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SPECIAL ISSUE-LETTER

Differential effects of press vs. pulse seawater intrusion on microbial communities of a tidal freshwater marsh

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Scientific Significance Statement

Seawater intrusion, via episodic events and long-term sea level rise, threatens tidal freshwater marshes (TFMs) and the ecosystem services they provide. Seawater intrusion can occur across a range of timescales, due to events like storm surges or drought, or long-term rising sea level. To date, there have been no large-scale, long-term studies that address the effects of episodic vs. continuous stressors on microbial communities in situ. Our multiyear field manipulation of brackish water inputs to a TFM suggests episodic seawater intrusion, while altering the microbial community composition, does not modify it in ways that affect ecosystem functioning. In contrast, continuous seawater intrusion leads to reduced microbial diversity and activity (soil respiration, extracellular enzyme activity) leading to a diminished ability to cycle carbon.

Abstract

Tidal freshwater marshes (TFMs) are threatened by seawater intrusion, which can affect microbial communities and alter biogeochemical processes. Here, we report on a long-term, large-scale manipulative field experiment that investigated continuous (press) and episodic (pulse, 2 months/yr) inputs of brackish water on microbial communities in a TFM. After 2.5 yr, microbial diversity was lower in press treatments than in control (untreated) plots whereas diversity in pulse plots was unaffected by brackish water additions. Sulfate reducer abundance increased in response to both press and pulse treatments whereas methanogens did not differ among treatments. Our results, along with other lab and field measurements that show reduced soil respiration and extracellular enzyme activity suggest that continuous seawater intrusion will decrease macrophyte C inputs that reduce bacterial diversity in ways that also diminish ecosystem carbon cycling.

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Author Contribution Statement: CC and MA designed the study. MA supported lab analysis (DNA extraction). SW conducted field work. JL and NW conducted statistical analyses. CM, NW, JL, MA, and CC wrote the paper.

Data Availability Statement: Sequence data are available on the NCBI SRA (BioProject PRJNA611801). Data, metadata, and code used to generate results are available on a Zenodo archive (doi: 10.5281/zenodo.4006673 of the GitHub repository https://github.com/LennonLab/MicroMarsh).

Additional Supporting Information may be found in the online version of this article.

Tidal freshwater marshes (TFMs) are threatened by rising seas, which are predicted to increase an additional 0.4–1.2 m by 2100 (Horton et al. 2014). TFMs will also experience episodic (pulse) seawater intrusion owing to climate change, which may manifest as more frequent and longer periods of drought, increased occurrence of storm surges, and decreased freshwater input from rivers (van Vliet et al. 2013). It is unclear how TFMs will respond to continuous vs. episodic incursions of seawater.

Salinity alters biogeochemical processes in TFMs, due in part to changes in the activity and composition of microbial communities (Reed and Martiny 2013). Seawater intrusion modifies the composition and availability of electron acceptors, such as sulfate (Capone and Kiene 1988). Increased sulfate can lead to shifts in microbial functional groups in TFMs, such as increased abundance of sulfate reducers and decreased abundance of methanogens (Dang et al. 2019).

Microbial diversity has been shown to be higher in freshwater compared to saline sediments (Wang et al. 2012). However, the response of microbial diversity in freshwater wetlands to salinity disturbance is unclear. For example, changes in microbial diversity in response to short-term (up to 55 d) salinity increases have been variable with some reporting increased (Jackson and Vallaire 2009), no change (Berga et al. 2017), or decreased diversity (Baldwin et al. 2006). In a reciprocal transplant experiment, both TFM and salt marsh communities remained more phylogenetically related to their "home" environment compared to the "away" environment after 40 d, suggesting that microbial communities are resistant to single, short-term perturbations (Morrissey and Franklin 2015). A longer-term (multiyear) study found that salinity reduced denitrification rates, likely through changes in the microbial community (Neubauer et al. 2019), but responses of individual microbial taxa were not measured. To our knowledge, there have been no studies that looked at short- vs. long-term effects of seawater intrusion on microbial diversity and community composition.

Our goal was to evaluate changes in the microbial community in response to episodic (pulse) and continuous (press) seawater intrusion in a controlled, large-scale, replicated field experiment: Seawater Addition Long Term Experiment (SALTEx). Our study is unique in that it spans a large spatial (2.5 m by 2.5 m replicated plots) and temporal scale (2.5 yr) and simulated realistic scenarios of seawater intrusion. There are few studies of this scale and duration that also combine high throughput sequencing to examine the effects of both press and pulse seawater intrusion on changes in microbial communities. Rather, studies to date involved either lab incubations (Edmonds et al. 2009, Jackson and Vallaire 2009) or transplant experiments (Morrissey and Franklin 2015, Dang et al. 2019). Unlike experiments that regularly (monthly) introduce seawater for short durations (24 h) (Servais et al. 2020), our experiment simulates episodic (several months) seawater intrusion associated with typical low river flow or drought conditions that are expected to occur as the climate warms and sea level rises.

Previous SALTEx studies revealed marked changes in biogeochemical cycling in response to continuous and episodic seawater intrusion. Continuous seawater intrusion led to plant senescence and death, decreased carbon dioxide (CO_2) and methane (CH_4) emissions (Herbert et al. 2018), and decreased root productivity and belowground biomass, resulting in soil subsidence (Solohin et al. 2020). Pore-water salinity, sulfate, sulfide, ammonium, nitrate, and phosphate were significantly higher in plots exposed to the press salinity treatment (Herbert et al. 2018, Widney et al. 2019). In contrast, episodic seawater intrusion, while producing temporary increases in pore-water salinity and sulfate, did not affect carbon (C) or nutrient cycling.

