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DEPTH AND TIME RELATED VARIATIONS OF MICROBIAL COMMUNIITES IN AN EMERGENT FRESHWATER WETLAND

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DEPTH AND TIME RELATED VARIATIONS OF MICROBIAL COMMUNIITES IN AN EMERGENT FRESHWATER WETLAND

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by

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ABSTRACT

Soils, and the microbial communities contained within them, are vital for most chemical, physical, and biological processes. This study investigated how microbial community structure responded to environmental changes, such as hydrology, across vertical space (depth) and time in an emergent fresh water wetland. Research was conducted in a non-tidal freshwater wetland along the James River (Charles City County, Virginia) by establishing plots in two areas that experienced different hydrologic regimes and plant communities. Soil cores (30 cm) were collected monthly from January 2008 to February 2009, and then every two to three months thereafter until October 2009, for a total of 17 sampling events. The soil cores were divided by depth (Top: 0 – 10 cm, Bottom: 20 – 30 cm) and analyzed for a variety of soil properties including: pH, organic matter (OM), water content (WC), C:N, redox, and root biomass. Additionally, above-ground plant communities were monitored during the growing seasons. Based on preliminary analysis, one date from each season (Winter, Spring, Summer, and Fall) from both sampling years were selected for in depth analysis of the microbial community structure via Terminal Restriction Fragment Length Polymorphism (T-RFLP) of 16S-rRNA. Analysis of variance (ANOVA) found significant differences were found between the environmental parameters in regards to site, depth, and season. Three physical-chemical variables (WC, OM, and redox) were different between sites, but the majority of environmental parameters were significantly different between depths and seasons. The dominant environmental effect on microbial communities was soil depth and, overall, no seasonal patterns were observed in the microbial communities. Further, archaeal communities were most strongly correlated to changes in water content, while redox was strongly correlated to changes across

depth in the bacterial communities. Collectively, these results demonstrate that wetland microbial communities are not a product of one separate variable or spatial scale, but result from various factors interlinked to shape microbial communities. More long-term studies are needed to investigate interactions between microbial community structure and environmental variables in these dynamic ecosystems.

INTRODUCTION

Wetlands are considered important ecosystems because of the vital ecological functions that they provide, such as sediment trapping, flood mitigation, water purification, and groundwater recharge. The significance of wetlands in terms of global health has recently begun to be recognized, as it has become apparent that their influence and importance far exceeds their size (Mitsch and Gosselink, 2000). Wetlands are very productive environments, globally producing an estimated 4 to 9 Pg of carbon per year, which makes them one of the largest components of the terrestrial carbon pool (Mitsch and Gosselink, 2000). In addition, wetlands are critical because of their ability to ameliorate the effects of nutrient pollution (e.g., nitrate from agricultural runoff), and act as a buffer between aquatic and terrestrial ecosystems. Given that microorganisms are an integral part of these and numerous other economic and ecological functions attributed to wetlands, an enhanced understanding of the environmental controls on microbial community structure and function is essential to a better understanding of biogeochemical cycling and the preservation of these habitats.

Hydric soils are the cornerstone for most chemical, physical, and biological activities in wetlands, and the resident microbial communities are the driving force for many of these processes. In freshwater wetland soils, the majority of the microbial research to date has either focused on the population dynamics of selected groups, such as methanogens (e.g., Utsumi *et al.* 2003, Chang and Yang 2003, Myrold 2005), or on overall biogeochemical processes (e.g., Thomas *et al.* 2009, Le Mer and Roger 2001, Bai *et al.* 2010). Significant knowledge has been

gained about each of these areas of study individually, providing a nascent understanding of the activity of microbial communities in wetlands, but our appreciation of the factors that control microbial community composition in freshwater wetlands is still unclear (Gutknecht *et al.* 2006). For example, wetland ecosystems often experience seasonal fluctuations in hydrology that can cause an alteration in the availability of oxygen in the soil. Changes in hydrology and oxygen are thought to influence microbial populations by decreasing the rates of decomposition and nutrient cycling (Bossio *et al.* 2006, Gutknecht *et al.* 2006, Hammer 1989).

One especially important area that is not well defined is how microbial community composition changes within vertical space (depth) as a potential result of variation in environmental parameters. For example, at greater depths, carbon substrate quality and quantity differs from the surface substrate due to the rapid decomposition of the labile fraction of the plant material, and the slow accumulation and burial of the resistant fraction over time (Bernal and Mitsch, 2008). Such differences in the carbon quality and availability over a depth gradient have been linked to shifts in microbial community composition in soils (Polymenakou *et al.* 2005, Fiere *et al.* 2003, Jackson *et al.* 2008). Similarly, redox values are affected by seasonal fluctuations in the hydroperiod and oxygen concentrations, specifically in terms of the availability of alternate terminal electron receptors, and are also related to shifts in microbial communities along a depth gradient (Wilms *et al.*, 2010, Pett-Ridge and Firestone, 2005). Such environmental variables, and many others, have the potential to influence the composition of the microbial communities, either individually or in tandem (Böer *et al.* 2009, Wilms *et al.* 2006, Thomas *et al.* 2009, Febria *et al.* 2009). The connection between changes in hydroperiod and the corresponding effects on the soil physiochemical parameters across a depth profile are likely critical to microbial community composition and the sediment ecosystem. Yet the interactions

between fluctuations in environmental variables, especially seasonal, and the microbial communities present across a depth profile is unclear (Bardgett and Shine 1999, Mentzer *et al.* 2006, Ahn *et al.* 2009, Peralta *et al.* 2010, Böer *et al.* 2009).

Seasonal variation includes changes in temperature, moisture regime, and potential alteration of the soil environment during the growing season caused by vegetation and inundation. Seasonal variation in soil saturation has the ability to influence environmental variables, such as redox and organic matter (OM) (Mitsch and Gosselink, 2000). Additionally, during the growing season, vegetation has a large effect on soil processes through root exudates and ventilation, potentially increasing microbial activity and affecting the community structure by providing additional sources of nutrients and creating micro-niches of oxygenated zones in the rhizosphere(Bachand and Horne 2000, Clement *et al.* 2002, Storm *et al.* 2003). However, the importance of plant community structure and biomass, especially its seasonal changes, on wetland soil microbial communities remains unclear (Kao *et al.* 2003).

Seasonal patterns have been observed in water column microbial community structure (Buesing *et al.* 2009) and sediment microbial activity (Gutknecht *et al.* 2006); however, very few studies have coupled the observation of seasonal microbial dynamics with a quantitative assessment of environmental parameters (Benner *et al.* 1986, Böer *et al.* 2009). Tighter coupling of endemic characteristics and microbial communities is essential if one is to determine which aspects of the environment are most important in controlling the composition of the soil microbial communities. Studies conducted in stream and coastal sediments found that temperature alone could not explain the seasonal shifts in microbial community structure, and these changes are more likely related to other unmeasured environmental parameters such as pH or redox potential (Böer *et al.* 2009, Wilms *et al.* 2010).

The research presented here sought to understand how environmental drivers influence microbial communities at various depths and in different hydrologic regimes in a young freshwater wetland. The objective was to elucidate the environmental variables that influence community composition during early succession, and to identify variables that have influence microbial communities over multiple growing seasons. Specifically, this study focused on the vertical and temporal patterns in sediment microbial communities, both bacteria and archaea, and quantitatively linked these patterns with changes in soil properties and plant community structure. This was done by monitoring two areas of an emergent wetland that experienced different hydrologic regimes and plant communities over a vertical depth within the rooting zone (0 - 40 cm). It was hypothesized that the different environmental conditions at these sites would have the greatest influence on the microbial communities present, and that depth would also affect microbial communities. Specifically, fluctuations in soil water content and redox were expected to have a greater effect on the microbial communities present at a dry site, relative to a wet site that maintained excessive saturated conditions. Within the wet site, plant communities were anticipated to have the largest effect on the microbial communities through the release of oxygen from the roots into the anaerobic soils. At both sites, variation across the depth profile was expected to correlate with differences in redox (aerobic to anaerobic zones) and organic matter (resource availability).

MATERIALS AND METHODS

1. Site Description

This research was conducted in an emergent freshwater wetland at Virginia Commonwealth University's Inger and Walter Rice Center for Environmental Life Sciences, located along the tidal freshwater portion of James River near Richmond, Virginia (USA) (Figure 1). The area of interest (~ 70 acres) was originally a forested wetland, which was cleared in 1927 and an earthen dam was erected to create Lake Charles. This area remained a lake until the Fall of 2006, when a storm surge breached the dam, draining a portion of the lake and recreating wetland habitat. Natural restoration of the freshwater wetland then began to occurr in the upper section of the former lake bed. Within a year, native wetland vegetation colonized the area and the original stream channel partially re-formed. Wetland soil conditions have been maintained since the original breach occurred.

