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Response of Soil Microbial Communities to Saltwater Intrusion in Tidal Freshwater Wetlands

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**RESPONSE OF SOIL MICROBIAL COMMUNITIES TO SALTWATER INTRUSION IN
TIDAL FRESHWATER WETLANDS**

A thesis submitted in partial fulfillment of the requirements for the Master of Science at Virginia
Commonwealth University

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Table of Contents

	PAGE
Title Page	I
Acknowledgements.....	II
List of Tables	IV
List of Figures	V
Abstract.....	1
Introduction.....	3
Methods	6
Results.....	10
Discussions	12
Conclusions.....	17
Literature Cited.....	19
Appendix.....	25
Vita.....	32

LIST OF TABLES

	PAGE
Table 1: Soil Properties	25
Table 2: Porewater Chemistry	26
Table 3: Extracellular Enzymes Activity	27

LIST OF FIGURES

	PAGE
Figure 1: Potential Rates of Methane and Carbon Dioxide Production	28
Figure 2: Bacterial, Archaeal, and Fungal Abundance	29
Figure 3: Methanogens and Sulfate Reducers Abundance	30
Figure 4: Principal Component Analysis of Gene Abundance, Gas Production, and Environmental Variables	31

ABSTRACT

RESPONSE OF SOIL MICROBIAL COMMUNITIES TO SALTWATER INTRUSION IN TIDAL FRESHWATER WETLANDS

By Chansotheary Dang, Master of Science

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2016

Major Director: Rima B. Franklin, Ph.D
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Saltwater intrusion due to global change is expected to have a detrimental effect on the biogeochemistry of tidal freshwater wetlands. Of particular concern is that fact that salinization can alter the role of these ecosystems in the global carbon cycling by causing shifts in microbial metabolism that alter greenhouse gas emissions and increase carbon mineralization rates. However, our understanding of how wetland microbial community dynamics will respond to saltwater intrusion is limited. To address this knowledge gap and increase our understanding of how microbial communities in tidal freshwater wetlands change over time (1, 3, 12, and 49 weeks) under elevated salinity conditions, an *in situ* soil transplant was conducted. Throughout the 49 weeks of saltwater exposure, salinity had no effect on soil quality (organic matter content and C:N ratio). In contrast, the concentration of porewater ion species (SO_4^{2-} , NO_3^- , and NH_4^+) considerably increased. The activity of hydrolytic enzymes, (β -1,4-glucosidase and 1,4- β -cellobiohydrolase) gradually decreased with prolonged exposure to saline conditions; by the final sampling event (49 weeks), activity was reduced by ~70% in comparison to the freshwater controls. Short term exposure to salinity (3 and 12 weeks) had a greater effect on phenol oxidase, decreasing activity by 10-20%. Saltwater exposure had an immediate (1 week) effect on potential rates of carbon

mineralization; overall, carbon dioxide production doubled and methane production decreased by ~20-fold. These changes in gas production were correlated to increased salinity and to changes in the abundance of methanogens and sulfate reducing bacteria, suggesting a shift in the terminal step in organic matter degradation from methanogenesis to sulfate reduction. Principal component analysis revealed distinct changes in soil environmental conditions and carbon metabolism within weeks, but the response of the microbial community was slower (months to a year). Taken together, results from this study indicate that the response of tidal freshwater wetlands to salinization is driven by complex interactions of microbial related processes and environmental changes that are dependent on the duration of exposure. Assessing the impact of environmental perturbation on ecosystem function may be better achieved by complementary analysis of both microbial community structure and function.

INTRODUCTION

Tidal freshwater wetlands (TFW) are highly dynamic ecosystems located at the interface of terrestrial and coastal landscapes. Due to their unique geomorphic position, TFW are hotspots for biogeochemical transformations and host an abundance of biological diversity (Simpson et al. 1983; Odum 1988; Barendregt et al. 2013). Although coastal wetlands comprise only a small portion of terrestrial landmass, these ecosystems sequester a disproportionately large amount of the global organic carbon due to their high primary productivity and the slow decomposition rates that are associated with their anoxic soil conditions (McLeod et al. 2011; Bernal and Mitsch 2012). As sea level continues to rise at an accelerated rate (IPCC 2014), TFW are becoming more susceptible to saltwater intrusion, which can impair the ability of these ecosystems to sequester carbon (Neubauer 2008; Craft et al. 2009). Recent studies have demonstrated that salinization of freshwater environments can have a significant impact on the carbon cycle through changes in vegetation assemblages (Sharpe and Baldwin 2012), variations in plant productivity (Spalding and Hester 2007; Sharpe and Baldwin 2012), shifts in anaerobic decomposition pathways (Weston et al. 2006; Chambers et al. 2011; Jun et al. 2011), and by altering microbial community dynamics (Jackson and Vallaire 2009; Nelson et al. 2014). Despite the relative importance of microbial communities in regulating the carbon biogeochemistry in TFW, there have been relatively few saltwater intrusion studies that have incorporated a microbial component (e.g., see Baldwin et al. 2006; Edmonds et al. 2009; Jackson and Vallaire 2009; Morrissey and Franklin 2015).

In a recent literature review of wetland salinization, Herbert et al. (2015) highlighted the variable results among studies addressing the effect of salinity on the carbon biogeochemistry. Most prior studies have focused on the terminal steps in anaerobic carbon degradation and compare methanogenesis with sulfate reduction. Under reducing conditions with limited terminal electron acceptors (characteristic of freshwater environments), methanogenesis is the dominant anaerobic pathway in organic matter (OM) degradation (Megonigal and Neubauer 2009; Poffenbarger et al. 2011). However, saltwater intrusion can increase the supply of terminal electron acceptors, particularly sulfate, which can alter the carbon

dynamics and the soil quality of TFW (Weston et al. 2006; Neubauer et al. 2013). Sulfate reduction is more energetically favorable than methanogenesis, thus microorganisms that are capable of reducing sulfate are expected to outcompete methanogens for similar carbon substrates (Capone and Kiene 1988). Therefore, saltwater exposure is expected to increase the total carbon mineralization in TFW (Craft 2009; Herbert et al. 2015; Weston et al. 2011). However, carbon metabolism in TFW is governed by a complex suite of biotic and abiotic interactions, and prior studies on the effect of elevated salinity on carbon mineralization provide contrasting results, especially in regards to the magnitude of changes in CO₂ emission or production (e.g., see Sutton-Grier et al. 2011; Weston et al. 2011; van Dijk et al. 2015).

