature marsh had higher RA of *Anaerolineae* (12% vs 5%, $p<0.0001$), *Desulfobacteria* (11% vs 8%, $p=0.012$), *Camplyobacteria* (4% vs 2%, $p = 0.041$) and *Spirochaetia* (2% vs 1.2%, $p=0.0115$), while *Gammaprotebacteria* (23% vs 18%. $p = 0.0385$), *Bacteroidia* (14% vs 9%, $p =0.0002$), *Desulfobulbia* (10% vs 5%, $p <0.0001$), and *Cyanobacteriia* (1.3% vs 0.4%) (Table 2.3, Figure 2.5) were more abundant in the Young Marsh.

Within the top 20 taxonomic classes in the Young Marsh, there were significant differences among the zones for 9 classes with RA > 1% (Table 4). Of note, the Upper zone had higher RA of *Bacteroidia*, *Planctomycetes*, *Acidimicrobiia*, *Actinobacteria* and *Cyanobacteriia* than the other zones ($p<0.05$). The Die-Off zone had the highest RA of *Desulfobacteria* (11%). The Edge zone had the highest RA of *Alphaproteobacteria* (12%); *Anaerolineae* and

Campylobacteria were greater in the Meadow (6.5±0.4 and 4.2±2, respectively) and the Die-Off (6.3±1.2 and 4.5±2, respectively) relative to other zones. *Cyanobacteriia* were two-fold greater in the Edge and Upper zones (2-3%) than the Meadow and Die-Off (<1%; Table 3). In the Mature Marsh, most of the dominant classes from the Young Marsh had similarly high RA, but were not different across zones. *Anaerolineae* trended toward significance (p=0.051) with highest RA in the Upper zone (18.4±1.9%). *Planctomycetes* was also slightly elevated in the Edge zone (p =0.054; 1.9±0.3%). *Acidimicrobiia* and *Spirochaetia* had the greatest RA in the Edge and Upper zones, respectively, relative to other zones (p<0.05; Table 2.3).

Microbial Diversity

For all indices computed, the Mature Marsh had higher alpha diversity than the Young Marsh (Figure 2.6; Table 2.4; $p < 0.001$ for all). Zones within the Mature Marsh were relatively homogeneous, with no significant differences although the Upper zone was slightly elevated (Table 2.4). The Young Marsh was more heterogeneous among zones, with more total observed features, and higher Shannon and Chao1 diversity at the Edge (p<0.05) relative to other zones (Figure 2.6; Table 2.4). In the post-hoc analyses, however, the Edge was statistically distinct only for Chao1 and Shannon indices (Figure 2.6). Faith's Phylogenetic Diversity was similar across zones.

Two Principal Coordinates were produced that together describe more than 40% of the variation within the data (Figure 2.7). The most distinctly different communities were found in the Upper portion of each marsh at the high marsh boundary. The Edge plots of the Mature Marsh share similarities with plots in the Young Marsh Meadow, perhaps suggesting a similarity in the ontogeny of the marsh. The Die-Off zone of the Mature Marsh appears more similar to the Upper zone, while the Young Die-Off is more similar to the Young Meadow zone. The Bray-Curtis dissimilarity cluster analysis illustrates that the Young Marsh is more heterogenous, with the Meadow and Die-Off being more similar to one another than the Young Edge and Upper zones that are very distinct from one another (Figure 2.8; Table 2.5). The Mature Marsh communities show a similar grouping of Meadow and Die-Off, with some additional similarity between Die-Off and Upper plots. In one subset of Young Marsh plots, both Die-Off and

Meadow are more similar to the Mature Marsh, and may represent a distinct Zone within the marsh where buried antecedent marsh is closer to the surface.

The two primary axes of the RDA explained 81.4% and 13.2% of the variability, and begin to illustrate the key sediment physicochemical drivers of the microbial community (Figure 2.9). On RDA1, high nutrient and carbon availability is linked with *Desulfobacteria*, *Campylobacteria*, *Anaerolineae*, *Delchaloccoidia*, *Thermoplasmata* and *Spirochaetia.* These bacterial classes are predominantly found in the Mature marsh. High BD and C:N group with the Young Marsh community, especially *Desulfolbia* and *Gammaproteobacteria*. Aboveground biomass loaded strongly positive on RDA2 (Figure 2.9).

The dominant identifiable metabolic function across the two marshes was chemoheterotrophy (Table 2.6). aerobic chemoheterotrophic was 1.5-fold greater in the Young Marsh (5.8%) than Mature Marsh (3.6%). While anaerobic chemoheterotrophy was greater in the Mature Marsh (1.9% versus 1.4%; $p<0.01$ for both; Table 2.6), and was likely comprised primarily of fermenting species (>90%; see Appendix 1(4-2)). Aromatic compound degradation was significantly more important in the Young Marsh vs Mature Marsh (0.23% vs 0.11%, $p<0.001$). The remaining key metabolic functions were primarily related to nitrogen and sulfur cycling, and were generally similarly represented across the two marshes (Table 2.6). Although not statistically different, sulfate respiration was slightly greater in the Mature Marsh, and cellulolysis was greater in the Young Marsh.

In the Young Marsh, several Metabolic function were significantly different across zones, including aerobic chemoheterotrophy, denitrification, cellulolysis and nitrogen fixation ($p<0.02$) (Table 2.7). Aerobic chemoheterotrophy in Edge sediments was two-fold greater than the Die-Off and about 1.5-fold greater than the Meadow. The Edge also had significantly higher denitrification than the other zones, with six-fold higher abundance relative to the Upper and Die-Off Zones (0.63% vs 0.10% and 0.11%) and more than 2-fold higher abundance relative to the Meadow (0.24%). Heterotrophic nitrogen fixation was absent in the Meadow, and very low in the Die-Off, and photosynthetic cyanobacteria followed a similar pattern ($p<0.01$ for both; Table 2.6 and Table 2.7), providing further evidence of the influence of a buried antecedent marsh supplying nutrients. Both the Edge and the Upper zones were similar to each other, and overall zonation mirrored %TN values (Table 2.1). In the Mature Marsh, all functions were similar across zones (Table 2.7).

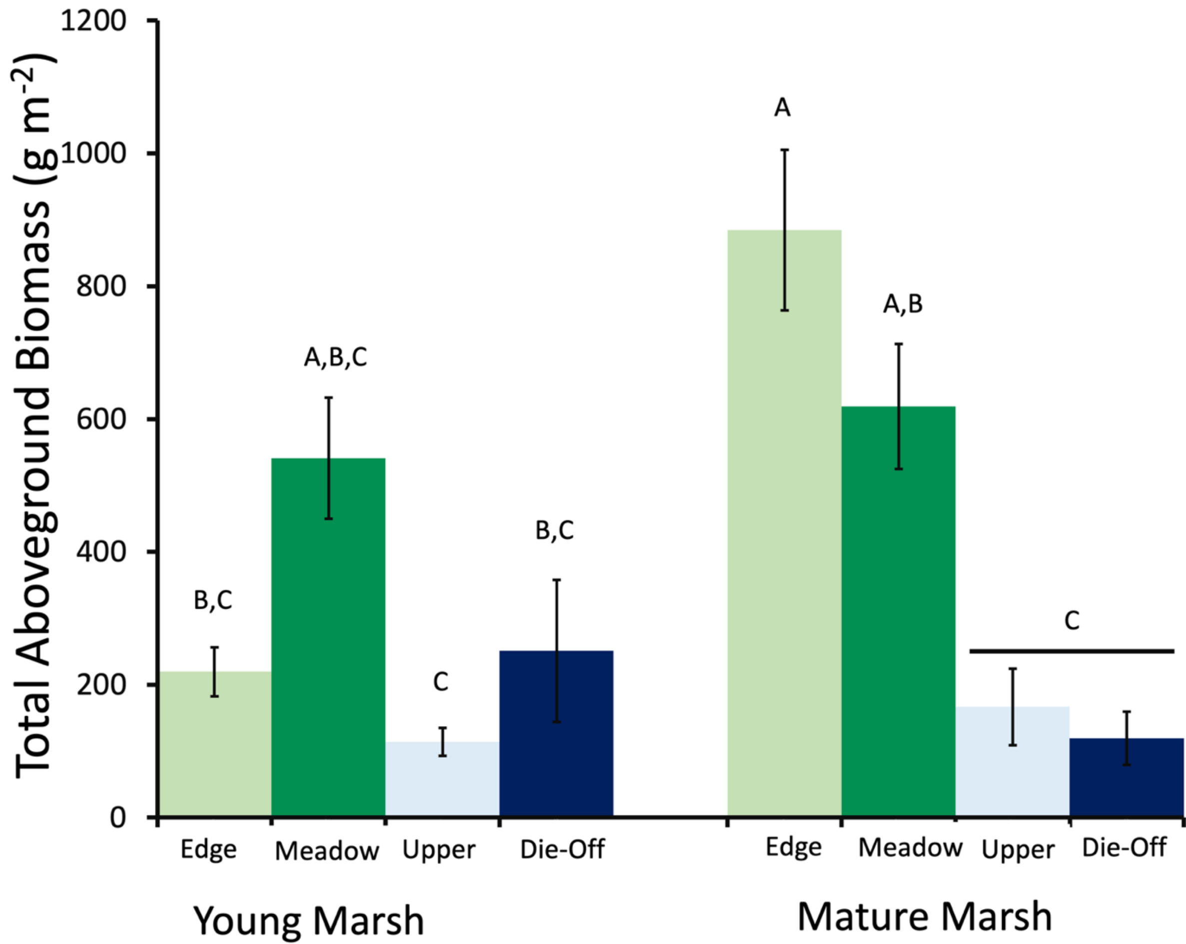

Figure 3.2- Total aboveground biomass for each zone in each marsh.(Mean ± Standard error (SE)). Unique letters above bars indicate statistical differences based on two-way ANOVA using Marsh, Zone and their interaction as fixed factors.

*Table 3.1- Plant and environmental characteristics across the four vegetation zones in each Marsh. (Mean ± S.E.). Unique superscripted letters next to values indicate significant differences based on a Two-Way ANOVA with site and zone as fixed factors. n =4-8 per zone. *indicates transformed data using reciprocal transformation and ** indicates transformed data using square root transformation, *** indicates log transformation and non-parametric post-hoc of Dunn's Joint Rank -All Pairs*

ENV Characteristic	Marsh	Edge	Meadow	Upper	Die-Off
Total Aboveground Biomass (g-m^{-2})	Young	220±37^{bc}	541±91^{abc}	113±21^{c}	251±107^{bc}
	Mature	884±120^{a}	618±94^{ab}	167±58^{c}	119±40^{c}
***S. alterniflora* (g m^{-2})****	Young	219±37^{bcd}	541±91^{abc}	44±16^{d}	251±107^{bcd}
	Mature	884±120^{a}	618±94^{ab}	167±58^{d}	119±40^{cd}
S. virginica* (g m^{-2})**	Young	0.46±0.46	0	69±29	0
	Mature	0	0	0	0
Elevation (m)	Young	-0.09±0.02^{b}	-	0.38±0.02^{a}	-0.17±0.1^{b}
	Mature	-0.09±0.05^{b}	-	-0.18±0.06^{b}	-
Salinity (ppt)	Young	39.50±2.53^{b}	38.6±1.36^{b}	50±0.71^{b}	40.75±2.14^{a}
	Mature	36.29±0.89^{b}	37±0.89^{b}	38.5±1.04^{b}	37.75±1.03^{b}
Bulk Density (g cm^{-3})	Young	1.32±0.01^{a}	1.07±0.12^{a}	1.16±0.03^{a}	0.93±0.17^{a}
	Mature	0.58±0.04^{b}	0.46±0.06^{b}	0.33±0.004^{b}	0.38±0.04^{b}
%Organic Matter*	Young	1.25±0.25^{b}	3.2±0.7^{b}	2±0^{b}	5±2^{b}
	Mature	8.1±0.5^{a}	8.8±0.5^{a}	8.5±0.3^{a}	8.75±0.3^{a}
% TC*	Young	0.28±0.03^{a}	0.64±0.06^{bc}	1.27±0.51^{b}	1.33±0.55^{bcd}
	Mature	2.86±0.42^{d}	2.57±0.04^{cd}	2.68±0.09^{cd}	2.66±0.08^{cd}
% TN*	Young	0.03±0.003^{a}	0.09±0.03^{b}	0.06±0.01^{b}	0.13±0.06^{bc}
	Mature	0.21±0.03^{c}	0.27±0.02^{c}	0.28±0.003^{c}	0.26±0.03^{c}
% TP**	Young	0.03±0.00^{b}	0.04±0.00^{b}	0.03±0.00^{b}	0.05±0.01^{b}
	Mature	0.07±0.00^{a}	0.08±0.01^{a}	0.07±0.00^{a}	0.07±0.01^{a}
Carbon Density (mg-m^{-2})	Young	369±36^{c}	1117±224^{ab}	739±61^{bc}	961±126^{abc}
	Mature	1633±197^{a}	1233±141^{ab}	857±21^{bc}	1006±76^{abc}
C:N*	Young	10.1±0.5^{a}	14.4±1.2^{bc}	13.3±0.3^{bc}	12.2±0.5^{abc}
	Mature	16.7±1.4^{c}	11.82±0.8^{ab}	10.8±0.2^{ab}	12.3±1.2^{abc}
N:P	Young	2.4±0.4^{c}	5.2±1.0^{abc}	4.3±0.6^{bc}	5.65±1.0^{abc}
	Mature	7.0±1.09^{ab}	8.0±0.4^{ab}	8.9±0.2^{a}	8.0±0.4^{ab}

Table 3.2- Environmental Factor- Statistical Analyses. Two-way ANOVAs examining the effects of Zone, Marsh and their interaction on plant biomass and environmental factors, significant effects are bolded (p<0.5).

	Zone		*Marsh*		*Zone x Marsh*	
	$F_{3,36}$	*p*	$F_{1,36}$	*p*	$F_{3,36}$	*p*
Aboveground Biomass (g-m^{-2})	12.4	**<0.0001**	6.2	**0.0183**	7.2	**0.0009**
***S. alterniflora* (g m^{-2})****	17.5	**<0.0001**	7.3	**0.0115**	5.1	**0.0059**
***S. virginica* (g m^{-2})**	7.2	**0.0009**	7.9	**0.0089**	7.2	**0.0009**
Elevation (m)	6.2	**0.0022**	7.3	**0.0114**	17.4	**<0.0001**
Salinity (ppt)	9.1	**0.0002**	24.1	**<0.0001**	4.9	**0.0069**
Bulk Density (g cm^{-3})	4.8	**0.0075**	142.6	**<0.0001**	1.1	0.36
%Organic Matter*	0.5	0.7	24.4	**<0.0001**	0.5	0.67
% TC*	23.4	**<0.0001**	133.3	**<0.0001**	23.4	**<0.0001**
% TN*	18.98	**<0.0001**	146.50	**<0.0001**	13.78	**<0.0001**
% TP**	2.2	0.115	98.3	**<0.0001**	0.92	0.45
Carbon Density (mg-m^{-2})	2.9	0.163	78.0	**0.0021**	8.7	**0.0009**
C:N*	0.43	0.73	0.10	0.75	14.0	**<0.0001**
N:P	2.9	**0.05**	35.3	**<0.0001**	0.93	0.44

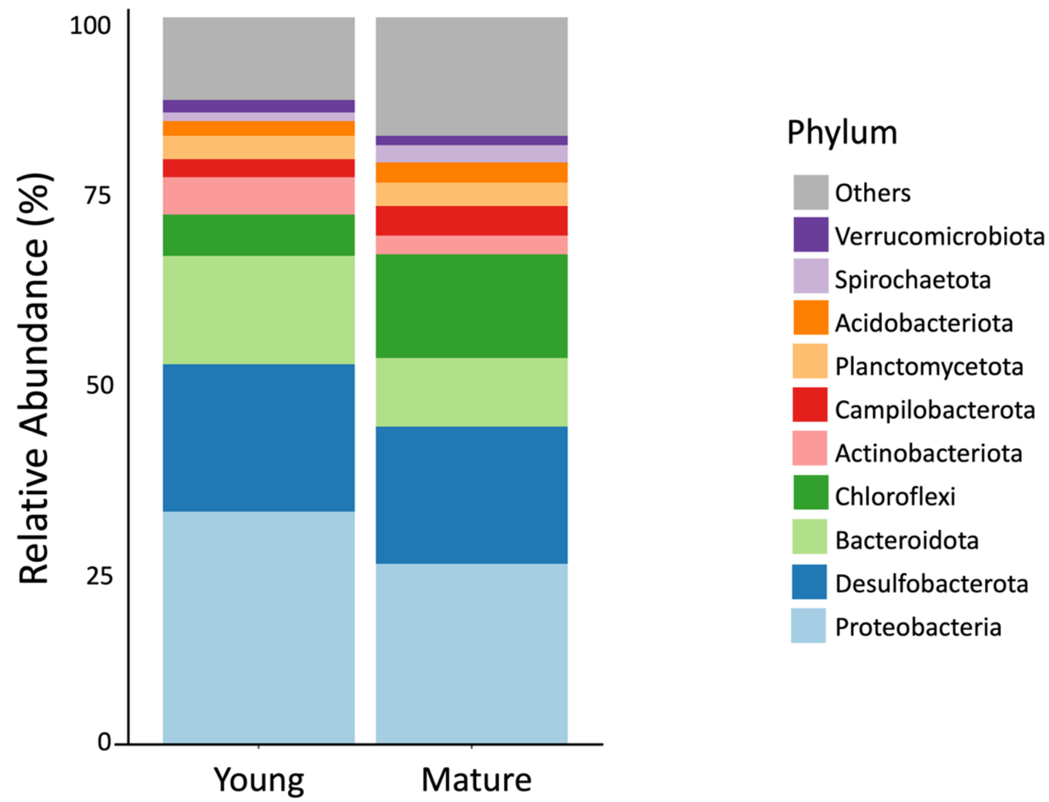

Figure 3.3-Relative Abundances of the top 10 Phyla in each Marsh.

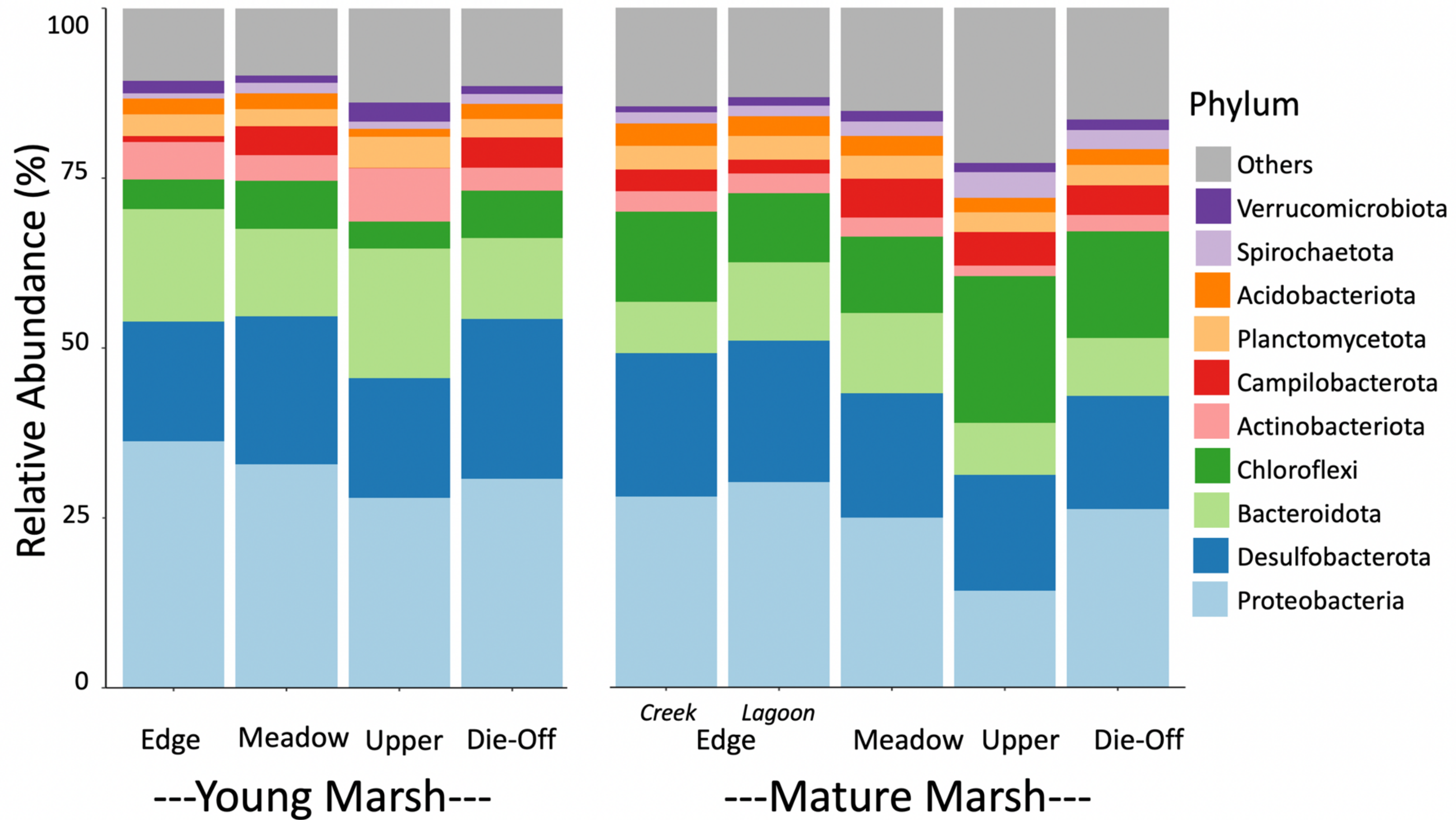

Figure 3.4:Relative Abundance of the top 10 phyla in each zone for the Young and Mature Marshes.

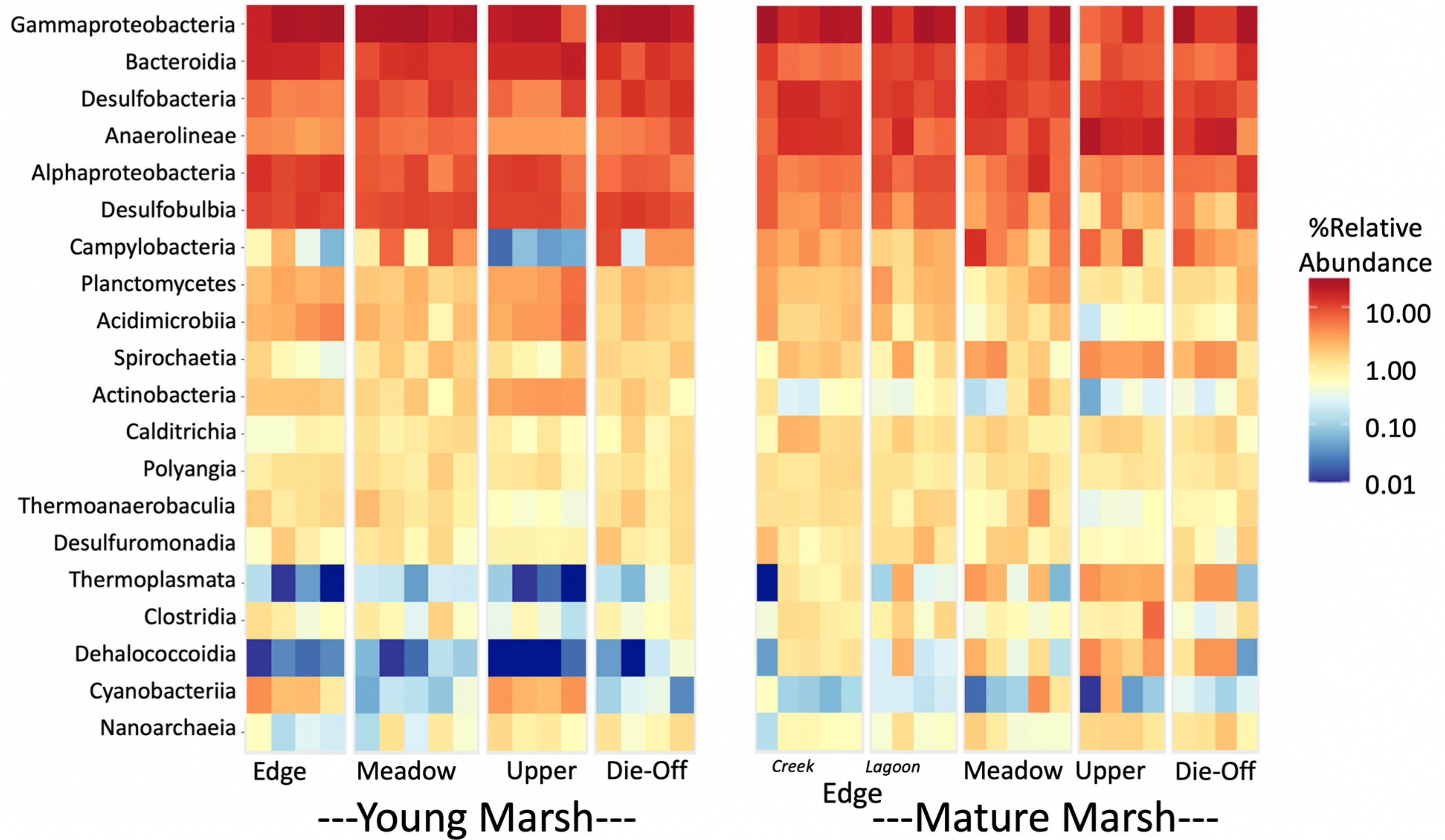

Figure 3.5-Heat Map of Relative Abundances at the taxonomic level of Class in each Marsh divided by Zone. Edge in Mature has been divided into Lagoon and Creek but further analyses group them as one zone.