2. Experimental Design

In the Winter of 2008, two sites were selected within the wetland based on apparent differences in microtopography and plant community composition, with the goal of comparing different hydrologic conditions. The sites were predominately groundwater fed, and the soils in each location were determined by hand analysis to be principally loamy clay (Richardson *et al*. 2001). The "Dry" site (37°20'11.1" N, 77°12'27.0" W) was established in an area of the wetland that would not typically be inundated above the soil surface, but maintained saturated soil conditions at depth, and the "Wet" site (37°20'13.9" N, 77°12'21.7" W) was established in

an area that usually maintained standing water on the soil surface (Figure 2). Three wetland plant species were common to both sites: *Juncus effusus, Leersia oryzoides,* and *Polygonum sagittatum,* with two additional species found at the Wet site (*Typha angustifolia* and *Murdannia keisak*) (Figure 3). These species are all obligate wetland indicators, except for *J. effusus,* which is a facultative wetland plant in this region of the country (USDA, NRCS. 2010).

At each site, a 5 m x 5 m plot was established and divided into 1 m x 1 m subplots using a square grid system. Sampling was conducted monthly from January 2008 to February 2009, and then every two to three months thereafter until October 2009, for a total of 17 sampling events. All sampling events included collection of 30-cm sediment cores for assessment of soil properties and microbial community structure; data on plant community composition and above-ground biomass were also gathered during the growing season (May through November of each year).

3. Sampling and Soil Analysis

For each sampling event, three subplots were randomly selected at each site. Within each subplot, a 30-cm sediment core was obtained using a Wildco® Hand Corer Sediment Sampler (5 cm x 50 cm Forestry Suppliers). Intact cores were kept upright and immediately transported at ambient temperature to the lab for processing. Because of the large number of samples collected over the duration of the study (17 sampling events with 3 cores each yielded 51 cores per site), it was necessary to sample some subplots more than once. However, a given subplot was never visited at two consecutive sampling events, and areas where cores had previously been collected were avoided.

Upon return to the lab, the soil cores were extracted from the sleeves and subdivided into "Top" (0 – 10 cm below the soil surface) and "Bottom" (20 – 30 cm below the surface) sections. All samples were homogenized by gentle hand mixing in an air-tight bag. Redox and pH measurements were immediately taken using a HANNA Combo pH and ORP probe (HANNA Instruments). Sub-samples to determine soil properties and for microbial community analysis were taken immediately, and stored at 4 and -20°C, respectively, until further processing. Processing of the soil for environmental parameters occurred within 24 h of sample collection.

4. Soil Characterization

To determine gravimetric water content (WC), a subsample of soil (20-35 g) was dried at 70°C for 72 h. The dry soil was then combusted at 450°C for 12 h to determine the OM content as mass loss on ignition (Klute, 1986). A subsample (2-3 g) was removed before combustion to determine the soil carbon to nitrogen ratio (C:N), and that subsample was stored at -20°C until acidification and analysis with a Series II CHNS/O Analyzer 2400 (Perkin Elmer).

5. Vegetation

During the growing season, estimates of above-ground plant biomass were obtained from the immediate vicinity of the sites by clipping to the ground all live plant material in three separate 1 m x 1 m sampling areas. Care was taken not to remove any vegetation in the main sampling plots. Plant material was then returned to the lab, sorted by species, and dried at 70°C for one week. Above-ground biomass (g dry weight (DW) per m^2) was then calculated for each species.

Root content of the soil (g root DW per g dry soil) was determined by soaking a subsample of ~30 grams of soil in 40 mL of 0.2 M Na_2HPO_4 for 24 to 48 h (Klute 1986). Roots were isolated by sieving the soil through two interlocking U.S.A. standard testing sieves (ATM Corp.); the top sieve contained 600 µm openings (#30 mesh) and the bottom sieve contained 355 µm openings (#45 mesh). The roots were then collected and rinsed by hand, and dried at 70°C for 48 to 72 h.

6. Microbial Community Analysis

After preliminary analysis of soil properties, eight dates were selected for microbial community structure analysis, which corresponded to one per season for the two-year duration of the study. For this subset of samples, bacterial and archaeal community composition was examined using "community fingerprinting" accomplished by terminal restriction fragment length polymorphism (T-RFLP) analysis (Liu *et al.* 1997).

6.1. DNA Extraction:

Whole-community DNA was extracted using a PowerSoil™ DNA Isolation Kit (MoBio Laboratories, Inc.) per the manufacturer's instructions. Successful extractions were identified by gel electrophoresis of 10 µl aliquots on a 1.5% agarose gel (100 volts, 1 h). Samples that did not show a band on the agarose gel were re-extracted from archived soil with the addition of a pre-extraction washing step to remove metal and humic inhibition (He *et al.* 2005). Specifically, ~ 0.25 grams of soil were added to 1.5 ml of 2 mM EDTA and vortexed at a low speed for one hour, after which time the samples were centrifuged at 10,000 rpms for 10 min to pellet the soil,

and the EDTA was decanted. DNA extraction then proceeded as above. DNA concentrations were determined using a Nanodrop 8000 Spectrophotometer (Thermo Scientific).

6.2. Polymerase Chain Reaction (PCR) Conditions:

For profiling the bacterial portion of the community, the 16S rRNA gene was amplified via PCR using bacteria-specific primers 27F (5'-AGA GTT TGA TCM TGG CTA G-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3'); the 27F primer was fluorescently labeled at the 5' end with FAM (Lane 2001). The total volume of each PCR reaction was 50 µl containing: 1 µl DNA template at the appropriate dilution, 5 U AmpliTaq DNA Polymerase, 1.5 mM $MgCl_2$ solution, 0.1 volume of GeneAmp 10X PCR Buffer II, 20 µg BSA, 1 mM each dNTP, and 0.3 µM primer of each primer. All reagents were supplied from Applied Biosystems except BSA, which was obtained from Roche. Most reactions contained between 10 and 12 ng of template DNA, though some required 15 to 20 ng of DNA. Thermal cycling was performed in a BioRad iCycler programmed for 95 ^{o}C for 5 min, 35 cycles of 94 ^{o}C for 1 min, 57 ^{o}C for 1 min, 72 ^{o}C for 2 min, and a final extension step of 72 ^{o}C for 8 min.

For profiling the archaeal members of the microbial community, PCR was performed using the primers 21F (5'-FAM-TTC CGG TTG ATC CYG CCG GA-3') and 958R (5'-YCC GGC GTT GCA MTC CAA TT-3') (Moesender *et al.*, 2001). Reaction conditions were similar to those used for bacteria with the following modifications: BSA was excluded, primer concentrations were increased to 2 µM, and dNTP concentrations were increased to 2.5 mM. Thermal cycling conditions were: 94^{o}C for 3 min, 30 cycles of 94 ^{o}C for 1 min, 55 ^{o}C for 1 min, 72 ^{o}C for 2 min, and a final extension step of 72 ^{o}C for 7 min.

6.3. Restriction Digests and Capillary Electrophoresis:

For each sample, triplicate 50-µl PCR reactions were combined, purified using a MinElute 96 UF™ PCR purification kit (Qiagen), and then digested using 40 U *Hha*I (New England Biolabs) at 37 ^{o}C for 6 h followed by 65 ^{o}C for 20 min. Digests were again purified using the MinElute 96 UF™ PCR purification kit (Qiagen), and 125 ng of the cleaned DNA was resolved using capillary electrophoresis with a MegaBACE 1000 fluorescent genotyper. Map Marker 400 ROX ladder (Bioventures) was included with each sample; injection was at 3000 V for 100 seconds, and run time was 100 min at 10,000 V. Following electrophoresis, T-RFLP peaks were scored as present/absent in the size range of 50 to 500 base pairs using Fragment Profiler (Version 1.2).

7. Statistical Analyses

7.1. Preliminary Analysis:

First, a series of Mantel tests were used to assess whether there was significant temporal autocorrelation for each environmental variable (Legendre and Legendre 1998). Data from all 17 sampling events were included. A distance matrix was constructed for time (as days since the first sampling event, t=0 to 625) and compared to a matrix of dissimilarity in each environmental parameter (generated using Gower's coefficient (Gower 1971)). r_m and p values were determined via randomization using 5000 permutations, and statistical significance was established using a step-down Bonferonni correction (Holm 1979) with an initial alpha of 0.05. When all data from all sites were considered simultaneously, no significant r_m values were obtained (Table 1), indicating little temporal autocorrelation in soil properties at the wetland scale. Given that the different sites and depths could respond differently to environmental

changes, a similar analysis was conducted for each separate sampling region, but the results demonstrate little temporal autocorrelation monthly (Table 1). These results are consistent with a visual examination of the time series graphs for each soil variable (Figure 4). Based on these results, it was decided to restrict subsequent analysis to an examination for seasonal trends, and to use eight selected sampling events for molecular characterization of the microbial community via T-RFLP. Dates were chosen to provide one sampling event per season for the two-year duration of the study, and to coincide with instances when full plant community data (i.e., total and species-specific biomass estimates) were available. All statistical comparisons described below were applied solely to the eight dates and focus on examination of seasonal patterns, and the potential for site and depth interactions.

