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Altered soil microbial community composition and function in two shrub-encroached marshes with different physicochemical gradients

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ABSTRACT

Important wetland functions, including regulating soil carbon (C) storage and water quality, are linked to biogeochemical processes mediated by soil microbes. Vegetation shifts such as shrub encroachment may alter the soil microbial community and result in changes in important biogeochemical processes, although few studies have examined this in subtropical marshes. Here, we used in-situ litter decomposition experiments, quantitative polymerase chain reaction, and laboratory assays on soil respiration, extracellular enzyme activity, and denitrification potential to determine differences in C storage and nitrogen (N) cycling between willow-encroached and non-encroached plots in two subtropical marshes (Moccasin Island and Lake Apopka, FL, USA). In both regions, encroached (willow or adjacent marsh) and non-encroached plots had distinctively different microbial communities, which were correlated with soil temperature and nutrient content. Greater enzyme activity, denitrification, and CO_2 production were observed in willow and/or adjacent marsh plots compared to control marsh plots at Moccasin Island. Conversely, lower enzyme activity, denitrification, and CO_2 production were detected in willow and/or adjacent marsh plots compared to control marsh plots at Lake Apopka. Despite differences in the response of biogeochemical processes and microbial community structure in the two study regions, in-situ decomposition rates were halved in willow litter compared to herbaceous litter in both regions, which was correlated with greater recalcitrant lignin content in willow litter. Ultimately, greater short-term litter C storage was observed in both study regions, but soil N cycling changes differed by region, potentially due to unique site characteristics such as hydroperiod and nutrient availability.

1. Introduction

Shrub encroachment, the shift from graminoid herbaceous plants to woody shrubs, is a phenomenon that has been rapidly increasing in occurrence worldwide over the last century in response to elevated atmospheric CO_2 levels, fire suppression, and hydrological alterations (Knapp et al., 2008). Pacala et al. (2001) report that 220–330 million hectares of grasslands have been shrub encroached in North America alone, consistent with rates of vegetation shifts in savannas, tundras, and other biomes worldwide (Eldridge et al., 2011; Maestre et al., 2016). The rapid rate of shrub encroachment in the last century are of concern as studies have shown that shrub encroachment can impact important ecosystem functions such as hydrological and biogeochemical cycling (Grover and Musick, 1990; Huxman et al., 2005; Schade and Hobbie, 2005; Knapp et al., 2008). However, the direction and magnitude of these changes are highly variable, depending on a variety of factors such as climate, encroaching shrub species, and land-use history (Jackson et al., 2002). The underlying causes behind reported environmental changes and how the soil microbial community responds to shrub encroachment are just a few of the many questions that remain unanswered (Maestre et al., 2016). Furthermore, recent reviews have highlighted another knowledge gap on shrub encroachment-little is known about shrub encroachment in aquatic systems, as most previous studies have focused on rangelands and deserts (Saintilan and Rogers, 2014).

Shrub encroachment is currently a global land management issue in wetlands as it may alter the critical role wetlands play in soil carbon (C) storage and nitrogen (N) cycling (Schuyt and Brander, 2004). For example, freshwater wetlands can store twice as much soil C as terrestrial systems and are commonly used to treat stormwater due to their ability to remove excess bioavailable N (38–83 g N m^{-2} yr^{-1}) via denitrification (Coveney et al., 2002; Mitsch et al., 2012; Adame et al., 2015). Marshes provide these services in large part due to the soil microbial community which acts as fundamental transformers of nutrients (Mitsch and Gosselink, 2007). Despite the important role soil microbes play in biogeochemical cycling, few studies have examined the effects

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of shrub encroachment on the soil microbial community and biogeochemical processes in subtropical marshes (Saintilan and Rogers, 2014). Considering shrub encroachment is a common disturbance in marshes, particularly subtropical marshes encroached by willows (Hall et al., 2017), it is critical to understand how soil microbial communities respond to shrub encroachment and its effect on important functions such as C storage and N cycling.

Terrestrial shrub encroachment studies, which comprise the bulk of the existing literature, have shown that shrub encroachment has the potential to alter the controlling factors of microbial communities, which include soil chemistry, nutrient input, and hydrology. Autogenic soil drying and nutrient redistribution have been associated with shrub encroachment due to the deeper and wider roots of shrubs compared to herbaceous species (Lee and Lauenroth, 1994; Pataki et al., 2008). Shrubs often redistribute nutrients from surrounding regions into the canopy, resulting in bare or unvegetated interspaces between shrub canopies (Schlesinger et al., 1996). Studies have also reported increased nutrient deposition and litterfall within shrub canopies, which can increase microbial community size and modify C storage (Dhillion, 1997; Duffy, 2014). The greater soil heterogeneity in shrub-encroached landscapes can promote microbial diversity and impact biogeochemical processes such as enzyme activity, CO_2 production and decomposition (Langenheder et al., 2006). Terrestrial studies have demonstrated different microbial communities and biogeochemical cycling between shrub-encroached and herbaceous lands, but it is unclear whether these responses occur synonymously in wetlands, which have vastly different hydrological regimes and biogeochemical processes than terrestrial studies (Montane et al., 2010; Yannarell et al., 2014).

An initial study by Ho & Chambers (unpublished data) characterized nutrient concentration differences in the soil, water, and leaf biomass between coastal plain willow shrub encroached plots and non-encroached marsh plots of two subtropical freshwater marshes (Moccasin Island and Lake Apopka) with different hydroperiods in the St. Johns River Watershed (FL, USA). Moccasin Island was a seasonally flooded marsh, while Lake Apopka was a permanently flooded marsh. Greater soil moisture and nutrients (C, N, and phosphorus (P)) were detected in willow and adjacent marsh plots at Moccasin Island, whereas no soil physicochemical differences were detected at Lake Apopka, suggesting biogeochemical processes such as C storage and N removal may also differ in these two shrub-encroached marshes. Therefore, this study's objectives were to identify differences in biogeochemical properties (enzyme activity, soil respiration, denitrification, N mineralization, and litter decay) and soil microbial community composition in willow plots, marsh plots adjacent to willows, and non-encroached control marsh plots. The two study regions were maintained to see if plot-level differences were consistent across the landscape. We hypothesized that microbial biomass and activity (enzyme activity, respiration, and denitrification) would be greatest in willow plots at Moccasin Island, following previous work indicating shrubs can create 'islands of fertility' (e.g., Schade and Hobbie, 2005) and the greater nutrient availability observed in those plots from the initial study. At Lake Apopka, microbial biomass and activity would not differ between plots as soil physicochemical properties did not differ. However, mass decay would be lowest in willow plots in both regions as willow litter contains more recalcitrant lignin than herbaceous litter in adjacent and marsh plots.

2. Materials and methods

2.1. Site description

Both study regions (Moccasin Island and Lake Apopka) are located within the St. Johns River Watershed (FL, USA) and were selected due to their varying hydroperiods and histories. The watershed is warm and wet with mean annual temperature and precipitation ranges around 21–22 °C and 1120–1160 mm (SJRWMD, 2018). The St. Johns River is a blackwater river that flows from south to north with < 10 m elevation changes throughout its 500-km length (SJRWMD, 2018). Moccasin Island is located near the headwaters of the river and is part of the River Lakes Conservation Area (5000 km^2; Brenner et al., 2001). Lake Apopka (3100 km^2) is a shallow lake that feeds into the St. Johns River (Schelske et al., 2005).