Microorganisms are critical decomposers in wetland ecosystems and studies of the structure and function of microbial communities may help us better understand these inconsistencies. For example, the initial steps of OM decomposition require the microbes to release extracellular enzymes to hydrolyze the complex macromolecules in plant detritus into simpler monomers for microbial uptake. The effect of salinity on these enzymes is not well understood, but studies that have examined enzyme activity generally find decreased activity with increased salinity (Frankenberger and Bingham 1982; Siddikee et al. 2011; Neubauer et al. 2013). However, just like with studies of carbon mineralization, there are contrasting results. Other studies found no relationship between salinity and the activity of enzymes involved in carbon degradation (Chamber et al. 2013; Morrissey and Franklin 2015; Chamber et al. 2016). Morrissey and Franklin (2015) suggested that changes in microbial community may occur gradually with exposure to elevated salinity, which could partially explain the inconsistent results across these various studies as they all use different exposure times. Notably, there have been a few wetland studies that include a temporal component when assessing the effect of saltwater intrusion; the few that do focus primarily on the biogeochemical aspects (Weston et al. 2011; Sutton-Grier et al. 2011; Neubauer et al. 2013; Marks et al. 2016). There have been studies that have looked at temporal changes in microbial communities in upland terrestrial soils (Grayson et al. 2000; Frey et al. 2008) as well as examining functional groups (e.g. sulfate reducers (Kearns et al. 2016); methanogens (Sun et al. 2012)) or specific phylogenetic groups (e.g. Archaeal *16S rRNA* (Kruger et al. 2005; Watanabe et al. 2007); Eubacterial *16S*

rRNA (Bernhard et al 2012)) in wetland soils. However, there are relatively fewer studies that address how environmental fluctuation affects the total wetland microbial communities composition and function over time (Mentzer et al. 2006; Reed and Martiny 2013).

Another striking omission in these prior studies is their failure to consider the fungal component of the microbial community. It is common to study the bacteria and archaea (usually via analysis of the *16s rRNA* gene as a phylogenetic marker (Edmonds et al. 2009; Reed and Martiny 2013; Morrissey et al. 2014; Morrissey and Franklin 2015)) as well as specific functional groups using process-specific genes (e.g., *mcrA* and *dsrA* (Beck et al. 2010; Morrissey and Franklin 2015); *nirK*, *nirS*, and *nosZ* (Bannert et al. 2011; Peralta et al. 2013; Prasse et al. 2015)) to characterize microbial communities in wetland ecosystems. In terrestrial ecosystems, fungi are well recognized for their essential role in aerobic carbon mineralization, and especially for their contribution to the degradation of recalcitrant carbon compounds such as lignins and phenolics from plant litter (Reddy et al. 2000; Thormann 2005). In contrast, the role of fungi in OM breakdown in wetlands has been largely ignored because it is assumed that the anaerobic conditions limit fungal growth (de Boer et al. 2005), but several recent studies have demonstrated that fungi are in fact significant contributors in wetlands carbon cycling (Hackney et al. 1999; Kuehn et al. 2000; 2001). Given this, it is likely that characterizing the total microbial community (archaeal, bacterial, and fungal) composition in TFW will allow for a better understanding on how saltwater intrusion will influence the global carbon pool.

To gain insight into how the microbial community in tidal freshwater wetlands changes with increasing exposure to saline conditions, an *in situ* soil transplant was utilized. The short term (1, 3, and 12 weeks) and long term (49 weeks) response was assessed using both functional genes and phylogenetic markers to study shifts in methanogens, sulfate reducers, and microbial community composition. These results were coupled to analysis of carbon transformations associated with microbial community function using extracellular enzyme assays and gas production assays. By examining how the composition of the microbial communities that regulates decomposition is impacted by salinity, we hope to elucidate how saltwater intrusion impacts overall carbon mineralization in TFW.

METHODS

Experimental Design:

To test for the effect on increased salinity on microbial communities from TFW, we performed a transplant experiment wherein we enclosed intact soil cores from a freshwater site in nylon mesh bags and relocated them downstream to the more saline site. This research was conducted in the Pamunkey/York River System (Virginia, USA) at Cumberland Marsh Preserve (37°33'25.921" N, 76°58'52.053" W), a pristine tidal freshwater marsh (salinity < 0.5 ppt) dominated by obligate freshwater macrophytes (e.g., *Peltandra virginica* and *Pontederia cordata*), and Taskinas Creek (37°24'52.994" N, 76°43'9.156" W), an oligohaline-mesohaline (5-19 ppt) tidal marsh with salt-tolerant vegetation (e.g., *Scirpus robustus* and *Spartina alterniflora*). The experimental manipulation was initiated in May of 2015 by establishing a 10 × 15 m plot (subdivided into 1 × 1 m quadrats) at each site. A random number generator was used to select quadrats within each plot from which to collect cores; no quadrat was sampled more than once. After the removal of the loose plant material from the surface of the soil, cores (10 cm diameter × 5 cm depth) were collected and encased in nylon bags (500 µm mesh, 15 × 25 cm). Encased cores were returned to their original location and allowed to pre-incubate for two weeks, after which time three experimental treatments were established: freshwater control (FC, freshwater cores incubated at the freshwater site), freshwater transplant (FT, freshwater cores relocated to the saline site), and saltwater control (SC, saltwater cores incubated at the saline site). The transplant represented time = 0 for all treatment groups. Five randomly selected bags per treatment were collected during low tide after 1, 3, 12, and 49 weeks of incubation. When transporting between sites or to the laboratory, soil samples were placed into a sealed airtight plastic bag and stored on ice.

Soil Characterization:

Soil conductivity (Hach Pocket Pro+ Multi 2, Loveland, CO, USA), pH, and redox (Laqua Act Portable pH/ORP/ION meter D-73, Irvine, CA, USA) were immediately measured upon return to the

laboratory. Soil was then homogenized and a subsample (~20 g) was removed for determining moisture content (gravimetrically, 100°C for 72 hr), organic matter (mass loss on ignition, 500°C for 5 hr), and C:N ratio (acidified with 0.10 M HCl, analyzed using a Perkin Elmer CHNS-O analyzer, Waltham, MA, USA). A subsample of soil was also archived for later genetic analysis (~ 5 g, stored at -80°C). Porewater was collected by centrifugation (~40 g of wet soil, 1500 × g for 15 min), filtered with a 0.22 µm pore-size mixed cellulose ester syringe filter, and stored at -20°C until it could be analyzed using ion chromatography (Dionex ICS-1000, Sunnyvale, CA, USA). The concentration of dissolved organic carbon (DOC) was also measured (Shimadzu TOC-V 5000, Columbia, MD, USA). The remainder of the soil was stored at 4°C until enzyme activity and gas production assays were complete (within one week).

Extracellular Enzyme Activity (EEA):

Soil slurries were prepared from approximately 1.0 g (\pm 0.2) of wet soil with 100 mL of sterilized deionized water and sonicated (15W for 2 min, Misonix Sonicator 3000, Farmingdale, NY, USA). Two hydrolytic enzymes, β -1,4-glucosidase (BG, cellobiose \rightarrow glucose) and 1,4- β -cellobiohydrolase (CHB, cellulose \rightarrow disaccharide) were measured fluorometrically using methylumbelliferone (MUB) labeled substrates with MES buffer (0.1 M, pH 6.1) following methods described in Morrissey et al. (2014). The assays were pre-incubated for 4 hr and 1 hr respectively at 30°C in the dark prior to fluorescent reading at 360 nm excitation and 460 nm emission for 6 hr. Phenol oxidase (POX, lignin \rightarrow oxidative lignin) was measured colorimetrically with L-3,4-dihydroxyphenylalanine (L-DOPA, 6.5 mM) substrate addition and sodium bicarbonate buffer (50 mM, pH 6.1) following methods described in Neubauer et al. (2013). The POX assay was pre-incubated at 30°C for 30 min in the dark and was measured at 460 nm wavelength for 6 hr. Fluorescent and colorimetric readings was done on a Synergy 2 plate reader (Biotek, Winooski, VT, USA).