7.2. In-depth Analysis of Selected Dates:

A correlation analysis was used to investigate the relationship between each environmental parameter, including soil properties and above- and below-ground plant biomass, using Spearman's rho and the PASW statistical package (Version 17). In addition, PASW was used to determine the differences between the means and potential effect of interaction between site, depth, and season on various environmental parameters using a three factor analysis of variance (ANOVA). Whenever significant interaction effects were detected, the contributing factors were separated by treatment for a series of individual one-factor ANOVAs. Differences between the seasons were determined using Tukey's post hoc comparison.

For the analysis of the microbial community composition, the bacterial and archaeal datasets were considered separately. T-RFLP peaks were binned according to size (base pairs, bp) with a minimum of three peaks required for each bin and minimum bin size of 0.5 bp.

Shoulder peaks, peaks outside the 50-500 bp range, and peaks less than 75 rfu in height were excluded. Peak data were then converted into a binary matrix with 1 denoting presence and 0 denoting absence of a particular terminal restriction fragment. The resulting matrix was analyzed using non-metric multidimensional scaling (NMDS) with the Jaccard similarity coefficient in PAST v. 2.01 (Hammer *et al.*, 2001). Significance of differences between groups were identified using ANOSIM (ANalysis Of SIMilarities), again with the Jaccard coefficient, corrected by PAST for multiple comparisons via step-down sequential Bonferroni approach (Clarke, 1993).

Additionally, canonical correspondence analysis (CCA) (PC- ORD vs. 5) was used to explore the relationship between the microbial community structure and environmental parameters. Row and column scores were standardized by centering and normalizing to yield bi-plot scaling with site scores rescaled to have a mean of zero and variance of one; plots were optimized so plot scores are weighted mean species scores (alpha = 1).

RESULTS

1. Environmental Parameters

1.1. Site, Depth, and Seasonal Differences in Soil Properties:

The soil parameters of redox, OM, and water content (WC) differed between the top 10 cm of the soil and the bottom 20-30 cm for most of the study (Figure 4); pH also differed with depth but less so than other parameters. Additionally, sites were generally less different than depths in regards to all environmental parameters. When the environmental parameters for the selected dates were graphed, differences between the sites were again not clear, while a difference was once again observed with depth (Figures 5 and 6). ANOVA was used to assess the statistical significance of each difference considering the factors of site, depth, and season (Table 2).

Overall, significant effects of site, depth, and season were observed for all soil parameters except for C:N (Table 2). The majority of environmental parameters were highly significantly different across depths and seasons (Table 2; Figures 5 and 6), and three variables (WC, OM, redox) also differed across sites. Several parameters displayed interaction effects between factors including: WC (where depth interacted with both site and season), pH and redox (which both showed a site-by-season interaction), and root content (which showed a site-by-depth interaction).

Organic matter: The ANOVA of OM revealed significant site, depth and season effects (Table 2). The Wet site had a higher OM (7.0 $\pm$ 0.6) than the Dry site (5.9 $\pm$ 0.5), and the tops of the soil cores were also found to have significantly higher OM (8.3 $\pm$ 0.5) than the bottoms (4.6

± 0.4). The percent OM was also different between the seasons ($p < 0.001$). Based on Tukey's HSD, OM was lowest in the Winter (4.3 ± 0.4 g) and increased significantly during the growing season (Spring: 5.1 ± 0.4 g; Summer: 6.6 ± 0.7 g). By the Fall, sampling events, OM was dramatically higher than all other times (9.8 ± 0.5 g).

pH: Season and site were found to have significant interaction in regards to pH (Table 2). No significant differences were observed between the averages at each site in Winter (5.9 ± 0.3), Spring (6.0 ± 0.2), and Summer (4.4 ± 0.1). Yet, during the Fall there was a significant difference ($p < 0.001$) in the pH's at each site, with the Wet site having higher average pH (6.6 ± 0.1) than the Dry site (5.3 ± 0.2). Additionally, pH was found to be significantly different between the top and bottom portions of the soil cores ($p = 0.035$) with the mean pH higher in the bottoms (5.8 ± 0.2) than the tops (5.3 ± 0.0).

Redox: Similar to pH, redox also had a significant interaction between season and site (Table 2). During the Spring and Fall, there was a significant difference in the redox at each site (Spring: $p < 0.001$, Fall: $p = 0.02$), with the Dry site having higher average redox (Spring: 189.0 ± 28.0, Fall: 301.5 ± 23.4) than the Wet site (Spring: 30.8 ± 28.0, Fall: 200.0 ± 33.1). At the Dry site, redox increased from the winter over the growing season (Winter = 179.8 ± 38.1, Spring = 189.0 ± 19.3, Summer = 276.8 ± 49.7, Fall = 301.5 ± 24.5); however, this increase was not statistically significant ($p = 0.36$). In the Wet site, high redox values were observed during the growing season (Winter = 130.0 ± 32.3, Spring = 30.8 ± 28.2, Summer = 278.0 ± 44.6, Fall = 200.0 ± 33.1) and significant differences were observed between the Winter/Spring and Summer/Fall ($p < 0.001$). Furthermore, redox was significantly different between the top and bottom portions of the soil cores ($p < 0.001$) with the mean redox lower in the bottoms (130.3 ± 14.7) than the tops (266.1 ± 14.7).

Water content: Water content had significant interactions between the depths and site and depth and season (Table 2). Regardless of site or season, there were no differences in the WC for the deep samples (site: $p = 0.77$, season: $p = 0.28$), and the overall average water content was 60.2% $\pm$ 3.0. However, when just the top layer of the soil is considered, the Wet top (92.0% $\pm$ 4.5) had significantly higher soil moisture compared to the Dry top (72.4% $\pm$ 4.0) ($p = 0.003$). For the tops, the only seasonal differences were between the Winter (98.2% $\pm$ 7.5) and Summer (65.4% $\pm$ 4.0) ($p < 0.001$).

1.2. Plant Community Properties:

Wetland vegetation also differed across sites, both in terms of biomass and community composition. Three plant species were common to both sites: *Juncus effusus, Leersia oryzoides,* and *Polygonum sagittatum*, with two additional species found at the Wet site (*Typha angustifolia* and *Murdannia keisak*). Figure 3 shows the clear differences in community composition across sites; when ANOSIM was applied to compare the presence or absence of each species in each plot, it was demonstrated that the sites were distinct from one another ($p=0.024$). Additionally, based on the ANOVA, a significant seasonal effect was observed ($p < 0.001$) where the Winter had no live plant above-ground biomass (0.0 $\pm$ 0.0 kg/m^2) but the above-ground biomass significantly increased over the growing season (Spring: 0.4 $\pm$ 0.03 kg/m^2; Summer: 1.1 $\pm$ 0.1 kg/m^2) and then decreased in the Fall (0.8 $\pm$ 0.2 kg/m^2).

When below-ground (root) biomass was considered, no significant seasonal effects were observed ($p=0.63$) (Table 2). However, a significant interaction effect was observed for site and depth ($p = 0.02$). In the bottoms, there are fewer roots and no effect of site (single factor ANOVA, $p =0.06$, overall average root for the bottoms 0.9 $\pm$ 0.8 mg per gram dry soil).

However, the Wet tops (7.5 $\pm$ 1.4 mg per gram dry soil) have less root matter than the Dry sites tops (14.7 $\pm$ 3.8 mg per gram dry soil) (single factor ANOVA, $p = 0.03$).

1.3. Spearman Rank Correlations (r_s):

Relationships among soil parameters were explored using Spearman Rank correlation and several significant relationships were found (Table 3). Of particular note, root content of the soil was correlated with nearly every other parameter measured. In contrast, soil C:N did not correlate significantly with any of the other variables. Above-ground plant biomass was strongly correlated with both soil OM and root content. Redox, OM, and soil water content were all highly correlated; pH was negatively correlated root content and redox.

When data collected for each depth were considered separately (results not presented), some differences emerged, and in general the tops were significantly correlated while the bottoms had few significant correlations. In particular, redox was most strongly correlated in the top layer of the soil with water content (top: $r_s = -0.55$, $p < 0.001$; and bottom: $r_s = 0.01$, $p > 0.05$) and OM (top: $r_s = 0.56$, $p < 0.001$; and bottom: $r_s = 0.22$, $p > 0.05$). A similar relationship was also found between pH and roots in the top layer (top: $r_s = -0.29$, $p<0.05$; bottom $r_s = -0.04$, $p<0.05$). At the surface the correlation between roots and plant biomass was stronger than at depth (top: $r_s = 0.94$, $p<0.05$; top: $r_s = 0.68$, $p<0.05$), where greater root biomass co-occurred with higher above-ground live plant biomass.