Moccasin Island (56 km^2; 28.2359° N, 80.8212° W) was historically a sawgrass marsh that was disturbed by levee construction for flood control in the 1900s. These hydrological alterations promoted bare moist soils for willow seed germination while hydrological restorations (levee removal) in the 1970s allowed mature willows necessary water for future expansion (Quintana-Ascencio et al., 2013). Currently, Moccasin Island is a sawgrass and willow-shrub dominated wetland. Based on aerial photography, willow coverage ranges from 30 to 90% in Moccasin Island and is dependent upon the frequency of dry periods (Hall et al., 2017). During this study, Moccasin Island was seasonally flooded (August–December).

Lake Apopka North Shore (80 km^2; 28.6946° N, 81.6586° W) was originally a sawgrass marsh, but drainage and nutrient enrichment from muck-farming practices in the late 1800s promoted cattail and willow-shrub dominance (Murphy, 2005). Restoration in the 1970s consisted of removing constructed levees and canals, planting native plants, and removing dissolved N and P from the lake using soil inversion and gizzard-shad removal (Schaus et al., 2010). Nutrient concentrations have been reduced by 80% since restoration began and have currently reached desired lake nutrient loads (Coveney, 2016). As willow was established prior to restoration, willows still cover > 40% of the shoreline (Murphy, 2005). Previous agricultural practices have resulted in highly compacted soils that are > 1 m below lake level, resulting in permanent flooding during the study (Conrow et al., 2011). These two study regions were selected because they are in the same watershed, were concurrently willow-encroached, have not experienced any management efforts to eradicate willow, but have unique hydroperiods and land histories.

2.2. Study design

In each region, a stratified random sampling design was used with three plot types: willow, adjacent marsh, and marsh. Using aerial photography, 50 potential plots (4 × 4 m) were designated based on vegetation type (shrub or herbaceous). Field verification consisted of splitting each plot into 16 quadrats (1 × 1m) and verifying that > 80% of the quadrants had aboveground coverage of the desired vegetation (willow or sawgrass/cattail). Herbaceous plots that passed field verification (~30 plots) were further separated as adjacent marsh (< 1 m from willows) or control marsh (> 10 m from willows) plots based on willow proximity. Adjacent marsh plots were used to test if biogeochemical differences were detected beyond willow canopies in willow-encroached marshes (e.g., the interspaces found in terrestrial studies), while control marsh plots were used as a reference for non-encroached marshes. Ultimately, each region was split into three plot types based on willow proximity and vegetation coverage: willow plots (> 80% willow), adjacent marsh plots (> 80% dominant herbaceous vegetation and < 1 m from willows, and marsh plots (same vegetation composition as adjacent marsh plots and > 10 m from willows on all sides). Within each region (Moccasin Island and Lake Apopka), five of each plot type were randomly selected for sampling using a Microsoft Excel random number generator.

2.3. Soil sampling and analysis

Sampling for all assays described below were completed from soil samples collected in the summer (July–August, 2017), except for potentially mineralizable nitrogen (PMN) rates, which was an additional study sampled in December 2017. For each analysis described below, three sampling points were randomly selected in each plot (five of each type). Prior to collecting the sample, soil temperature and redox

potential was measured at the surface (1 cm depth) of each sampling point using a Platinum-tipped electrode, volt meter, and calomel reference electrode for redox, and a Fisherbrand Long-Stem Digital Thermometer for temperature. A correction factor of 250 mV was applied to redox measurements to convert readings to Eh (Thomas et al., 2009). Two 0–10 cm soil cores were collected in 10 cm diameter polycarbonate tubes around each sampling point using the push core technique, extruded in the field, and composited into one sample to account for soil heterogeneity. All soil cores were collected in sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI) and transported on dry ice to the University of Central Florida for where they were homogenized by hand and stored at 4 °C until analysis.

Soil moisture, total C and total N were determined on triplicate homogenized 0–15 cm soil cores collected in every plot. Samples were oven dried at 70 °C for 72 h and weighed for gravimetric water content. A dried subsample was ground on a SPEX Sample Prep Mixer Mill 8000M (Metuchen, NJ), and analyzed on the Elementar Vario Micro Cube (Elementar Americas Inc., Mount Laurel, NJ) for soil C and N.

2.4. Microbial community size and composition

2.4.1. Microbial biomass carbon and dissolved organic carbon

Duplicate samples (~5 g of wet soil) were prepared in 40 mL centrifuge vials. One was designated as the non-fumigate sample and was extracted with 25 mL of 0.5 M K_2SO4 and incubated at 25 °C for one hour. The other was designated as the fumigate sample and was chloroform fumigated for 24 h prior to addition of 0.5 M K_2SO_4 and incubation at 25 °C for an hour (Vance et al., 1987). Both the incubated non-fumigate and fumigate samples were centrifuged at 150 rpm for 10 min, vacuumed filtered using Supor 0.45 μM filters, preserved using double distilled (DD) H_2SO_4, and stored at 4 °C until analysis. Organic flocculants formed after filtration were separated from the supernatant and dissolved in 1 M H_2SO_4. Both the supernatant and dissolved flocculants were analyzed for dissolved organic C (DOC) on a Shimadzu TOC-L analyzer (Shimadzu Instruments, Kyoto, Japan). The non-fumigated sample represents soil extractable DOC. Soil microbial biomass-C was calculated as the difference in DOC between the fumigate and non-fumigate sample.

2.4.2. Viability qPCR

Waterlogged wetland soils can result in slower decay of dead microbial DNA and lead to the overestimation of certain bacteria taxa (Emerson et al., 2017). To obtain an accurate representation of the current soil microbial community, a viability quantitative polymerase chain reaction (qPCR) method was implemented to only quantify viable microorganisms. Propidium monoazide (PMA) dye, which only intercalates to damaged cell membranes, is used to prevent downstream amplification and quantification of non-viable DNA (Nocker et al., 2007). As no previous studies have examined the soil microbial community of these study sites in detail, domain-specific primers for bacteria, archaea, and fungi were used to quantify the proportion of each domain in the soil. Furthermore, six of the most common soil bacteria phyla were analyzed using phyla-specific primers to gain a better understanding of the general microbial community structure in these marshes (Fierer et al., 2007).