*Table 3.3: Relative abundance (RA) of the top 10 bacterial classes across all zones in each marsh. Chi square results of a Kruskall-Wallis test comparing the two marsh sites (Between site comparison). Chi-square and p-values for each individual marsh evaluating differences among zones (Within Site) are also shown. Values for each zone are shown in Figure 4 & 5. * indicates significantly higher RA within indicated Marsh relative to the other. Bolded p-value indicates significance difference between groups.*

Class	**Site**	**Whole Site RA**	**Between Site**		**Within Site**	
			X^2	***p***	X^2	***p***
Gammaproteobacteria	**Young***	23.9±2	4.3	**0.039**	3.7	0.29
	Mature	16.3±1			7.8	0.051
Bacteroidia	**Young***	13.6±0.8	13.4	**0.0002**	9.5	**0.023**
	Mature	9.0±0.7			2.9	0.41
Desulfobacteria	**Young**	8.5±0.7	6.3	**0.012**	9.1	**0.028**
	Mature*	11.6±1			1.9	0.6
Anaerolineae	**Young**	5.1±1	18.1	**<0.0001**	12.5	**0.006**
	Mature*	12.3±0.8			7.8	**0.051**
Alphaproteobacteria	**Young***	9.0±0.7	4.9	**0.027**	8.6	**0.035**
	Mature	6.9±0.6			6.6	0.087
Desulfobulbia	**Young***	10.4±0.5	25.3	**<0.0001**	1.7	0.645
	Mature	4.7±0.4			4.7	0.197
Campylobacteria	**Young**	2.6±0.8	4.2	**0.041**	10.3	**0.016**
	Mature*	4.1±0.7			2.9	0.414
Planctomycetes	**Young**	2.5±1.1	3.7	0.055	10.8	**0.013**
	Mature	1.9±0.9			7.7	0.054
Acidimicrobiia	**Young***	2.7±1.4	10.5	**0.012**	11.8	**0.008**
	Mature	1.3±0.8			10.4	**0.015**
Spirochaetia	**Young**	1.2±0.1	6.4	**0.012**	4.3	0.23
	Mature*	2.3±0.3			8.9	**0.031**

Table 3.4:Diversity Indices for each Marsh. Chi square results of a Kruskall-Wallis test comparing the two marsh sites (Between site comparison). Chi-square and p-values for each individual marsh evaluating differences among zones (Within Site) are also shown. Bolded p-values are significant p<0.05

Diversity Index	**Site**	**Mean±SE**	**Between Site**		**Within Site**	
			X²	***p***	***X²***	***p***
Total Observed Features	**Young**	1060±42	9.4	**0.0022**	8.6	**0.035**
	Mature	1300±47			4.2	0.237
Shannon Diversity (H')	**Young**	6.0±0.05	18.8	**<0.0001**	9.9	**0.020**
	Mature	6.4±0.04			2.1	0.557
Chao1	**Young**	1179±54	8.2	**0.0044**	8.9	**0.031**
	Mature	1452±60			4.2	0.240
Faith's Phylogenetic Diversity	**Young**	73.5±1.4	17.6	**<0.0001**	6.7	0.083
	Mature	89.7±2.7			5.4	0.145

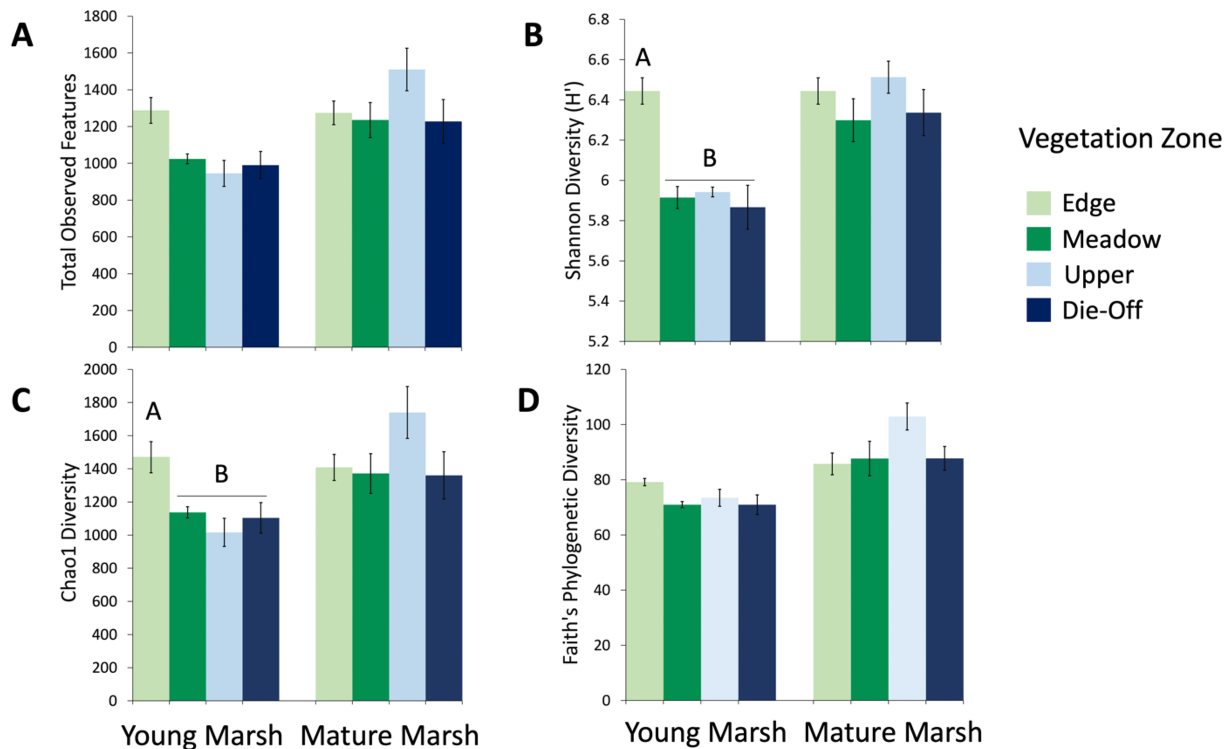

Figure 3.6- Microbial diversity in each of the Marshes separated by vegetation zone. A) Total observed features, B) Shannon Diversity (H'), C) Chao1, D) Faith's PD

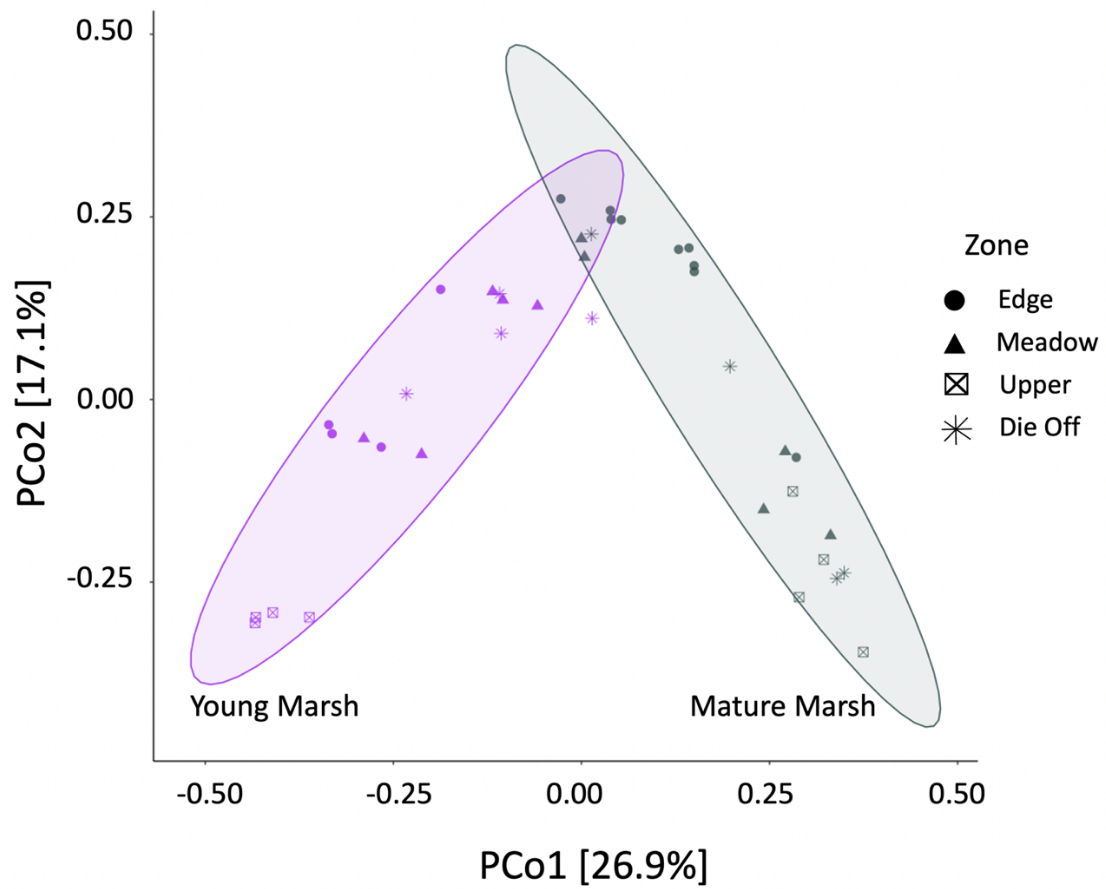

Figure 3.7-Principal coordinates analysis (PCoA) of bacterial community structure. Shapes and colors represent the two marshes and their respective vegetation zones.

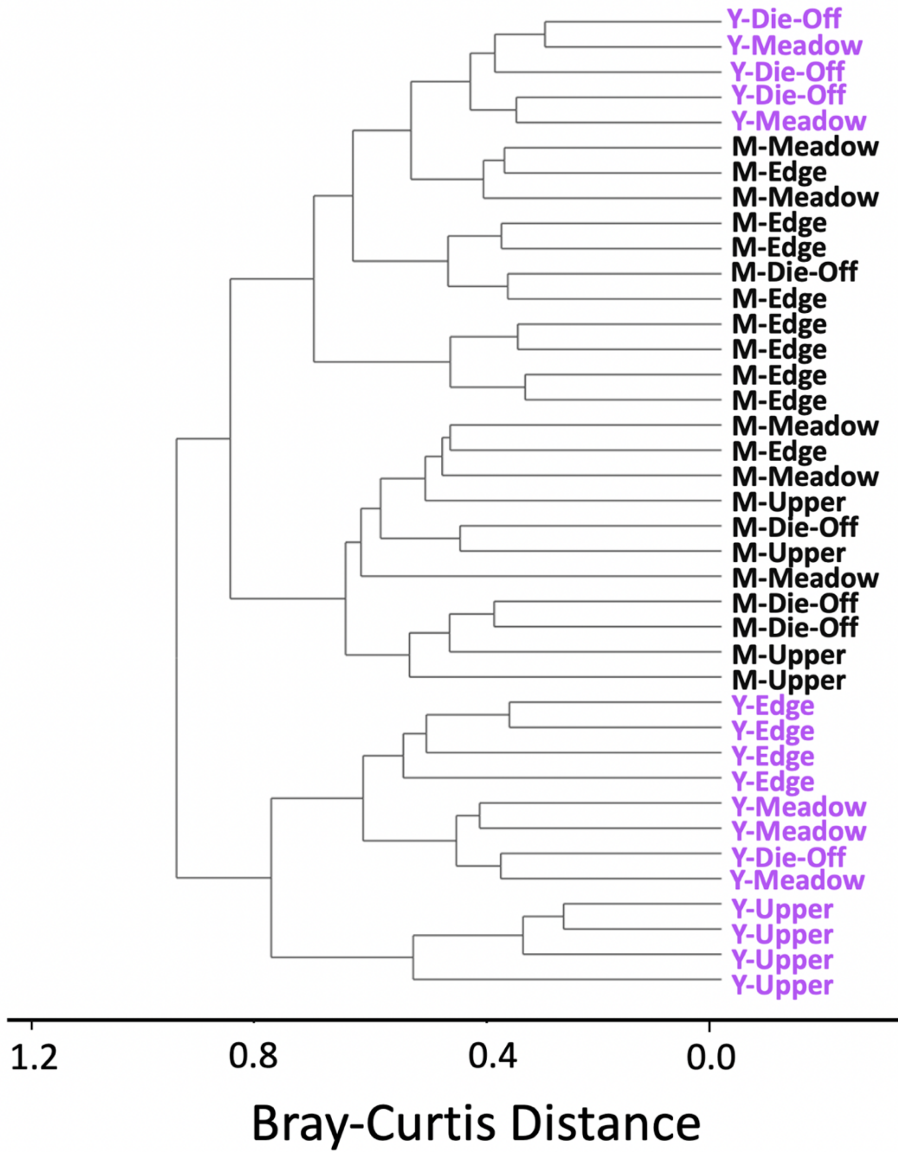

Figure 3.8: Bray Curtis Cluster Plot depicting both Marshes and their vegetation Zones.

Table 3.5:Bray-Curtis Distance with PERMANOVA results for each pairwise comparison of marshes and zones. Bold p-values indicate a significant difference at p < 0.05.

Marsh-Zone	**F**	**R^2**	***p***
Y-Edge vs M-Edge	5.92	0.35	**0.003**
Y-Edge vs Y-Meadow	3.08	0.31	**0.018**
Y-Edge vs Y-Meadow	4.31	0.38	**0.008**
Y-Edge vs Y-Upper	5.13	0.46	**0.033**
Y-Edge vs M-Upper	7.15	0.54	**0.024**
Y-Edge vs Y-Die-Off	3.51	0.37	**0.025**
Y-Edge vs M-Die-Off	4.58	0.43	**0.031**
M-Edge vs Y-Meadow	4.78	0.29	**0.001**
M-Edge vs M-Meadow	2.38	0.17	**0.015**
M-Edge vs Y-Upper	11.54	0.51	**0.001**
M-Edge vs M-Upper	5.55	0.34	**0.001**
M-Edge vs Y-Die-Off	3.92	0.26	**0.003**
M-Edge vs M-Die-Off	2.56	0.19	**0.027**
Y-Meadow vs M-Meadow	2.78	0.26	**0.011**
Y-Meadow vs Y-Upper	6.42	0.48	**0.006**
Y-Meadow vs M-Upper	6.12	0.47	**0.014**
Y-Meadow vs Y-Die-Off	0.60	0.08	0.894
Y-Meadow vs M-Die-Off	3.46	0.33	**0.006**
M-Meadow vs Y-Upper	7.46	0.52	**0.006**
M-Meadow vs M-Upper	1.82	0.21	0.052
M-Meadow vs Y-Die-Off	2.21	0.24	0.067
M-Meadow vs M-Die-Off	0.80	0.10	0.545
Y-Upper vs M-Upper	9.63	0.62	**0.028**
Y-Upper vs Y-Die-Off	7.19	0.55	**0.036**
Y-Upper vs M-Die-Off	7.59	0.56	**0.04**
M-Upper vs Y-Die-Off	5.37	0.47	**0.027**
M-Upper vs M-Die-Off	1.19	0.17	0.292
Y-Die-Off vs M-Die-Off	2.86	0.32	**0.037**

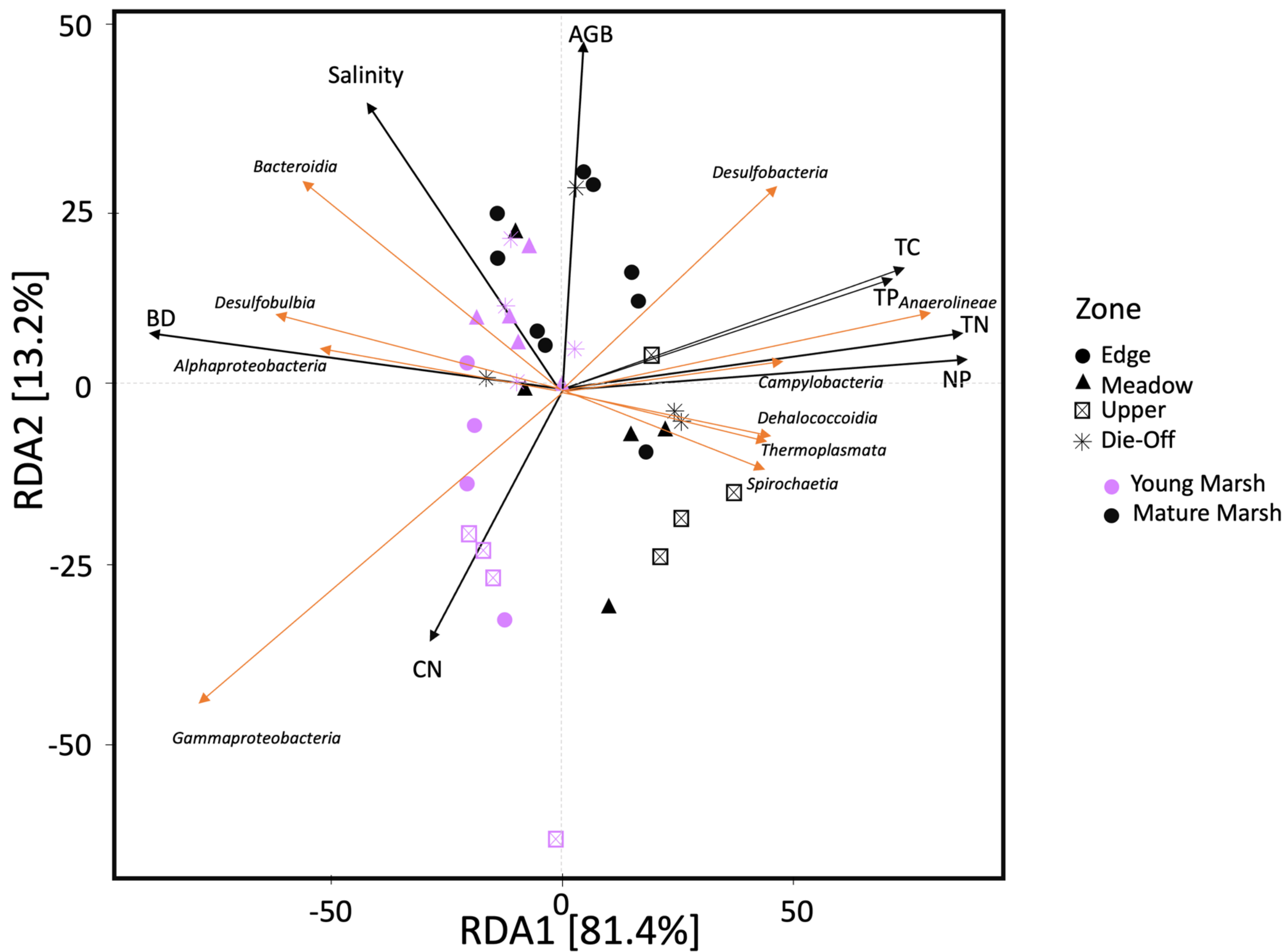

Figure 3.9-RDA depicting Environmental Factors driving sediment microbial communities.

Table 3.6-Metabolic function in each Marsh and within each Marsh showing the Chi square results of the Kruskall-Wallis test comparing between the two marsh sites (Between Site comparison) and within each individual marsh across zones (Within Site). Bolded values indicate the zone within each marsh with highest relative importance for each function where a significant difference among zones was observed

Metabolic function	**Site**	**Percent**	**Between Site**		**Within Site**	
			X^2	***p***	***X^2***	***p***
Aerobic Chemoheterotrophy	**Young**	5.8±0.5[a]	10.9	**0.001**	12.6	**0.006**
	Mature	3.6±0.4[b]			4.9	0.18
Anaerobic Chemoheterotrophy	**Young**	1.4±0.1[b]	9.3	**0.002**	2.1	0.55
	Mature	1.9±0.1[a]			5.8	0.12
Fermentation	**Young**	1.5±0.1[b]	9.4	**0.002**	5.7	0.13
	Mature	1.9±0.1[a]			7.02	0.071
Denitrification (nitrate reduction)	**Young**	0.27±0.1	1.7	0.19	10.6	**0.0142**
	Mature	0.15±0.03			6.9	0.075
Aromatic compound	**Young**	0.23±0.03[a]	12.3	**0.0005**	6.6	0.087
	Mature	0.11±0.02[b]			3.8	0.29
Respiration of sulfur compounds	**Young**	0.31±0.04	1.59	0.21	3.9	0.27
	Mature	0.41±0.05			1.98	0.58
Dark oxidation of sulfur compounds	**Young**	0.11±0.06	0.0008	0.98	1.4	0.70
	Mature	0.10±0.01			4.6	0.203
Nitrogen fixation	**Young**	0.14±0.04	0.84	0.36	12.96	**0.0047**
	Mature	0.07±0.01			0.61	0.89
Cellulolysis	**Young**	0.48±0.07	2.2	0.14	11.3	**0.01**
	Mature	0.32±0.04			5.3	0.15
Dark oxidation of Sulfur compounds	**Young**	0.11±0.02	0.0008	0.977	1.4	0.70
	Mature	0.10±0.01			4.6	0.20
Non-Photosynthetic Cyanobacteria	**Young**	0.16±0.02	0.01	0.93	1.0	0.80
	Mature	0.17±0.02			5.4	0.14
Photosynthetic Cyanobacteria	**Young**	1.4±0.3[a]	6.88	**0.009**	12.6	**0.006**
	Mature	0.5±0.1[b]			1.9	0.6

Table 3.7-Metabolic function by percent in each of the zones and in each Marsh. Superscripted letters indicate similar grouping of zones based on post hoc analysis.