2. Soil Microbial Community Analysis

Patterns in the microbial community structure elucidated with T-RFLP DNA fingerprinting were visualized using NMDS. All possible combinations of site, depth, and time were visualized

graphically, and the strongest pattern observed was between site and depth regardless of time for both communities. The data were analyzed in three dimensions, and the two axes that demonstrated the greatest variation were graphed (Figures 7 and 8).

2.1. Archaeal Community:

All data points regardless of site, depth or season were summarized graphically using the two major axes from the 3D analysis, these axes had a stress level of 0.15 and accounted for 61% of the variability in the data (Figure 7). In this graph, the data points separated primarily by depth (Axis 1: 44%) and then by site (Axis 2: 17%). Each sampling site and depth were significantly different from one another based on a one-way ANOSIM (all $p < 0.001$).

Most seasonal samples were not significantly different from one another (Figure 8; ANOSIM: all $p > 0.05$ except Fall 2009 to Spring 2008 ($p < 0.001$) and Winter 2009 ($p < 0.001$).

2.2. Bacterial Community:

As with the archaeal communities, all bacterial data points regardless of site, depth or month were summarized graphically with the two major axes based on the 3D analysis (stress = 0.18, total variability = 61%, Figure 7). In this graph, the data points again separated out primarily by depth (Axis 1: 38%) and then by site (Axis 3: 23%). Each sampling site and depth was significantly different from one another based on a one-way ANOSIM (all $p < 0.05$).

Similar to the archaeal communities, no consistent seasonal patterns or groupings were observed in any of the combinations of depth or site in the bacterial communities (Figure 8). None of the seasons were found to be significantly different via one-way ANOSIM (all $p > 0.05$).

3. Canonical Correspondence Analysis

In the CCA biplot of the archaea data, Axis 1 explained only 9.7% of the variation and Axis 2 explained 4% (less than NMDS results). The samples clustered by depth in a similar manner to the patterns observed in the NMDS, so the graphs are not reproduced here. Water content, OM, redox, and roots per gram dry weight correlated with Axis 1 and were significant (Table 4, Pearson $r = 0.85$, Monte Carlo $p < 0.001$).

Similarly, for bacteria, little total variation (10.5%) was explained by the CCA (Axis 1: 7.5, Axis 2: 3%), and the samples clustered by depth comparable to the NDMS results presented in Figure 5. Once again, water content, OM, redox, and roots per gram dry weight were correlated with Axis 1 and were significant (Table 4, Pearson $r = 0.72$, Monte Carlo $p < 0.001$). Additionally, for both microbial communities average live plant biomass and C:N were not strongly correlated to either axis in the CCA analysis (Table 4).

DISCUSSION

In the present study, it was hypothesized that environmental conditions at different sites within the wetland would have the greatest influence on the microbial communities present, and then, within the sites, depth would be a distinguishing factor between communities. However, soil depth was the most important factor in driving environmental conditions and in determining microbial community structure during this study period. In addition, microbial communities within each site and depth did not follow a clear seasonal trend.

Saturated soils have little aeration and, thus, reduced redox status, which in turn constrained decomposition creating an accumulation of OM (Bossio *et al.* 2005; Gutknecht *et al.* 2006; Hammer 1989). This expected pattern was observed over the sites and depth gradient studied here; with the wet site and top 10 cm of the soil having higher OM and WC, and lower redox, all of which were significantly correlated with one another (Table 2). Surprisingly, the only significant interaction found between site and depth was in relation to soil WC with bottoms having lower WC than tops, specifically wet tops had a higher average WC than dry site the bottoms. This is caused the low porosity in compacted depths of loamy clay . As a result, bottom sediments contained less than half of the WC than top sediments. Fluctuations in hydrology and aeration would have been limited at 20-30 cm depth to changes in the water table, while the top 10 cm experienced large shifts in surface water inputs, creating differences in physiochemical properties of the sediment.

Differences between sites and depths based on OM were not surprising. As expected, the wetter site experienced a higher accumulation of OM, especially in the top 10 cm of soil, which was most likely caused by slower decomposition rates. Another possibility is that above-ground

productivity differed across sites, creating large differences in the amount of OM that would be added to the soil surface. However, this is not likely based on ANOVA results, which found no significant differences between the sites based on above-ground plant biomass ($p = 0.40$). In addition, OM increased significantly over the growing season, which could be from an accumulation of root biomass in the soil as plants grow. However, when the soil OM was re-calculated after removing the contribution of root weight, and then reanalyzed via ANOVA, the pattern did not change. Non-root soil OM increased significantly in the Fall (9.2 ± 0.9 mg) versus the other seasons (Spring: 4.4 ± 0.3 mg, Summer: 5.9 ± 0.6 mg, Winter: 3.9 ± 0.7 mg). One potential explanation for this observed increase in OM is the physical breakdown of surface litter into small particles throughout the growing season, which was then incorporated into the soil matrix and reached a significant accumulation by the Fall.

Plant communities are assumed to indirectly influence soil pH and redox potentials by releasing exudates and oxygen through roots, which in turn influence soil microbial communities (Thomas *et al.* 2009, Ehrenfeld *et al.* 2005). Influence of the plant community is anticipated in the rooting zone of the soil, typically the top 40 cm (Mitsch and Gosselink 2000). Plants differ in their influence on soil physiochemical properties. For example, *Typha latifolia* (a similar species to the type of *Typha* found in this wetland) releases twice as much oxygen through its roots as J*uncus effusus* (another dominant plant in this wetland), though both effectively create aerobic micro-niches that have the potential to influence redox and pH (Webner *et al.* 2002). Here, more roots were found in the top layers of the soil than at depth, this is most likely due to the age of the wetland and recent re-colonization of this area by wetland vegetation. A strong effect of the plant roots was observed in the top 10 cm of the soil as demonstrated by the correlation between roots and redox and pH (Table 3).

Additional seasonal differences were observed in the environmental parameters of pH, redox, and average live plant biomass. As previously mentioned, pH and redox values can be affected by the plant community through root exudates, which likely experience seasonally fluctuations (Ehrenfeld *et al.* 2005, Thomas *et al.* 2009). However, this study used bulk soil measurements to determine the soil properties; smaller scale measurements would better indicate rhizosphere-induced changes in pH and redox throughout the seasons.

Overall, seasonal and depth differences were significant in the environmental parameters considered. One concern with the observed differences with depth was that the 20-30 cm soil layer may have been from the original wetland, and the surface sediments deposited while this area was a lake. However, all soil sampled was deposited while this area was a lake (VCU Rice Center Site Assessment report by Draper Aden Associates, 2003).

The dominant environmental parameters effecting microbial communities was soil depth. Archaea communities in surficial sediments were more similar than archaea in the bottom sediments, but the opposite was observed for the bacterial communities. Even though these patterns were distinct, the two subsets of the microbial community were still highly correlated (Mantel test comparing similarity in bacterial communities to similarity in archaea communities, $r_M = 0.23$, $p < 0.001$). Soil saturation is a dominant factor in wetlands and controls archaeal and bacterial microbial populations (Gutknecht *et al.* 2006, Mentzer *et al.* 2006, Balasooriya *et al.* 2008). Though little is known about how environmental parameters influence the total archaea community, methanogenesis rates (a process mediated by anaerobic archaea and thought to be very important in freshwater wetlands) are linked to water level and WC in sediments (Coles and Yavitt, 2004). Even though little of the variation observed in the total archaeal communities in this study was explained by the environmental variables measured, the variation that could be

explained in the CCA was strongly correlated with WC and moderatley correlated with redox, OM, and root content (Table 4). In peat sediments, archaea abundance increases with depth and is presumed to be caused by anoxic conditions (Debysh *et al.* 2006; Jackson *et al.* 2009), which is affected by the WC of the soils. This difference in water alone may be the cause of the distinct communities observed between the depths (Hansel *et al.*, 2008), though other factors involved with WC and water source (e.g., dissolved organic carbon from soil and plant leachate) might also contribute (Jackson *et al.* 2009; Kemnitz *et al.*, 2007).

Bacteria communities are also influenced by WC and redox in soils (Balasooriya *et al.* 2008, Gutknecht *et al.* 2006), and, similar to the archaea communities, what little variation could be explained in these bacterial communities was correlated with soil WC and redox values. Redox was strongly correlated to changes across depth in bacterial communities in the CCA ($r = 0.80$). In marine benthic sediments and tropical soils, redox has also been found to be related to bacterial community structure (Edlund *et al.* 2008, Pett-Ridge and Firestone 2005). However, redox potentials are also a function of the microbial communities present and are influenced by nutrient availability, carbon availability, water table fluctuations (Thomas *et al.* 2009).