To isolate only viable DNA from the soil, PMA dye was used (Nocker et al., 2007). Optimization of PMA utilized viable and non-viable *Staphylococcus aureus* (*S. aureus*) (provided by the University of Central Florida's Cole Lab), which was added to sterile soil samples. Non-viable *S. aureus* was created by immersing *S. aureus* in a 90 °C water bath for 15 min and then culturing on luria broth agar overnight at 37 °C to verify that the culture was not viable. Quantification of viable and non-viable *S. aureus* was determined on a BioTek Synergy HTX multi-mode reader at 600 nm (BioTek Instruments, Winooski, VT). To determine optimal PMA conditions, sets (consisting of sterilized soil spiked with either viable or non-viable *S. aureus*) were designated with different PMA concentrations (20–70 μM) and photoactivation times (15–50 min) using a 600 W LED lamp. After photoactivation, all samples were extracted using the DNeasy PowerSoil kit (Qiagen, Hilden, Germany). DNA samples were quantified through qPCR on a CFX96 Touch Real-Time PCR detection system (BioRad, Hercules, CA). Each 20 μL reaction consisted of 10 μL of PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA), 10 μM of universal bacteria primers Eub338 and Eub518, 2.5 ng of template DNA, and qPCR grade water (Fierer et al., 2005). Samples were analyzed in triplicate in polypropylene 96-well plates with an absolute standard curve (serial dilutions of *S. aureus*). All plates were run for 15 min at 95 °C, then 40 cycles of 1 min at 95 °C, 30 s at 53 °C, and 1 min at 72 °C, and then a melt curve was programmed. Sets with high PMA concentrations and/or long photoactivation times resulted in low recovery rates of live *S. aureus* detection due to prolonged or intense intercalation of viable DNA with PMA. Out of the two sets with > 90% recovery rates of viable *S. aureus*, the set with minimal experiment manipulations (20 μM PMA dye and 15 min of photoactivation) was selected as optimal PMA conditions (Nocker et al., 2007; Desneux et al., 2015).

Soil microbial composition quantification followed the protocol described below. Soil samples were wet sieved (2 mm mesh) and injected with 20 μM PMA dye and photoactivated for 15 min prior to DNA extraction with the DNeasy PowerSoil kit. Extracted DNA samples were run in triplicate on a CFX96 qPCR System using the same reaction composition from PMA optimization. Following a modified phyla-specific qPCR method for quantification of six of the most common bacteria phyla found in soil, samples were run for 15 min at 95 °C, 40 cycles of 95 °C for 1 min, 30 s at specified annealing temperature in Table 1, 72 °C for 1 min, followed by a programmed melt curve (Fierer et al., 2005; Cadillo-Quiroz et al., 2006; Table 1). Microbial DNA specific to each microbial pyla were extracted and quantified to create standard curves for absolute quantification during qPCR (Carolina Biological Supply, Burlington, NC; Table 1). Post-amplification products were run on a 2% agarose gel to verify amplicon length.

All bacteria, fungi and archaea abundances were calculated as the total amount of gene copies in each soil sample from each assay using

Table 1
qPCR Cycle Properties for targeted microbial communities and bacterial taxa. Table modified from Fierer et al. (2005).

Target group	DNA Standard	Forward/Reverse primers[a]	Amplicon length (bp)	Annealing temp (°C)
All Bacteria	*Staphylococcus aureus*	Eub338/Eub518	200	53
Alpha-proteobacteria	Rhodospirllum rubrum	Eub338/Alf685	365	60
Acidobacteria	Acidobacterium capsulatum	Acid31/Eub518	500	50
Beta-proteobacteria	Spirillum volutans	Eub338/Bet680	360	60
Actinobacteria	Micrococcus luteus	Actino235/Eub518	300	60
Firmicutes	*Staphylococcus aureus*	Lgc353/Eub518	180	60
Bacteroidetes	Novosphingobium capsulatum	Cfb319/Eub518	220	65
All Fungi	Eurotium chevalieri	5.8s/ITSlf	300	53
All Archaea	Halobacterium salinarum	Arch967F/Arch-1060R	140	60

[a] Nucleotide sequence of primers provided in Fierer et al. (2005) and Cadillo-Quiroz et al. (2006).

domain-specific universal primers. Six common bacteria phyla observed in soils were quantified using phyla-specific primers (Fierer et al., 2005; Table 1). Bacteria composition of each phyla was calculated as the gene copy number for the specific bacteria phyla over total number of all bacteria gene copies. Bacteria classified as "unspecified bacteria" was calculated as the remaining amount of all bacteria gene copies not identified as one of the six bacteria phyla quantified.

2.5. Microbial functions

2.5.1. Enzyme activity

Alkaline phosphatase, β-1-4-glucosidase, β-N-acetylglucosaminidase, β-xylosidase and cellobiose were measured with methylumberiferone (MUF) substrate assays within 24 h of field collection (Freeman et al., 1995). A soil slurry (~0.5 g wet soil and 39 mL of autoclaved distilled water) was incubated at 25 °C for 1 h on an orbital shaker. 150 μL of the slurry and 100 μL of substrate (MUF-x) were incubated at 25 °C for 24 h and fluorescence activity was measured at excitation/emission wavelengths 360/460 nm on a BioTek Synergy HTX multi-mode reader at the 0 and 24-h mark (BioTek Instruments, Winooski, VT). Enzyme activity rate was determined as the difference between the final and initial fluorescence over 24 h.

2.5.2. Soil carbon production rates

To determine potential soil production rates for CO_2 and CH_4, a microcosm was created in a 120 mL serum bottom using ~5 g of fresh wet soil and 10 mL of N_2 purged site water. All serum bottles were closed with a rubber septa and aluminum crimp and evacuated with N_2 gas. Samples were placed in the orbital shaker at 25 °C for 96 h. At every sampling time (12, 24, 48, 72, and 96-h), headspace gas samples and pressure readings were taken to determine total gas production at a constant temperature using Henry's Law. The headspace gas was analyzed for CO_2 and CH_4 using a Shimadzu Gas Chromatograph (Shimadzu, Kyoto, Japan). Standard curves were made with known concentrations of pure mixtures of gases and a linear regression was fitted to measure the production rate of every soil sample.

2.6. Litter decay

Each plot was split into 9 quadrats and ~10 live mature leaves from the dominant plant species in the plot (willow, cattail, or sawgrass) were randomly collected at eye level in each quadrat in March 2017. All leaf tissue was rinsed with nanopore water before being oven dried at 60 °C for 72 h. A subset of fresh leaf tissue from each plot was ground and total C and N content were determined. Insoluble lignin content was also analyzed using the acetyl bromide method (Moreira-Vilar et al., 2014). Twelve litter bags (dimensions: 31 × 31 cm; 2 mm mesh) were filled with ~1 g of dried leaf tissue representative of the plot's vegetation composition and anchored in a circular pattern to the ground using wooden stakes in March 2017. Four random bags from each plot were collected in June 2017 and brought back to the University of Central Florida for analysis. Litter was rinsed with deionized water and dried at 60 °C for 72 h before being weighed. Mass decay rates were determined as the mass loss over time (Wieder and Lang, 1982). Nutrient (total C, insoluble lignin, and total N) content was determined on the litter mass remaining using the same methods described for soils. In September 2017, the study regions were affected by Hurricane Irma, preventing the collection of litter bags beyond the four-month collection and the calculation of longer term mass decay rates.