Carbon Mineralization Potential:

Anaerobic production of CO₂ and CH₄ was measured as described in Neubauer et al. (2005). Anaerobic conditions were maintained using an N₂ filled glove box. Briefly, approximately 7.0 g (\pm 0.2) of homogenized soil was combined with 7.0 mL of deoxygenated site-specific porewater (filtered with GF/F and GF/C filters) in a 60 mL serum bottle; two technical replicates were prepared for each sample. Bottles were pre-incubated overnight and thoroughly flushed the next morning with N₂ (15 min). Gas samples were then collected after 0, 8, 24, 36, and 48 hr by briefly vortexing the soil slurry, injecting 5 mL of N₂, and immediately withdrawing 5 mL of gas from the headspace of each bottle. Concentrations of CO₂ and CH₄ were determined using a Shimadzu GC-14A gas chromatograph with methanizer and flame ionization detector (Shimadzu Scientific Instruments, Columbia, MD, USA).

Molecular Analyses:

Prior to DNA extraction, soil samples (~0.5 g wet weight) were centrifuged (10,000 \times g, 1 min) and excess water was pipetted off. To help remove humic acids, each sample was then amended with 1.5 mL of EDTA (0.2 mM, pH 8.0), vortexed at low speed (15 min), and centrifuged (2,500 \times g, 10 min). The supernatant was discarded and DNA was then extracted from the residual soil using the MoBio PowerSoil DNA Isolation Kit (Carlsbad, CA, USA) following manufacturer's instructions. Successful extraction was verified by agarose gel (1.0%) electrophoresis and ethidium bromide staining. The purity and concentration of the DNA was assessed using a Nanodrop-ND 1000 (Thermo Scientific, Wilmington, DE, USA) and final extracts were stored at -20°C.

Quantitative PCR (qPCR) assays were used to assess the relative abundance of several microbial groups. Bacteria (eubacteria) and archaea were studied using the 16s *rRNA* gene with the EUB 338/EUB 517 and the Arch 967F/Arch 1060R primer sets respectively. The assays followed the protocols outlined in Morrissey et al. (2014) with a slight modification of the thermal cycling conditions for archaea: 95°C for 5 min followed by 40 cycles of 20 sec at 95°C, 30 sec at 59°C, and 30 sec at 72°C. Fungal abundance

was quantified by targeting the second internal transcribed spaced (ITS2) region using primers designed by Taylor (2004), 5.8s_Fun and a shorted version of ITS4_Fun, from which two base pairs were removed from the 5' end (yielding: 5' CCT CCG CTT ATT GAT ATG CTT AAR T 3'). Reaction mixtures included 6 ng of template DNA and both primers at a concentration of 0.75 μ M. Thermal cycling conditions were: 95°C for 6 min followed by 35 cycles of 15 sec at 95°C, 30 sec at 55°C, and 1 min at 72°C and a final extension at 72°C for 5 min. To study methanogens, the mlas/mcrA-rev primer pair was used to target the methyl coenzyme-M reductase (*mcrA*) gene following Morrissey et al. (2014). The abundance of sulfate reducing bacteria was assessed by targeting the dissimilatory sulfite reductase (*dsrA*) gene using the dsrA 290F/dsrA 660R primers following Morrissey and Franklin (2015) but using 2 ng of template DNA and both primers at a concentration at 0.5 μ M. All qPCR reactions (15 μ L) were performed using a BioRad CFX 384 Real Time System and SsoAdvanced™ Universal SYBR® Green Supermix (BioRad, Hercules, CA, USA). Three technical replicates were prepared for each sample for each assay, and data were analyzed with BioRad CFX Manager (Version 3.1). Standard curves were prepared used genomic DNA from the following ATCC stains (Manassas, VA, USA): *Desulfavibrio desulfuricans* (Strain 27774, for eubacteria and dsrA), *Methanococcus voltae* (Strain A3, for archaea and mcrA), and *Saccharomyces cerevisiae* (Strain S288c, for fungi). All reaction efficiencies were between 96 and 103% and all $r^2 \geq 0.995$.

Statistical Analyses:

All data analysis was performed using R 3.2.0 (R core team 2015) with an α of 0.05. Technical replicates were average prior to statistically analysis. Gas production rates and gene abundances were log (X + 1) transformed prior to statistical analyses. A two-way analysis of variance (ANOVA, treatment and time as main effects) was performed on each measured variable. Whenever a significant interaction effect was encountered, separate one-way ANOVAs were run for each time point to determine treatment effects. When significant differences were found, a Tukey's HSD post hoc test was used for pairwise comparisons. Spearman's correlation (ρ) was used to assess the relationship between environmental

parameters, process rates, enzyme activities, and microbial abundances. Principal component analysis (PCA) was used to visualize the overall variation in microbial abundance, process rates, and environment factors across treatments and over time using the PAST statistical package (Version 3, Hammer et al. 2001).

RESULTS

Soil Parameters and Porewater Chemistry:

Soil salinity was reflective of the host environment, where the freshwater control (FC) always had the lowest salinity (≤ 0.1 PSU) compared to the soils hosted at the brackish marsh (1.3 - 6.5 PSU) (Table 1). There was little variability in salinity at the freshwater site over the course of the study (one-way ANOVA within the FC treatment: $p = 0.35$). Salinity varied across time for the FT and the SC samples, however there were no differences between these two treatments ($p > 0.14$ for all times). For all sampling events, the soil originating from the freshwater site (FC and FT) had the higher OM and lower C:N compared to the SC. Soil redox potential was always negative and significantly lower for SC (-160 ± 18 mV) compared to soils of freshwater origin (overall mean for FC and FT: -78 ± 13 mV) ($p < 0.001$). No meaningful trend was observed for soil pH, which ranged from 5.1 - 6.9 across all treatments and times. The concentration of DOC varied with both treatment and time (Table 2). Specifically, DOC concentrations in the short term experiment (1-12 weeks) were, on average, ~ 3.5 -fold greater than the values recorded for the long term sampling event (49 weeks). For most sampling events (except week 12), the porewater DOC concentration was greater for the SC than for the FC (significant differences were found in only weeks 3 and 49, $p \leq 0.001$). Porewater concentrations of NH_4^+ , NO_3^- , and SO_4^{2-} were higher in soils hosted at the brackish marsh (i.e., FT and SC) by approximately 11, 5, and 65-fold, respectively (Table 2).

Extracellular Enzyme Activities (EEA):

To specifically determine the response of freshwater soils to increased salinity, EEA was measured for the FC and FT treatments (Table 3). Due to differences in soil OM content between the freshwater marsh and the brackish marsh, EEA was not measured for the SC treatment. In general, hydrolytic enzymes regulating the degradation of cellulose were slightly suppressed under higher saline conditions for the short term experiment (~20% during weeks 1-12). This effect was much greater with long-term exposure (49 weeks), which reduced BG and CHB activity by nearly 70%. For POX activity, we were unable to collect measurement for week 1. Results indicated the length of saltwater exposure had a significant effect on POX activity. Salinity had the strongest effect on POX activity in the short term incubation, and suppressed activity by 20% during week 3 and 10% during week 12. We observed no effect of salinity on POX activity at week 49.

Carbon Mineralization Potential:

A treatment effect was observed for CH₄ production, but differences over time were not statistically significant (Figure 1). Overall, the potential rates of CH₄ production were higher in the FC than in the SC by approximately 82-fold. Exposure to saline conditions suppressed CH₄ production in the FT treatment to levels comparable to the SC; post-hoc tests revealed no significant differences between these treatments for the overall study. In contrast, CO₂ production was stimulated with exposure to saline conditions. During the first week of incubation, CO₂ production increased approximately 2-fold (FC to FT) but did not reach levels observed for SC. For all subsequent sampling events, CO₂ production of the FT was not statistically different from the SC.