Metabolic function	Zone	Young Marsh	Mature Marsh
Aerobic Chemoheterotrophy	***Edge***	8.4±0.3^{a}	3.9±0.5
	Meadow	4.6±0.7^{b}	4.1±0.8
	Upper	6.9±0.4^{ab}	2.2±0.2
	Die-Off	3.8±0.4^{b}	3.7±1.3
Anerobic Chemoheterotrophy	***Edge***	1.4±0.1	1.9±0.1
	Meadow	1.6±0.2	1.7±0.3
	Upper	1.5±0.1	2.2±0.1
	Die-Off	1.3±0.1	1.6±0.1
Fermentation	***Edge***	1.7±0.05	1.95±0.13
	Meadow	1.61±0.2	1.8±0.2
	Upper	1.3±0.06	2.2±0.1
	Die-Off	1.3±0.12	1.5±0.04
Nitrate reduction	***Edge***	0.63±0.1^{a}	0.20±0.03
	Meadow	0.24±0.05^{ab}	0.19±0.05
	Upper	0.10±0.04^{b}	0.04±0.02
	Die-Off	0.11±0.05^{b}	0.12±0.1
Aromatic compound degradation	***Edge***	0.27±0.02	0.12±0.02
	Meadow	0.17±0.05	0.15±0.05
	Upper	0.31±0.08	0.07±0.00
	Die-Off	0.18±0.02	0.12±0.03
Respiration of sulfur compounds	***Edge***	0.35±0.08	0.38±0.07
	Meadow	0.27±0.12	0.38±0.09
	Upper	0.37±0.02	0.59±0.14
	Die-Off	0.28±0.05	0.34±0.2
Dark oxidation of sulfur compounds	***Edge***	0.16±0.09	0.11±0.02
	Meadow	0.11±0.02	0.13±0.02
	Upper	0.07±0.05	0.07±0.03
	Die-Off	0.08±0.03	0.08±0.03
Nitrogen fixation	***Edge***	0.25±0.07^{ab}	0.07±0.02
	Meadow	0^{b}	0.07±0.04
	Upper	0.31±0.04^{a}	0.09±0.04
	Die-Off	0.03±0.03^{ab}	0.05±0.03
Cellulolysis	***Edge***	0.63±0.1^{a}	0.23±0.06
	Meadow	0.26±0.07^{ab}	0.34±0.12
	Upper	0.84±0.09^{ab}	0.37±0.07
	Die-Off	0.23±0.03^{b}	0.44±0.02
Dark oxidation of Sulfur compounds	***Edge***	0.16±0.08	0.11±0.02
	Meadow	0.11±0.02	0.13±0.02
	Upper	0.07±0.05	0.07±0.03
	Die-Off	0.08±0.03	0.08±0.03
Non-Photosynthetic Cyanobacteria	***Edge***	0.18±0.03	0.21±0.03
	Meadow	0.17±0.05	0.14±0.05
	Upper	0.15±0.06	0.18±0.05
	Die-Off	0.13±0.03	0.10±0.01
Photosynthetic Cyanobacteria	***Edge***	2.2±0.3	0.43±0.05
	Meadow	0.4±0.1	0.70±0.31
	Upper	2.7±0.1	0.34±0.17
	Die-Off	0.6±0.2	0.44±0.07

Discussion

The two different-aged marshes on Hog Island are distinct in physical, chemical, and biological characteristics. The physicochemical environment of the Mature Marsh, as previously established (Osgood and Zieman 1993, Tyler and Zieman 1999, Tyler et al. 2003), suggests a mostly homogeneous system with high carbon and nutrients, low bulk density, and moderate salinity that supports a gradient in *S. alterniflora* biomass but is at the same time degrading at the edges and in the interior. The Young Marsh supports similar biomass in the interior, but the lower nutrients, expanding edges of *S. alterniflora*, and heterogeneity suggest a more complex mosaic of the Young Marsh overlying buried marsh that may be exacerbating stress in a patchwork fashion. These factors, in turn, create positive and negative feedbacks with the vegetation, and drive the overall composition of the microbial community in terms of specific functional groups of organisms along with biodiversity.

Overall, microbial diversity increased with marsh age (Table 2.4 and Figure 2.6), suggesting a greater array of microbial niches in the Mature marsh. The measured variation in C, N and P stock and bulk density, along with previously assessed variation in sulfide and redox potential (Tyler and Zieman 1999, Goldsmith et al. 2020) in sediments across ages, suggest strong differences in the relative importance of different biogeochemical pathways. In the Young Marsh, *Gammaproteobacteria*, *Bacteroidia*, *Alphaproteobacteria* and *Acidimicrobiia* are among the most dominant groups. *Gammaprotebacteria* is a highly diverse class of bacteria containing both obligate anaerobes and obligate aerobes, and is one of the most abundant bacterial classes found in wetlands (Williams et al. 2010). This class made up nearly one quarter of the bacteria in the Young Marsh and was relatively evenly distributed across zones (Figure 2.5 and Table 2.3). Functionally, *Gammaproteobacteria* are important to various Metabolic function which mostly require oxygen, thus the greater relative importance in the Young Marsh where the less anoxic sediments allow proliferation of oxygen dependent species (Table 2.6; Williams et al. 2010). The additional dominant species may also require the less anoxic environment and higher bulk density (Figure 2.9). *Anaerolineae* was found with greater RA in the Mature Marsh. This class of bacteria is known for occurring in areas of high TP (Xia et al. 2016, Mai et al. 2021). The other class of bacteria found in higher RA in the Mature Marsh is *Spirochaetia*, which is known to

degrade cellulose and chitin (Piazza et al. 2019), but this is contrary to the higher abundance of cellulose metabolism found at this site (Table 2.6).

Sulfur plays a very important role in biogeochemistry and metabolism of salt marshes, with sulfate reduction as the dominant metabolic pathway in some systems (Howarth 1984). While sulfate reducers comprised at least 15-20% of the total microbial community, differences in the relative abundance of two groups of sulfate reducers between wetlands suggest different controls on this metabolic pathway(Kuever 2014). *Desulfobacteria* were higher in the Mature Marsh, while the Young Marsh had higher RA of *Desulfobulbia* indicates different biogeochemical processes surrounding sulfate reducers. The metabolic pathways within the top processes included respiration of Sulfur compounds which were slightly greater in the Mature Marsh. (Table 2.6; Sulfate respiration, as a subset of sulfur respiration showed a greater between site difference, see Appendix 1 (4.2). *Desulfobacteria* is typically found in anoxic conditions and is associated with phosphite oxidation (Ewens et al. 2021). The greater P availability of the Mature Marsh, along with the lower redox potential may promote abundance of this group. In contrast, *Desulfobulbia*, a group known as 'cable bacteria', tend to proliferate more where oxygen and sulfide are both present (Pfeffer et al. 2012, Larsen et al. 2015). The high abundance of this chain forming species in the Young Marsh, especially in the Meadow and Die-Off zones, suggests that there is a significant source of sulfide in these zones. At these sites, within ten centimeters of the surface, the sediment switches from sandy to very fine-grained (Tyler, personal observation), suggesting that new marsh platform overlays the fine sediment and rich organic layer of the buried marsh, as observed elsewhere in the Hog Island Chronosequence (Osgood and Zieman 1993). Although we sampled only at the surface, these chain-forming bacteria may be able to access sulfide from deeper in the sediment profile, and thereby proliferate. We suggest that the plots grouping with the Mature Marsh in the Bray Curtis analysis (Figure 2.8) are likely plots where an older mature marsh is very close to the surface.

Nitrogen was higher in the Mature marsh than the Young Marsh, especially in comparison to the Young Edge and Upper zones. The more moderate concentration of nitrogen in the Young Meadow and Die-Off zones may be a function of both increased age (relative to the Edge) and the presence of the buried old marsh beneath the surface. Bacterial groups associated with nitrogen cycling, especially *Cyanobacteriia,* common autotrophic nitrogen fixers in marine systems, had higher relative abundances in the Young Marsh versus the Mature Marsh, and were

especially high in the Edge and Upper zones. The abundance of these bacterial classes matches with the analysis of function, where there were distinct differences in the functional abundance nitrogen fixation in these low N zones (Table 2.5). This result corroborates the findings of Tyler et al. (2003), who determined that rates of N fixation were higher in the younger marshes, where N limited *S. alterniflora* production (Tyler et al. 2003), and may imply that as N accumulates in the sediment, that N fixation becomes less advantageous and is eclipsed by other processes.

Conclusion

While resilience to stress is often thought to be facilitated by high species and functional diversity, salt marsh to climate change may also be predicated on the interaction between the biotic and abiotic components. While the Mature Marsh has higher diversity, the gradual rise in sea level, the warming of the ocean and air, and the variability in climate may exacerbate conditions in the Mature Marsh especially expanding the areas of Die-Off. An addition of sediment to the Mature Marsh may supply sediment on top of nutrient rich marsh platform that could potentially allow for increased platform elevation and better adaptation to these changes. The Young Marsh is more heterogenous and portions of it are on top of an old buried marsh that appears to be supplying nutrients and sulfur to plants and surface microbes. This portion may be more resilient to SLR and the warming ocean due to sediment grain size allowing greater advection of oxygen rich waters to the subsurface, and upward movement of nutrients. The Young Marsh had lower microbial diversity in the surface sediments that were sampled, but if deeper depth profiles were sampled, we may see higher diversity of soil microbes that drive key biogeochemical cycling. As such, the plots overlaying buried antecedent marsh may not be as representative of a naturally developing back-barrier system as those at the Edge or elsewhere in the Meadow.

The results of this study suggest that when restoring salt marshes, we should focus on rapidly pushing succession to mid-age systems. At middle age, the marsh has a developed sediment organic matter and nutrient pool, but lower levels of anoxia that may compound with waterlogging to kill *S. alterniflora* and thereby further limit the metabolic function to anaerobic processes. Younger systems, with greater heterogeneity in soil characteristics and biotic communities may better withstand the push of stressors from climate change and anthropogenic

disasters such as oil spills. This could potentially be done by “seeding” key microbes into restored marshes and using soil amendments to modify soil physiochemical properties.

Chapter 4 Succession and soil management in freshwater depressional wetlands

Introduction

Continued loss of inland freshwater wetlands has led to a decrease in provision of ecosystem services such as biogeochemical cycling, carbon sequestration, flood mitigation and wildlife habitat. Wetland creation and restoration is the required solution to replace these essential ecosystem services. Traditional restoration approaches in freshwater wetlands rely on engineering hydrology and plant communities to reestablish carbon and nutrient cycling and other ecosystem functions associated with wetlands. However, the resulting ecosystems often do not fully replicate the functions and services of natural wetlands (Zedler 2000, Yu et al. 2017, Xu et al. 2019, Scott et al. 2020). Understanding how to manipulate abiotic components to promote biotic factors is therefore critical. Researchers, project managers and government entities should expand wetland construction and management approaches to more fully provide the sought-after ecosystem services (Zedler 2000, Moreno-Mateos et al. 2012, Zedler et al. 2012).

Freshwater wetlands vary based on their hydrology and are classified as one of seven hydrogeomorphic classification (Brinson 1993). There are two dominant freshwater HGM wetlands in Finger Lakes Region of New York State: Depressional and Riverine. Each of these are then classified by their vegetation type. The focus of this research is on Depressional wetlands and they are classified further based on vegetation composition which are emergent wetlands, wet meadows, and wooded wetlands (swamps) (Mitsch and Gosselink 2007). Each of these have ecosystem functions based on the interactions of the hydrology, vegetation, soil physico-chemical characteristics and soil microbial communities (Zak et al. 2003). Each of these wetland types also pose their own challenges and timelines for restoration. The two types of interest in this study are emergent wetlands and wooded wetlands. Each of these receive materials from different types of plants with different decomposition rates and litter chemistry resulting in different rates of carbon contributions to the soil (Stoler and Relyea 2020). Studies have shown how C:N levels, which are an indicator of organic matter lability, can impact key processes in the carbon and nitrogen cycle (Brady and Weil 2008). In created wetlands the right balance of carbon and nitrogen can therefore impact the trajectory of wetland succession, promoting carbon accumulation and the development of a functional nitrogen cycle similar to

natural wetlands (Atkinson and Cairns 2001, McGowan 2020). Each of these wetland types also have different successional trajectories following creation due to differences in maturation rate of the target vegetation, with wooded wetlands potentially taking decades for trees to mature and form closed canopies. These differences may also define differences in soil microbial abundances and diversity between the different wetland types based on biogeochemical processes present in different stages of wetland succession.

Soil microbial community development during succession has primarily been studied in ecosystems such as the forefields of glaciers, sand dunes, old-fields and other similar chronosequence systems, with fewer studies on wetlands (Johnson and Miyanishi 2008, Hahn and Quideau 2013, Bokhorst et al. 2017, Zhong et al. 2018, Orland et al. 2020). Microbial abundance and diversity may peak at the early to mid-stages of succession, in correlation with plant diversity (Turner et al. 2019). Studies in wetland succession and soil microbial diversity are few and usually don't encompass comparing wetlands of different ages or wetland types (Mitsch et al. 2012, Li et al. 2019). While a comparison of soil microbial communities between created and natural wetlands has been done, the focus has been on why created wetlands are not functionally similar to natural wetlands, as opposed to successional changes in communities as created wetlands mature (Ansola et al. 2014). An understanding of how microbial communities develop over time in wetlands, in relationship to the other biotic components of the ecosystem is critical.

Higher diversity, abundance, and overall composition of soil microbial communities within wetlands has been linked to a higher number of ecosystem functions (Ansola et al. 2014, Louca et al. 2018). Metabolic function important to function in freshwater wetlands include both anaerobic and aerobic pathways with the anaerobic pathways dominating function. These include fermentation, nitrate reduction, and methanogenesis (Schlesinger and Bernhardt 2020). These functions are driven by key microbial phyla, including *Proteobacteria, Bacteroidetes, Firmicutes,* and *Actinobacteria* (Mellado and Vera 2021, Abadi et al. 2021). *Bacteroidetes* is crucial to nitrogen fixation while *Firmicutes* is important to denitrification (Inoue et al. 2015, Cheng et al. 2022). The exception is anammox which is carried out by *Nitrososphaeria* and *Planctomycetes* (Mellado and Vera 2021). In the phylum Euryarchaeota, methanogenesis, a key ecosystem function with important climate and carbon cycling contributions, occurs in seven orders, *Methanopyrales*, *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*,

Methanocellales, *Methanosarcinales*, and *Methanomassiliicoccales*, with more recent genomic analysis suggesting that this metabolic pathway may be more widespread(Evans et al. 2019)

A functionally sound freshwater wetland ecosystem not only depends on soil microbes driving biogeochemical cycles, but also on the plant community. Plant diversity and floristic quality are essential components of successful ecosystem restoration (Doherty et al. 2011, Brown and Norris 2018) and high biodiversity is commonly thought to be essential to most ecosystems. For more diverse systems, floristic quality and the coefficient of conservation are important to consider when assessing the success of a restored ecosystem (Bried et al. 2013, Deberry and Perry 2015) and are essential to plant-microbe interactions (Faber-Langendoen 2018). Plants deemed of high floristic quality are typically endemic, rare, or found within a narrow range of environmental conditions. These higher-valued plants are intolerant to anthropogenic pressures and if are present in an ecosystem will indicate a healthier environment. Low quality, invasive plants may interfere with establishment of native plant species (Matthews and Endress 2010, Ballantine et al. 2012, Brown and Norris 2018), impairing restoration outcomes, shifting biogeochemical cycles, and leading to low plant species diversity (Anderson and Mitsch 2006, Mitsch et al. 2012, Wardle 2016).

Environmental degradation from invasive species, urbanization, increased or decreased precipitation, and excess nutrient runoff from agriculture makes protecting, restoring, and creating wetlands challenging (Gilby et al. 2020). Exotic plants may change soil nutrient composition due to their differences in chemistry, morphology, and phenology (Ehrenfeld 2003, Zedler and Kercher 2004, Hannah et al. 2020). In the freshwater wetlands of the Northeast United States, invasive species such as *Phragmites australis* invades as a monoculture and provides fewer benefits than a wetland with high biodiversity of native plant species(Duke et al. 2015). In North America, due to altered hydrologic regimes and nutrient inputs, *Typha* spp. – native *Typha latifolia*, non-native *Typha angustifolia*, along with their hybrid *Typha* x glauca - have become the dominant feature in wetlands preventing other native plants and animals to thrive in productive wetland systems (Bansal et al. 2019). Opportunistic invasive plants may thrive in degraded wetlands where nutrient availability is high and soils are poorly developed (Duke et al. 2015, Bansal et al. 2019, Johnson et al. 2021).

Soil development during succession is a very slow process taking centuries to thousands of years, varying with parent material, climate, biota, and topography (Brady and Weil 2008).

Wetland soils are typically hydric and may develop slowly due to anaerobic conditions and slower decomposition rates, with rates dependent on the hydrology or the wetland type (Mitsch and Gosselink 2007, Ballantine and Schneider 2016)Soil organic matter (SOM) in wetlands is key to wetland soil development, driving nutrient availability to plants, increasing bulk density, and creating a soil system ideal for soil biota. In depressional wetlands, soil development is slow initially due to the lack of outside or allochthonous inputs. After 55 years, the SOM of restored depressional wetlands has been found to be less than 50% of that in natural reference sites (Ballantine and Schneider 2016). This is in line with previous research indicating carbon storage of restored wetlands is approximately 26% lower than their natural reference counterparts (Moreno-Mateos et al. 2012). Therefore, management techniques that add additional carbon may drive succession toward desired outcomes in created or restored wetlands. Carbon addition may also change soil microbial communities to promote biogeochemical cycling.

Soil amendments such as biochar, leaf litter compost, clay, and manure, may aid in the trajectory of a successful created wetland by changing soil properties such as carbon sequestration and nutrient retention (Ballantine et al. 2012, Scott et al. 2020, Rubin et al. 2020). Further outcomes may include improved biogeochemical processes, increased soil microbial diversity, and increased plant diversity (Ballantine et al. 2012, Maietta et al. 2020, Scott et al. 2020). Created wetlands have impaired nutrient cycles and soil amendments can aid in restoring these cycles, however, not all soil amendments are created equal and some can result in increased leaching of nutrients, invasive species encroachment, and increased greenhouse gas emissions (Ballantine et al. 2015, Maietta et al. 2020, Scott et al. 2020). Leaf litter compost has been shown to aid in the trajectory of succession by increasing soil C:N and potential denitrification rates (McGowan 2020). Approaching restoration by only using soil amendments is not the ultimate solution, but may be a component of successful wetland restoration.

The objective of the work presented in this chapter was to assess whether soil amendments accelerate wetland succession toward reference natural wetland conditions in emergent and wooded wetlands. We hypothesized that experimental soil amendments would increase soil organic matter, carbon density, and nutrient content resulting in an increased diversity of soil microbes and an increase in diversity and floristic quality of plants comparable to a natural reference wetland. Studying soil characteristics along with soil microbial and plant community structure and function at different successional stages and under different

experimental soil carbon amendment regimes will allow us to better understand the trajectory of wetland development, and how management action may push the system towards the mature, desired outcome.

Methods

Experimental soil amendments were carried out at three created freshwater wetlands in Western New York State. At each site, a mature reference wetland was paired with a created wetland, where experimental soil amendments took place. The three created wetlands vary in hydrogeomorphic type (two are forested depressional wetlands and one is an emergent depressional wetland), and in age, although all wetlands were less than 12 yrs old at the time of the study. In the summer of 2020, plant and microbial community structure and soil characteristics were assessed in amended and unamended plots in the created wetland and in its paired reference wetland.

Site Description and Experimental Design

The emergent wetland and one of the forested wetlands were at High Acres Nature Area (HANA), 101 ha of natural and created wetlands in Perinton, New York managed and owned by Waste Management of New York (Figure 1). This site consists of grassland, forest, and a series of wetlands that vary from deep emergent ponds, vernal pools, and forested wetlands. Previously, the land was used for row crop agriculture, pasture, and gravel mining. The land was later used by Waste Management as mitigation for wetlands that were filled during landfill expansion. Two areas, Cady Wetlands South, an emergent wetland created in 2009 on former row crop soil, and Packard Wetlands area A, a forested wetland created in 2012 on former pasture, were under annual soil amendment from 2014-2019 and 2015-2020, respectively. The Cady Wetland is primarily dominated by *Typha* spp., *Phalaris arundinacea*, and *Persicaria* spp. The Packard site has been somewhat dry in recent years and is dominated primarily by wet meadow species (McGowan 2020). Each site was paired with a mature reference site, which were identified as having been relatively untouched for at least 75 years. The natural emergent site is in the Eastern Wetland complex, and consists of primarily *Typha* spp. The natural wooded site, Newt Pond, was dominated by *Salix* spp. and *Equisetum hyemale.*

Mill Seat Wetland Restoration Area is a 79-ha upland-wetland complex with 35 hectares currently under active restoration and management by Waste Management of NY. It is located in the Town of Riga and after 10 years of management, Waste Management will give the ownership to the town. A section of forested wetland created in 2019 (Phase 1) was used for the soil amendment, which began in 2019 and continued annually thereafter. A mixed species cover crop seed mix was spread initially, and black willow live stakes were planted (Mill Seat Scope, 2019). A mature, forested swamp at least 100 yr old was chosen as the reference site at Mill Seat and the dominant species are *Acer rubrum* and *Fraxinus pensylvanica*.

At the High Acres created wetlands, five pairs of 2 x 30 m zones (60 m^2 each) were established at the initiation of the experimental amendment period (Williams 2021; Figure 2). In Phase 1 at Mill Seat, 2 pairs of larger zones (4 x 50 m) were established to minimize edge effects (Figure 3). For all compost experimental areas, each year at the beginning of the growing season (typically late May to early June) leaf litter compost was applied to half of the transects at each site. The compost composition was 28 %C, 1.8 %N, with a C:N of 18.7. Approximately 5-7 cm of compost was applied across the entire transect resulting in an addition of approximately 2.0 kg C m^{-2} and 0.13 kg N m^{-2} yr^{-1} (McGowan 2020). Within each zone, two (HANA) or four (MS) permanent 1 m^2 sampling plots were established for vegetation, microbe, and soil sampling. In the natural reference wetlands, 6 sampling plots were established by haphazardly choosing plots at least 20 meters apart to best represent the site.

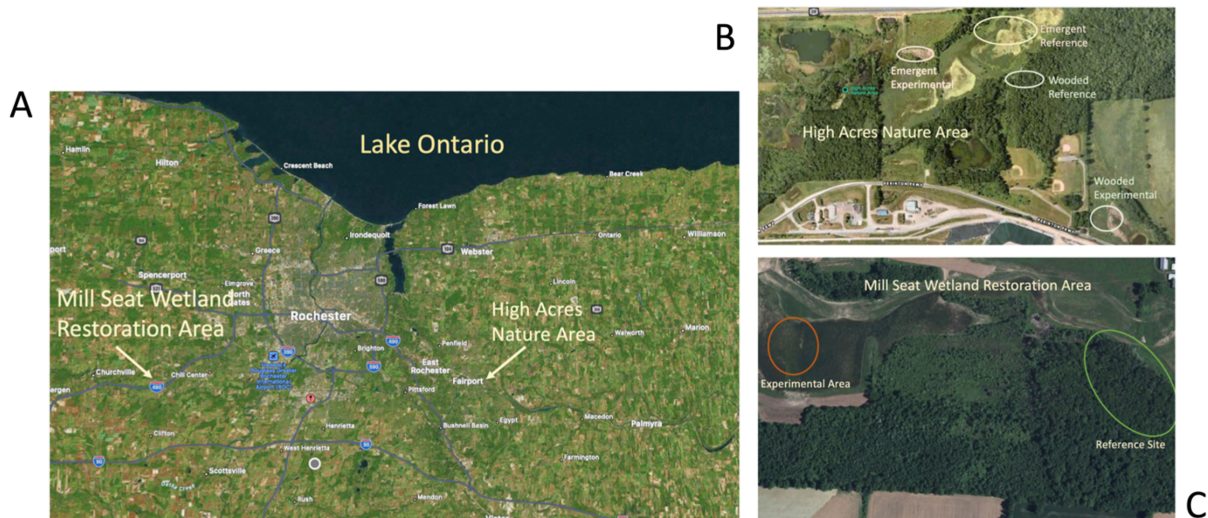

Figure 4.1-Location of Experimental and Reference sites at High Acres and Mill Seat Restoration Areas. A) Greater Rochester, NY B) High Acres Nature Area, C) Mill Seat Wetland Restoration Area

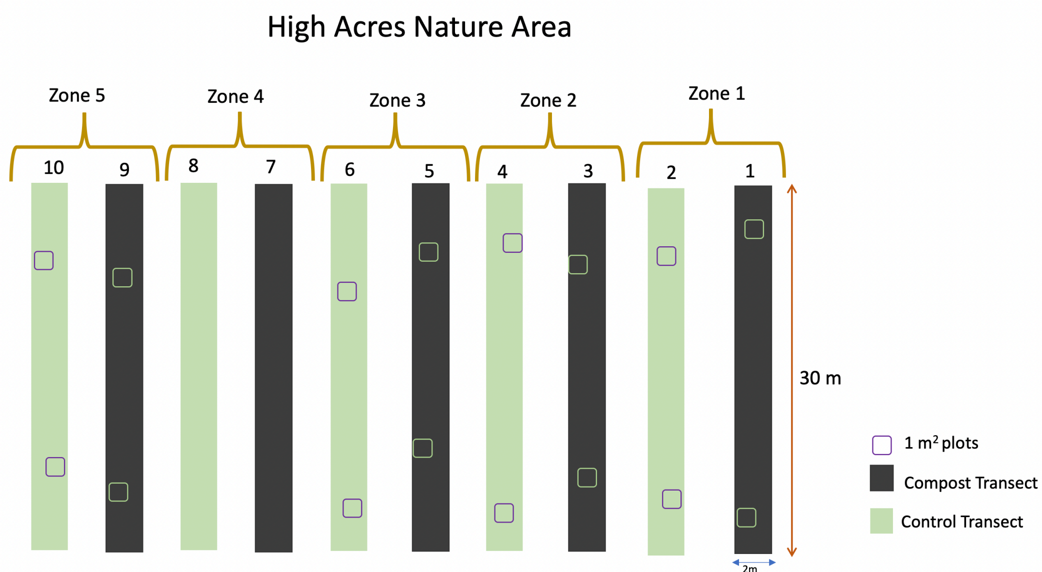

Figure 4.2-High Acres Nature Area Experimental Design. Five paired blocks were created with one zone with compost added and the other with no compost addition. In each zone, permanent sampling plots were established where vegetation, microbes and soil characteristics were measured.