As previously discussed, pH can be influenced by plant communities through roots, but it can also be affected by the physical characteristics of the soil. In mineral wetlands with low amounts of OM, such as in this study, pH is typically slightly acidic to neutral (Hammer 1989; Mayes *et al.* 2009). While the influence of pH on microbial communities is not yet clearly understood (Gutknecht *et al.* 2006), the values recorded in this study were fairly constant and not extreme enough to strain the microbial communities.

Contrary to the environmental parameters, no clear seasonal patterns were observed in the microbial communities, potentially because a strong relationship between the plant

community and microorganims has not yet been established. Additionally, due to the recent history of this wetland, the hydrology of the system was very stochastic throughout the study. This may have influenced the microbial communities, not only through fluctuations in WC but also by affecting other environmental factors such as the source and availability of nutrients (Treves *et al.* 2003; Zhou *et al.* 2002), and possibly prevented any sort of predictable seasonal patterns from emerging at this time. Additionally, at longer temporal scales, wetlands experience an accumulation of OM, succession in plant community structure, and changes in hydrological flow. The accumulation of these changes causes cascading effects that are important to microbial community structure that may not have been in effect in this wetland due to its age. Environmental heterogeneity increases through time with succession and is very important to microbial community structure in wetlands and benthic habitats (Buesing *et al.* 2009, Hullar *et al.* 2006, Böer *et al.* 2009, Wilms *et al.* 2006).

In this study, little of the variation observed in the microbial community was explained by the environmental parameters examined. Other studies that have examined depth-related changes in the communities have succeeded in linking such patterns to environmental parameters such as redox, carbon content and even predation (First *et al.* 2010, Böer *et al.* 2009, Edlund *et al.* 2008, Fierer *et al.* 2003, Wilms *et al.* 2006). Yet the influence of such environmental variables over time is still not clearly understood (Böer *et al*. 2009, Mentzer *et al.* 2006, Wilms *et al.* 2006, Hullar *et al*. 2006). Future work that includes a broader scope of environmental parameters over a more intense and longer sampling period is needed in order to clarify the interaction between environmental parameters and seasonal dynamics and microbial community structure.

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APPENDIX

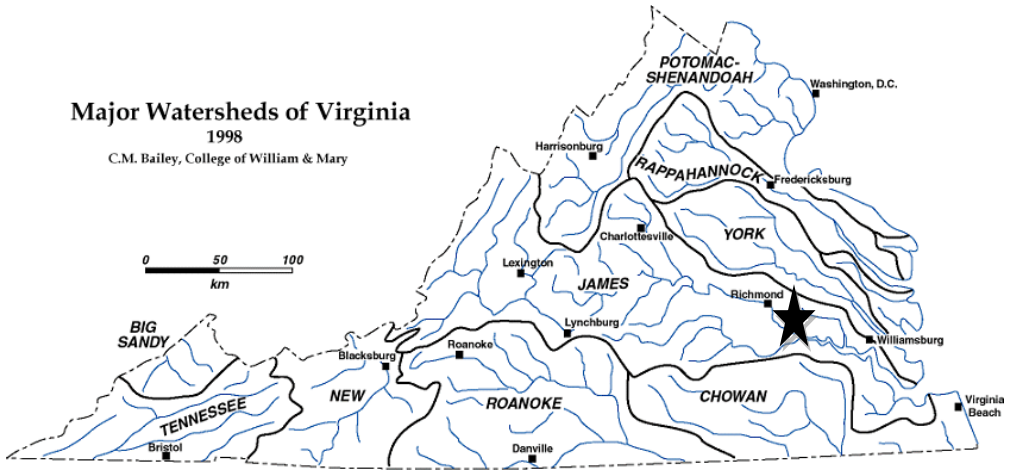

Figure 1: Location of study site, Virginia Commonwealth University's Inger and Walter Rice Center for Environmental Sciences, located along the tidal freshwater portion of James River near Richmond, Virginia (USA).

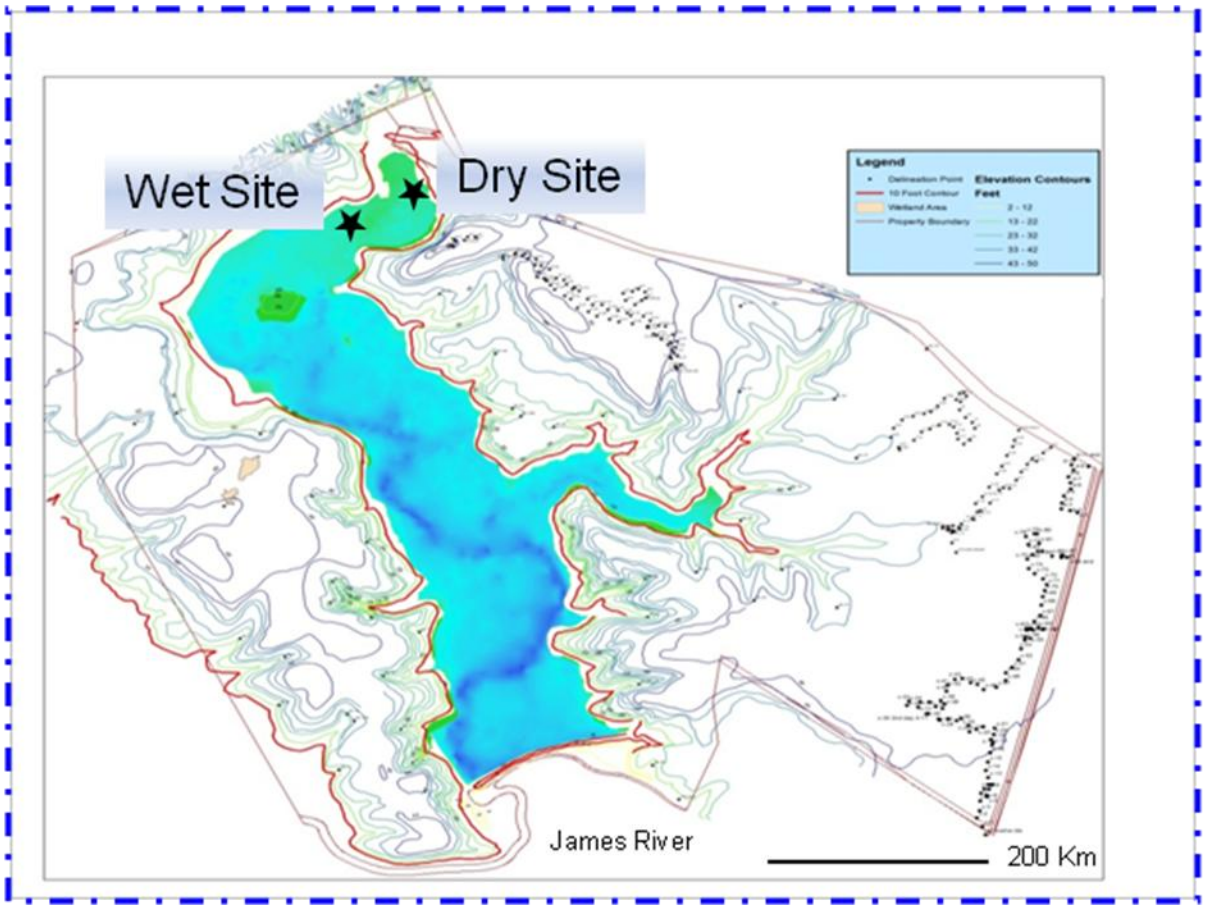

Figure 2: A map of former Lake Charles, with the study sites indicated, Virginia Commonwealth University's Inger and Walter Rice Center for Environmental Sciences, located along the tidal freshwater portion of James River near Richmond, Virginia (USA).

Table 1: Mantel Temporal Correlations

	All samples		**Wet Tops**			**Wet Bottoms**		**Dry Tops**		**Dry Bottoms**	
	r	p	r	p		r	p	r	p	r	p
pH	0.05	0.03	0.15	0.0004	*	0.004	0.40	0.11	0.03	0.12	0.02
Redox	0.05	0.03	- 0.03	0.70		- 0.03	0.66	0.04	0.17	- 0.08	0.93
OM	0.05	0.02	0.02	0.31		0.01	0.41	0.03	0.31	- 0.06	0.78
WC	0.06	0.03	0.15	0.01		0.09	0.12	0.08	0.15	0.04	0.27

* Significant following sequential step-down Bonferroni correction with an initial alpha of 0.05.