Gene Abundance:

Overall, higher bacterial (*16s rRNA*) abundance was observed in soils hosted at the brackish marsh (FT and SC), however this trend was only statistically significant for week 3 and week 49 (Figure 2). Conversely, archaeal (*16s rRNA*) abundance decreased with elevated salinity. Fungal abundance

(*ITS2*) was influenced by soil origin, in which the SC had the highest abundance. When compared to the bacterial abundance, fungal abundance was much lower (~1000-fold). Soil origin had the greatest effect on methanogens (*mcrA*) abundance, in which soils from the freshwater site (FT and FP) were approximately 40-fold higher than the SC (Figure 3). In contrast, the abundance of sulfate reducers (*dsrA*) was highest in the SC and lowest in the FC. The abundance of sulfate reducers in FT increased over time and, by week 12, matched the levels found in SC (Figure 3). In general, gene abundances tended to be higher in week 49, though the increase was not significant for bacteria or *dsrA*.

Overall Response to Increased Salinity

Principal component analysis was used to visualize the overall response of the soils to salinity exposure based on the environmental data, gas production results, and qPCR assays (Figure 4). Approximately 50% of the variability in the data set is represented using the first and second principal components (PC). Differences in the FC and SC treatment were largely driven by soil properties and porewater chemistry (PC1). The soils from the FT treatment clustered between the two control treatments and showed a consistent temporal trend. Across all treatments, week 1, 3, and 12 microbial abundance was lower than that of week 49, which drove separation on PC2.

DISCUSSION

Under environmental perturbation, changes in microbial communities can vary across a temporal scale. Thus, studies that only examine these short term (weeks to months) or long term (years) changes may not fully document the mechanisms that drive microbial community change, especially with regards to the biogeochemical functions. The experiment described here sought to address the effects of both short (1, 3, and 12 weeks) and long(er) (49 weeks) term exposure to salinity by transplanting the microbial community from freshwater wetland soil downstream to a brackish marsh. Given that rates of OM decomposition in TFW depend on different biological and physical feedback cycles, addressing how

factors regulating carbon cycling change across a temporal scale is important in understanding the impact of saltwater intrusion.

Soil enzymes play a critical role in OM decomposition by depolymerizing macromolecules into labile carbon substrates for microbial uptake (Schimel and Weintraub 2003; Schimel and Schaeffer 2012). In this experiment, we focused on comparing the FC and FT, which allowed us to isolate the effect of salinity without confounding effects of changing OM content, as would be the case if we included an analysis of soils originating from the saline site. Overall, exposure to saline conditions negatively affected enzyme activity. This effect was most pronounced for BG and CHB in response to long term saltwater exposure (49 weeks). Conversely, POX activity was most affected in the short term exposure. These findings are similar to other saltwater intrusion studies that have documented a moderate decrease in enzyme activity (Jackson and Vallaire 2006; Neubauer et al. 2013). Neubauer et al. (2013) hypothesized that this decrease resulted from changes in soil carbon pool as a result of 3.5 years of simulated saltwater intrusion. After approximately one year of elevated salinity exposure, we observed no change in either soil OM or C:N ratio (Table 1) but it may be that differences such as these only manifest with more prolonged saltwater exposure. In a transplant study, Morrissey and Franklin (2015) also found salinity did not alter soil carbon properties after a relatively short period of exposure (five weeks). The moderate decrease in soil enzyme activity observed in this study may be attributed to osmotic stress placed on the microbial community. Under stressed conditions, organisms may shift from growth and maintenance to survival mechanisms, which may alter resource allocations (Schimel et al. 2007). The mechanisms regulating enzyme activity may be dependent on the length of saltwater exposure. Additionally, since the production of extracellular enzymes is performed by a broad and diverse group of microorganisms, this function of the community may be more resistant due to functional redundancy (Schimel et al. 2007), compared to functions that are performed by only a small group of organisms with specialized physiological pathways such as methanogens (Morrissey et al., 2014).

Salinity can also affect soil organic carbon lability and sorption dynamics that can potentially influence the availability of carbon substrates for microbial metabolism. For example, it has been shown

that initial exposure to increased ionic strength (such as would be found in saltwater) may enhance the bioavailability of organic substrates and make organic particles more accessible for decomposition (Wong et al. 2008; Singh 2016). Some studies have suggested that high quality and more labile OM content and low C:N ratio of soils may be important in alleviating some of the adverse effect of salinity on the microorganisms and enzyme activities (Liang et al. 2003; Wichern et al. 2006), which may also explain why enzyme activity was only moderately reduced in this study. Soil physico-chemical properties are an important component in determining microbial community composition and stability under environmental perturbation (Griffiths and Philippot 2012). A large contributor of soil OM is the decomposition of wetland plants (Kayranli et al. 2010). Carbon inputs from freshwater macrophytes and root exudates are generally considered easier to decompose than saltwater macrophytes due to lower C:N ratio and lignin content (Odum 1988). However, salinity can have a negative impact on freshwater macrophytes (Baldwin and Mendelssohn 1998; Sharpe and Baldwin 2012; Sutter et al. 2013) and chronic exposure to salinity can shift plant communities to include species that are more adapted to saline conditions (Perry and Hershner 1999; Nielsen et al. 2003; Craft 2007). These sorts of changes in plant community composition can lead to changes in the OM quality and quantity (Spalding and Hester 2007; Hopfensperger et al. 2014), all of which have been shown to have an effect on the microbial community (Faulwetter et al. 2009; Sutton-Grier and Megonigal 2011). Since the length of salinity exposure appears to have varying effects on soil properties and microbial process rates, it is imperative to assess the effect of saltwater exposure on factors regulating carbon mineralization on time scales longer than just one year and to consider whole ecosystem responses.

Despite the moderate decrease in enzyme activities, saltwater intrusion can increase the availability of more energetically favorable terminal electron acceptors (e.g., sulfate). This increase, combined with greater bioavailability of organic material (discussed above), may stimulate overall rates of carbon mineralization. Similar to several other salinity manipulation studies (Weston et al. 2006; Chambers et al. 2011; Marton et al. 2012; van Dijk et al. 2015), we found that elevated salinity had a profound effect on gas production rates. Overall, CH₄ production was suppressed in FT, and there was a