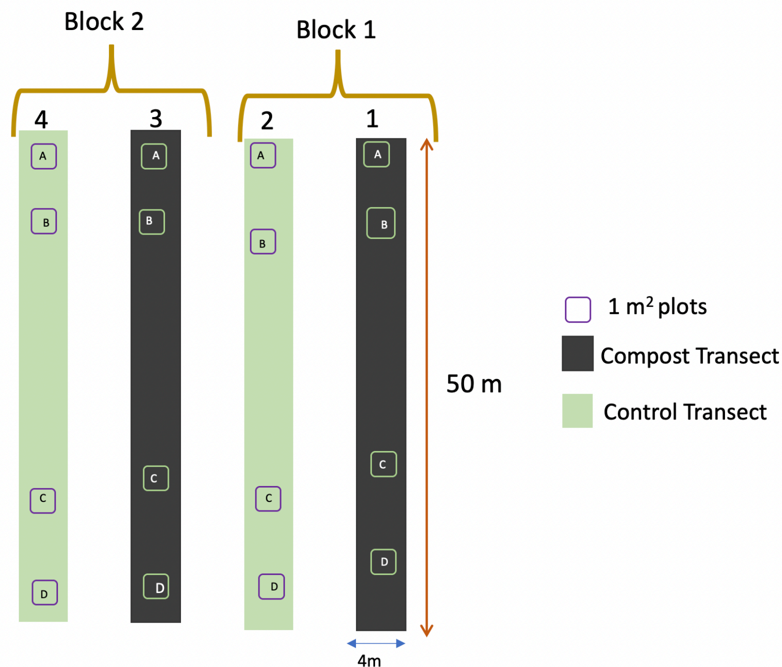

Figure 4.3-Compost Experimental Design at Mill Seat Wetland Restoration Area. Two paired blocks were created with one zone with compost added and the other with no compost addition. In each zone, permanent sampling plots were established where vegetation, microbes and soil characteristics were measured.

Vegetation

Plant surveys were conducted in the permanent 1m^2 plots at each site in July, 2020. Estimated percent cover was recorded for each plant species present with at least two observers. This included the herbaceous layer, shrubs, and where present overstory species were noted. Shannon Diversity was calculated using the following formula (Shannon and Weaver 1949). Species were also categorized as Native and/or Invasive by using New York Flora Atlas (New York Flora Association n.d.). All *Typha* spp. were categorized as invasive even though they are considered native to North America (Bansal et al. 2019). Each species was assigned a coefficient of conservatism assigned by the Northeast Regional Floristic quality assessment tool (Bried et al. 2012, Faber-Langendoen 2018, Faber-Langendoen et al. 2019). This information then was used to calculate the Floristic Quality Index (FQI) at each plot. The following equation was applied at each plot (USGS n.d.). CC_i is the coefficient of conservatism of the plant and N is the number of native species in each plot.

Equation 1-Floristic Quality Index

$$FQI_{std} = \left(\frac{\sum(CC_i)}{\sqrt{N_{native\ species}}}\right)$$

In addition to plant diversity and FQI, at each plot percent cover of native plants and invasive plants were determined.

Soil Sampling

At each site, soil samples were collected from the permanent plots in July 2020. Two 5 cm diameter by 10 cm deep soil cores were extracted from each plot with a tulip bulb corer. From one core, three 5 cc syringes of soil were collected at 0-1 cm depth and placed in a 15 ml centrifuge tube for microbial analysis. Centrifuge tubes were stored at -80 ^{o}C freezer for later DNA extraction. Both cores were then subdivided into two strata (0-5 cm and 5-10 cm), placed in zipper top sample bags, and frozen at -20 ^{o}C until analysis. An additional sample for bulk density assessment was collected using a metal core approximately 9 cm in depth and 5 cm in diameter.

Laboratory Methods

Immediately after sampling in the field, pH was measured using a small subsample from each 5 cm depth section. Approximately 5 cm^3 of soil was homogenized and mixed with 5 ml of deionized water. After 10 minutes, a Mettler Toledo pH meter was used to measure the pH (Gelderman and Mallarino 2012).

Bulk density and soil moisture content were measured from the bulk density core by obtaining the wet mass, drying at 60 ^{o}C for 48 hr, and reweighing. Dry mass divided by sample volume gives the bulk density. Soil moisture was calculated based on the percent mass loss between wet and dry.

Soil organic matter content (%OM) was assessed by drying one core (both depth sections) at 60 ^{o}C for 48 hr and homogenizing using a mortar and pestle. A portion was stored in scintillation vials for further analyses (percent C, N and P) and the remainder was placed in tins, ashed in a muffle furnace at 550°C for 4 hr, and the percent organic matter (%OM) determined based on the mass loss on combustion (Heiri et al. 2001).

Soil total phosphorus (%TP) was determined by adding 50% w/v magnesium nitrate to 0.1 g of oven-dried soil and ashing the sample for 2 hr at 550°C in a muffle furnace. Once cool, 10 mL of 1 M HCl was added, samples were then shaken for 16 hr and allow to settle overnight. Samples were diluted and phosphate was measured using the ammonium molybdate photometric method (Murphy and Riley 1962) and a Shimadzu UV 1900 Spectrophotometer (Aspila et al. 1976). The remainder of the dried sample was analyzed using Perkin Elmer 2400 Elemental Analyzer for Carbon (%TC) and Nitrogen (%TN). All analyses were conducted in duplicate or triplicate. Carbon density was calculated based on the %TC and the soil bulk density; elemental ratios of C, N and P were calculated as the molar ratio.

The second core (both sections) were thawed and used for extractable ammonium and nitrate analysis. Two – 5 g subsamples were weighed out from each sample bag. One sample was dried at 60 °C for 48 hr and the other moist soil sample was mixed with 50 mL 2M KCl for 30 min. The wet samples were then centrifuged, the supernatant decanted, filtered (0.45 µm), and placed into whirl-pak bags (Knepel 2012). Filtered samples were frozen at -20°C prior to analysis. Extracted nitrate was measured using a Shimadzu UV 1900 Spectrophotometer at 540 nm with the procedure outlined in Doane and Horwáth (2003). Extractable ammonium was quantified using the phenol hypochlorite method (Maynard et al. 2008) and sample absorbance was read at 630 nm. The dried samples were used to obtain the wet-dry ratio of the soil, and final values were expressed as mg kg^{-1} dry weight.

Microbial Analyses

DNA was extracted from sediment using DNeasy PowerSoil Kit (QIAGEN 2021) per manufacturer's instructions (n=88). The DNA purity and quantity was confirmed by using a Nanodrop 2000 spectrophotometer. DNA sequence data was generated using Illumina paired-end sequencing at the Environmental Sample Preparation and Sequencing Facility (ESPSF) at Argonne National Laboratory. The V4 region of the 16S rRNA gene (515F-806R) was PCR amplified with region-specific primers that include sequencer adapter sequences used in the Illumina flowcell (1, 2). For the PCR reaction, each 1 µL DNA sample was combined with 9.5 µL of MO BIO PCR Water (Certified DNA-Free), 12.5 µL of QuantaBio's AccuStart II PCR ToughMix (2x concentration, 1x final), 1 µL Golay barcode tagged Forward Primer (5 µM concentration, 200 pM final), 1 µL Reverse Primer (5 µM concentration, 200 pM final). The reaction conditions were: 94 °C for 3 minutes to denature the DNA, with 35 cycles at 94 °C for

45 s, 50 °C for 60 s, and 72 °C for 90 s; with a final extension of 10 min at 72 °C to ensure complete amplification. Amplicons were then quantified using PicoGreen (Invitrogen) and a plate reader (Infinite 200 PRO, Tecan) and then pooled into a single tube so that each amplicon was in equimolar amounts. This pool was cleaned up using AMPure XP Beads (Beckman Coulter), and quantified using a fluorometer (Qubit, Invitrogen). After quantification, the molarity of the pool was determined and diluted down to 2 nM, denatured, and then diluted to a final concentration of 6.75 pM with a 10% PhiX spike for sequencing on the Illumina MiSeq. Amplicons were then sequenced on a 151 bp x 12bp x151bp MiSeq run (Caporaso et al. 2011, 2012)

The resulting 16s rRNA sequences were demultiplexed in QIIME2 (Bolyen et al. 2019) and the final 5,051,843 sequences were denoised with the DADA2 plugin for QIIME2 (Callahan et al. 2016). Taxonomy was assigned using SILVA database (Quast et al. 2013). R-Studio and R were used to convert the artifacts into a *phyloseq* object (McMurdie and Holmes 2013, R Core Team 2022, RStudio Team 2022) and then transformed into a 'microtable' for Microeco. All ASVs not assigned to Archaea or Bacteria, and those assigned to "mitochondria" or "chloroplast" were removed. The *tidy_dataset* function was used to trim the dataset to eliminate samples with 0 artifacts and the microtable was rarified to a sample size of 10,000 sequences per sample (only one sample was removed). From this final data, we calculated relative abundance at the Kingdom, Phylum, Class, Order, Family, Genus, and Species level. All data visualizations were performed using the ggplot2 package in Microeco for R (Wickham et al. 2019, Liu et al. 2021). Alpha diversity metrics for each site as a whole, and for each treatment within each site were calculated using Microeco, including species richness, evenness, total observed features, and Shannon, Chao1, and Faith's Phylogenetic Diversity Indices. Beta diversity analyses were performed in Microeco to evaluate community structure among the two marshes and their respective zones. Dissimilarity was visualized using Principal Coordinate Analyses using pair-wise Bray-Curtis dissimilarity estimates, and relationships visualized using a cluster graph. Using the Bray-Curtis distance, a RDA was performed show correlations among the microbial taxa level of class and the associated environmental factors: plant diversity, FQI, BD, SM, pH, CD, %TP, %TC, C:N, N:P, NO_3^-, and NH_4^+. Highly autocorrelated variables assessing similar parameters (e.g. %TC and %OM) were reduced to a single variable (%TC). The top metabolic

functions found in each plot was determining by using the package FAPROTAX within the Microeco package for R (Louca et al. 2016, Liu et al. 2021).

Statistical Analyses

Differences in plant diversity, FQI, %Invasive and %Native plant cover, and soil characteristics were evaluated using a one-way analysis of variance (ANOVA) in JMP Pro 16 for each wetland group. All environmental data were assessed for normality (Anderson-Darling) and homogeneity of variance (Levene's test) prior to analysis to ensure adherence with the assumptions of the test. A one-way ANOVA was performed on the environmental data for each wetland site. When there was significance a post-hoc Tukey (HSD) test was applied. A Kruskall-Wallis test was also used on the 10 most abundant microbial taxa at the level of class, the diversity indices, and the Metabolic function to evaluate differences between treatments in each wetland group. When there was a significant difference, a Dunn's post hoc test with a Bonferroni adjustment was applied. A PERMANOVA using Bray-Curtis dissimilarity was used to assess Beta Diversity.

Results

Vegetation

Plant diversity did not vary with treatment within the HANA Emergent and HANA Wooded wetlands, however, there were significant differences in FQI ($p=0.002$ and $p = 0.013$; Figure 3.4, Table 3.1 and Table 3.2). The highest FQI was found in control and compost areas for HANA Emergent with twice as much as the reference (FQI=9.4-9.6 and FQI=4.9±0.9). At the HANA Wooded site, FQI at the compost and control sites was 30-40% lower than the reference site (Table 3.2). There were significant differences in plant diversity between treatments for the Mill Seat Wooded wetlands ($p = 0.031$), with the compost plots having the highest diversity and the reference the lowest (H'= 1.1±0.1; H'=0.7±0.1, Table 3.3, Figure 3.4). Native plant cover did not differ across treatments at any of the three sites (typically >80% for HANA Emergent, >65% HANA Wooded and >90% Mill Seat Wooded), however, percent invasive plant cover was significantly higher in the HANA Emergent reference site ($p<0.01$, Table 3.1) and lower in the reference site at Mill Seat Wooded ($p<0.001$, Table 3.3). This is likely attributed to the high *Typha* spp. cover in reference wetlands at both sites (Table 3.1,3.2, 3.3 Figure 3.4).

Soil Physicochemical Characteristics

Across all three wetland areas, pH and TP were significantly different among treatments ($p<0.01$). Soils were more acidic in the reference sites (6.7 – 7.1) compared to the created control plots (7.1 – 7.3) and compost soils were slightly more acidic than controls, but not significantly so (Table 3.1, 3.2, 3.3). Percent TP was greater in the reference sites for all wetland groups. Differences in TP between treatments were largest at the HANA Wooded site, with 0.6% in the reference wetland, almost three-fold higher than the compost or the control. For all other environmental factors, each wetland site showed unique patterns across treatments.

At the HANA Emergent site, NO_3^- ($p<0.05$), N:P ($p<0.001$) and bulk density ($p<0.05$) were significantly different between treatments (Table 3.1). For both NO^-_3 and N:P, the compost site was 2-3-fold higher than the reference, with the control falling between the other sites. Bulk density was similar in compost and reference sites and 1.5-fold higher in the control site. Organic matter and %TC were higher in the compost, but also highly variable and the differences weren't significant (Table 3.1).

At the HANA Wooded site, the compost addition led to clear differences in soil characteristics between treatments (Table 3.2). Organic matter content, % TC and carbon density were approximately 2-fold higher in the compost site than the reference or control sites ($p=0.004$, $p=0.0003$, $p=0.01$, respectively). Total nitrogen content (%TN) was also substantially higher in the compost site than the control or reference ($p<0.0001$), with values of 1% compared to 0.4-0.5%. In contrast, N:P was highest in the reference site and lowest in the control, with the compost falling between ($p<0.0001$). Soil moisture (%SM) was also intermediate in the compost sites, with a ~6% difference between the lowest %SM measured at the reference site and the highest value measured at the control ($p=0.04$, Table 3.2).

At the Mill Seat Wooded site there were also large differences in soil characteristics between treatments, driven by differences between the reference site and the two created sites (Table 3.3). Soil carbon and nitrogen characteristics were generally highest the reference site with %OM, %TC, %TN, N:P and NH_4^+ concentrations approximately 2-fold higher in reference soils compared to control and compost soils ($p<0.001$). In contrast, C:N was lowest in the reference, at 15 compared to 20 in the compost and control ($p<0.001$). Soil moisture (%SM) and bulk density were also lower in the reference site ($p=0.008$, $p=0.005$), with nearly 50% lower moisture and bulk density compared to the control site. Compost did not have a significant effect on any of the soil variables.

Microbial Community Composition

The pattern in relative abundance (RA) of the top 10 most abundant phyla show that there are similarities across all wetlands, with *Proteobacteria* (25-30%) being most abundant in all sites. There were differences within other dominant groups, especially *Firmicutes* (5-30%), *Actinobacteriota* (2-19%), and *Bacteroidota* (3-20%). *Firmicutes* was greater (15-30%) in the compost treatments across all wetlands, and *Verrumicrobiota* and *Desulfobacterota* were lower (Figure 3.5). Additional analysis at the class level within each wetland shows that the three wetlands differed somewhat in RA at the class level. There was somewhat higher *Alphaproteobacteria* RA at HANA Emergent and Mill Seat Wooded sites ($p=0.02$), lower *Bacteroidia* and *Polyangia* ($p=0.01$; $p=0.03$, respectively) at the Mill Seat Wooded site, and greater *Planctomycetes*, and *Clostridia* at the HANA Emergent site ($p<0.05$, Table 3.5).

The general lack of difference among the three wetland groups as a whole is likely derived from the strongly distinct communities within each treatment group (Table 3.4). The microbial community in HANA Emergent sites differs significantly across the three treatments, with differences in RA observed within the top ten classes (Figure 3.6, Table 3.5). *Gammaproteobacteria* was highest in the compost treatment at 17.1% and lowest in the reference at 12.6% (p=0.002). Similarly, *Bacilli* was highest in the compost (8.4%) versus the control (2.9%) and reference (3.1%; p=0.0001). Other groups with greater RA in the compost treatment were *Actinobacteria* and *Planctomycetes* (4% and 5.1%, respectively; p<0.0001). The reference site had the highest RAs of *Bacteroidia, Verrucomicrobiae* and *Desulfuromonadia* (9.4%, 4.5% & 2.6%, respectively; p<0.007) (Table 3.5).

Community patterns for the HANA Wooded Wetland group were similar to HANA Emergent, with the exception of *Gammaproteobacteria*, which was substantially higher in the Reference site (19%) relative to the control (1.7% and compost 11.7%; p=0.0108; Table 5 and Figure 7). *Bacilli* was four-fold greater in the compost (18.7%) than control and reference (4.5% and 1%, respectively; p=0.0012). *Actinobacteria* in compost (5.8%) were nearly two-fold greater relative to the control (3.3%) and six-fold greater than the reference (0.8%, p=0.0077). *Clostridia* was also nearly two-fold higher in the compost (4.2%) than the control and reference (2.3% and 2.8%; p =0.0072). However, the control had three-fold higher *Vicinamibacteria* than the compost, and more than eight-fold that of the reference (9.7%, 3.2% and 1.3%, respectively; p = 0.0034). Compost was similar to the reference for the bacteria class *Anaerolineae* (5.3% and 5.8%; p =0.0077) while the control had a RA of 1.7%. The reference had higher amounts of *Desulfuromonadia* at 3.5% with the control at 2.8% and the compost substantially lower (0.8%; p=0.0175; Table 3.5).

Community patterns of RA at the Mill Seat Wetlands were slightly different than the other two wetlands. *Alphaproteobacteria* was lowest in the compost treatment (~14%) while the control and the reference had 18.3% and 17.4% RA, respectively (p= 0.0017). Also, of note, *Bacilli,* as in the other wetland groups was much higher in the compost treatment (15.4%), relative to the control (3.2%) and reference (1.9%; p<0.0001). *Anaerolineae* and *Clostridia* were in the compost treatment (5.7% and 4.8%), relative to 1.8% for the reference site (for both) and 3.7% and 2.8% for the control, respectively (p=0.0001 & p<0.0001). RA for *Thermoleophilia* and *Acidimicrobiia* for the reference site (6.2% and 3.4%, respectively) were greater than either

of the created treatments. *Thermoleophilia* in the reference site was two-fold larger in RA compared to the compost and control treatments ($p<0.0001$); *Acidimicrobiia* was about 1.5-fold higher in the reference versus the control and compost ($p<0.0001$). Of interest, the nitrogen fixing group *Cyanobacteriia* was higher in the control at 3.6% versus the compost at 2.1%, and not present in the reference. ($p=0.0021$; Table 3.5).

Microbial Diversity

Four alpha diversity indices (observed features, Shannon (H'), Chao1 and Faith's Phylogenetic diversity) were used to characterize differences across the three wetland sites and within each of the three sites (Figure 3.7). Alpha diversity was significantly different between the three wetland sites ($p<0.0001$) for all four indices with HANA Emergent higher than both wooded sites (Table 3.6). Within the HANA Emergent site, there were no significant differences among treatments (Table 3.7). For the HANA Wooded site, the reference was higher for all indices, except for Shannon (H') where there were no post hoc differences identified in spite of overall significance ($p=0.05$; Table 3.7). At the Mill Seat Wooded site, Shannon (H') and Faith's Phylogenetic Diversity ($p<0.05$) were greatest for the compost treatment and lowest for the reference (Table 3.7).

The PCoA illustrates distinctly different grouping within each of the three wetlands. For the HANA Emergent site (Figure 3.8) the control and compost are strongly positive for PCo1 (26%) and group together, while the reference site loads strongly negative. On PCo2 (12.9%), the control is very tightly clustered while the compost ranges substantially from -0.50 to 0.25. The reference wetland plots all fall at about zero for PCo2. For the HANA Wooded Wetland (Figure 3.8), the compost and control are separated on PCo1 (30.3%), and again the compost occupies a much greater range of values (-0.75 - +0.25) than either the control or reference. The reference is similar to the compost for PCo1, but highly divergent and negative on PCo2 (12.9%), where the values for compost and control are positive and more similar to one another. In the Mill Seat Wooded Wetlands (Figure 11), the three treatments group distinctly on PCo1 (29.6%), with the reference site strongly negative and the compost strongly positive, and the control at roughly zero. On PCo2 (16.5%), the compost and reference sites generally overlap in the positive range, with more spread for the compost treatment. The control is strongly negative on PCo2.

The Bray-Curtis dissimilarity cluster illustrates that HANA Emergent (Figure 3.9) shows overlap of control and compost samples while the reference is more distant from the other treatments. For HANA Wooded, the Bray-Curtis dissimilarity cluster shows that all three treatments are distinct (Figure 3.9), while Mill Seat Wooded Bray-Curtis dissimilarity cluster shows some overlap with reference and compost (Figure 3.9).

The two primary axes of the RDA for HANA Emergent explained 42.1% and 24.1% of the variability between groups, and begin to illustrate the key sediment physicochemical drivers of the microbial community differences (Figure 3.10). On RDA1, high nutrient and carbon availability is linked with *Actinobacteria*, while *Gammaproteobacteria* is correlated with plant diversity. Reference wetland plots were associated with *Acidobacteriae, Verrucomicrobiae,* and *Bacteroidia* as well as the environmental factors of total phosphorus (TP) and invasive plant cover (Figure 3.10). The two primary axes of the RDA for HANA Wooded explain 56.5% and 31.7% of variability. On RDA1 high nutrients are associated with the compost plots, while control plots negatively load on RDA2 and correlate with *Gammaproteobacteria* and *Bacteroidia*. Reference plots are positive for RDA2 and associated with *Vicinamibacteria*, and *Thermoleophilia*.(Figure 3.10-B). The two primary axes of the RDA for Mill Seat Wooded site (Figure 3.10) explain 58.4% and 15.9% with nutrients positive for RDA1 but strongly negative for RDA2. On RDA 2, the following microbial classes correlate with Nitrate (NO^{-}_{3}) and are strongly negative for RDA2: *Cyanobacteriia*, *bacteriap25, Thermoleophilia, and Vicinamibacteria*. *Gammaproteobacteria, Alphaproteobacteria,* and *Bacteroidia* are positive on RDA1, and associate with the reference sites.

Metabolic functional Groups

The relative importance of different Metabolic function was assessed across wetlands and treatment groups and the top twelve most important Metabolic function were identified (Table 3.8, 3.9). While aerobic chemoheterotrophy was dominant across all three wetlands, it was significantly higher in the Wooded sites, with values of 15-16%, compared to 14% in the HANA Emergent site ($p<0.003$; Table 3.8). A similar same pattern was seen with anaerobic chemoheterotrophy, except that the HANA Emergent was highest at 3.4% and was distinctly different from the Mill Seat Wooded at 3% ($p<0.02$; Table 3.8). The other metabolic functions that differed significantly between the three wetland groups were methylotrophy ($p=0.0002$),

hydrocarbon degradation ($p=0.0004$), methanotrophy ($p=0.0004$) and methanogenesis ($p<0.02$) and these processes represented 0.5 – 1.6% of the community. For all of these processes except methanogenesis, HANA Emergent was the highest, with values of that were approximately 2-folder higher than the Mill Seat Wooded site. For methanogenesis, the Mill Seat Wooded site had the lowest abundance (0.5%) and the HANA Wooded had the highest (0.8%).

There were differences in the relative importance of key metabolic functions across treatments for some, but not all sites. At the HANA Emergent site there were no significant differences across treatments for any of the top 12 Metabolic function. At the HANA Wooded site there were substantial differences in metabolic functions between the reference and control plots, with the compost plots generally falling between the other groups ($p<0.05$) (Table 3.8 and 3.9). The reference site had higher values for many metabolic functions including anaerobic chemoheterotrophy, methylotrophy, methanotrophy, fermentation, phototrophy, hydrocarbon degradation, methanogenesis and photosynthetic cyanobacteria (Table 3.8 and 3.9). Two metabolic functions, aerobic chemoheterotrophy and dark hydrogen oxidation, were lower in the reference site compared to the control site and in the case of aerobic chemoheterotrophy, also significantly lower than the compost site($p=0.014$) (Table 3.8 and 3.9).