2.7. Nitrogen cycling

2.7.1. Potentially mineralizable nitrogen rates

Potentially mineralizable nitrogen (PMN) rates were determined through laboratory incubations (White and Reddy, 2000). A subset of fresh wet soil (~5 g) was extracted with 2M KCl and incubated on an orbital shaker for 1 h at 25 °C prior to centrifuging at 150 rpm for 10 min. Supernatant was vacuum filtered using Supor 0.45 μM filters, preserved using double distilled DD H_2SO4, and stored at 4 °C until analysis for NO_3^- and NH_4^+ on a Seal AQ2 Discrete Analyzer (Seal Analytical, Mequon, WI) for initial extractable N content using EPA methods EPA-127 and EPA-103 respectively. A duplicate subset was placed in a 120 mL glass serum bottle and crimp sealed with a rubber septa and aluminum cap. Samples were purged with N_2 for 10 min and injected with 10 mL of N_2 purged distilled H_2O to create anaerobic conditions and incubated for ten days at room temperature before being injected with 2M KCl. Samples were incubated at 25 °C for 1 h and the supernatant was analyzed for NH_4^+ on an AQ2 Discrete Analyzer for final extractable N concentrations. PMN rates were calculated as the difference between the initial and final extractable N concentrations over time.

2.7.2. Potential denitrification rates

To determine denitrification potential, a microcosm was created in a 120 mL serum bottom using ~5 g of fresh wet soil, 17 mL of C_2H_2 and 10 mL of 50 ppm nitrate glucose solution. Acetylene was used to stop denitrification at N_2O, which was measured using a Shimadzu 2014 Gas Chromatograph equipped with an electron capture detector (Yoshinari and Knowles, 1976). Samples were incubated on an orbital shaker at 25 °C for 96 h. The headspace gas and pressure measurements were collected at 12, 24, 48, 72, and 96-h for analysis on the gas chromatograph. Standard curves were made with known concentrations of pure mixtures of gases and a linear regression (all $R^2 > 0.95$) was fitted to measure the change in gas concentration over time. The gas pressure, constant temperature, and headspace gas concentration were used to calculate total gas volume and thus, the denitrification potential of every soil sample.

2.8. Statistical analyses

Each study region was statistically analyzed independently in R statistics (R Foundation for Statistical Computing, Vienna, Austria). For every measured property, the three samples in each plot were averaged for a mean plot value to be used in statistical analyses. Therefore, all data presented had an $n = 5$ for each plot type. All data sets were tested for normality (Shapiro-Wilk) and homogeneity (Levene's test). For data that did not meet the assumptions (all extracellular enzyme activity rates for both regions), data was log transformed to meet the assumptions. Differences between measured properties and plot types were analyzed using a one-way ANOVA ($F_{(2,12)}$ for all ANOVAs; stats package). If significant differences were detected between plot types, Tukey's test was used to determine which plots differed from each other (lsmeans package). All results are reported as the untransformed mean ± SE and p-values shown are from ANOVA models. Relationships between soil physicochemical properties and the measured responses in this study were analyzed using linear regressions ($df = 13$) at a significance of $p < 0.05$. The physicochemical property data used for correlations were collected using the same plot design set-up and equipment as in this study.

3. Results

3.1. Soil microbial community size and composition

Microbial biomass C and viability qPCR showed no changes in microbial community size or general composition (total bacteria:archaea:fungi) between plot types at Moccasin Island. Half of the bacteria gene copies in this region were designated as unspecified bacteria (bacteria gene copies that were not sub-identified as any of the six bacteria phyla studied). However, out of the six bacteria phyla quantified, major compositional shifts within bacteria was detected (Table 2). Both Bacteriodetes and Betaproteobacteria gene copy

Table 2
Mean ± SE % bacteria composition at Moccasin Island by plot type. Letters represent significantly different means according to plot type determined by Tukey's HSD test.

Taxon	Control	Adjacent	Willow
Alpha-proteobacteria	1.75 ± 0.71	1.24 ± 0.89	0.85 ± 0.29
Beta-proteobacteria	1.44 ± 0.07^{b}	0.56 ± 0.04^{a}	2.82 ± 0.15^{c}
Acidobacteria	39.8 ± 26.0	39.7 ± 23.7	30.9 ± 24.6
Actinobacteria	3.76 ± 0.89	2.37 ± 1.21	3.42 ± 0.47
Bacteriodetes	7.68 ± 0.93^{b}	3.84 ± 1.80^{a}	5.43 ± 1.71^{a}
Firmicutes	0.08 ± 0.04	0.03 ± 0.02	0.04 ± 0.01
Unspecified bacteria	45.6 ± 6.50^{a}	52.3 ± 9.74^{ab}	56.6 ± 1.50^{b}

Table 3
Correlation table of important relationships for Moccasin Island and Lake Apopka. Bold p values represent significant relationships.

Relationship	Region	Linear equation	R^2 value	P value
Bacteriodetes - Temperature				
	Moccasin Island	−35.1x +1E3	0.53	< **0.01**
	Lake Apopka	−34.9x +1E3	0.12	0.20
Beta-proteobacteria - Temperature				
	Moccasin Island	−162x +515	0.70	< **0.01**
	Lake Apopka	−0.288x +102	0.14	0.17
Actinobacteria - DOC				
	Moccasin Island	1280x + 160	0.32	**0.03**
	Lake Apopka	157x – 2240	0.71	< **0.01**
CO_2 – Soil C				
	Moccasin Island	149x + 114	0.61	< **0.01**
	Lake Apopka	4.72x + 10.1	0.36	**0.02**
Phosphatase - Moisture				
	Moccasin Island	0.761x – 22.2	0.17	0.13
	Lake Apopka	−0.005x + 0.386	0.58	< **0.01**
Mass Decay – Leaf lignin				
	Moccasin Island	455x – 3.17	0.78	< **0.01**
	Lake Apopka	175x – 20.8	0.66	< **0.01**
Denitrification - Moisture				
	Moccasin Island	0.006x – 0.12	0.66	< **0.01**
	Lake Apopka	0.005x – 0.04	0.57	< **0.01**

abundance were lowest in adjacent marsh plots and were inversely related to soil temperature (r = −0.73 and −0.84, respectively; Table 3).

At Lake Apopka, willow plots had the greatest microbial community size, due to the significant increase in archaea abundance ($p < 0.001$; Fig. 1). Fungi were found in small quantities (< 5000 gene copies g^{-1} soil) and mainly in willow and adjacent marsh plots. Despite no significant differences in total bacteria community size, shifts in major bacteria phyla abundance was observed by plot type, specifically in Actinobacteria and unspecified bacteria abundance ($p < 0.001$; $p = 0.01$; Fig. 2). Willow and adjacent marsh plots had more unspecified bacteria gene copies than marsh plots ($p < 0.001$). Actinobacteria gene copy abundance was lower in adjacent marsh and willow plots (21 ± 0.8% bacteria gene copies; 18 ± 0.4% bacteria gene copies) compared to marsh plots (53 ± 0.4% bacteria gene copies) and

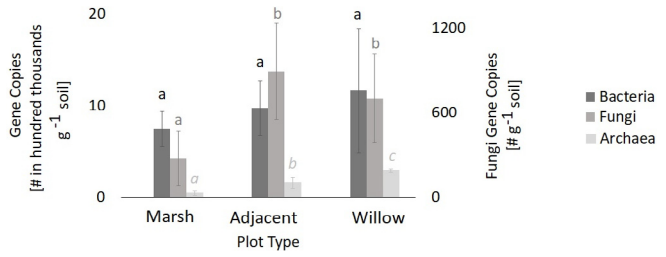

Fig. 1. Mean gene copy number ± SE for all bacteria and archaea in Lake Apopka by plot type. Letters signify significantly different mean rates as determined by Tukey's HSD test.