negative correlation with salinity across all treatments (Spearman's correlation: $r_{\text{CH}_4} = -0.49$, $p < 0.001$). In contrast, CO_2 production increased when salinity was higher ($r_{\text{CO}_2} = 0.42$, $p < 0.001$) and, within only three weeks, production in the FT reached levels comparable to those found in the SC (Figure 1). This simultaneous increase in CO_2 and decrease in CH_4 production has been attributed to terminal electron availability (Weston et al. 2011; Neubauer et al. 2013; Hoffensperger et al. 2014), which causes a shift in the microbial community from one dependent on methanogenesis to one that relies more on sulfate reduction. The moderate correlation we observed between gas production rates and functional gene abundance supports this hypothesis ($r_{\text{CH}_4 - \text{mcrA}} = 0.68$, $p < 0.001$; $r_{\text{CO}_2 - \text{dsrA}} = 0.33$, $p = 0.01$). Moreover, the abundance of these functional genes was also correlated to salinity ($r_{\text{mcrA}} = -0.53$, $p < 0.001$; $r_{\text{dsrA}} = 0.65$, $p < 0.001$). Interestingly, the abundance of sulfate reducers did not increase until 12 weeks of saltwater exposure (Figure 3). Given that sulfate reducers are a phylogenetically and metabolically diverse group of organisms that are ubiquitous in freshwater environments, one possible explanation for this finding is that the increased availability in sulfate during salinization may have activated the existing freshwater community that was dormant, and it simply took several weeks for the community to fully acclimate. Both Edmonds et al. (2009) and Kearns et al. (2016) found that saltwater intrusion did not alter the community of sulfate reducers after 5 weeks and 1 year, respectively, which also suggests that sulfate reducers found in freshwater soils may be resistant to environmental perturbation. However, both of these studies utilized laboratory incubations with artificial saltwater, so the lack of change in the sulfate-reducing community may not be reflective of environmental conditions where the influx of salt-tolerant sulfate reducers would likely accompany a natural salinization event. Though the abundance of sulfate reducers did not increase until week 12 (Figure 3), CO_2 production increased within only 1 week (Figure 1). This result is consistent with the hypothesis that the increase availability of sulfate activated a dormant freshwater community, which was able to quickly begin mineralizing carbon but took some time to increase in abundance.

Another possible explanation for this pattern is that the initial increase in CO_2 was due to iron reducing bacteria. Although not as well studied in the context of saltwater intrusion, microbial iron (III)

reduction can also be important in regulating carbon mineralization in both freshwater and brackish environments (Neubauer et al. 2005). The increase in ionic strength from saltwater can increase the availability of labile iron, stimulating iron reduction (Baldwin et al. 2006; Bongoua-devisme et al. 2012; van Dijk et al. 2015; Weston et al. 2006). For example, Weston et al. (2006) reported an initial increase in iron reduction within 7 days of elevated salinity; however, by 12 days, sulfate reduction became the dominant anaerobic carbon mineralization pathway. The decline in iron reduction could be due to the abiotic interactions with sulfide compounds and the immobilization of reduced iron into pyrite (Neubauer and Craft 2009). A similar event could have occurred in this study, and the transient period of activity by iron reducers could explain the increased rates of CO₂ production that occurred during the lag period before the abundance of sulfate reducers increased. This hypothesis is consistent with the findings of Morrissey and Franklin (2015), who performed a similar transplant experiment using the same sites that we did over a five week incubation period. They found *Geobacter* (a putative iron reducer) persisted in transplanted soils (analogous to our FT), and abundance was only reduced by 35% relative to the freshwater controls (our FC). Though iron reduction is performed by a phylogenetically diverse group of microorganisms (Lovely et al. 2004; Li et al. 2011), and their tolerance for saline conditions is well documented (Bongoua-Devisme et al. 2012), the abundance of *Geobacter* in the soils from the saltwater site (SC) was only ~0.5% of that found in the freshwater soils and no other known iron reducers were detected. Over this same time frame, Morrissey and Franklin (2015) observed no change in abundance of sulfate reducers in the transplanted soils, which is also consistent with our work.

Across all treatments, we found the microbial community to be dominated by bacteria (Figure 2), with an abundance of *16S rRNA* genes that was approximately 10-fold higher than was found for archaea. The abundance of archaea *16S rRNA* genes was strongly correlated with *mcrA* abundance ($r = 0.67$, $p < 0.001$) which, together with the fact that the ratio of the abundance of these two genes was approximately 1.0, suggest that a large fraction of archaea found in our freshwater samples (FC and FT) were likely methanogens. Since methanogens are adversely affected by osmotic stress (Chambers et al. 2011) and are likely to be poor competitors with sulfate reducers (Capone and Kiene 1988; Megonigal et al. 2004;

Sutton-Grier et al. 2011), we expected exposure to saline conditions to decrease methanogen abundance in the FT treatment. Interestingly, abundance remained high and comparable to the FC (Figure 3), which may indicate that the methanogen community became metabolically inactive with saltwater exposure but did not die. Several studies have demonstrated that under sulfate reducing conditions, methanogenesis is able to proceed by utilizing non-competitive carbon substrates (Oremland and Polcin 1982; Weston et al. 2011; Yuan et al. 2014).

Fungi were detected in all of our soil samples, though at a much lower abundance than bacteria (~5000-fold across all treatments). However, considering the highly reduced conditions of our soils cores, this is not necessarily surprising. Often, fungal communities in wetlands are associated with standing plant litter and decaying detritus (Kuehn et al. 2000; Buchan et al. 2003; Kuehn et al. 2011), and there have been few studies that have considered the bulk soil. A recent study by Mohamed and Martiny (2011) found that fungal community composition was primarily driven salinity; while we did not study composition, we did observe an increase in fungal abundance in the SC soils and a modest correlation with salinity ($r = 0.36$, $p = 0.005$), which suggests salinity is a driver in our study as well.

CONCLUSIONS

Overall, the higher salinity and elevated concentrations of sulfate at the brackish marsh, combined with the relatively higher OM content at the freshwater site, drove the differences in gene abundance across the two sites (compare FC and SC in Figure 4). These differences corresponded to consistent changes in carbon mineralization across the sites. Interestingly, the transplanted soils (FT) cluster between the two controls treatments in our PCA, showing a clear progression toward the more saline site (SC) with prolonged incubation. Differences in gas production and microbial abundance between the short term and long term incubations suggest that saltwater intrusion changes the microbial community dynamics gradually over time and these differences are, at least in part mediated, by soil origin (i.e., the initial soil properties and freshwater microbial community). This is consistent with several

recent studies that point to site history as the major determinant of microbial community structure and function (Drenovsky et al. 2003; Nelson et al. 2014; Morrissey and Franklin 2015) as well as a recent meta-analysis by Allison and Martiny (2008), which revealed that the effects of disturbance on microbial community composition can persist for years. In our study system, longer exposure to saltwater intrusion may result in larger ecosystem scale changes such as shifts in vegetation assemblages and different physiochemical soil properties, which may negate the initial site history effect on the microbial community.

The findings of this study indicate that saltwater intrusion can dramatically affect microbial community dynamics and factors regulating anaerobic carbon mineralization in TFW, which could impair the ecological function of these ecosystems as a carbon reservoir. The magnitude of changes in these processes is dependent on the complex interaction between the soil microbial community and the length of saltwater exposure. Although previous studies have suggested that simply increasing sulfate availability will cause the microbial community to shift from methanogenesis to sulfate reduction, and our own results show an immediate change in gas production that is consistent with this hypothesis, we found that changes in the abundance of methanogens and sulfate reducers are actually much slower. These findings highlight the complexity of terminal anaerobic OM degradation and the microorganisms associated with these processes in regards to saltwater intrusion. The results also suggest that assessing saltwater exposure across multiple time points may provide a better predictive potential to address small and large scale changes in ecosystem functions, and that additional studies that consider even longer exposure times are needed.

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APPENDIX

Table 1: The mean (\pm standard error, n=5 per group) of soil parameters for the freshwater control (FC), freshwater transplant (FT), and saltwater control (SC) treatments after 1, 3, 12, and 49 weeks of *in situ* incubation. Two way ANOVA results for interaction (treatment \times time), treatment, and time effect. Whenever a significant interaction effect was observed ($p < 0.05$), separate one-way ANOVAs were performed to compare each treatment at each time point. A p -value ≤ 0.05 is consider significant and NS denotes not significant.