The Mill Seat Wooded site also exhibited significant differences in metabolic functions across treatments, however, at this site the differences were often associated with the compost treatment ($p< 0.01$)(Table 3.8 and 3.9). Several key metabolic functions were higher in the compost site, with significantly higher presence of methanotrophs and methylotrophs in compost plots compared to control plots and higher nitrate reduction and hydrocarbon degradation in compost plots compared to both reference and control plots (Table 3.8 and 3.9). There were also key differences in nitrogen fixing organisms, with higher photosynthetic cyanobacteria and lower heterotrophic nitrogen fixers in the control and compost plots compared to the reference plots (Table 3.8 and 3.9). There were also a few key differences between just the reference and control plots, with dark hydrogen oxidation being higher and phototrophy lower in the reference compared to the control, a pattern that was opposite that observed in the HANA Wooded site (Table 3.8 and 3.9).

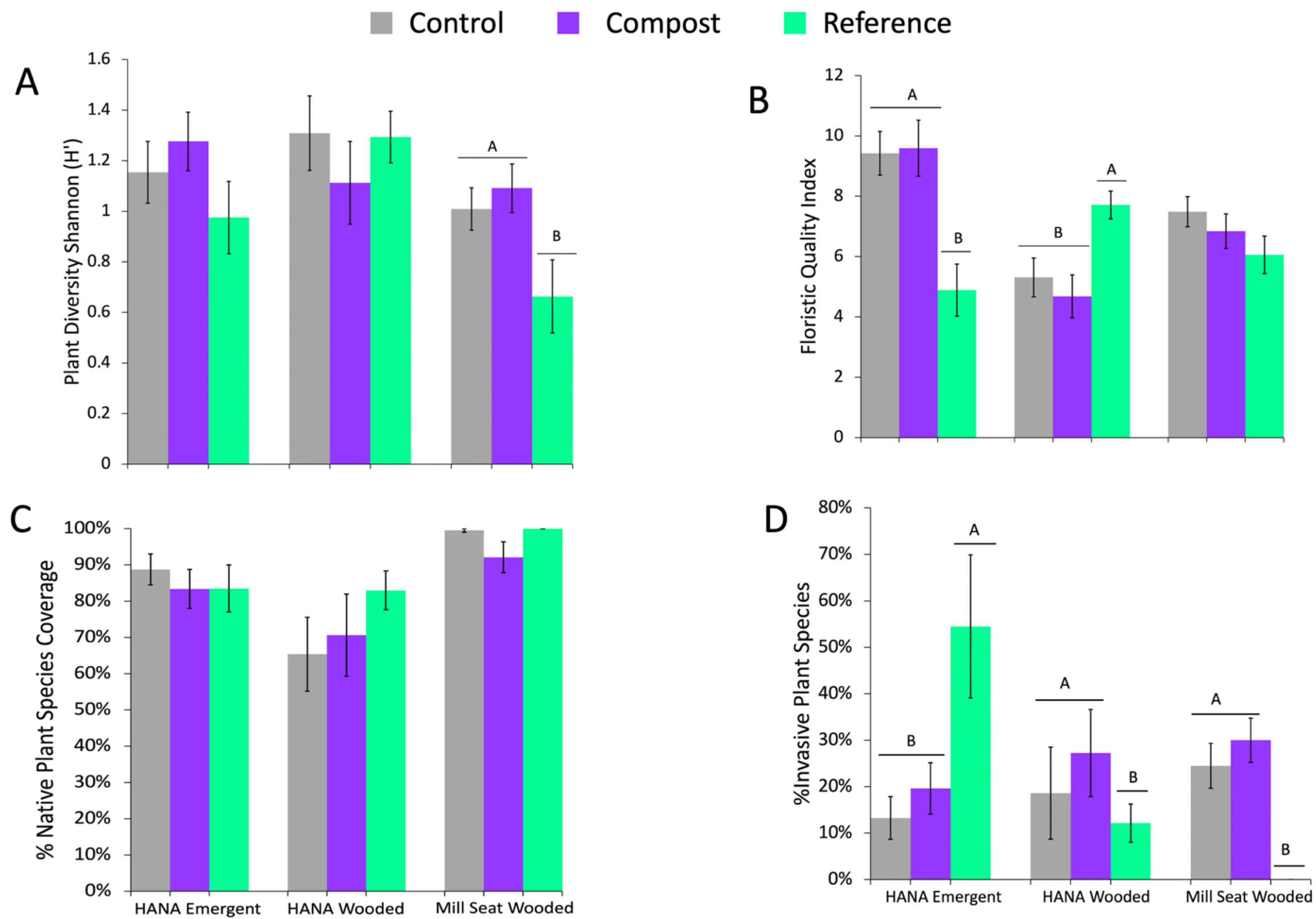

Figure 4.4-Vegetation characteristics in control, compost and reference sites at the three wetland locations. Mean ± SE, n= 6-8, (A) Shannon diversity index (B), Floristic Quality Index (FQI), (C) % native plant cover and (D) % invasive plant cover. Letters indicate significant differences between treatments within a wetland location.

Table 4.1-Environmental factors for HANA Emergent. Mean ±SE for the 3 treatments and One-Way ANOVA. Bolded p-values are significant. Connected letters are from HSD Tukey's test

HANA Emergent	Treatment	Mean ± S.E	F	p-value
Plant Diversity (H')	***Control*** ***Compost*** ***Reference***	1.2±0.1 1.3±0.1 1.0±0.7	$1.3_{(2,21)}$	0.28
FQI	***Control*** ***Compost*** ***Reference***	9.4±0.7[a] 9.6±0.9[a] 4.9±0.9[b]	$8.9_{(2,21)}$	**0.0019**
% Native Plant Cover	***Control*** ***Compost*** ***Reference***	89±4 83±5 84±6	$0.35_{(2,21)}$	0.71
% Invasive Plant Cover	***Control*** ***Compost*** ***Reference***	13±8[b] 20±8[b] 55±9[a]	$6.2_{(2,21)}$	**0.0086**
Bulk Density (g cm^{-2})	***Control*** ***Compost*** ***Reference***	0.51±0.05[a] 0.32±0.02[b] 0.35±0.07[b]	$5.8_{(2,21)}$	**0.0108**
%SM	***Control*** ***Compost*** ***Reference***	43±3 34±2 35±6	$2.6_{(2,21)}$	0.1015
pH	***Control*** ***Compost*** ***Reference***	7.16±0.04[a] 7.10±0.06[a] 6.71±0.05[b]	$15.8_{(2,21)}$	**<0.0001**
%OM	***Control*** ***Compost*** ***Reference***	31±5 47±7 32±3	$2.6_{(2,21)}$	0.1026
%TC	***Control*** ***Compost*** ***Reference***	15±3 17±1 13±1	$0.92_{(2,17)}$	0.42
%TN)	***Control*** ***Compost*** ***Reference***	1.1±0.3 1.3±0.1 1.02±0.1	$0.65_{(2,17)}$	0.53
%TP	***Control*** ***Compost*** ***Reference***	0.2±0.0[b] 0.2±0.0[b] 0.3±0.0[a]	$7.9_{(2,17)}$	**0.0045**
NO^-_3 ug/g	***Control*** ***Compost*** ***Reference***	0.025±0.01[ab] 0.032±0.01[a] 0.01±0.01[b]	$4.7_{(2,17)}$	**0.0223**
NH_4^+ ug/g	***Control*** ***Compost*** ***Reference***	67.2±22.5 83.8±18.1 39.4±7.2	$1.6_{(2,17)}$	0.23
C:N	***Control*** ***Compost*** ***Reference***	16.1±0.5 15.7±0.4 14.9±0.2	$2.4_{(2,17)}$	0.13
Carbon Density	***Control*** ***Compost*** ***Reference***	0.11±0.04 0.41±0.11 0.31±0.04	$2.6_{(2,17)}$	0.11
N:P	***Control*** ***Compost*** ***Reference***	2.33±0.34[ab] 3.02±0.22[a] 1.74±0.14[b]	$6.6_{(2,17)}$	**0.0089**

Table 4.2-Environmental Factors for HANA Wooded Wetlands. Mean ±SE for the 3 treatments and One-Way ANOVA. Bolded p-values are significant. Connected letters are from HSD Tukey's test

HANA Wooded	Treatment	Mean ± S.E	F	p-value
Plant Diversity (H')	***Control***	1.3±0.1	$0.58_{(2,21)}$	0.58
	Compost	1.1±0.2		
	Reference	1.3±0.1		
FQI	***Control***	$5.3±0.6^{b}$	$5.6_{(2,21)}$	**0.0126**
	Compost	$4.7±0.7^{b}$		
	Reference	$7.7±0.5^{a}$		
% Native Plant Cover	***Control***	65±10	$0.75_{(2,21)}$	0.49
	Compost	71±11		
	Reference	83±5		
% Invasive Plant Cover	***Control***	19±9	$0.7_{(2,21)}$	0.51
	Compost	27±9		
	Reference	12±10		
Bulk Density (g cm^{-2})	***Control***	0.633±0.02	1.98	0.17
	Compost	0.489±0.05		
	Reference	0.569±0.09		
%SM	***Control***	$64±2^{a}$	$3.98_{(2,21)}$	**0.0359**
	Compost	$59±2^{ab}$		
	Reference	$53±5^{b}$		
pH	***Control***	$7.3±0.1^{a}$	$7.2_{(2,21)}$	**0.0047**
	Compost	$7.2±0.2^{a}$		
	Reference	$6.7±0.0^{b}$		
%OM	***Control***	$17±1^{b}$	$7.5_{(2,21)}$	**0.004**
	Compost	$31±1^{a}$		
	Reference	$17±6^{b}$		
%TC	***Control***	$7±0^{b}$	$15.7_{(2,15)}$	**0.0003**
	Compost	$15±1^{a}$		
	Reference	$6±2^{b}$		
%TN	***Control***	$0.5±0.0^{b}$	$20.8_{(2,15)}$	**<0.0001**
	Compost	$1.0±0.0^{a}$		
	Reference	$0.4±0.2^{b}$		
%TP	***Control***	$0.18±0.0^{b}$	$58.8_{(2,15)}$	**<0.0001**
	Compost	$0.23±0.0^{a}$		
	Reference	$0.06±0.0^{c}$		
NO^{-}_{3} ug/g	***Control***	0.52±0.25	$1.6_{(2,15)}$	0.24
	Compost	0.68±0.22		
	Reference	0.097±0.03		
NH_4^{+}) ug/g	***Control***	21.1±9.0	$3.3_{(2,15)}$	0.0704
	Compost	35.7±8.8		
	Reference	69.4±23.2		
C:N	***Control***	16.7±0.3	$0.042_{(2,15)}$	0.96
	Compost	17.6±0.6		

	Treatment	Mean ± S.E	F	p-value
	Reference	17.2±5.5		
Carbon Density	***Control***	$0.38±0.02^{ab}$	$6.4_{(2,15)}$	**0.0119**
	Compost	$0.57±0.09^{a}$		
	Reference	$0.22±0.06^{b}$		
N:P	***Control***	$6.14±0.26^{c}$	$27.6_{(2,15)}$	**<0.0001**
	Compost	$9.75±0.73^{b}$		
	Reference	$15.12±1.5^{a}$		

Table 4.3-Mill Seat Wooded Wetlands. Mean ±SE for the 3 treatments and One-Way ANOVA. Bolded p-values are significant. Connected letters are from HSD Tukey's test

Mill Seat Wooded	Treatment	Mean ± S.E	F	p-value
Plant Diversity (H')	***Control***	$1.0±0.1^{ab}$	$4.2_{(2,21)}$	**0.031**
	Compost	$1.1±0.1^{a}$		
	Reference	$0.7±0.1^{b}$		
FQI	***Control***	7.5±0.5	$1.5_{(2,21)}$	0.25
	Compost	6.85±0.6		
	Reference	6.1±0.6		
% Native Plant Cover	***Control***	99.5±1	$2.7_{(2,21)}$	0.091
	Compost	92±4.2		
	Reference	100±0.0		
% Invasive Plant Cover	***Control***	$25±5^{a}$	$12.4_{(2,21)}$	**0.0004**
	Compost	$30±4^{a}$		
	Reference	$0±0^{b}$		
Bulk Density (g cm^{-2})	***Control***	$0.47±0.07^{a}$	$7.0_{(2,21)}$	**0.0053**
	Compost	$0.34±0.05^{ab}$		
	Reference	$0.18±0.02^{b}$		
%SM	***Control***	$46±5^{a}$	$10.6_{(2,21)}$	**0.0008**
	Compost	$41±3^{a}$		
	Reference	$23±1^{b}$		
pH	***Control***	$7.4±0.07^{a}$	$7.0_{(2,21)}$	**0.0052**
	Compost	$7.3±0.06^{ab}$		
	Reference	$7.1±0.02^{b}$		
%OM	***Control***	$26±5^{b}$	$12.1_{(2,21)}$	**0.0004**
	Compost	$28±3^{b}$		
	Reference	$52±2^{a}$		
%TC	***Control***	$16±1^{b}$	$58.4_{(2,17)}$	**<0.0001**
	Compost	$15±1^{b}$		
	Reference	$27±1^{a}$		
%TN	***Control***	$0.85±0.1^{b}$	$80.3_{(2,17)}$	**<0.0001**
	Compost	$0.96±0.1^{b}$		
	Reference	$2.1±0.1^{a}$		
%TP	***Control***	$0.14±0.0^{b}$	$12.5_{(2,17)}$	**0.0006**
	Compost	$0.17±0.0^{b}$		
	Reference	$0.23±0.0^{a}$		
NO^{-}_{3} ug/g	***Control***	0.01±0.00	$0.87_{(2,17)}$	0.44
	Compost	0.01±0.01		
	Reference	0.02±0.01		
NH_4^+ ug/g	***Control***	$39.9±8.1^{b}$	$5.0_{(2,17)}$	**0.0216**
	Compost	$41.7±5.4^{b}$		

	Reference	91.8±20.6[a]		
C:N	***Control***	19.9±0.3[a]	$85.7_{(2,17)}$	**<0.0001**
	Compost	19.6±0.3[a]		
	Reference	14.8±0.3[b]		
Carbon Density	***Control***	0.47±0.05	$0.52_{(2,17)}$	0.61
	Compost	0.41±0.09		
	Reference	0.38±0.03		
N:P	***Control***	14.0±0.8[b]	$22.2_{(2,17)}$	**<0.0001**
	Compost	12.5±0.8[b]		
	Reference	20.2±1.0[a]		

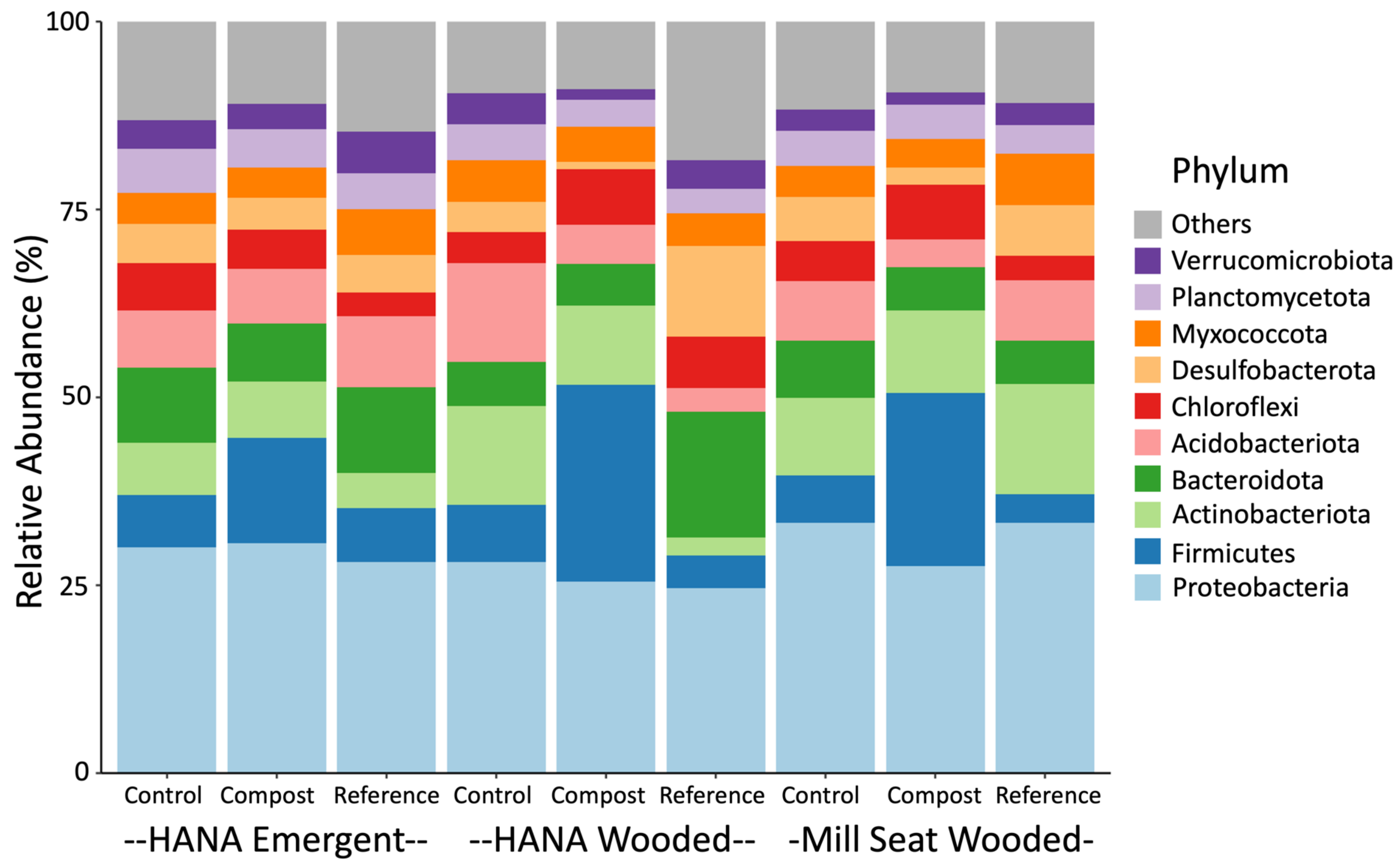

Figure 4.5-Relative Abundances of the top 10 Phyla across treatments in each of the wetlands.

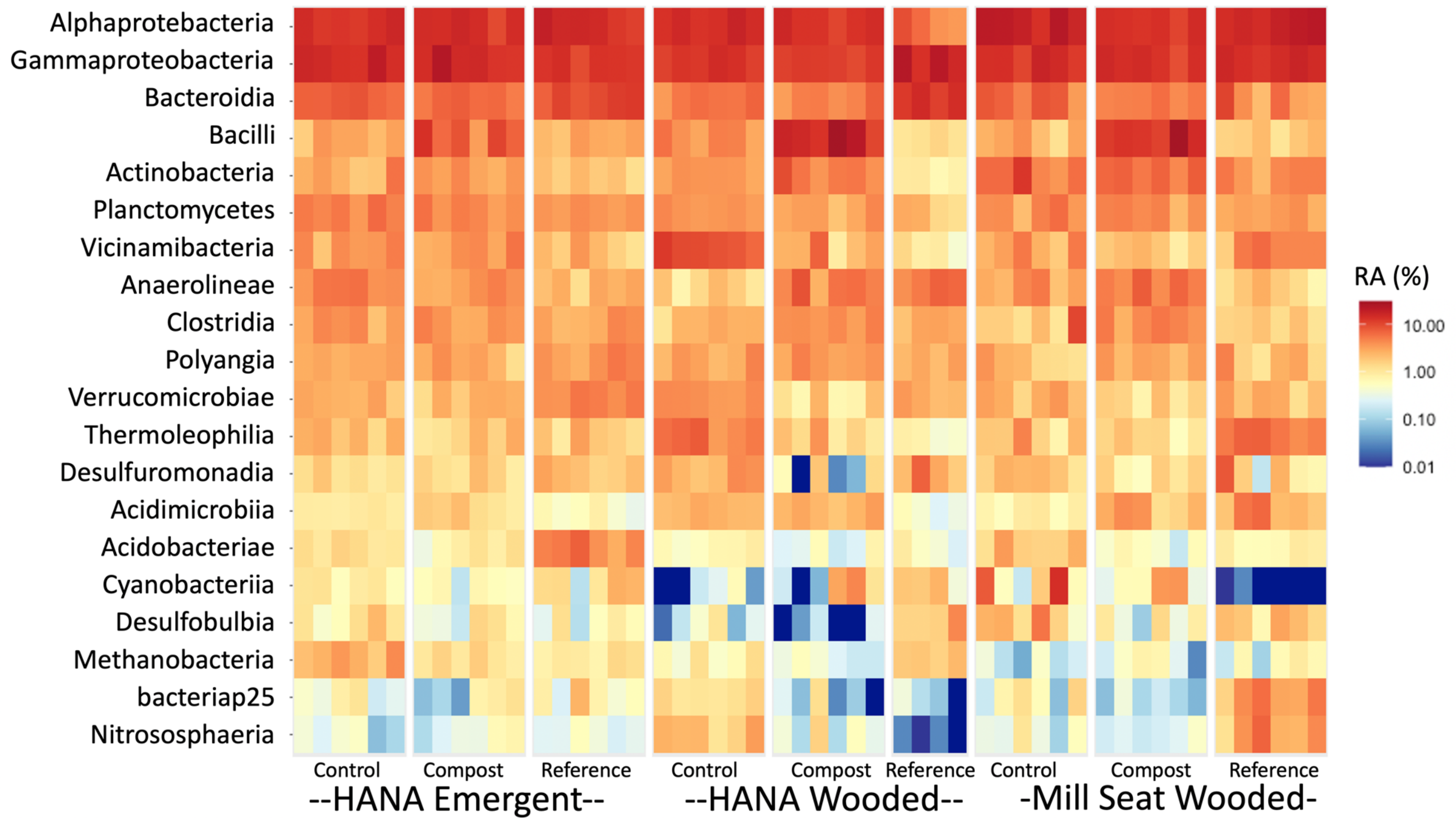

Figure 4.6-Heat map of the top 20 microbial classes, including both Bacteria and Archaea, across treatments in each of the wetlands.

*Table 4.4-Relative abundances (RAs) of the top 10 bacterial classes across all treatments in each wetland. Chi square results of a Kruskall-Wallis test comparing the three wetland sites (Between site comparison). Chi-square and p-values for each individual wetland site evaluating differences among Treatments (Within Site) are also shown. Values for each treatment are shown in Table 5. * indicates significantly higher RA within indicated wetlands relative to the other. Bolded p-value indicates significance difference between groups.*

Class	**Site**	**Whole Site RA**	**Between Site**		**Within Site**	
			$X^2_{2,51}$	***p***	$X^2_{2,1}$	***p***
Alphaproteobacteria	**HANA**	15±0.5[ab]	8.8	**0.01**	0.78	0.7
	HANA Wooded	12±1[b]			9.2	**0.01**
	Mill Seat	17±1[a]			8.7	**0.01**
Gammaproteobacteria	**HANA**	15±1	3.9	0.14	5.6	0.06
	HANA Wooded	14±1			8.3	**0.02**
	Mill Seat	14±0.5			1.1	0.59
Bacteroidia	**HANA**	8.1±0.5[a]	9.1	**0.01**	9.98	**0.007**
	HANA Wooded	7.4±1[ab]			9.4	**0.009**
	Mill Seat	5.7±0.6[b]			3.0	0.22
Bacilli	**HANA**	4.7±0.8	1.0	0.6	10.2	**<0.01**
	HANA Wooded	8.8±2.2			13.2	**0.001**
	Mill Seat	6.8±1.8			12.8	**0.002**
Actinobacteria	**HANA**	2.8±0.3[b]	18.9	**<0.01**	9.1	**0.011**
	HANA Wooded	3.7±0.6[b]			11.8	**0.003**
	Mill Seat	5.9±0.5[a]			5.98	0.050
Planctomycetes	**HANA**	4.5±0.2[a]	10.5	**<0.01**	5.5	0.064
	HANA Wooded	3.2±0.2[b]			5.2	0.07
	Mill Seat	3.9±0.3[ab]			3.9	0.14
Anaerolineae	**HANA**	3.7±0.3	0.37	0.83	9.9	**<0.01**
	HANA Wooded	4.1±0.6			10.7	**0.005**
	Mill Seat	3.5±0.5			12.1	**0.002**
Vicinamibacteria	**HANA**	3.3±0.3	0.31	0.86	6.9	**0.032**
	HANA Wooded	4.9±1.0			11.8	**0.003**
	Mill Seat	3.3±0.4			6.2	**0.046**
Clostridia	**HANA**	3.5±0.2	4.5	0.10	0.74	0.69
	HANA Wooded	3.1±0.2			11.3	**0.003**
	Mill Seat	3.0±0.6			8.3	**0.016**
Polyangia	**HANA**	3.3±0.2	4.9	0.09	4.2	0.12
	HANA Wooded	3.3±0.2			3.1	0.21
	Mill Seat	2.5±0.3			2.1	0.35

Table 4.5-Relative abundances of the top 10 bacterial classes across treatment in each wetland. (Mean ± SE). Unique letters indicate significant differences based on a Wilcoxon/Kruskal-Wallis test and Dunn's post hoc test with a Bonferroni adjustment.