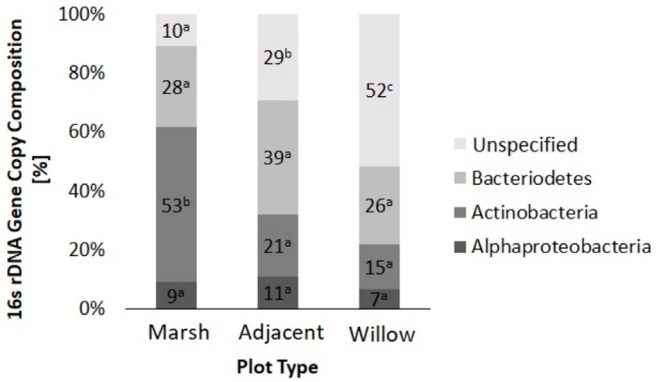

Fig. 2. Mean gene copy % for bacteria taxa in Lake Apopka by plot type. Value represents mean gene copy % of total bacteria and letters signify significantly different means between plot types as determined by Tukey's HSD test. Both Betaproteobacteria and Firmicutes comprised > 1% of total bacteria and are not shown in this figure.

directly related to soil dissolved organic C (DOC) concentrations (r = 0.84; Table 3).

3.2. Soil microbial functions

At Moccasin Island, both β-1-4-glucosidase and β-N-acetylglucosaminidase activity followed an increasing activity gradient from marsh to willow plots ($p = 0.05$; Fig. 3a and b). Willow plots had greater alkaline phosphatase activity than all other plot types ($p < 0.01$; Fig. 3c). Extracellular enzyme activity was not highly correlated to any soil physicochemical property, but in general, β-1-4-glucosidase activity increased with soil DOC concentrations while β-N-acetylglucosaminidase and alkaline phosphatase activity were directly related to soil moisture content (Table 3). Like enzyme activity rates, willow and adjacent marsh plots had the highest CO_2 production rates ($p < 0.01$; 1.9 ± 0.2 μg CO_2 g^{-1} soil h^{-1}; 2.1 ± 0.2 μg CO_2 g^{-1} soil h^{-1}) while marsh plots had the lowest CO_2 production rates (1.1 ± 0.1 μg CO_2 g^{-1} soil h^{-1}). CO_2 production was directly related to soil C content (r = 0.88; Table 3). Methanogenesis was minimal in all plots (< 0.1 μg CH_4 g^{-1} soil h^{-1}).

At Lake Apopka, adjacent marsh and/or willow plots generally had

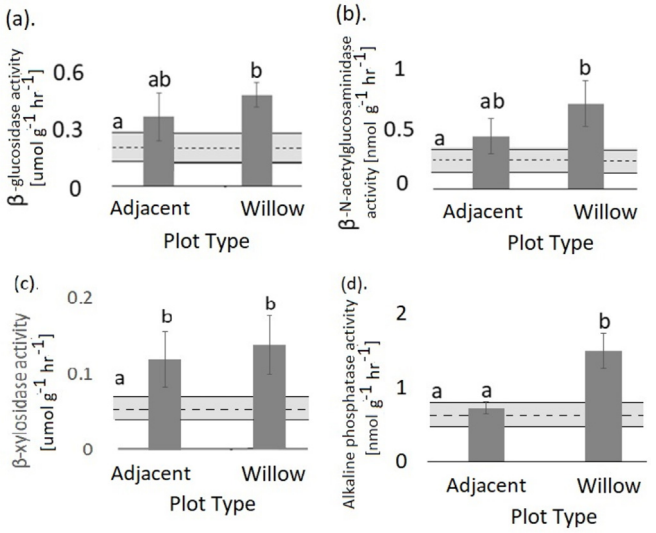

Fig. 3. Mean value ± SE for extracellular enzyme activity rates in Moccasin Island by plot type. Horizontal bar represents mean value ± SE for marsh plots (i.e. controls). Letters signify significantly different activity rates as determined by Tukey's HSD test.

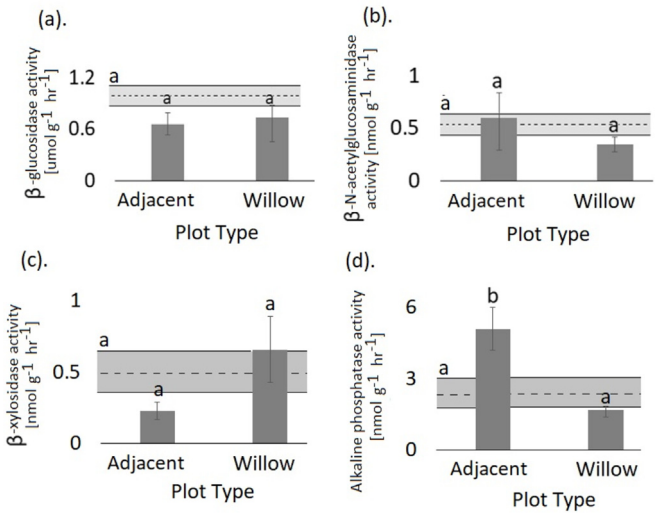

Fig. 4. Mean value ± SE for extracellular enzyme activity rates in Lake Apopka by plot type. Horizontal bar represents mean value ± SE for marsh plots (i.e. controls). Letters signify significantly different activity rates as determined by Tukey's HSD test.

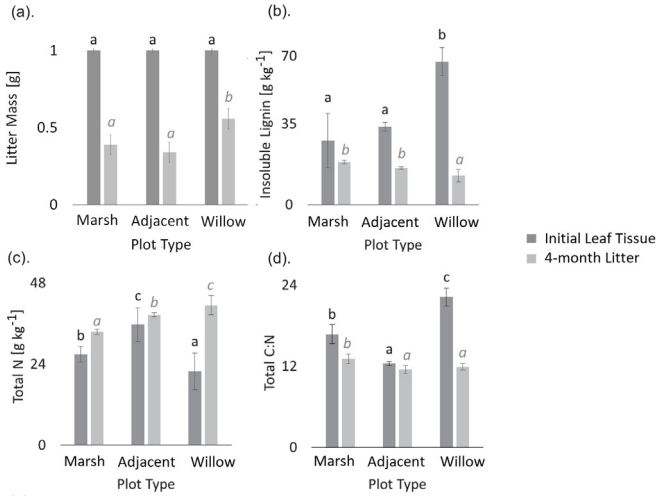

Fig. 5. Mean value ± SE for initial leaf tissue and 4-month litter in Moccasin Island by plot type. Letters represent significantly different means determined by Tukey's HSD test for initial leaf tissue. Italicized letters represent significantly different means determined by Tukey's HSD test for 4-month litter.