Soil Properties	Week	Mean (\pm SE)			ANOVA Results	
		FC	FT	SC	Factor	p
Salinity (PSU)	1	0.06 (0.01)	1.89 (0.17)	2.39 (0.25)	Interaction	< 0.001
	3	0.04 (0.00)	2.57 (0.31)	3.19 (0.46)	Treatment	-
	12	0.07 (0.02)	5.00 (0.68)	5.49 (0.31)	Time	-
	49	0.05 (0.01)	4.19 (0.22)	4.31 (0.40)		
Organic Matter (%)	1	35.7 (1.2)	35.7 (1.5)	25.2 (0.5)	Interaction	0.21 (NS)
	3	32.7 (0.6)	34.5 (1.7)	27.1 (1.7)	Treatment	< 0.001
	12	36.3 (0.6)	35.6 (1.6)	31.2 (1.8)	Time	0.08 (NS)
	49	35.6 (2.6)	36.3 (1.1)	25.2 (1.0)		
Redox (mV)	1	- 55 (25)	- 35 (31)	- 165 (42)	Interaction	0.22 (NS)
	3	- 86 (40)	- 69 (75)	- 181 (30)	Treatment	< 0.001
	12	- 26 (7)	- 84 (11)	- 73 (14)	Time	0.002
	49	- 84 (2)	- 182 (29)	- 225 (4)		
pH	1	6.1 (0.2)	5.1 (0.2)	5.6 (0.3)	Interaction	< 0.001
	3	5.8 (0.2)	5.3 (0.3)	5.8 (0.4)	Treatment	-
	12	5.5 (0.1)	6.0 (0.1)	5.6 (0.2)	Time	-
	49	5.4 (0.1)	6.8 (0.1)	6.8 (0.2)		
C:N	1	12.6 (0.2)	12.5 (0.4)	15.2 (0.5)	Interaction	0.37 (NS)
	3	11.9 (0.5)	12.1 (0.5)	13.8 (0.2)	Treatment	< 0.001
	12	13.8 (0.2)	14.9 (0.4)	18.3 (1.6)	Time	< 0.001
	49	12.6 (0.3)	13.2 (0.2)	14.6 (0.9)		

Table 2: The mean (\pm standard error, $n=5$ per group) of porewater DOC ($\text{mg} \cdot \text{L}^{-1}$) and ion species (mM) for the freshwater control (FC), freshwater transplant (FT), and saltwater control (SC) treatments after 1, 3, 12, and 49 weeks of *in situ* incubation. Two way ANOVA results for interaction (treatment \times time), treatment, and time effect. Whenever a significant interaction effect was observed ($p < 0.05$), separate one-way ANOVAs were performed to compare each treatment at each time point. A p -value ≤ 0.05 is considered significant and NS denotes not significant.

Porewater	Week	Mean (\pm SE)			ANOVA Results	
		FC	FT	SC	Factor	p
DOC	1	35 (9)	25 (6)	52 (7)	Interaction	< 0.001
	3	36 (3)	48 (3)	81 (10)	Treatment	-
	12	41 (6)	59 (7)	27 (3)	Time	-
	49	7 (1)	19 (3)	18 (2)		
NH_4^+	1	0.04 (0.01)	0.43 (0.04)	0.41 (0.05)	Interaction	0.31 (NS)
	3	0.01 (0.00)	0.84 (0.33)	0.48 (0.05)	Treatment	0.04
	12	0.01 (0.00)	0.39 (0.02)	0.33 (0.03)	Time	0.04
	49	0.11 (0.09)	0.00 (0.00)	1.09 (0.96)		
NO_3^-	1	0.13 (0.05)	0.40 (0.06)	0.61 (0.06)	Interaction	0.65 (NS)
	3	0.08 (0.01)	0.63 (0.10)	0.57 (0.02)	Treatment	< 0.001
	12	0.10 (0.02)	0.68 (0.03)	0.76 (0.04)	Time	0.32 (NS)
	49	0.26 (0.03)	0.54 (0.06)	1.15 (0.63)		
SO_4^{2-}	1	0.10 (0.01)	13.7 (1.4)	17.1 (1.2)	Interaction	0.002
	3	0.07 (0.02)	18.6 (2.1)	13.7 (1.9)	Treatment	-
	12	0.09 (0.03)	14.7 (1.1)	17.0 (1.0)	Time	-
	49	0.61 (0.08)	10.9 (1.6)	8.9 (2.3)		

Table 3: The mean (\pm standard error, n=5 per group) rates of extracellular enzyme activity ($\mu\text{mol substrates} \cdot \text{hr}^{-1} \cdot \text{g OM}^{-1}$) for the freshwater control (FC) and freshwater transplant (FT) treatments after 1, 3, 12, and 49 weeks of *in situ* incubation. Two way ANOVA results for interaction (treatment \times time), treatment, and time effect. Whenever a significant interaction effect was observed ($p < 0.05$), separate one-way ANOVAs were performed to compare each treatment at each time point. A p-value ≤ 0.05 is consider significant and NS denotes not significant. “ND” denotes instances where no data are available.

Enzymes	Week	Mean (\pm SE)		ANOVA Results	
		FC	FT	Factor	p
BG	1	22.2 (1.9)	18.5 (1.5)	Interaction	0.07 (NS)
	3	18.4 (3.1)	12.6 (1.7)	Treatment	0.001
	12	19.5 (3.2)	17.4 (2.0)	Time	0.22 (NS)
	49	23.3 (4.6)	7.2 (2.6)		
CHB	1	1.26 (0.22)	0.74 (0.12)	Interaction	0.07 (NS)
	3	1.05 (0.04)	1.13 (0.10)	Treatment	0.01
	12	1.89 (0.17)	1.49 (0.34)	Time	0.07 (NS)
	49	2.64 (0.82)	0.89 (0.27)		
POX	1	ND	ND	Interaction	0.16 (NS)
	3	459 (19)	361 (24)	Treatment	0.05
	12	318 (16)	288 (36)	Time	< 0.001
	49	308 (13)	309 (35)		