Bacterial Class	Treatment	HANA Emergent	HANA	Mill Seat
Alphaproteobacteria	***Control***	14±1	15±1[a]	19±1[a]
	Compost	14±1	14±1[a]	14±1[b]
	Reference	15±1	5.9±1[b]	18±1[ab]
Gammaproteobacteria	***Control***	16±1	13±1[ab]	14±1
	Compost	16±2	12±0.4[b]	14±1
	Reference	13±1	19±2[a]	15±1
Bacteroidia	***Control***	7.5±0.4[ab]	5.6±0.5[ab]	6.9±0.9
	Compost	6.4±0.4[b]	5.0±0.7[b]	5.1±0.3
	Reference	10±1[a]	14±1[a]	5.0±1
Bacilli	***Control***	2.9±0.3[b]	4.3±0.5[ab]	2.7±0.4[b]
	Compost	8.5±2[a]	18±3[a]	16±3[a]
	Reference	2.8±0.2[b]	1.2±0.1[b]	1.8±0.2[b]
Actinobacteria	***Control***	3.1±0.1[ab]	3.6±0.2[ab]	6.7±1.3
	Compost	3.5±0.3[a]	5.7±1.0[a]	6.5±0.5
	Reference	1.9±0.2[b]	0.8±0.1[b]	4.3±0.5
Planctomycetes	***Control***	5.1±1	3.8±0.2	4.2±0.4
	Compost	4.5±0.3	3.2±0.4	4.2±0.6
	Reference	3.7±0.2	2.0±0.4	3.2±0.2
Anaerolineae	***Control***	4.9±0.4[a]	1.5±0.2[b]	3.4±0.5[a]
	Compost	3.6±0.4[ab]	5.5±0.9[a]	5.6±0.6[a]
	Reference	2.5±0.3[b]	5.9±0.7[a]	1.6±0.3[b]
Vicinamibacteria	***Control***	3.9±0.5[a]	9.5±0.8[a]	3.5±0.6[a]
	Compost	3.8±0.5[a]	3.1±0.9[ab]	1.8±0.2[a]
	Reference	2.2±0.9[a]	0.91±0.2[b]	4.6±0.7[a]
Clostridia	***Control***	3.6±0.4	2.4±0.3[b]	3.1±1.5[b]
	Compost	3.7±0.4	4.1±0.2[a]	4.4±0.4[a]
	Reference	3.3±0.4	2.8±0.3[b]	1.6±0.2[b]
Polyangia	***Control***	2.9±0.4	3.2±0.4	2.1±0.4
	Compost	2.8±0.4	3.6±0.3	3.0±0.3
	Reference	4.0±0.4	2.7±0.3	2.6±0.3

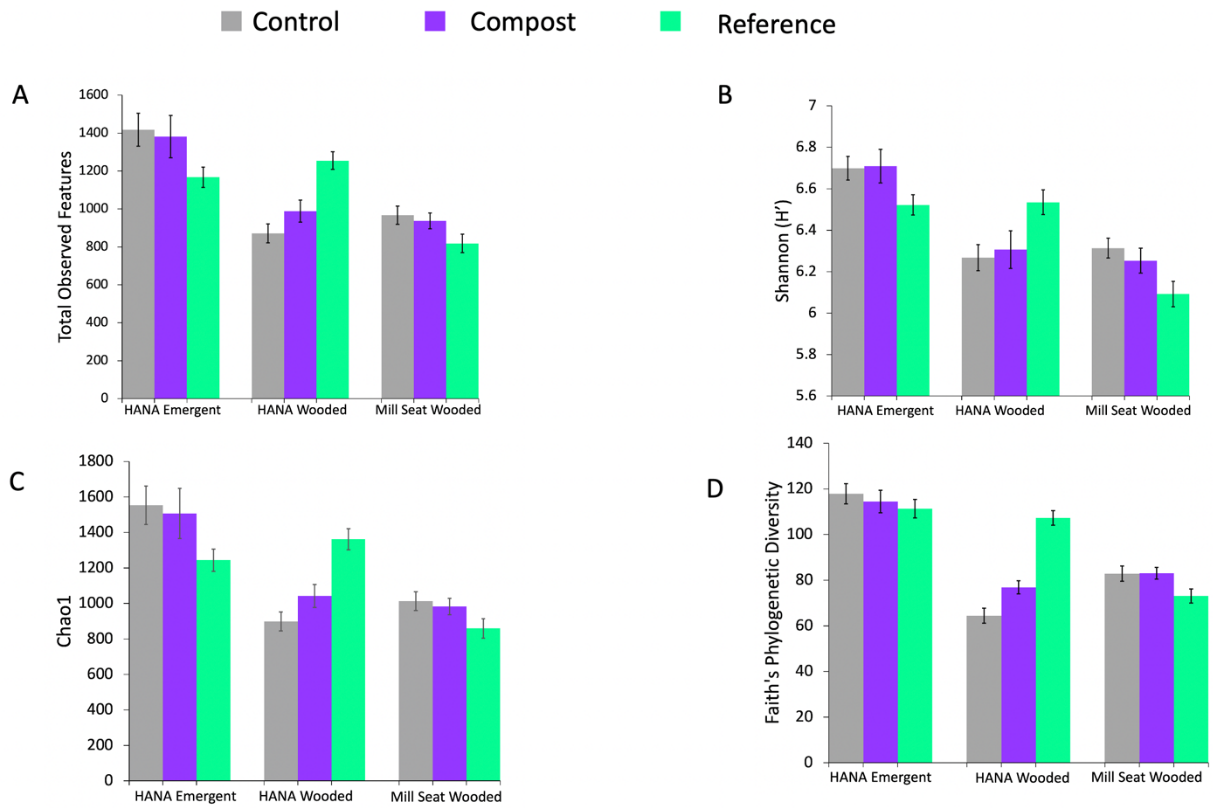

Figure 4.7- Alpha diversity metrics in control, compost and reference sites at the three wetland locations. Mean ± SE A) Total observed features, B)Shannon diversity index, C) Chao1 index, and D) Faith's Phylogenetic Diversity.

Table 4.6-Microbial Diversity between wetlands and within each wetland.Using Kruskall-Wallis test and Dunn's post hoc test with Bonferroni adjustment

Diversity Index	**Site**	**Whole Site**	**Between Site**		**Within Site**	
			$X^2_{2,51}$	*p*	$X^2_{2,1}$	*p*
Total Observed Features	**HANA**	1332±65	21.9	**<0.001**	4.9	0.09
	HANA Wooded	1013±48			9.96	**0.007**
	Mill Seat	880±43			4.4	0.11
Shannon (H')	**HANA**	6.67±0.05	24.5	**<0.001**	6.0	**0.05**
	HANA Wooded	6.35±0.05			6.3	**0.04**
	Mill Seat	6.19±0.06			5.6	0.06
Chao1	**HANA**	1440±78	21.4	**<0.001**	5.2	0.07
	HANA Wooded	1067±56			9.9	**0.007**
	Mill Seat	918±47			3.6	0.17
Phylogenetic Diversity	**HANA**	114±3	23.3	**<0.001**	2.3	0.32
	HANA Wooded	89±5			11.8	**0.003**
	Mill Seat	80±3			5.1	0.08

Table 4.7- Microbial Diversity across the three treatments at each site. using Kruskall-Wallis test and Dunn's post hoc test with Bonferroni adjustment.

Diversity	**Treatment**	**HANA**	**HANA**	**Mill Seat**
Total observed features	***Control***	1401±110	867±49[b]	974±39
	Compost	1469±103	996±56[ab]	920±80
	Reference	1125±84	1260±40[a]	746±74
Shannon (H')	***Control***	6.7±0.1	6.3±0.1	6.3±0.1
	Compost	6.8±0.1	6.3±0.1	6.2±0.1
	Reference	6.5±0.1	6.5±0.05	6.0±0.1
Chao1	***Control***	1527±135	897±53[b]	1019±44
	Compost	1596±123	1039±63[ab]	957±86
	Reference	1196±100	1364±37[a]	778±84
Phylogenetic Diversity	***Control***	116±5	72±3[b]	86±4
	Compost	118±5	86±3[ab]	85±5
	Reference	107±6	119±3[a]	70±5

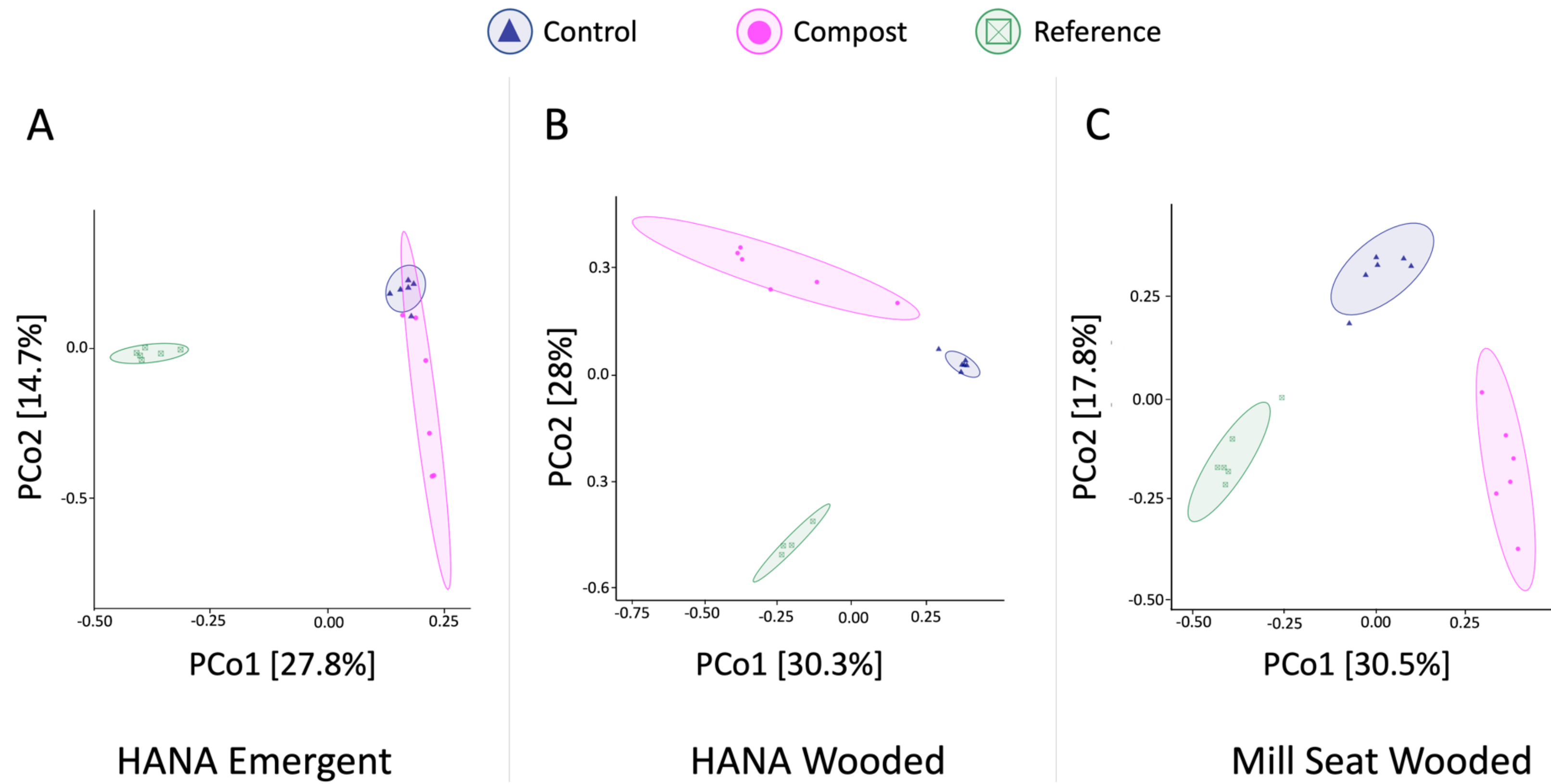

Figure 4.8-PCoA of microbial communities across treatments. A) HANA Emergent B) HANA Wooded and C)Mill Seat Wooded

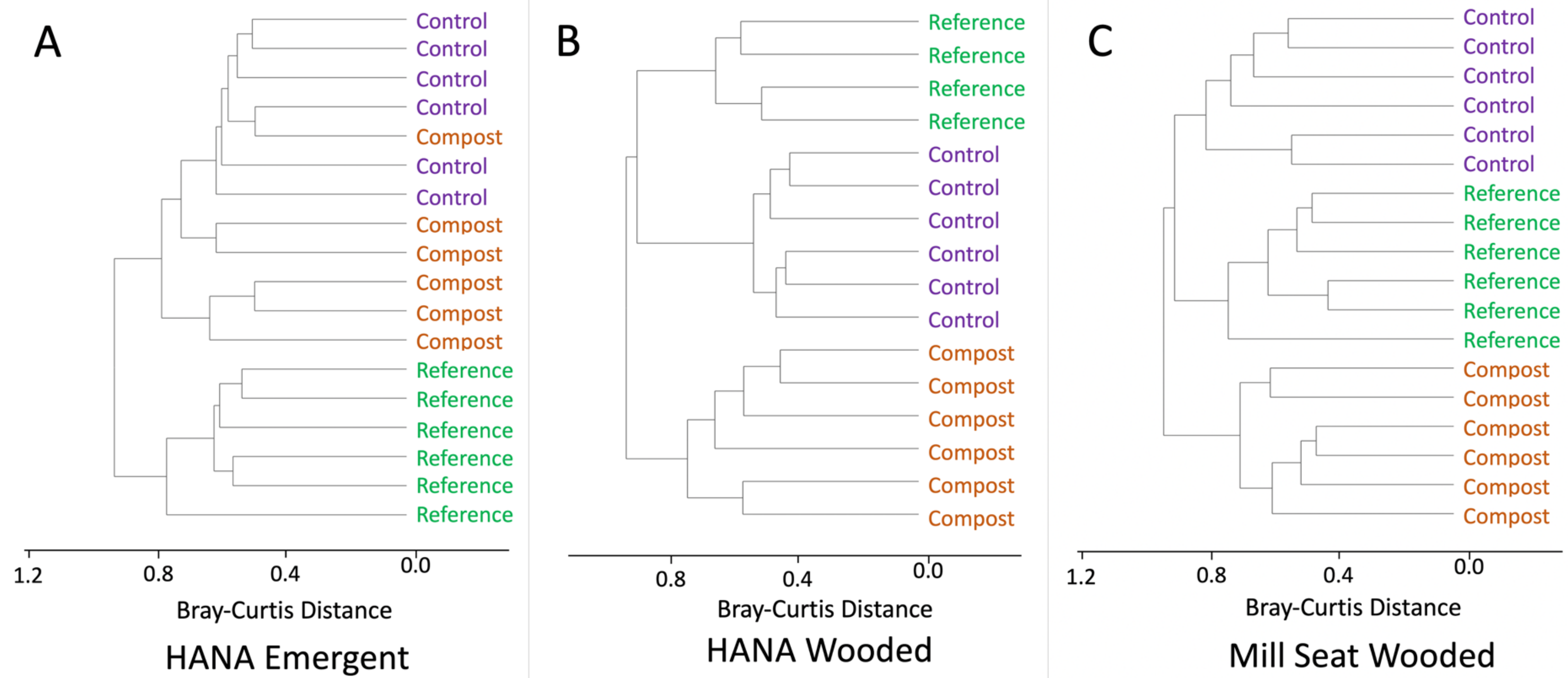

Figure 4.9-Bray-Curtis Cluster of microbial communities sampled in control, compost, and reference plots. A) HANA Emergent B) HANA Wooded and C) Mill Seat Wooded wetlands

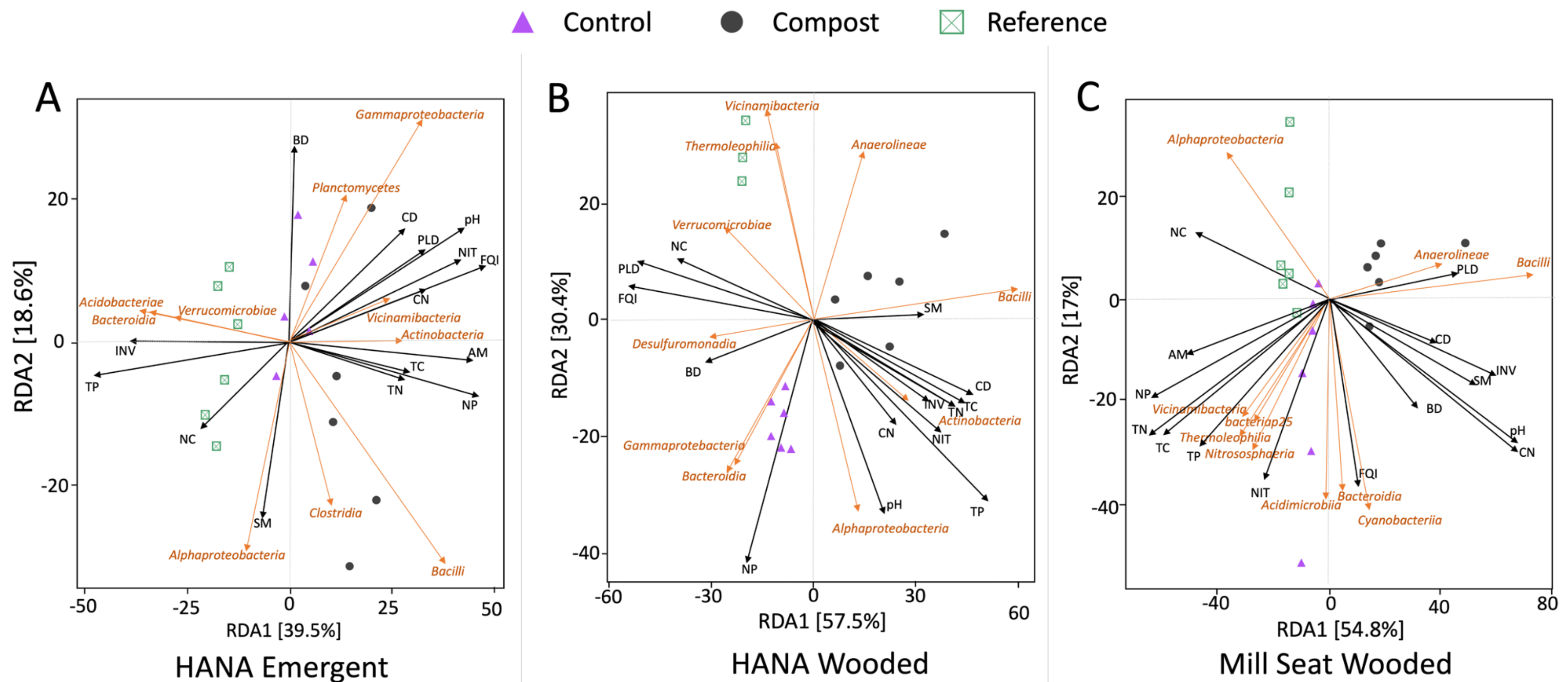

Figure 4.10-RDA of environmental factors correlated with key bacterial classes. Samples collected in control, compost and reference plots at A) HANA Emergent B) HANA Wooded and C)Mill Seat Wooded wetlands. PLD=plant diversity, FQI=floristic quality Index, NC= native plant cover, INV= Invasive plant cover, NIT=nitrate, AM=Ammonium, TN=total nitrogen, TC=total carbon, TP=total phosphorus, BD=bulk density, NP=molar N:P, CN=molar C:N, SM=soil moisture, CD = carbon density

Table 4.8-Metabolic function in each wetland and across treatments within each wetland. Showing the Chi square results of the Kruskall-Wallis test comparing between the three wetland sites (Between Site comparison) and between treatments within a wetland (Within Site).

Metabolic function	**Site**	**Whole Site**	**Between Site**		**Within Site**	
		%	$X^2_{2,51}$	***p***	$X^2_{2,1}$	***p***
Aerobic Chemoheterotrophy	**HANA**	14±0.3[b]	11.82	**0.0027**	1.08	0.58
	HANA Wooded	15±0.9[a]			8.5	**0.014**
	Mill Seat	16±0.5[a]			3.5	0.17
Anaerobic Chemoheterotrophy	**HANA**	3.4±0.2[a]	7.8	**0.0198**	1.16	0.56
	HANA Wooded	3.0±0.4[ab]			13.2	**0.001**
	Mill Seat	2.6±0.2[b]			3.8	0.15
Dark Hydrogen Oxidation	**HANA**	1.6±0.1	2.6	0.27	1.3	0.5
	HANA Wooded	1.9±0.2			10.1	**0.006**
	Mill Seat	1.8±0.1			9.5	**0.009**
Fermentation	**HANA**	1.9±0.1	3.6	0.16	2.3	0.31
	HANA Wooded	1.7±0.2			9.01	**0.011**
	Mill Seat	1.7±0.1			1.5	0.48
Phototrophy	**HANA**	1.4±0.1	1.2	0.55	2.1	0.35
	HANA Wooded	1.2±0.2			7.8	**0.02**
	Mill Seat	1.9±0.5			11.4	**0.003**
Methylotrophy	**HANA**	1.6±0.1[a]	16.7	**0.0002**	1.2	0.55
	HANA Wooded	1.2±0.2[ab]			13.2	**0.001**
	Mill Seat	0.7±0.1[b]			12.8	**0.002**
Hydrocarbon degradation	**HANA**	1.4±0.1[a]	15.5	**0.0004**	1.2	0.55
	HANA Wooded	1.0±0.2[ab]			11.4	**0.003**
	Mill Seat	0.6±0.1[b]			10.5	**0.005**
Methanotrophy	**HANA**	1.3±0.1[a]	15.8	**0.0004**	1.7	0.42
	HANA Wooded	0.89±0.2[b]			13.3	**0.001**
	Mill Seat	0.54±0.1[b]			12.5	**0.002**
Photosynthetic Cyanobacteria	**HANA**	0.72±0.09	0.35	0.84	1.07	0.58
	HANA Wooded	0.69±0.16			7.1	**0.028**
	Mill Seat	1.3±0.4			11.8	**0.003**
Nitrate Reduction	**HANA**	0.72±0.04	0.71	0.7	1.14	0.56
	HANA Wooded	0.79±0.06			4.2	0.12
	Mill Seat	0.72±0.05			7.4	**0.025**
Nitrogen Fixation	**HANA**	0.95±0.07	4.1	0.13	1.6	0.45
	HANA Wooded	0.73±0.12			2.3	0.31
	Mill Seat	0.96±0.18			9.0	**0.011**
Methanogenesis	**HANA**	0.71±0.04[ab]	8.25	**0.0162**	0.32	0.85
	HANA Wooded	0.82±0.10[a]			6.5	**0.039**
	Mill Seat	0.53±0.07[b]			5.3	0.70

Table 4.9-Metabolic function by percent in each of the treatments within each wetland. Within each zone, superscripted letters indicate similar grouping of treatments based on post hoc analysis.