lower β-1-4-glucosidase, β-N-acetylglucosaminidase and β-xylosidase activity rates than marsh plots ($p = 0.2$; Fig. 4a–c). Only alkaline phosphatase activity significantly differed by plot type ($p < 0.001$). Adjacent marsh plots had greater alkaline phosphatase activity ($5.1 \pm 0.9\,\mu mol\,g^{-1}$ soil h^{-1}) than all other plot types (Fig. 4d). In general, adjacent marsh plots ($1.8 \pm 0.2\,\mu g\ CO_2\ g^{-1}$ soil h^{-1}) produced less CO_2 than marsh ($3.2 \pm 0.8\,\mu g\ CO_2\ g^{-1}$ soil h^{-1}) and willow plots ($2.5 \pm 0.2\,\mu g\ CO_2\ g^{-1}$ soil h^{-1}; $p = 0.1$) and was directly correlated to soil C content ($r = 0.77$; Table 3). Methanogenesis was minimal in all plots ($< 0.1\,\mu g\ CH_4\ g^{-1}$ soil h^{-1}). Soil physicochemical properties explained little of the variance in enzyme activity or respiration rates except for alkaline phosphatase activity, which was indirectly related to soil moisture content ($r = -0.75$; Table 3).

3.3. Litter decay

At Moccasin Island, short-term (4 month) mass decay was lowest for willow litter compared to sawgrass litter in adjacent marsh and marsh plots ($p < 0.01$). Mass decay rates were directly related to leaf lignin content ($r = 0.88$; Table 3; Fig. 5a). Initially, greater C and lignin content was observed in willow leaf tissue compared to sawgrass leaf tissue, resulting in lower litter quality (i.e., lignin:N and C:N; $p = 0.01$). After only four months of decomposition, major chemical changes were detected in the litter. Lignin content drastically decreased by 75% in willow litter ($p = 0.02$; Fig. 5b). Following four months of in-situ decomposition, willow litter at Moccasin Island had the greatest litter N concentrations ($p < 0.001$) and as a result, willow plots contained the lowest total C:N and lignin:N ratios ($p = 0.05$; $p = 0.04$; Fig. 5b–d).

Similarly, at Lake Apopka, mass decay was lower in willow plots compared to marsh plots ($p < 0.001$; Fig. 6a). Greater lignin and total N content were detected in willow litter compared to herbaceous litter after four months of decomposition ($p < 0.01$; Fig. 6b–c). Resultantly, remaining willow litter had greater or similar litter quality (C:N and lignin:N) as cattail litter in marsh plots (Fig. 6d).

3.4. Nitrogen cycling

At Moccasin Island, PMN and denitrification rates differed by plot type ($p = 0.02$; $p = 0.001$). Specifically, adjacent marsh ($16.1 \pm 1.3\,mg\,g^{-1}$ soil day^{-1}) and willow plots ($16.2 \pm 2.1\,mg\,g^{-1}$ soil day^{-1}) had greater PMN rates than marsh plots ($12.8 \pm 2.0\,mg\,g^{-1}$ soil day^{-1}). Adjacent marsh soils had greater

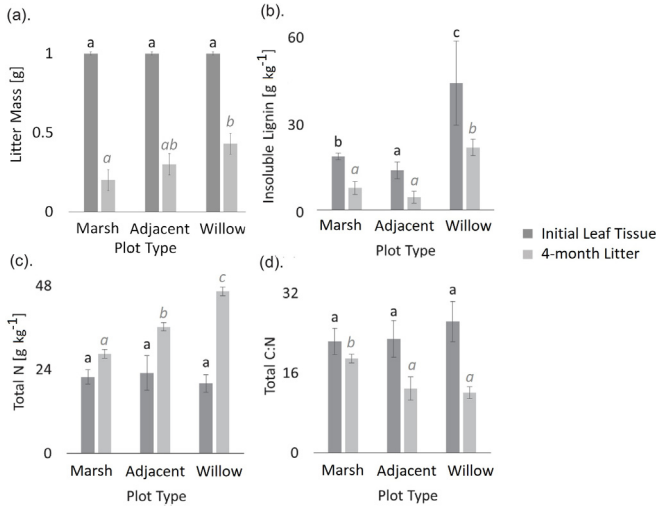

Fig. 6. Mean value ± SE for initial leaf tissue and 4-month litter in Lake Apopka by plot type. Letters represent significantly different means determined by Tukey's HSD test for initial leaf tissue. Italicized letters represent significantly different means determined by Tukey's HSD test for 4-month litter.

potential denitrification rates ($0.31 \pm 0.05\,\mu g\,N\ g^{-1}$ soil h^{-1}) compared to willow ($0.22 \pm 0.04\,\mu g\,N\ g^{-1}$ soil h^{-1}) and marsh soils ($0.16 \pm 0.05\,\mu g\,N\ g^{-1}$ soil h^{-1}). Denitrification potential was directly related to soil moisture content with the greatest denitrification and moisture in adjacent marsh and willow plots ($r = 0.81$; Table 3).

At Lake Apopka, PMN rates were similar in all plots, but differences in denitrification rates were observed ($p < 0.001$). Willow and adjacent marsh plots had lower denitrification rates ($0.22 \pm 0.05\,\mu g\,N\ g^{-1}$ soil h^{-1}; $0.24 \pm 0.04\,\mu g\,N\ g^{-1}$ soil h^{-1}) than marsh plots ($0.34 \pm 0.02\,\mu g\,N\ g^{-1}$ soil h^{-1}). Denitrification potential was directly related to soil moisture content ($r = 0.74$; Table 3).

4. Discussion

This study emphasizes the importance of understanding the relationships between shifting vegetation communities, wetland soil physicochemical properties, and soil microbial activity. Major differences in soil microbial activity and biogeochemical processes were detected within encroached marshes, relative to their respective control

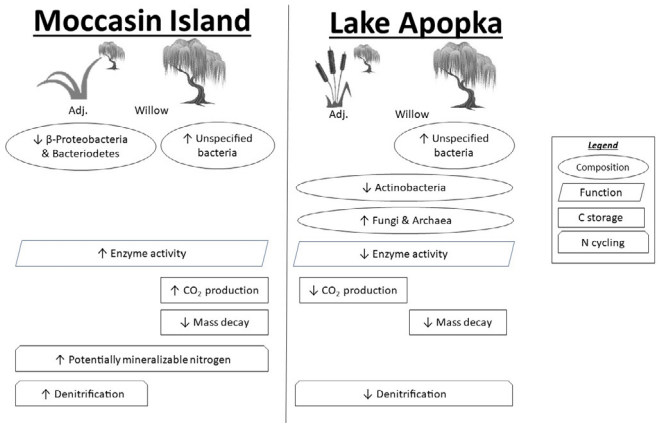

Fig. 7. Key differences in biogeochemical processes in willow and adjacent marsh plots compared to marsh plots by region. Boxes overlaying both adj. and willow plots signify differences in both plot types ($p < 0.05$).

plots, and were correlated to soil physicochemical properties (Fig. 7). At Lake Apopka, greater microbial biomass, particularly of fungi and archaea, were detected in adjacent marsh and willow plots. However, less microbial activity (denitrification, respiration, etc.) were observed in adjacent marsh and/or willow plots. Conversely, at Moccasin Island, microbial biomass size was similar among all plot types but major shifts between bacteria phyla (Bacteriodetes and beta-Proteobacteria) were detected. Also, greater denitrification, CO_2 respiration, and enzyme activity were observed in adjacent marsh and/or willow plots.