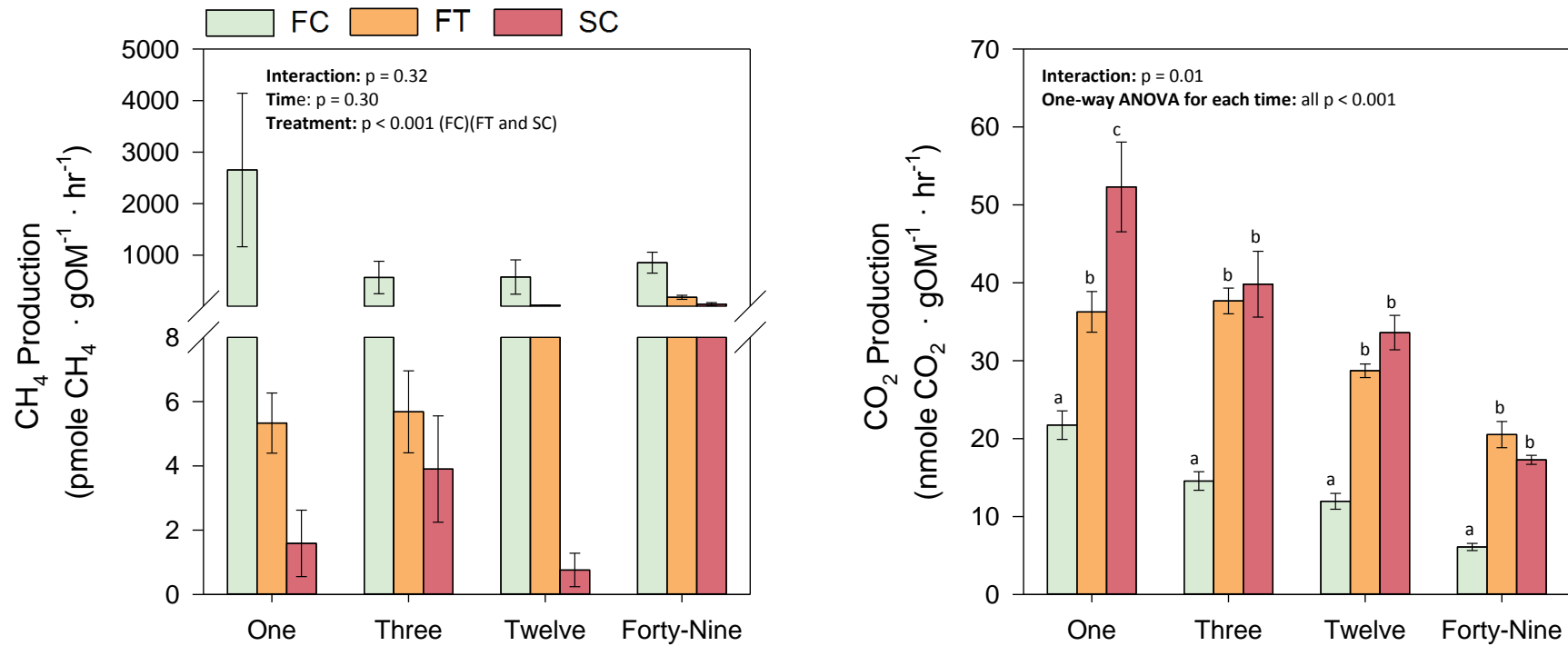


Figure 1: Mean (\pm standard error, $n=5$) gas production rates for the freshwater control (FC), freshwater transplant (FT), and saltwater control (SC) treatments measured after 1, 3, 12, and 49 weeks *in situ* incubation. For each dataset, a two-way ANOVA (treatment \times time) was performed. In cases where the interaction effect was not significant ($p>0.05$), main effects were interpreted and post-hoc comparisons were performed using Tukey's HSD; groups that are significantly different overall are distinguished using parentheses at the top of each graph. Whenever a significant interaction effect was observed ($p\leq 0.05$), separate one-way ANOVAs were performed to compare each treatment at each time point and the results of those post-hoc tests are displayed using different lowercase letters within each sampling event.

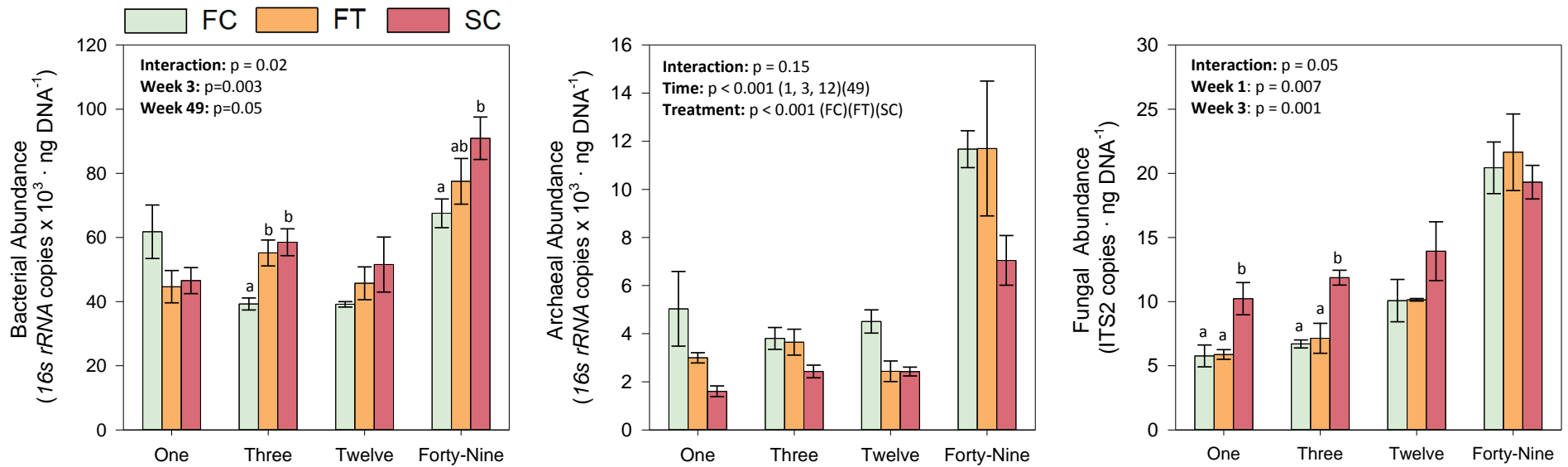


Figure 2: Mean abundance (\pm standard error, $n=5$) of bacteria, archaea, and fungi for the freshwater control (FC), freshwater transplant (FT), and saltwater control (SC) treatments measured after 1, 3, 12, and 49 weeks *in situ* incubation. For each dataset, a two-way ANOVA (treatment \times time) was performed. In cases where the interaction effect was not significant ($p > 0.05$), main effects were interpreted and post-hoc comparisons were performed using Tukey's HSD; groups that are significantly different overall are distinguished using parentheses at the top of each graph. Whenever a significant interaction effect was observed ($p \leq 0.05$), separate one-way ANOVAs were performed to compare each treatment at each time point and the results of those post-hoc tests are displayed using different lowercase letters within each sampling event.