Metabolic	Treatment	HANA Emergent	HANA Wooded	Mill Seat
Aerobic Chemoheterotrophy	***Control***	14±0.5	17±0.4^{a}	17±0.8
	Compost	14±0.8	17±0.3^{a}	16±0.7
	Reference	14±0.4	9.5±1.2^{b}	16±1
Anaerobic Chemoheterotrophy	***Control***	3.3±0.1	1.8±0.2^{b}	2.7±0.4
	Compost	3.4±0.3	2.8±0.1^{ab}	2.9±0.2
	Reference	3.5±0.4	5.2±0.4^{a}	2.2±0.2
Dark Hydrogen Oxidation	***Control***	1.5±0.1	2.3±0.1^{a}	1.4±0.1^{b}
	Compost	1.7±0.1	2.0±0.2^{ab}	1.8±0.1^{ab}
	Reference	1.7±0.2	1.0±0.1^{b}	2.2±0.1^{a}
Fermentation	***Control***	1.8±0.1	1.1±0.1^{b}	1.9±0.2
	Compost	1.7±0.1	1.4±0.2^{ab}	1.5±0.2
	Reference	2.1±0.2	3.0±0.3^{a}	1.6±0.1
Phototrophy	***Control***	1.4±0.1	0.6±0.2^{b}	3.5±1.1^{a}
	Compost	1.2±0.2	1.1±0.3^{ab}	1.9±0.5^{ab}
	Reference	1.6±0.3	2.2±0.3^{a}	0.29±0.1^{b}
Methylotrophy	***Control***	1.8±0.2	0.39±0.1^{b}	0.7±0.1^{ab}
	Compost	1.6±0.3	1.1±0.1^{ab}	1.1±0.1^{a}
	Reference	1.4±0.2	2.5±0.3^{a}	0.4±0.06^{b}
Hydrocarbon degradation	***Control***	1.4±0.2	0.45±0.08^{b}	0.53±0.06^{b}
	Compost	1.6±0.3	0.82±0.09^{ab}	0.86±0.04^{a}
	Reference	1.2±0.2	2.2±0.3^{a}	0.48±0.09^{b}
Methanotrophy	***Control***	1.3±0.2	0.2±0.1^{b}	0.52±0.08^{ab}
	Compost	1.5±0.3	0.75±0.1^{ab}	0.85±0.04^{a}
	Reference	1.1±0.2	2.1±0.3^{a}	0.25±0.03^{b}
Photosynthetic Cyanobacteria	***Control***	0.75±0.1	0.28±0.13^{b}	2.6±1^{a}
	Compost	0.62±0.1	0.58±0.23^{ab}	1.4±0.5^{a}
	Reference	0.81±0.2	1.47±0.3^{a}	0.03±0.02^{b}
Nitrate Reduction	***Control***	0.72±0.08	0.84±0.13	0.66±0.05^{a}
	Compost	0.80±0.07	0.88±0.09	0.91±0.07^{a}
	Reference	0.63±0.11	0.58±0.05	0.60±0.1^{a}
Nitrogen Fixation	***Control***	0.92±0.1	0.47±0.09	1.3±0.2^{a}
	Compost	0.86±0.13	0.94±0.27	1.2±0.4^{a}
	Reference	1.1±0.1	0.82±0.18	0.4±0.1^{b}
Methanogenesis	***Control***	0.7±0.1	0.59±0.04^{a}	0.31±0.04
	Compost	0.7±0.1	0.72±0.15^{a}	0.69±0.15
	Reference	0.7±0.0	1.3±0.1^{a}	0.58±0.14

Discussion

Created wetlands may follow two pathways of succession: either convergence towards a desirable reference wetland or divergence from that reference (Matthews and Spyreas 2010). Studies have shown that when creating wetlands, initial conditions over the first few years may be desirable, but in time, plant species composition may change in response to underlying hydrology and soil conditions and this change will continue in situations with poor or no management (Vanw inkle 2021). This may translate into the types of changes in soil microbial communities that we observed in this study. Although the studied created wetlands may be on a trajectory that is alternate to the intended goals, they may eventually reach the reference stage through a meandering path toward convergence or an acceptable deviation from expectations. However, in many cases, created wetlands end up in an alternative state that is not similar to a reference wetland (Suding et al. 2004). In this research, we affirmed the expectation that created wetlands may be on a trajectory to a state distinct from the desired mature reference wetland, with highly site-specific drivers that are dependent on antecedent land use, site management history, and wetland type.

The three wetland groups in this study are unique from one another in both abiotic and biotic characteristics. While the differences between emergent and wooded wetlands were anticipated based on a priori hydrogeomorphic classification, the difference between the two wooded wetlands was less expected. The emergent wetlands had relatively high organic matter and nutrients and higher plant diversity. This variation was reflected in the microbial diversity, with significantly greater values for all diversity metrics in emergent relative to wooded sites. While the diversity between the two wooded sites was similar, there was greater soil carbon, and nutrients at the Mill Seat site. These differences were also reflected in the relative abundance of microorganisms. For example, *Actinobacteria*, key organisms in decomposition of refractory organic matter, were much greater in the Mill Seat wetlands and likely reflect the much older nature of the reference site (Table 3.4). In other cases, the two HANA sites group together; *Polyangi*a are typically more associated with terrestrial soils (Garcia and Müller 2014) and may reflect the more recent transition from terrestrial land use to wetland, even for the reference sites at HANA. Within both wooded reference wetlands, but especially at Mill Seat, invasive plant species and ash die-off due to invasion by the emerald ash borer may also be altering soil properties and the soil microbial community. Within each site, the site-specific drivers of

microbial community structure are unique and begin to illustrate the complexity of applying a one-size-fits all model to wetland restoration.

The overarching goal of wetland creation is to drive succession toward the desired reference wetland. But these reference wetlands are constantly changing due to outside factors such as nutrient enrichment from nearby farms, residential and commercial operations, and the ongoing push (temperature change) and pulse (erratic interannual precipitation). Internally, both natural reference and created wetlands are also impacted by invasive species, creating restoration setbacks as well as a shifting target reference state. In the HANA Emergent site, the created wetland is in many ways becoming a better representative of an emergent wetland than the reference wetland, with high organic matter, nutrient content, and higher floristic quality (Table 3.1). The reference wetland is under very little management, with minor invasive plant control efforts that include *Lythrum salicaria* management through annual, manual cutting and the appearance of the *Gallerucella* beetle introduced to New York State as a biocontrol agent (Grevstad 2006), and management of beaver populations to prevent flooding behind dams. Water levels have varied substantially over the past decade, particularly during drought years (e.g. 2017), but have been more consistent in recent years (Tyler unpub. data). The high *Typha* spp. cover leads to lower plant diversity overall, as water levels and climate variability seem to consistently favor cattails. In contrast, the abundance of native species at the created sites, which may be related to intensive management during the permit period for the wetland, and continued application of glyphosate to control monoculture forming invasive plants, suggests the formation of a diverse habitat. The site was routinely treated with glyphosate through the fall of 2019, when the permit period ended.

The soil microbial community in the HANA Emergent created wetland had higher RA of *Gammaproteobacteria* and *Anaerolineae* versus the reference wetland. Since *Gammaproteobacteria* is large group including taxa involved in many key ecosystem functions, the reason for these differences should be further explored (Williams et al. 2010). *Anaerolineae* on the other hand is known to occur in areas of high TP (Xia et al. 2016), however the reference site has higher TP. While the form of P was not measured here, perhaps the more recent history of row crop agriculture at this site leads to greater availability of orthophosphate in the soils (Table 3.1 and Table 3.5) (Perillo et al. 2021). Further, the high nitrate levels at the created site

may promote bacteria within the *Anaerolineae* capable of nitrate reduction (Gonzalez-Gil et al. 2015). Thus, the residual impacts of more recent farming may play a role in community composition at the created emergent wetland.

In the HANA Wooded wetland, the created wetland has had a complex restoration history, which has impacted its successional trajectory. Roughly two years after construction, pumping of water from the adjacent aquifer at the nearby landfill resulted in a rapid drying of this wetland. While pumping had ceased at the time of this study, and remedial ditching was completed to restore hydrologic connectivity, this history resulted in the development of a plant community more characteristic of a wet meadow (Appendix 2). This is in stark contrast to the HANA Wooded reference site, which is a natural vernal pool found in a forested section of HANA. While it is not far from the created wetlands, its soil characteristics are distinctly different from the created wetland (Table 3.1), and beneath the more organic surface layer the soils are sandy and very low in carbon and nutrients (Appendix 2). These differences are reflected in the soil microbial community, which has higher RA of both *Gammaproteobacteria* and *Bacteroidia* as well as higher percentages of anerobic chemoheterotrophy and lower percentages of aerobic chemoheterotrophy compared to the created wetlands.

At the Mill Seat Wooded site, the created wetlands are very young and at the time of sampling did not display any similarities to the reference, except in native plant cover (Table 3.1). However, it may be too soon to evaluate the impacts of compost addition on the successional trajectory of this wetland as compost additions only occurred twice before the time of sampling. Differences in soil nutrients including high %TN, %TP, %TC and low C:N at the reference site may be driving differences in the microbial community between the created wetlands and the reference wetland (Table 3.1). *Vicinamibacteria* has higher RA in the reference than the created wetland, perhaps because of the known role of *Vicinamibacteria* in nitrogen and phosphorus removal (Kristensen et al. 2021). Photosynthetic cyanobacteria, which are known for fixing nitrogen, were also higher in the control plots compared to the reference, where low nitrogen availability and relatively high light availability may have favored their establishment. The lack of a closed canopy in the young created wetlands may also explain the higher percentage of phototrophs compared to the mature wooded reference site.

In all three created wetlands we see that after multiple years of compost addition, the soil organic matter and carbon was higher in amended plots. At the Mill Seat site this increase was less substantial, due to the shorter time since the initiation of compost addition, however it still shows a trend of increase %OM and TC. Along with carbon, nitrogen and phosphorus levels were also elevated in composted amended plots, although to a lesser extent. Prior studies have shown that this has contributed to an increase in potential denitrification in the two HANA wetlands (McGowan 2020, Huang 2021). This difference in function is expected to be associated with a shift in the soil microorganisms involved in nitrate reduction, however, our metabolic function analysis only showed significant increases in the percentage of nitrate reducing microbes in the Mill Seat Wooded compost plots. We did observe that across all wetlands, *Bacilli,* a group are known to include denitrifiers (Zhang et al. 2016), were higher in compost plots than either control or reference plots, supporting prior observations of higher potential denitrification rates in HANA compost soils and suggesting that higher rates of denitrification may also occur in compost amended plots at Mill Seat.

There were additional microbial community differences in the compost plots that suggest a shift towards the reference microbial community. At the HANA Wooded site many of the percentages for metabolic functions in compost plots fell between the reference and control plots, suggesting a shift towards the reference state. This pattern was also observed for dark hydrogen oxidation and phototrophy at the Mill Seat Wooded site. Further, *Anaerolineae* had higher RA in both the reference and the compost plots compared to the control at the HANA Wooded site. This is potentially due to compost and reference soils having more bioavailable TP, nitrate, or higher soil moisture, which support the nutrient and anaerobic conditions associated with this group of organisms (Gonzalez-Gil et al. 2015).

Our results suggest that slight changes in current management practices can drive the successional trajectory of created wetlands towards a reference state. However, even this small study suggests that based on site characteristics and stochastic events (such as precipitation, or lack of), adaptive management plans need to be developed to meet restoration goals. For instance, leaf litter compost could be applied from nearby source (nearby wetland area) rather than residential leaf litter compost, providing a source of organic matter more like a natural wetland. This could further alter soil nutrients, introduce local wetland microbes, and more

rapidly shift the microbial community towards a reference state. Use of hyper localized compost may also deter invasive species and promote establishment of native plant species.

Conclusions

Creating appropriate and functional wetlands to mitigate unavoidable loss of wetlands elsewhere requires a three-part strategy. Prior to construction, proper siting includes determining the appropriate hydrology, soil conditions and vegetation present. During construction, soil amendments and seeding for appropriate vegetation may be key strategies to ensure success. Finally, monitoring of hydrology and vegetation, with appropriate adaptive steps to promote the trajectory of wetland structural and functional development towards that of the desired performance goals outlined in the original mitigation plan (Zedler 2000). Current wetland management approaches should be adapted based on underlying conditions of the created wetland and the desired goals specific to plant species mix, nutrient pools and carbon sequestration. Ongoing studies should continue to aid the trajectory of the created wetland. Management of these systems is labor intensive and time consuming, therefore goals should be focus on the long term, with yearly assessments adapting to the needs of individual created wetlands.

Appendices

Appendix 1- Salt Marsh

Table A.10-Grain Size portions for each of the vegetation zones.

Soil Grain Size	Vegetation Zone	Young Marsh	Mature Marsh
%Sand	**Edge**	94%	15%
	Meadow	32%	33%
	Upper	96%	14%
	Die off	82%	10%
%Silt	**Edge**	2%	50%
	Meadow	36%	32%
	Upper	0%	44%
	Die off	8%	47%
%Clay	**Edge**	4%	47%
	Meadow	32%	35%
	Upper	4%	42%
	Die off	10%	43%

Table A.11-Young Marsh all Metabolic functions.

	Young Marsh							
	Edge	**+/- SE**	**Meadow**	**+/- SE**	**Upper**	**+/- SE**	**Die-Off**	**+/- SE**
chemoheterotrophy	9.77	0.37	6.19	0.66	8.37	0.38	5.08	0.29
photoautotrophy	2.67	0.37	0.83	0.09	2.96	0.06	1.10	0.18
phototrophy	2.73	0.35	0.83	0.09	2.99	0.04	1.10	0.18
oxygenic photoautotrophy	2.23	0.33	0.40	0.05	2.74	0.13	0.56	0.17
nitrate reduction	0.63	0.09	0.24	0.05	0.10	0.04	0.11	0.05
sulfate respiration	0.32	0.09	0.25	0.12	0.19	0.04	0.26	0.04
respiration of sulfur compounds	0.35	0.08	0.27	0.12	0.37	0.02	0.28	0.05
predatory or exoparasitic	0.91	0.18	0.44	0.08	0.71	0.15	0.45	0.15

anoxygenic photoautotrophy S oxidizing	0.45	0.04	0.41	0.04	0.22	0.08	0.51	0.08
anoxygenic photoautotrophy	0.45	0.04	0.43	0.05	0.22	0.08	0.53	0.07
intracellular parasites	0.72	0.24	0.23	0.07	0.63	0.14	0.18	0.09
dark hydrogen oxidation	0.16	0.09	0.00	0.00	0.03	0.03	0.03	0.03
dark sulfur oxidation	0.14	0.06	0.08	0.02	0.03	0.03	0.08	0.03
hydrocarbon degradation	0.27	0.02	0.16	0.05	0.32	0.11	0.06	0.03
methanogenesis by CO2 reduction with H2	0.04	0.04	0.00	0.00	0.00	0.00	0.03	0.03
hydrogenotrophic methanogenesis	0.04	0.04	0.04	0.04	0.00	0.00	0.03	0.03
methanogenesis	0.04	0.04	0.04	0.04	0.00	0.00	0.03	0.03
dark sulfite oxidation	0.10	0.02	0.08	0.02	0.00	0.00	0.08	0.03
chitinolysis	0.15	0.03	0.08	0.02	0.11	0.01	0.10	0.01
knallgas bacteria	0.04	0.04	0.00	0.00	0.00	0.00	0.00	0.00
xylanolysis	0.15	0.03	0.10	0.03	0.13	0.02	0.10	0.01
human pathogens all	0.08	0.03	0.02	0.02	0.00	0.00	0.03	0.03

human associated	0.10	0.02	0.02	0.02	0.00	0.00	0.03	0.03
animal parasites or symbionts	0.28	0.05	0.14	0.05	0.18	0.02	0.11	0.08
ureolysis	0.06	0.04	0.00	0.00	0.00	0.00	0.00	0.00
sulfite respiration	0.09	0.01	0.06	0.04	0.00	0.00	0.03	0.03
thiosulfate respiration	0.06	0.02	0.08	0.03	0.18	0.04	0.05	0.05
dark sulfide oxidation	0.02	0.02	0.04	0.02	0.07	0.05	0.00	0.00
aliphatic non methane hydrocarbon degradation	0.08	0.00	0.04	0.02	0.13	0.05	0.03	0.03
nitrate respiration	0.06	0.04	0.07	0.03	0.00	0.00	0.03	0.03
nitrogen respiration	0.06	0.04	0.07	0.03	0.00	0.00	0.03	0.03
methanotrophy	0.02	0.02	0.00	0.00	0.00	0.00	0.00	0.00
methanogenesis by reduction of methyl compounds with H2	0.00	0.00	0.04	0.04	0.00	0.00	0.00	0.00
methanol oxidation	0.00	0.00	0.04	0.02	0.08	0.03	0.00	0.00
methylotrophy	0.02	0.02	0.08	0.04	0.08	0.03	0.00	0.00
aerobic ammonia oxidation	0.06	0.02	0.00	0.00	0.06	0.03	0.00	0.00

nitrification	0.06	0.02	0.00	0.00	0.06	0.03	0.00	0.00
sulfur respiration	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
nitrate denitrification	0.00	0.00	0.02	0.02	0.00	0.00	0.00	0.00
nitrite denitrification	0.00	0.00	0.02	0.02	0.00	0.00	0.00	0.00
nitrous oxide denitrification	0.00	0.00	0.02	0.02	0.00	0.00	0.00	0.00
denitrification	0.00	0.00	0.02	0.02	0.00	0.00	0.00	0.00
nitrite ammonification	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
nitrite respiration	0.00	0.00	0.02	0.02	0.00	0.00	0.00	0.00
dark thiosulfate oxidation	0.00	0.00	0.00	0.00	0.05	0.05	0.00	0.00
manganese respiration	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
human gut	0.02	0.02	0.00	0.00	0.00	0.00	0.00	0.00
mammal gut	0.02	0.02	0.00	0.00	0.00	0.00	0.00	0.00
iron respiration	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
photoheterotrophy	0.06	0.02	0.00	0.00	0.03	0.03	0.00	0.00

Table A.12-Mature Marsh all Metabolic functions.

	Mature Marsh							
Metabolic Process	**Edge**	**+/- SE**	**Meadow**	**+/- SE**	**Upper**	**+/- SE**	**Die-Off**	**+/- SE**
chemoheterotrophy	5.73	0.40	5.88	0.70	4.45	0.21	5.24	1.26
photoautotrophy	0.94	0.07	1.06	0.28	0.63	0.19	0.82	0.11
phototrophy	0.97	0.07	1.11	0.29	0.63	0.19	0.83	0.11
oxygenic photoautotrophy	0.43	0.05	0.70	0.31	0.34	0.17	0.44	0.07
nitrate reduction	0.20	0.03	0.19	0.05	0.04	0.02	0.12	0.10
sulfate respiration	0.36	0.06	0.38	0.09	0.58	0.15	0.24	0.09
respiration of sulfur compounds	0.38	0.07	0.38	0.09	0.59	0.14	0.34	0.18
predatory or exoparasitic	0.35	0.04	0.47	0.11	0.30	0.08	0.27	0.12
anoxygenic photoautotrophy S oxidizing	0.50	0.04	0.37	0.03	0.29	0.04	0.37	0.05
anoxygenic photoautotrophy	0.50	0.04	0.37	0.03	0.29	0.04	0.37	0.05
intracellular parasites	0.36	0.06	0.48	0.07	0.46	0.06	0.38	0.11
dark hydrogen oxidation	0.01	0.01	0.00	0.00	0.02	0.02	0.00	0.00
dark sulfur oxidation	0.08	0.00	0.10	0.01	0.07	0.03	0.06	0.02
hydrocarbon degradation	0.10	0.01	0.00	0.00	0.02	0.02	0.02	0.02
methanogenesis by CO2 reduction with H2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
hydrogenotrophic methanogenesis	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00
methanogenesis	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00
dark sulfite oxidation	0.08	0.00	0.10	0.01	0.07	0.03	0.06	0.02
chitinolysis	0.11	0.03	0.08	0.03	0.07	0.00	0.07	0.02
knallgas bacteria	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

xylanolysis	0.11	0.03	0.08	0.03	0.10	0.01	0.07	0.02
human pathogens all	0.07	0.02	0.10	0.04	0.05	0.02	0.06	0.04
human associated	0.07	0.02	0.10	0.04	0.07	0.02	0.06	0.04
animal parasites or symbionts	0.16	0.03	0.12	0.04	0.10	0.03	0.10	0.04
ureolysis	0.00	0.00	0.03	0.03	0.00	0.00	0.00	0.00
sulfite respiration	0.15	0.03	0.12	0.05	0.17	0.06	0.12	0.05
thiosulfate respiration	0.03	0.02	0.04	0.03	0.05	0.03	0.07	0.07
dark sulfide oxidation	0.03	0.02	0.01	0.01	0.00	0.00	0.02	0.02
aliphatic non methane hydrocarbon degradation	0.01	0.01	0.00	0.00	0.02	0.02	0.00	0.00
nitrate respiration	0.07	0.02	0.05	0.02	0.00	0.00	0.07	0.07
nitrogen respiration	0.07	0.02	0.05	0.02	0.02	0.02	0.07	0.07
methanotrophy	0.07	0.02	0.00	0.00	0.00	0.00	0.02	0.02
methanogenesis by reduction of methyl compounds with H2	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00
methanol oxidation	0.01	0.01	0.00	0.00	0.00	0.00	0.02	0.02
methylotrophy	0.07	0.02	0.01	0.01	0.00	0.00	0.04	0.02
aerobic ammonia oxidation	0.05	0.01	0.02	0.02	0.00	0.00	0.00	0.00
nitrification	0.05	0.01	0.02	0.02	0.00	0.00	0.00	0.00
sulfur respiration	0.00	0.00	0.00	0.00	0.02	0.02	0.04	0.04
nitrate denitrification	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00
nitrite denitrification	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00
nitrous oxide denitrification	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00

denitrification	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00
nitrite ammonification	0.00	0.00	0.00	0.00	0.02	0.02	0.00	0.00
nitrite respiration	0.00	0.00	0.01	0.01	0.02	0.02	0.00	0.00
dark thiosulfate oxidation	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00
manganese respiration	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.02
human gut	0.00	0.00	0.00	0.00	0.02	0.02	0.00	0.00
mammal gut	0.00	0.00	0.00	0.00	0.02	0.02	0.00	0.00
iron respiration	0.00	0.00	0.00	0.00	0.02	0.02	0.02	0.02
photoheterotrophy	0.03	0.01	0.05	0.02	0.00	0.00	0.02	0.02

Appendix 2-Freshwater Wetlands

Plant Species List by Site

Table A.13-HANA Emergent Plant Species List.

	HANA Emergent	
Control	**Compost**	**Reference**
Alisma subcordatum	*Alisma subcordatum*	*Acer rubrum*
Caltha palustris	*Caltha palustris*	*Bidens cernua*
Eleocharis obtuse	*Leersia oryzoides*	*Carex lurida*
Ludwigia palustris	*Ludwigia palustris*	*Leersia oryzoides*
Lycopus americanus	*Lycopus americanus*	*Lemna minor*
Lythrum salicaria	*Lythrum salicaria*	*Ludwigia palustris*
Persicaria amphibium	*Persicaria amphibium*	*Lythrum salicaria*
Phalaris arundinaceae	*Phalaris arundinaceae*	*Persicaria pennsylvanica*
Phragmites australias	*Phragmites australias*	*Scirpus cyperinus*
Pontederia cordata	*Rumex crispus*	*Solanum dulcamara*
Rumex crispus	*Sagittaria latifolia*	*Typha latifolia*
Sagittaria latifolia	*Schoenoplectus acutus*	
Schoenoplectus tabernaemontani	*Solidago canadensis*	
Solidago canadensis	*Symphyotrichum puniceum*	
Typha latifolia	*Typha angustofolia*	
	Typha latifolia	

Table A.14-HANA Wooded Plant Species List.

	HANA Wooded	
Control	**Compost**	**Reference**
Acer negundo	*Acer negundo*	*Arctium minus*
Acer rubrum	*Arctium minus*	*Carex crinita*
Alisma subcordatum	*Carex alopecoidea*	*Eleocharis obtuse*
Arctium minus	*Eutrochium maculatum*	*Equisteum hymele*
Asclepias incarnata	*Galium trifidum*	*Galium trifidum*
Carex alopecoidea	*Lactuca canadensis*	*Lemna minor*
Daucus carota	*Leersia oryzoides*	*Lythrum salicaria*
Lactuca canadensis	*liverwort*	*Persicaria amphibium*
Leersia oryzoides	*Lythrum salicaria*	*Populus deltoides*
Ludwigia palustris	*Persicaria pennsylvanica*	*Salix sericea*
Lythrum salicaria	*Phalaris arundinaceae*	*Schoenoplectus tabernaemontani*
Persicaria pennsylvanica	*Phragmites australias*	*Typha latifolia*
Phalaris arundinaceae	*Ranunculus sceleratus*	*Utricularia vulgaris*
Ranunculus sceleratus	*Solanum dulcamara*	
Schoenoplectus acutus	*Solidago canadensis*	
Solanum dulcamara	*Typha angustofolia*	
Solidago canadensis	*Unknown grass*	
Trifolium pratense	*Vitis aestivalis*	
Typha angustofolia		
Unknown grass		
Unknown weed		

Table A.15-Mill Seat Wooded Plant Species List.