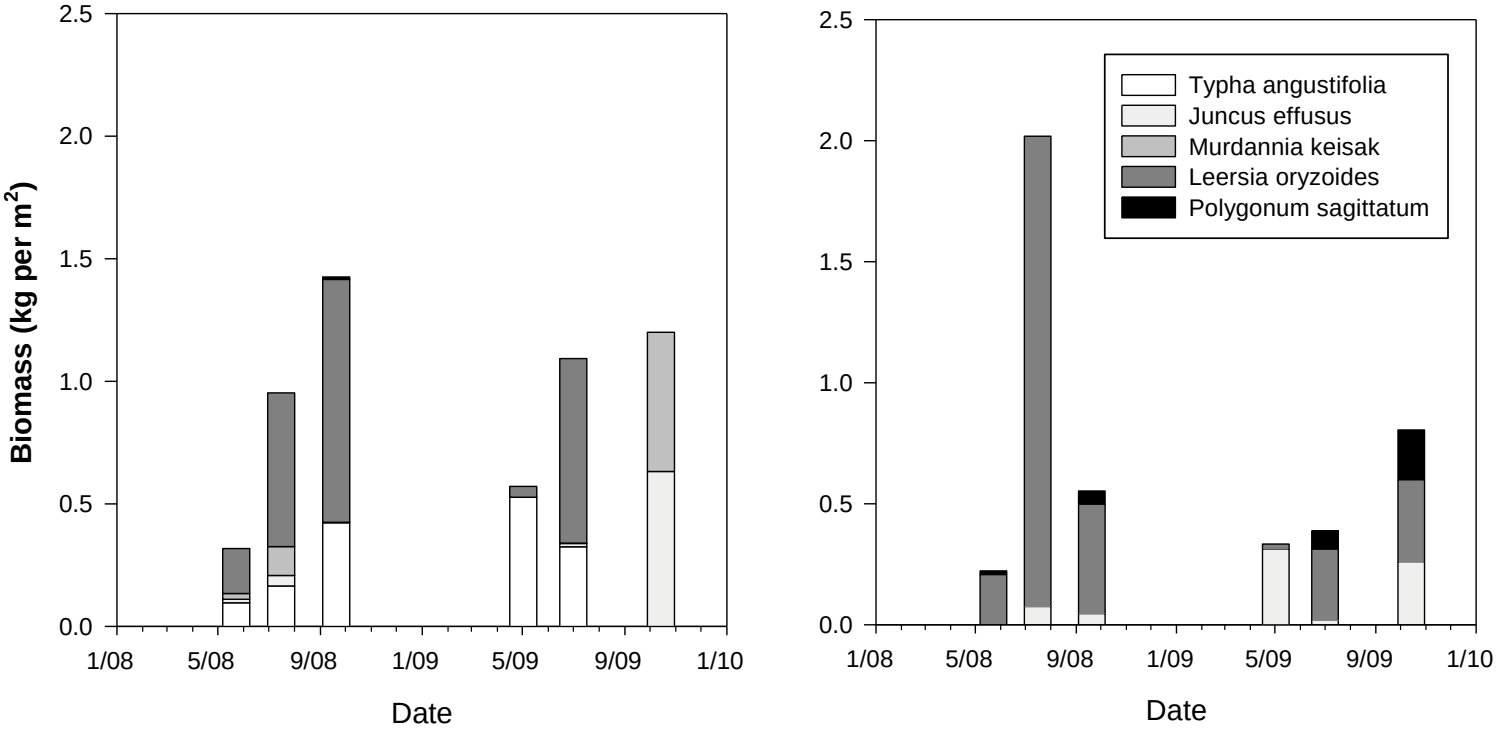

Figure 3: Average kg per m^2 of live plant biomass by species present. A.) Wet site B.) Dry site. Three wetland plant species were common to both sites: *Juncus effusus*, *Leersia oryzoides*, and *Polygonum sagittatum*, with two additional species found at the wet site (*Typha angustifolia* and *Murdannia keisak*)

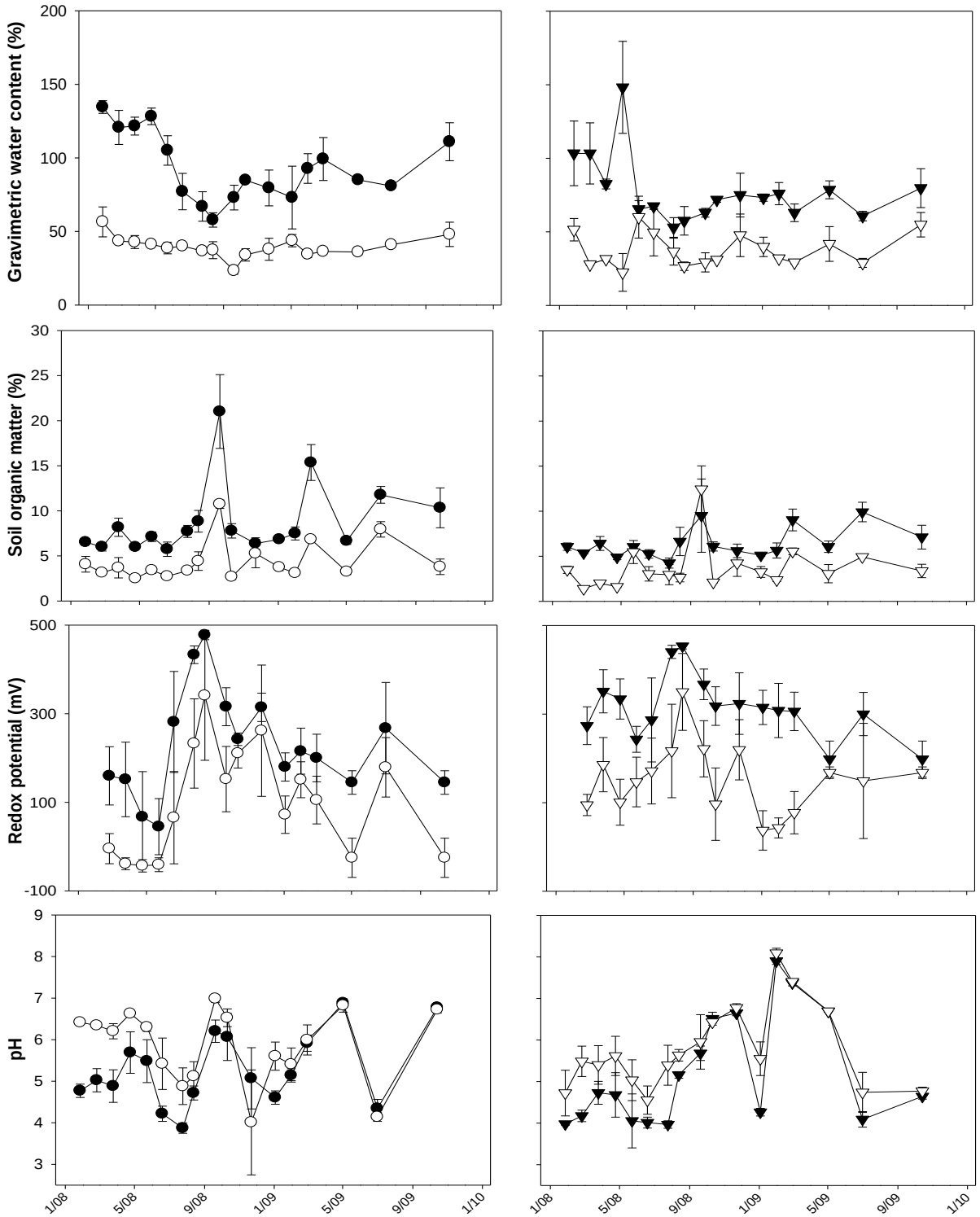

Figure 4: Selected environmental parameters for all dates sampled (mean $\pm$ 1 S.E.), Circles are the Wet site while triangles are the Dry site; filled shapes are the top 10 cm of the soil core while open shapes are the bottom 20-30 cm of the soil core.

Table 2: ANOVA Test Statistics

		C:N		OM		pH		Redox		WC		Roots		Biomass	
	df	F	p	F	p	F	p	F	p	F	p	F	p	F	p
Site	1	0.00	0.94	4.77	0.03*	1.27	0.26[a]	14.17	<0.001** [a]	7.57	0.007* [a]	3.54	0.06 [a]	6.03	0.441
Depth	1	1.29	0.26	43.59	<0.001**	7.90	0.006*	44.22	<0.001**	172.86	<0.001** [a]	35.27	0.001* [a]	N/A	N/A
Season	3	0.44	0.73	19.55	<0.001**	18.51	<0.001**[a]	14.93	<0.001** [a]	4.27	0.007* [a]	0.58	0.63	68.29	<0.001**
Site x Depth	1	0.67	0.42	2.30	0.13	0.02	0.87	0.28	0.59	9.51	0.003*	5.33	0.02	N/A	N/A
Site x Season	3	0.74	0.54	0.72	0.54	7.70	<0.001**	2.82	0.04*	0.33	0.803	0.49	0.69	7.51	0.428
Depth x Season	3	2.23	0.09	0.43	0.73	0.55	0.65	0.81	0.49	4.62	0.005*	0.70	0.55	N/A	N/A
Site x Depth x Season	3	0.17	0.91	0.56	0.64	0.29	0.83	0.78	0.50	1.01	0.39	0.45	0.72	N/A	N/A