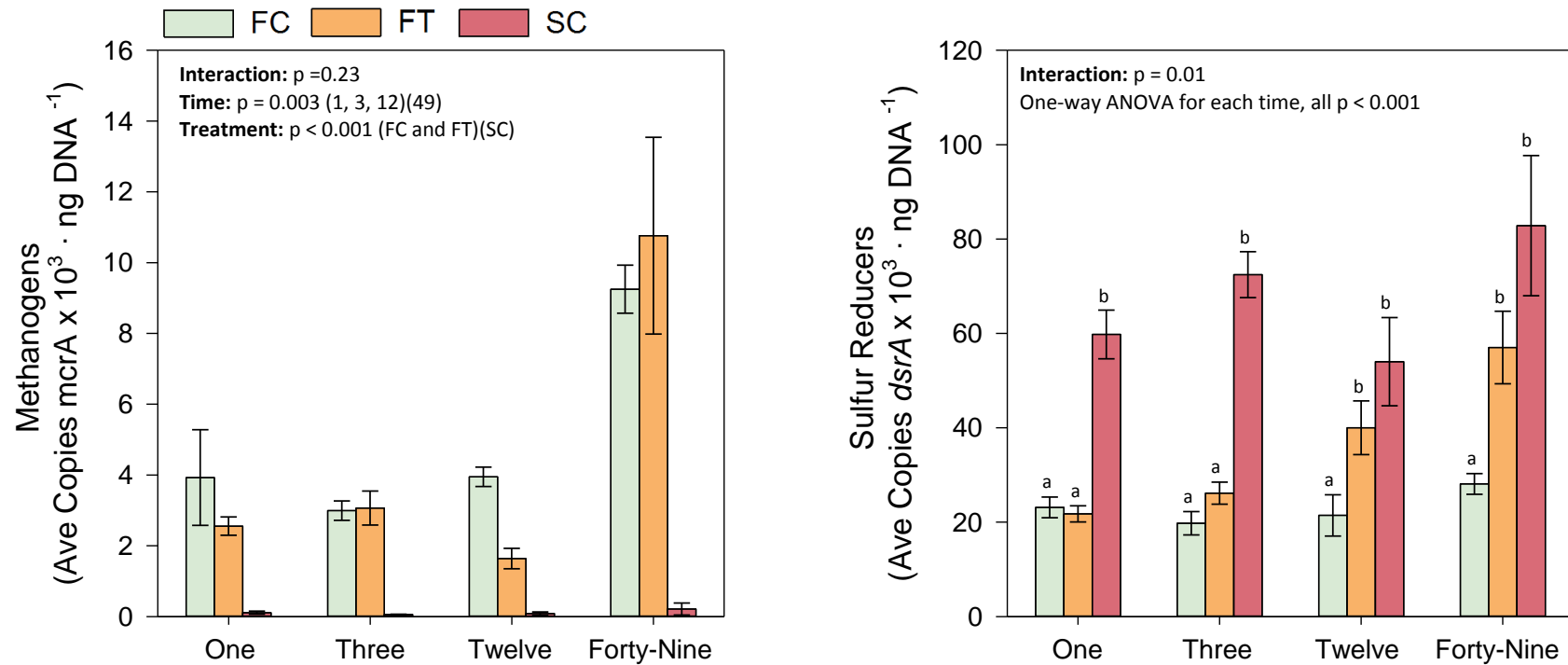


Figure 3: Mean functional gene abundance (\pm standard error, $n=5$) of methanogens and sulfate reducers for the freshwater control (FC), freshwater transplant (FT), and saltwater control (SC) treatments measured after 1, 3, 12, and 49 weeks *in situ* incubation. For each dataset, a two-way ANOVA (treatment \times time) was performed. In cases where the interaction effect was not significant ($p>0.05$), main effects were interpreted and post-hoc comparisons were performed using Tukey's HSD; groups that are significantly different overall are distinguished using parentheses at the top of each graph. Whenever a significant interaction effect was observed ($p<0.05$), separate one-way ANOVAs were performed to compare each treatment at each time point and the results of those post-hoc tests are displayed using different lowercase letters within each sampling event.

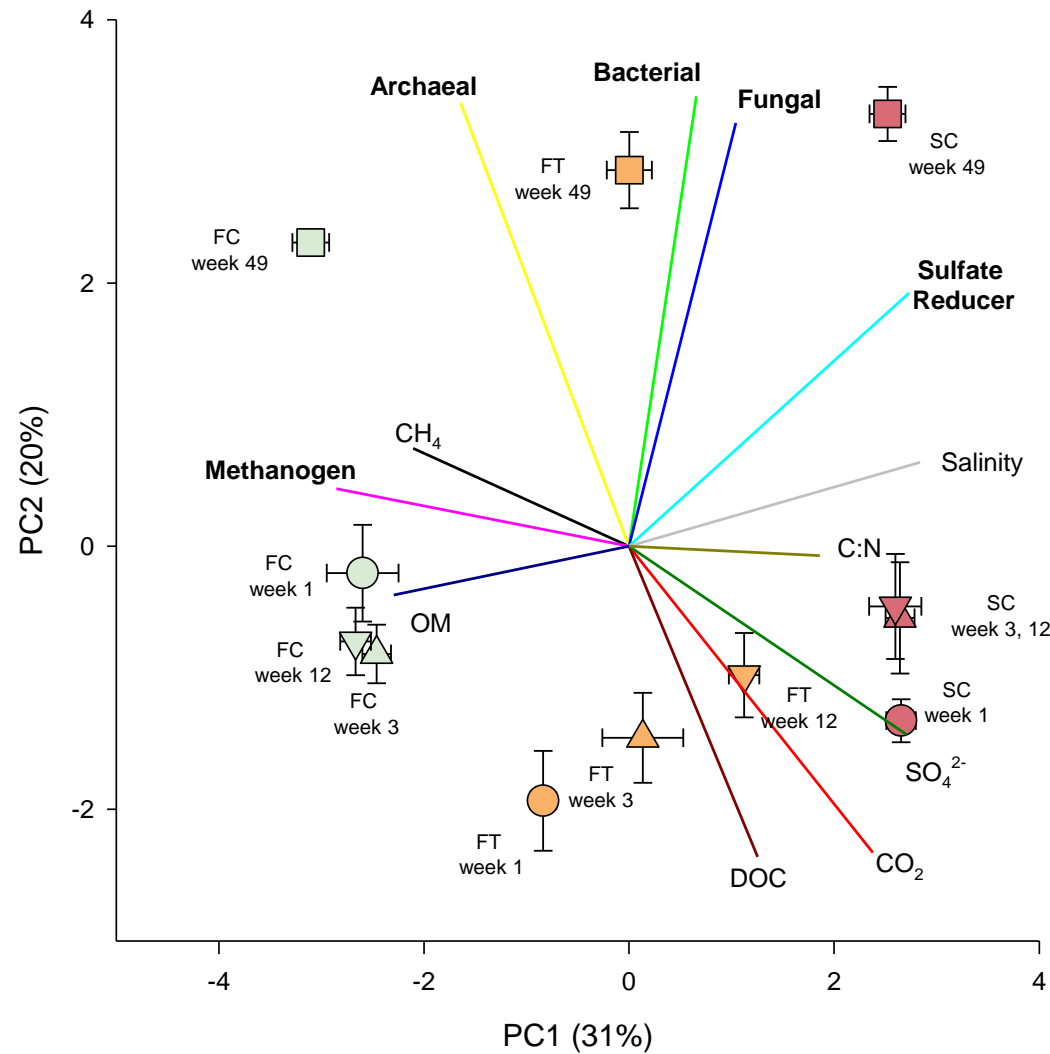


Figure 4: Principal component analysis with gene abundance, gas production rates, and environmental parameters as input variables. Separation along PC1 was driven by soil properties and porewater chemistry as well as differences in process rates. Separation along PC2 was driven by gene abundance by all groups (*16s rRNA*, *ITS2*, *mcrA*, *dsrA*), in which week 49 had the highest gene abundance.

VITA

Chansotheary Dang was born on August 11, 1988 in Richmond, Virginia. A first generation Asian American she graduated from Manchester High School, Midlothian, Virginia in 2007 and became the first member in her family to complete high school and attend college. She received dual degrees in Bachelor of Science in Biology and Chemistry from Virginia Commonwealth University in 2012. She was a drug screener for a toxicology lab for a year before returning to academia. During her time in graduate school, Chansotheary received honorable mention for the Department of Biology's Outstanding Teaching Assistant Award and was the recipient of the VCU Rice Center Student Research Award. In 2016, she received a Master of Science in Biology from Virginia Commonwealth University.