Mill Seat Wooded		
Control	**Compost**	**Reference**
Alisma subcordatum	*Alisma subcordatum*	*Acer rubrum*
Phalaris arundinaceae	*Bidens frondosa*	*Carex crinita*
Schoenoplectus tabernaemontani	*Daucus carota*	*Carex lacustris*
Sium suave	*Ludwigia palustris*	*Equisteum fluviatile*
Sparganium americanum	*Phalaris arundinaceae*	*Fraxinus pennsylvanica*
Typha angustofolia	*Populus deltoides*	*Parthenocissus quinquefolia*
Typha latifolia	*Schoenoplectus tabernaemontani*	*Persicaria pennsylvanica*
	Sium suave	*Rosa palustris*
	Sparganium americanum	*Toxicodendron radicans*
	Typha angustofolia	
	Typha latifolia	

Table A.16-Soil Grain Size for all treatments in each Wetland.

Soil Grain Size	Treatment	HANA Emergent	HANA Wooded	Mill Seat Wooded
%Sand	**Control**	52%	48%	47%
	Compost	52%	48%	47%
	Reference	51%	73%	68%
%Silt	**Control**	33%	37%	26%
	Compost	33%	37%	26%
	Reference	31%	18%	6%
%Clay	**Control**	15%	15%	27%
	Compost	15%	15%	27%
	Reference	19%	9%	26%

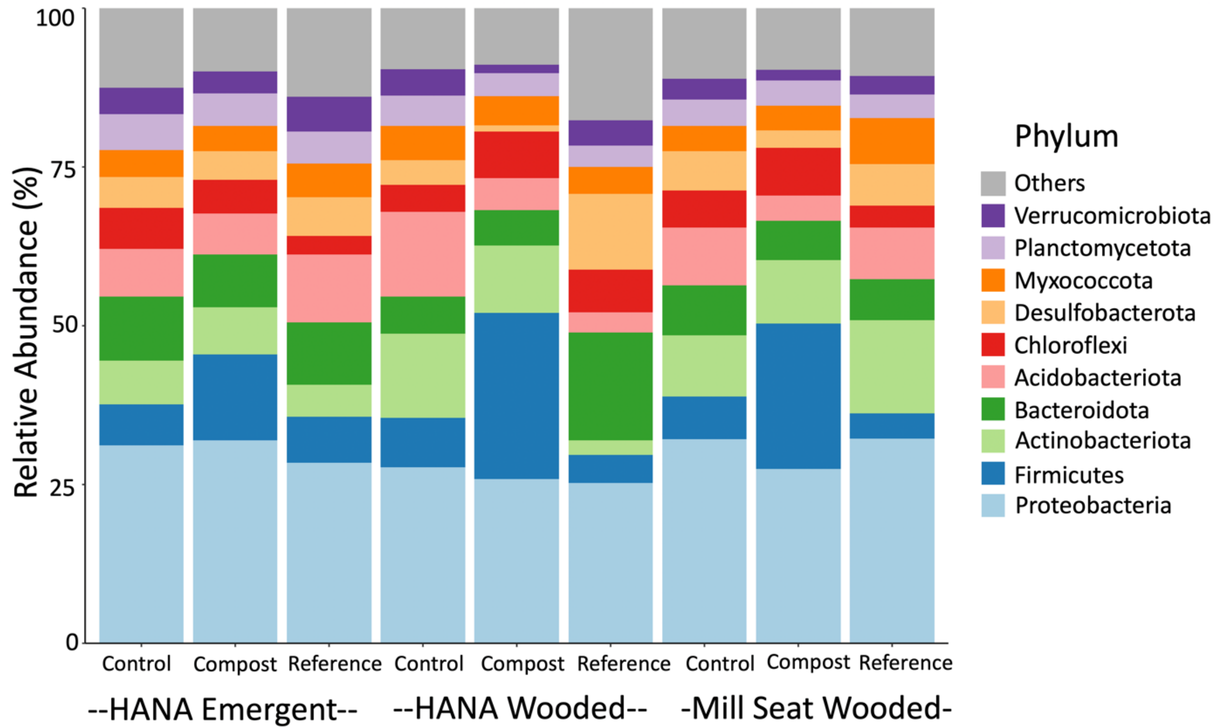

Figure A.11-Relative Abundance on replicates of Soil Amendment experiment.

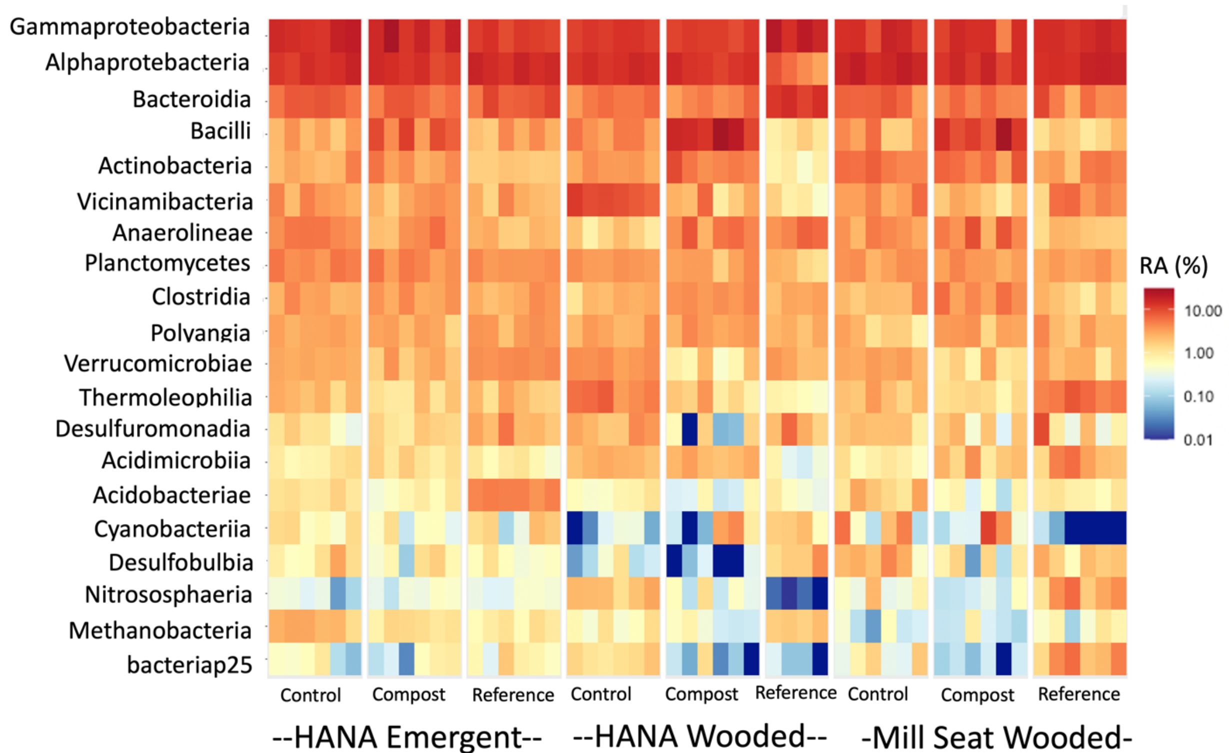

Figure A.12-Heat Map of Relative Abundances of Class for Soil Amendment replicates.

Table A.17-HANA Emergent -All Metabolic functions.

	HANA Emergent					
Metabolic Process	**Control**	**+/- SE**	**Compost**	**+/- SE**	**Reference**	**+/- SE**
chemoheterotrophy	17.06	0.47	17.93	0.77	17.12	0.46
aerobic chemoheterotrophy	13.81	0.53	14.49	0.71	13.65	0.42
anaerobic chemoheterotrophy	3.25	0.15	3.44	0.35	3.47	0.43
methylotrophy	1.59	0.17	1.79	0.29	1.44	0.23
methanotrophy	1.32	0.17	1.54	0.25	1.14	0.22
hydrocarbon degradation	1.41	0.16	1.60	0.26	1.20	0.23
dark hydrogen oxidation	1.49	0.12	1.66	0.10	1.73	0.20
phototrophy	1.41	0.15	1.16	0.17	1.62	0.30
fermentation	1.78	0.09	1.73	0.14	2.12	0.24
nitrogen fixation	0.92	0.10	0.86	0.13	1.07	0.14
predatory or exoparasitic	1.45	0.07	1.32	0.03	1.96	0.10
intracellular parasites	2.15	0.27	1.91	0.27	2.15	0.22
photoautotrophy	0.95	0.12	0.82	0.14	1.26	0.29
photosynthetic cyanobacteria	0.75	0.11	0.62	0.11	0.81	0.22
oxygenic photoautotrophy	0.75	0.11	0.62	0.11	0.81	0.22
animal parasites or symbionts	0.69	0.13	0.71	0.08	0.42	0.07
xylanolysis	0.04	0.02	0.33	0.07	0.05	0.03
nitrate reduction	0.72	0.08	0.80	0.07	0.63	0.11
human associated	0.33	0.08	0.36	0.05	0.22	0.04
nonphotosynthetic cyanobacteria	0.56	0.02	0.65	0.11	0.74	0.09
ureolysis	0.27	0.03	0.35	0.04	0.44	0.14
methanogenesis	0.73	0.10	0.70	0.08	0.72	0.05
human pathogens all	0.33	0.08	0.34	0.04	0.20	0.04
iron respiration	0.31	0.02	0.33	0.02	0.36	0.05
hydrogenotrophic methanogenesis	0.63	0.08	0.64	0.07	0.57	0.04
cellulolysis	0.08	0.03	0.18	0.05	0.24	0.03
photoheterotrophy	0.50	0.06	0.37	0.07	0.43	0.05
methanogenesis by CO2 reduction with H2	0.48	0.06	0.51	0.06	0.43	0.05

aromatic compound degradation	0.42	0.06	0.38	0.03	0.35	0.04
methanol oxidation	0.18	0.03	0.22	0.06	0.29	0.08
aerobic ammonia oxidation	0.31	0.07	0.37	0.09	0.30	0.05
nitrification	0.31	0.07	0.37	0.09	0.30	0.05
respiration of sulfur compounds	0.47	0.03	0.33	0.06	0.68	0.09
nitrogen respiration	0.25	0.09	0.27	0.05	0.23	0.05
anoxygenic photoautotrophy S oxidizing	0.20	0.06	0.20	0.07	0.40	0.07
anoxygenic photoautotrophy	0.20	0.06	0.20	0.07	0.46	0.08
sulfate respiration	0.39	0.03	0.25	0.05	0.59	0.08
sulfur respiration	0.08	0.01	0.07	0.01	0.08	0.03
sulfite respiration	0.15	0.02	0.11	0.02	0.21	0.04
nitrite ammonification	0.00	0.00	0.02	0.01	0.02	0.02
nitrite respiration	0.06	0.03	0.07	0.03	0.08	0.05
dark oxidation of sulfur compounds	0.17	0.06	0.11	0.04	0.02	0.02
human pathogens pneumonia	0.05	0.01	0.06	0.01	0.02	0.02
human gut	0.00	0.00	0.02	0.01	0.02	0.02
mammal gut	0.00	0.00	0.02	0.01	0.02	0.02
dark iron oxidation	0.01	0.01	0.03	0.02	0.00	0.00
nitrate respiration	0.25	0.09	0.25	0.06	0.22	0.05
fumarate respiration	0.03	0.01	0.06	0.01	0.01	0.01
reductive acetogenesis	0.05	0.02	0.05	0.01	0.00	0.00
methanogenesis using formate	0.00	0.00	0.00	0.00	0.01	0.01
methanogenesis by reduction of methyl compounds with H2	0.13	0.02	0.10	0.02	0.15	0.02
dark sulfite oxidation	0.00	0.00	0.00	0.00	0.00	0.00
thiosulfate respiration	0.01	0.01	0.01	0.01	0.01	0.01
nitrate denitrification	0.06	0.03	0.04	0.03	0.07	0.03
nitrite denitrification	0.06	0.03	0.04	0.03	0.07	0.03
nitrous oxide denitrification	0.06	0.03	0.04	0.03	0.07	0.03
denitrification	0.06	0.03	0.04	0.03	0.07	0.03
chitinolysis	0.02	0.01	0.01	0.01	0.04	0.02
knallgas bacteria	0.00	0.00	0.00	0.00	0.00	0.00
nitrate ammonification	0.00	0.00	0.00	0.00	0.00	0.00

dark sulfide oxidation	0.13	0.05	0.04	0.03	0.02	0.02
dark thiosulfate oxidation	0.00	0.00	0.01	0.01	0.00	0.00
manganese oxidation	0.01	0.01	0.01	0.01	0.01	0.01
manganese respiration	0.00	0.00	0.00	0.00	0.00	0.00
aromatic hydrocarbon degradation	0.07	0.04	0.04	0.02	0.03	0.02
aliphatic non methane hydrocarbon degradation	0.07	0.04	0.04	0.02	0.03	0.02
chlorate reducers	0.02	0.01	0.03	0.01	0.02	0.02

Table A.18-HANA Wooded All Metabolic functions.

	HANA Wooded					
Metabolic Process	**Control**	**+/- SE**	**Compost**	**+/- SE**	**Reference**	**+/- SE**
chemoheterotrophy	18.53	0.45	19.50	0.45	14.76	1.00
aerobic chemoheterotrophy	16.72	0.36	16.74	0.36	9.54	1.24
anaerobic chemoheterotrophy	1.82	0.17	2.76	0.17	5.23	0.39
methylotrophy	0.39	0.09	1.07	0.09	2.45	0.27
methanotrophy	0.20	0.07	0.75	0.07	2.12	0.28
hydrocarbon degradation	0.45	0.08	0.82	0.08	2.17	0.27
dark hydrogen oxidation	2.34	0.09	1.96	0.09	1.00	0.11
phototrophy	0.57	0.16	1.07	0.16	2.20	0.25
fermentation	1.10	0.14	1.36	0.14	3.03	0.30
nitrogen fixation	0.47	0.09	0.94	0.09	0.82	0.18
predatory or exoparasitic	1.15	0.15	1.15	0.15	1.27	0.23
intracellular parasites	0.66	0.05	0.59	0.05	1.22	0.23
photoautotrophy	0.46	0.15	0.78	0.15	1.90	0.23
photosynthetic cyanobacteria	0.28	0.13	0.58	0.13	1.47	0.25
oxygenic photoautotrophy	0.28	0.13	0.58	0.13	1.47	0.25
animal parasites or symbionts	0.51	0.07	0.39	0.07	0.59	0.13
xylanolysis	0.11	0.05	0.59	0.05	0.06	0.04

nitrate reduction	0.84	0.13	0.88	0.13	0.58	0.05
human associated	0.11	0.03	0.17	0.03	0.34	0.03
nonphotosynthetic cyanobacteria	0.27	0.08	0.63	0.08	0.60	0.07
ureolysis	0.41	0.08	0.55	0.08	0.23	0.07
methanogenesis	0.59	0.05	0.72	0.05	1.32	0.14
human pathogens all	0.11	0.03	0.17	0.03	0.34	0.03
iron respiration	0.47	0.04	0.23	0.04	0.43	0.04
hydrogenotrophic methanogenesis	0.55	0.05	0.63	0.05	1.19	0.15
cellulolysis	0.21	0.03	0.21	0.03	0.02	0.02
photoheterotrophy	0.22	0.05	0.40	0.05	0.30	0.03
methanogenesis by CO2 reduction with H2	0.44	0.04	0.53	0.04	0.98	0.13
aromatic compound degradation	0.87	0.04	0.48	0.04	0.18	0.04
methanol oxidation	0.07	0.04	0.25	0.04	0.18	0.01
aerobic ammonia oxidation	0.96	0.08	0.33	0.08	0.04	0.02
nitrification	0.96	0.08	0.33	0.08	0.04	0.02
respiration of sulfur compounds	0.21	0.03	0.13	0.03	1.11	0.15
nitrogen respiration	0.31	0.07	0.32	0.07	0.28	0.03
anoxygenic photoautotrophy S oxidizing	0.18	0.04	0.20	0.04	0.43	0.03
anoxygenic photoautotrophy	0.18	0.04	0.20	0.04	0.43	0.03
sulfate respiration	0.09	0.03	0.06	0.03	0.92	0.14
sulfur respiration	0.12	0.01	0.10	0.01	0.10	0.02
sulfite respiration	0.07	0.02	0.06	0.02	0.40	0.06
nitrite ammonification	0.05	0.03	0.02	0.03	0.00	0.00
nitrite respiration	0.21	0.03	0.25	0.03	0.00	0.00
dark oxidation of sulfur compounds	0.17	0.02	0.11	0.02	0.20	0.07

human pathogens pneumonia	0.05	0.02	0.00	0.02	0.08	0.03
human gut	0.00	0.00	0.00	0.00	0.00	0.00
mammal gut	0.00	0.00	0.00	0.00	0.00	0.00
dark iron oxidation	0.07	0.02	0.04	0.02	0.00	0.00
nitrate respiration	0.31	0.07	0.32	0.07	0.28	0.03
fumarate respiration	0.06	0.03	0.03	0.03	0.04	0.02
reductive acetogenesis	0.00	0.00	0.02	0.00	0.08	0.00
methanogenesis using formate	0.00	0.00	0.00	0.00	0.04	0.02
methanogenesis by reduction of methyl compounds with H2	0.12	0.01	0.10	0.01	0.18	0.02
dark sulfite oxidation	0.00	0.00	0.00	0.00	0.02	0.02
thiosulfate respiration	0.00	0.00	0.03	0.00	0.04	0.02
nitrate denitrification	0.16	0.04	0.21	0.04	0.00	0.00
nitrite denitrification	0.16	0.04	0.21	0.04	0.00	0.00
nitrous oxide denitrification	0.16	0.04	0.21	0.04	0.00	0.00
denitrification	0.16	0.04	0.21	0.04	0.00	0.00
chitinolysis	0.22	0.06	0.28	0.06	0.02	0.02
knallgas bacteria	0.05	0.03	0.02	0.03	0.00	0.00
nitrate ammonification	0.05	0.03	0.02	0.03	0.00	0.00
dark sulfide oxidation	0.12	0.01	0.02	0.01	0.20	0.07
dark thiosulfate oxidation	0.00	0.00	0.00	0.00	0.00	0.00
manganese oxidation	0.00	0.00	0.02	0.00	0.00	0.00
manganese respiration	0.00	0.00	0.03	0.00	0.00	0.00
aromatic hydrocarbon degradation	0.25	0.03	0.05	0.03	0.00	0.00

aliphatic non methane hydrocarbon degradation	0.25	0.03	0.07	0.03	0.00	0.00
chlorate reducers	0.04	0.02	0.02	0.02	0.08	0.00

Table A.19-Mill Seat Wooded All Metabolic functions.

	Mill Seat Wooded					
Metabolic Process	**Control**	**+/- SE**	**Compost**	**+/- SE**	**Reference**	**+/- SE**
chemoheterotrophy	20.15	0.51	18.56	0.68	17.79	1.16
aerobic chemoheterotrophy	17.49	0.78	15.66	0.65	15.59	1.00
anaerobic chemoheterotrophy	2.67	0.37	2.90	0.19	2.20	0.20
methylotrophy	0.67	0.08	1.06	0.05	0.38	0.06
methanotrophy	0.52	0.08	0.85	0.04	0.25	0.03
hydrocarbon degradation	0.53	0.06	0.86	0.04	0.48	0.09
dark hydrogen oxidation	1.43	0.12	1.79	0.11	2.21	0.14
phototrophy	3.53	1.14	1.92	0.52	0.29	0.12
fermentation	1.94	0.22	1.52	0.18	1.56	0.15
nitrogen fixation	1.32	0.44	1.17	0.20	0.39	0.08
predatory or exoparasitic	1.21	0.11	0.94	0.10	1.05	0.15
intracellular parasites	0.68	0.15	0.75	0.09	1.40	0.19
photoautotrophy	3.02	1.07	1.66	0.47	0.16	0.07
photosynthetic cyanobacteria	2.61	1.03	1.43	0.47	0.04	0.02
oxygenic photoautotrophy	2.61	1.03	1.43	0.47	0.04	0.02
animal parasites or symbionts	0.83	0.08	0.64	0.10	0.78	0.14
xylanolysis	0.12	0.03	0.60	0.04	0.08	0.04
nitrate reduction	0.66	0.05	0.91	0.07	0.60	0.10
human associated	0.54	0.09	0.41	0.08	0.28	0.12
nonphotosynthetic cyanobacteria	0.53	0.12	0.81	0.11	0.19	0.06
ureolysis	0.83	0.14	0.46	0.05	0.26	0.11

methanogenesis	0.31	0.04	0.69	0.15	0.58	0.14
human pathogens all	0.54	0.09	0.39	0.09	0.28	0.12
iron respiration	0.46	0.03	0.39	0.06	0.36	0.07
hydrogenotrophic methanogenesis	0.30	0.04	0.67	0.14	0.52	0.11
cellulolysis	0.17	0.03	0.13	0.02	0.03	0.03
photoheterotrophy	0.53	0.08	0.33	0.05	0.16	0.06
methanogenesis by CO2 reduction with H2	0.24	0.03	0.59	0.12	0.40	0.09
aromatic compound degradation	0.94	0.08	0.60	0.15	0.93	0.12
methanol oxidation	0.20	0.08	0.17	0.02	0.02	0.02
aerobic ammonia oxidation	0.48	0.16	0.28	0.05	1.15	0.16
nitrification	0.48	0.16	0.28	0.05	1.15	0.16
respiration of sulfur compounds	0.51	0.06	0.43	0.11	0.33	0.05
nitrogen respiration	0.27	0.05	0.32	0.05	0.18	0.07
anoxygenic photoautotrophy S oxidizing	0.41	0.12	0.24	0.04	0.12	0.06
anoxygenic photoautotrophy	0.41	0.12	0.24	0.04	0.12	0.06
sulfate respiration	0.39	0.06	0.26	0.08	0.25	0.07
sulfur respiration	0.10	0.00	0.10	0.04	0.09	0.03
sulfite respiration	0.17	0.04	0.10	0.02	0.11	0.02
nitrite ammonification	0.00	0.00	0.02	0.02	0.00	0.00
nitrite respiration	0.02	0.02	0.13	0.04	0.03	0.03
dark oxidation of sulfur compounds	0.38	0.05	0.13	0.02	0.33	0.07
human pathogens pneumonia	0.09	0.03	0.05	0.02	0.08	0.04
human gut	0.00	0.00	0.02	0.02	0.00	0.00
mammal gut	0.00	0.00	0.02	0.02	0.00	0.00
dark iron oxidation	0.02	0.02	0.00	0.00	0.00	0.00

nitrate respiration	0.27	0.05	0.30	0.05	0.18	0.07
fumarate respiration	0.05	0.02	0.07	0.02	0.00	0.00
reductive acetogenesis	0.02	0.02	0.00	0.00	0.00	0.00
methanogenesis using formate	0.00	0.00	0.00	0.00	0.00	0.00
methanogenesis by reduction of methyl compounds with H2	0.06	0.02	0.08	0.03	0.12	0.03
dark sulfite oxidation	0.00	0.00	0.00	0.00	0.00	0.00
thiosulfate respiration	0.02	0.02	0.07	0.03	0.00	0.00
nitrate denitrification	0.02	0.02	0.12	0.03	0.03	0.03
nitrite denitrification	0.02	0.02	0.12	0.03	0.03	0.03
nitrous oxide denitrification	0.02	0.02	0.12	0.03	0.03	0.03
denitrification	0.02	0.02	0.12	0.03	0.03	0.03
chitinolysis	0.38	0.07	0.18	0.07	0.13	0.03
knallgas bacteria	0.00	0.00	0.00	0.00	0.00	0.00
nitrate ammonification	0.00	0.00	0.00	0.00	0.00	0.00
dark sulfide oxidation	0.27	0.07	0.02	0.02	0.30	0.07
dark thiosulfate oxidation	0.00	0.00	0.00	0.00	0.00	0.00
manganese oxidation	0.04	0.02	0.00	0.00	0.00	0.00
manganese respiration	0.00	0.00	0.00	0.00	0.00	0.00
aromatic hydrocarbon degradation	0.02	0.02	0.02	0.02	0.23	0.07
aliphatic non methane hydrocarbon degradation	0.02	0.02	0.02	0.02	0.23	0.07
chlorate reducers	0.04	0.02	0.00	0.00	0.00	0.00

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