In the process of analyzing these plot-level differences among the two study regions, we found that despite the divergent results between the two regions, several key relationships remained consistent. Microbial compositional changes were highly related to DOC concentrations and temperature, while nutrient availability (soil C) and soil moisture greatly impacted microbial functions (e.g., denitrification, CO_2 production, and some enzyme activities; Table 3). These relationships between microbial activity, biogeochemical processes and soil physicochemical properties were present in both study regions, despite the fact that the underlying gradients were nearly reversed (i.e., total soil nutrient storage increased in willow and adjacent plots at Moccasin Island, but generally decreased at Lake Apopka; Ho and Chambers, unpublishd data; Table 4). These underlying relationships between microbial communities and soil properties represent transferable findings that can be tested in other systems and provide some of the first data on how shrub-encroached wetlands, particularly with unique histories, may differ in nutrient cycling. This also suggests soil physicochemical property differences may drive major changes in microbial community structure and function, resulting in differences in C storage and N cycling in willow-encroached marshes of this study. Teasing apart the cause-and-effect relationship (i.e., did willow encroachment produce the physicochemical gradients, or did the physicochemical gradients supersede, or even encourage, willow encroachment?) is outside the scope of this project, but would be a worthwhile follow-up study to pursue using an experimental or before-after-impact-control (BACI) study design.

4.1. Microbial compositional changes between plot types

The relative abundance of bacteria, fungi and archaea is important to biogeochemical functioning because in nutrient-limited environments, microorganisms compete for the same resources. Therefore, shifts in the relative abundance of certain microbes (i.e. fungi vs. bacteria) with different utilization efficiencies, can result in alterations to nutrient cycling through decomposition, respiration, and enzyme activity (Fierer et al., 2007). In this study, only total fungi and archaea dominance significantly differed between plots, and only at Lake Apopka. Fungi were undetectable at Moccasin Island and observed in small quantities at Lake Apopka within willow and adjacent marsh plots. The detection of fungi at Lake Apopka is of interest to C cycling as fungi are well known for specializing in the decomposition of recalcitrant C sources and greater fungi:bacteria can result in slower C turnover in the long term (Waldrop et al., 2000; Busse et al., 2009). Archaea, which were more abundant in willow and adjacent marsh plots at Lake Apopka, are common nitrifiers and methanogens (Gubry-Rangin et al., 2010). Since methanogenesis was minimal at Lake Apopka, these archaea could be nitrifiers, playing an essential role in N cycling that should be further investigated. Lastly, while total bacteria abundance did not differ between plots, major bacteria phyla shifts were observed between Actinobacteria at Lake Apopka and mesophilic bacteria at Moccasin Island (Fig. 7).

Correlations between soil microbial composition and physicochemical properties in the study regions demonstrated that litter quality and temperature changes, and hydroperiod indirectly, were related to bacteria community composition differences (Table 3). Soil DOC, often considered the labile-C pool microbes can easily access for metabolism, was observed as a correlate of Actinobacteria abundance (Gonet and Debaska, 2006). Some studies have shown no relationship between oligotrophs like Actinobacteria to labile-C sources (Fierer et al., 2005), while Actinobacteria increased in response to labile-C amendments in other studies (Goldfarb et al., 2011). At both Lake Apopka and Moccasin Island, Actinobacteria abundance was directly related to soil DOC concentrations. This was notable in Lake Apopka, where significantly less Actinobacteria were observed in adjacent marsh and willow plots where willow litter (significantly higher in recalcitrant lignin than cattail) may have reduced leachable DOC, subsequently diminishing Actinobacteria abundance. As both fungi and Actinobacteria can specialize in breaking down recalcitrant C sources, there may be competition between the two groups in nutrient-limited environments. The combination of microbial competition for recalcitrant C and low DOC availability may have led to greater fungal abundance and less Actinobacteria in the willow and adjacent marsh plots compared to control plots at Lake Apopka (Lewin et al., 2016).

At Moccasin Island, there was large variability in Acidobacteria

Table 4
Mean ± SE for soil physiochemical properties in Moccasin Island and Lake Apopka by plot type.

Study Region	Plot Type	Soil Temp [°C]	Redox Potential [mV]	Soil moisture [%]	Total C [kg kg^{-1} soil]	Total N [g kg^{-1} soil]	Total P [g kg^{-1} soil]
Moccasin Island							
	Control	28.1 ± 0.6^a	82 ± 13^b	52 ± 9^a	0.2 ± 0.0^a	21 ± 4^a	5 ± 1^a
	Adjacent	30.4 ± 0.7^b	−6 ± 13^a	79 ± 2^b	0.4 ± 0.1^b	33 ± 1^b	7 ± 1^b
	Willow	28.0 ± 0.6^a	23 ± 21^a	86 ± 1^c	0.4 ± 0.1^b	32 ± 1^b	8 ± 1^b
Lake Apopka							
	Control	30.9 ± 1.2	120 ± 22	63 ± 3^b	0.3 ± 0.1^b	18 ± 5^b	4 ± 1^a
	Adjacent	31.0 ± 1.2	67 ± 36	57 ± 4^b	0.2 ± 0.0^b	16 ± 3^b	4 ± 1^a
	Willow	29.3 ± 1.3	77 ± 22	47 ± 5^a	0.1 ± 0.0^a	10 ± 1^a	4 ± 0^a

Superscript letters represent significantly different means by Tukey's HSD test at $p < 0.05$.

abundance within plots, possibly obscuring bacteria abundance shifts. Studies have reported greater Acidobacteria variability in the rhizosphere (Fierer et al., 2007; Uksa et al., 2015). Therefore, small differences in the abundance of roots within our field replicate samples may have led to increased variability in Acidobacteria, relative to other bacteria groups. However, major shifts between bacteria phyla were still detected for two out of the six bacteria phyla analyzed. Adjacent marsh plots had the lowest abundance of β-Proteobacteria and Bacteriodetes. The shifts in microbial community between plot types was correlated with soil temperature (Table 3). The greatest soil temperatures were detected in adjacent marsh plots, possibly due to irradiance being reflected from willow canopies into adjacent areas (Ho & Chambers, unpublished data). Soil temperature was inversely correlated to β-Proteobacteria and Bacteriodetes abundance, where less gene copies from these phyla were observed in adjacent marsh plots which also had the highest temperatures. Previous studies have shown both phyla to be considered mesophilic bacteria (Green et al., 2006). Temperature likely played a larger role at Moccasin Island than at Lake Apopka due to Moccasin Island's seasonal flooding status, permitting greater direct radiance onto the soil surface. By comparison, surface water levels were always > 30 cm at Lake Apopka and surface temperatures did not differ between plots. Direct radiance was not measured in this study but should be investigated in future studies to determine possible co-occurring drivers of microbial community shifts. In sum, C quality (DOC concentration) or soil temperature were major indicators of bacteria abundance shifts in willow-encroached marshes; flooding status may have indirectly impacted bacteria composition by influencing soil temperature (Table 3).