*Significant at the 0.05 level
**Significant at the 0.001 level
[a] Output from three way ANOVA

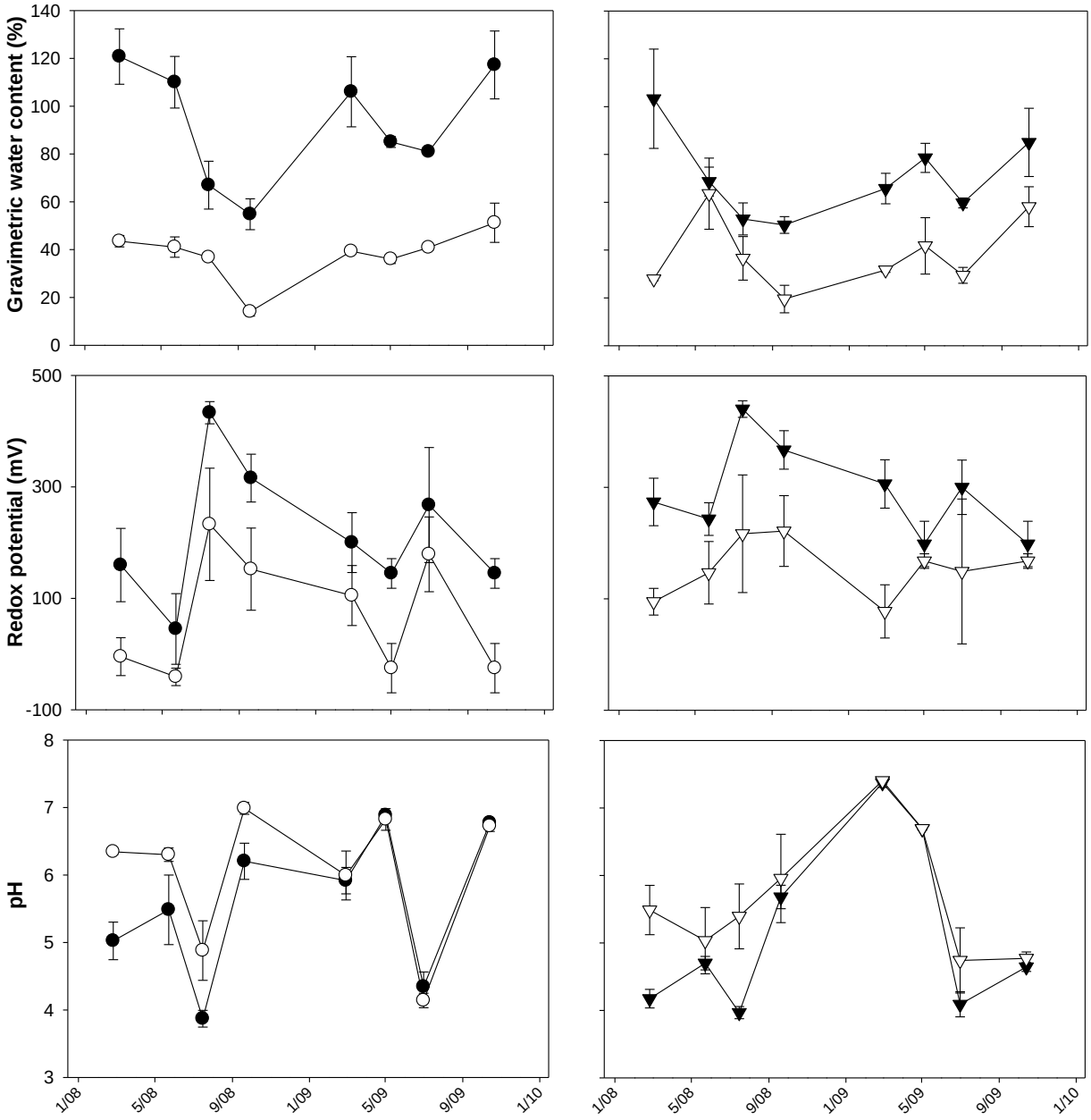

Figure 5: Soil parameters (water content, pH, redox) for the eight selected dates (mean $\pm$ 1 S.E.). Circles are the Wet site while triangles are the Dry site; filled shapes are the top 10 cm of the soil core while open shapes are the bottom 20-30 cm of the soil core.

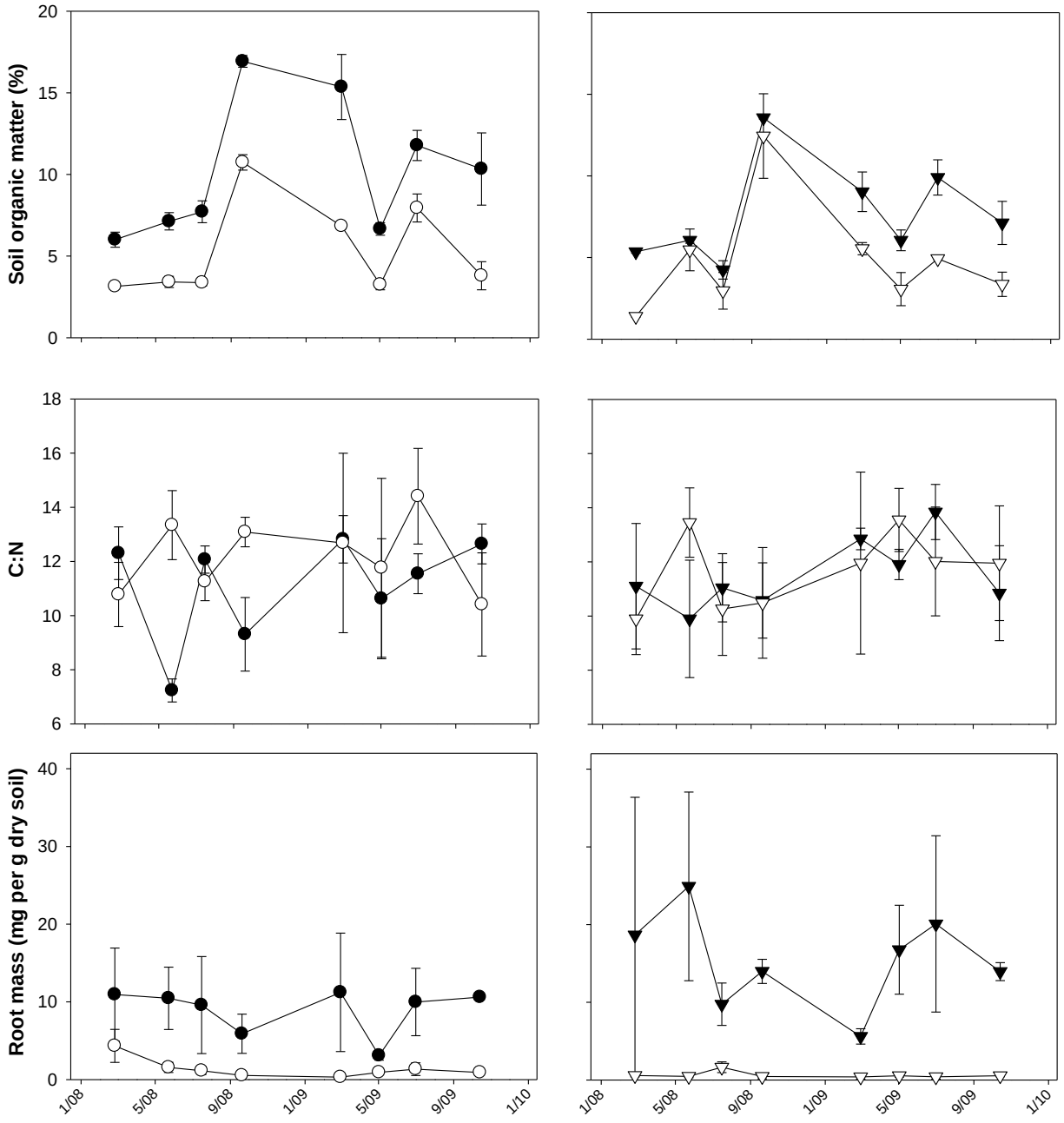

Figure 6: Soil parameters (OM, C:N, roots per gram dry soil) for the eight selected dates (mean ± 1 S.E.). Circles are the Wet site while triangles are the Dry site; filled shapes are the top 10 cm of the soil core while open shapes are the bottom 20-30 cm of the soil core.