4.2. Microbial functional differences between plot types

In this study, greater enzyme activity and respiration rates were observed in willow and/or adjacent marsh plots at Moccasin Island while the lowest enzyme activity occurred in willow plots at Lake Apopka. The differing relationship between microbial activity and willow encroachment between regions was associated with nutrient availability and soil moisture. At Lake Apopka, willow plots had lower nutrient availability and moisture than control plots (Table 4), and also had generally lower enzyme activity rates. High nutrient availability may have promoted greater microbial community size, enzyme activity, and respiration rates in this study, as suggested by the positive relationship between soil C availability and CO_2 production in both regions (Hartman et al., 2008, Table 3). Similarly, denitrification potential was related to soil moisture at both Lake Apopka and Moccasin Island. Greater soil moisture content can inhibit O_2 diffusion, promoting reduced soils conditions (−50 to 50 mV) and denitrification (Weier et al., 1993). Overall, greater microbial activity occurred where larger soil nutrient pools were detected, and denitrification activity was linked to soil moisture, which agree with the relationships between soil physicochemical properties and biogeochemical processes found by others (Langenheder et al., 2006; Peng et al., 2007).

4.3. Litter decay and C storage

Relationships between C-cycling enzymes (β-1-4-glucosidase, β-xylosidase and cellobiose), CO_2 production, and mass decay rates did not show any strong correlations, potentially due to the different time frames of each assay. Individually, CO_2 production and mass decay rates were depressed in Lake Apopka's willow plots, highlighting the potential for greater C storage in willow plots at Lake Apopka. While Moccasin Island's willow plots had greater C-extracellular enzyme activity and a slight increase in CO_2 production, short-term mass decay was significantly less than the control plots, suggesting the potential for litter C storage. In both regions, mass decay rates were highly correlated with recalcitrant lignin-C content in the plots' respective litter. Other studies have also found similar results; litter quality decreases with shrub encroachment and depresses decomposition rates (Duffy, 2014). However, it is important to note that the rates reported in this study and many others only demonstrate short-term decomposition rates. It has been assumed that long-term decomposition rates may be magnitudes slower than short-term decomposition rates, as labile-C in fresh litter breaks down first, and the remaining recalcitrant-C sources degrade more slowly (Bazter and Sharitz, 2014). However, this study reports litter chemistry changes that suggest willow litter quality may *improve* in as little as four months of decomposition (as indicated by lignin:N and C:N), potentially speeding up the rate of decomposition in the long-term. The greater fungi abundance in willow and adjacent marsh plots may aid in quicker turnover of recalcitrant litter in willow plots, and the increased abundance of unspecified bacteria (i.e., those phyla not identified as one of the six major soil phyla used as primers) may suggest the emergence of specialized phyla that are more efficient at lignin degradation. Therefore, this study indicates willow-encroached marshes have a greater potential to serve as short-term C sinks due to willow's lower initial litter quality, but litter chemistry changes and microbial composition differences may reduce the long-term C sink potential. Future studies on long-term decomposition rates are needed to elucidate these findings.

4.4. Nitrogen cycling

Freshwater marshes are commonly at risk of eutrophication from agriculture and urbanization, making it important to consider how shrub encroachment can alter N and P cycling. Greater N mineralization and lower denitrification in willow plots may result in eutrophication and decreased water quality over time as less net bioavailable N is removed from the water. At Moccasin Island, greater N cycling (β -N-acetylglucosaminidase activity, N mineralization, denitrification) was detected in willow and/or adjacent marsh plots, while Lake Apopka's willow plots had lower denitrification potential and β-N-acetylglucosaminidase activity than marsh plots. These trends in each respective marsh are consistent with previous studies on N cycling and nutrient amendments indicating N mineralization and denitrification rates were directly related to soil nutrient availability and moisture content, respectively (Peng et al., 2007; Barnes et al., 2012). Therefore, rather than shrub encroachment producing a consistent directional change in N cycling, it was highly dependent upon the study region and associated soil physicochemical properties. Similarly, the relationship between soil total P and encroachment mirrors that of alkaline phosphatase activity at Moccasin Island, but not a Lake Apopka (Table 4). As P availability can also contribute to eutrophication, future investigations should be done on the forms of available P to help explain the observed patterns of P cycling.

Structural differences between sawgrass and cattail may also contribute to N cycling differences between regions. The presence of hypertrophied lenticels and secondary aerenchyma in cattail and willows can promote oxidation hotspots via radial oxygen loss, allowing for coupled nitrification/denitrification in the rhizosphere (Pereira and Kozlowski, 1977; Pauliukonis and Schneider, 2001; Randerson et al., 2011; Hall et al., 2017). At Lake Apopka, no differences in N mineralization nor redox potential were observed when the native herbaceous species being compared to willow was cattail. However, at Moccasin Island, both willow and adjacent marsh plots (potentially due to willow root penetration into the adjacent plot) had greater N mineralization rates and lower redox potentials than sawgrass control plots (Table 4). Future studies on radial oxygen loss between these three species will need to be done to corroborate this as an explanation for the diverging effects of willow encroachment on N cycling between the two study regions.

5. Conclusions

Overall, major differences in soil microbial composition and

function were observed in willow and adjacent marsh plots, resulting in consistently lower short-term litter decomposition rates in both study regions and changes in N cycling (N mineralization, denitrification, etc.) that varied by region. Differences in the microbial community (fungi, archaea, Actinobacteria, etc.) and biogeochemical processes in willow-encroached marshes were highly dependent upon physicochemical properties, such as litter quality, nutrient availability, soil temperature, and soil moisture. Specifically, soil temperature and DOC gradients correlated with microbial community composition, while soil C and moisture content correlated with soil respiration and denitrification rates, respectively. These relationships were significant in both study regions regardless of dominant vegetation, suggesting the underlying physicochemical gradients may have as much, or more, influence on soil microbial community and activity than shrub encroachment. Further elucidation of the major drivers involved in these microbial shifts are still needed, but this study is one of the first to have focused on the relationships between vegetation shifts, physicochemical properties, and soil microbial community and activity in shrub-encroached wetlands. Additionally, this study underlines the importance of site-specific investigations in order to understand the impact of willow-encroachment on microbial and biogeochemical processes. The two study regions we investigated were both located within the same watershed, but had unique site histories and hydroperiods influencing landscape-scale soil properties, which may have been further modified by willow-encroachment. Land managers should consider how willow-encroachment alters biogeochemical functions in their specific system. For example, if land managers are trying to optimize C storage, it may be beneficial to maintain healthy willow populations, at least in the short term, due to slow mass decay of litter. However, if removal of bioavailable N is the goal, the appropriate response may depend on the regions' nutrient availability and soil moisture gradients. Based on these results, and findings from future studies, land managers can make better informed decisions on whether to actively exclude willow or maintain willow populations based on desired wetland functions (soil C storage, water quality improvement, etc.).

Declarations of interest

None.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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