Table 3: Spearman Rank Correlations between All Environmental Parameters

	WC	Redox	pH	OM	Roots	C:N
Redox	0.27**					
pH	-0.17	-0.52**				
OM	0.50**	0.38**	-0.16			
Roots	0.49**	0.34**	-0.30**	0.42**		
C:N	-0.04	0.01	-0.01	0.02	-0.19	
Biomass	0.03	0.12	-0.12	0.56**	0.28**	0.07

*Significant at the 0.05 level
**Significant at the 0.001 level

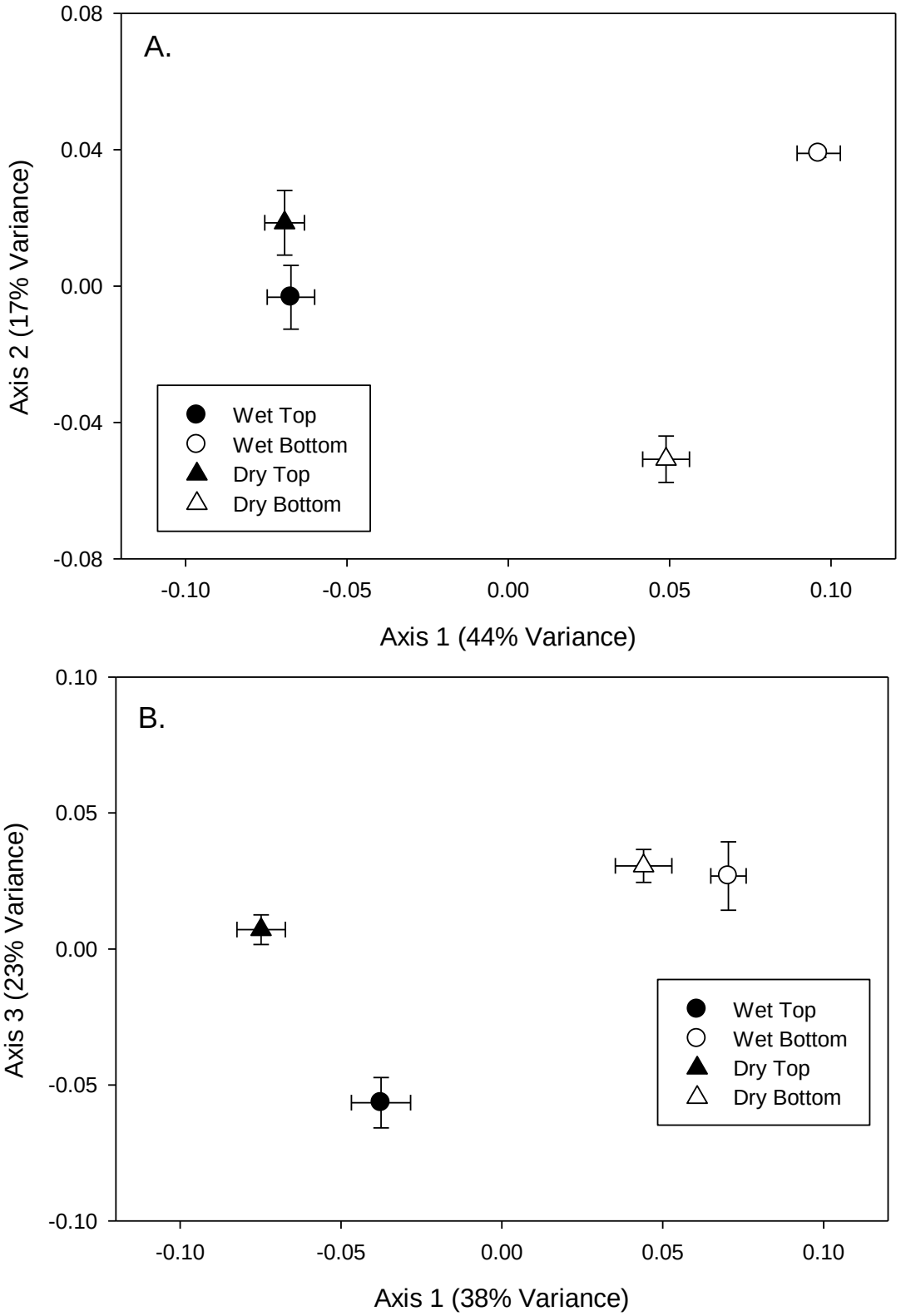

Figure 7: NMDS of the microbial communities (mean ± 1 S.E.) all samples by site and depth. A.) Archaea Communities and B.) Bacterial Communities.

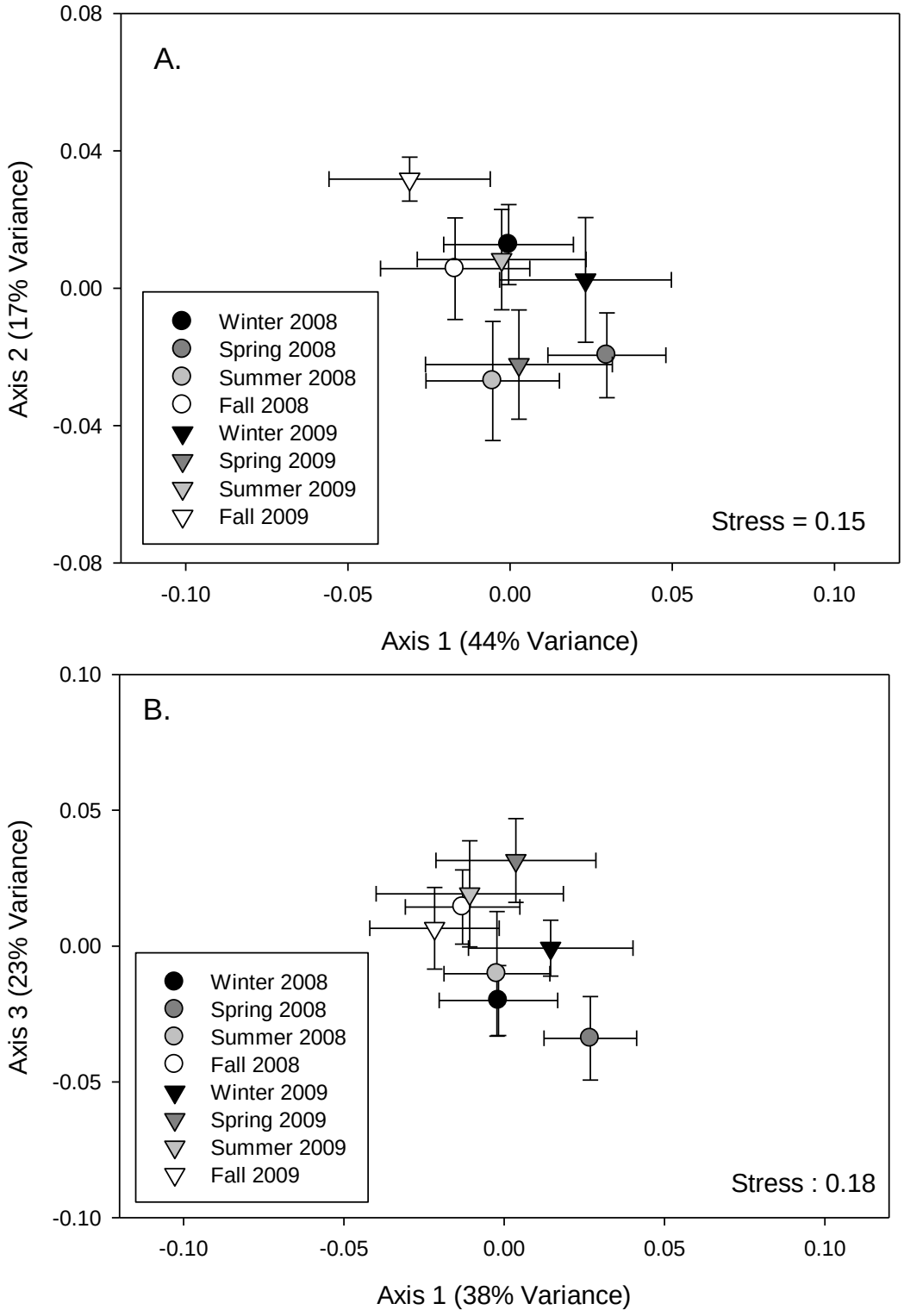

Figure 8: NMDS of the microbial communities (mean ± 1 S.E.) all samples by season for both years sampled A.) Archaea Communities and B.) Bacterial Communities.

Table 4: CCA of the Microbial Communities. Correlation values for each environmental parameter to Axis 1 (significant based on the Monte Carlo test with $p < 0.001$).

	Archaea	**Bacteria**
pH	-0.09	-0.44
Redox	0.52	0.80
OM	0.58	0.52
Roots	0.64	0.58
WC	0.85	0.59
Live Average Plant Biomass	-0.15	-0.34
C:N	0.11	0.24

Vita

Amy S. Jenkins was born on May 25, 1984 in Blacksburg, Va. She graduated from Springfield Southeast High School in 2001 and then she became a Rotary Exchange Student to Eislingen, Germany. She graduated from Lincoln Land Community College with a A.S in Biology in 2004 and from Florida State University with a B.S. in 2006.