Our aim was to discern the effects of episodic vs. continuous seawater intrusion on microbial diversity and community composition. We hypothesized that microbial diversity would be lower in plots receiving continuous and episodic seawater intrusion relative to control and freshwater treatments. We also hypothesized the abundance of sulfate reducers would increase and methanogens decrease in plots receiving seawater additions relative to plots not receiving seawater additions.

Methods

Site description and experimental design

The experiment was conducted in a TFM on the Altamaha River, Georgia. The site is dominated by giant cutgrass, *Zizaniopsis miliacea* Michx, and experiences twice-daily tidal inundations of freshwater with an average flooding depth of 25 cm at high tide. We established 2.5 × 2.5 m replicated (n = 6 per treatment) plots, which were randomly assigned to one of the following treatment groups: control, fresh, pulse salinity, and press salinity. From April 2014 through December 2017, more than 3.5 yr, press plots were dosed four times per week with treatment water, seawater and fresh river water mixed onsite (salinity of ~15) to maintain pore-water at target salinities of 2–5. Pulse plots were dosed four times per week with treatment water during September and October of each year to mimic periods when river flow is typically low, such as occurs during drought or hurricane storm surges, and with fresh river water for the remainder of the year. Fresh plots were dosed with river water four times per week. Control (untreated) plots did not receive water additions but, like all treatments, were regularly inundated by the tides. Details of the full multiyear experiment (3.5 yr) can be found in Herbert et al. (2018) and Widney et al. (2019).

Sample collection and environmental variables

We collected soils (0–10 cm) from four of the six replicate plots from each treatment on 24 October 2016, 2.5 yr after treatments were initiated. Soils were shipped frozen to Indiana University and stored at −80°C until DNA extraction.

We measured pore-water salinity, sulfides, ammonium (NH_4^+), nitrate (NO_3^-), dissolved reactive phosphorus (DRP), and soil surface temperature quarterly, including 24 October

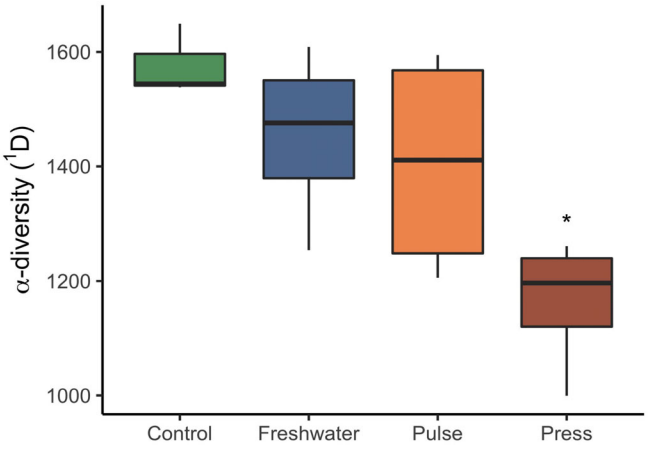

Fig. 1. Alpha diversity of the total DNA community in each treatment group. * indicates press is different from control ($p = 0.017$).

2016 to identify potential drivers of community structure. Pore-water results and methods are described in more detail elsewhere (Herbert et al. 2018; Widney et al. 2019). As the press plots began to lose vegetation from seawater additions (Fig. S1), we measured soil surface temperature using an infrared thermometer when soils were not inundated. Temperature was measured at four locations within each plot and averaged (Craft 2016). Two-way ANOVA based on treatment and sampling date was conducted to determine differences in

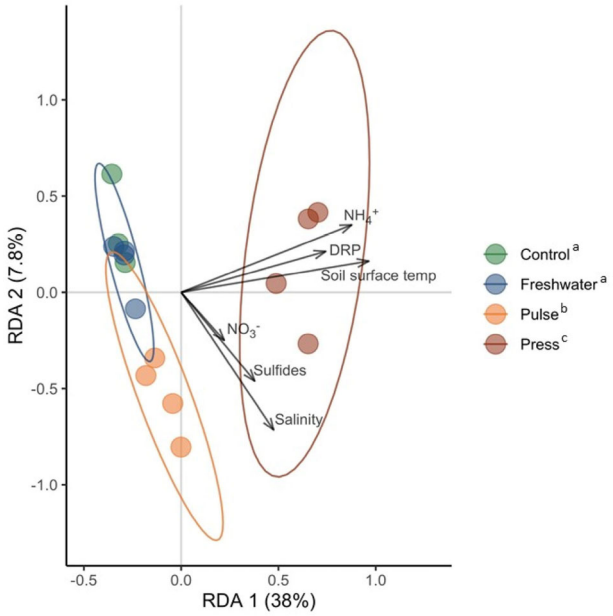

Fig. 2. Redundancy analysis of microbial community structure with vectors depicting environmental variables along axes RDA1 and RDA2. Circles represent 95% confidence ellipses. Treatments sharing the same letter are not significantly different from each other ($p < 0.05$) according to a pairwise permutational multivariate ANOVA (PERMANOVA) for treatment effects. Environmental data are from Herbert et al. (2018) and Widney et al. (2019).

Table 1. Mean October 2016 pore-water chemistry, soil surface temperature, carbon flux, and carbon-acquiring extracellular enzyme activity (EEA)* data for each treatment with standard errors in parentheses. Pore-water salinity, sulfide, ammonium (NH_4^+), and nitrate (NO_3^-) data came from Widney et al. (2019). Pore-water soluble reactive phosphorus (SRP), dissolved organic carbon (DOC), ecosystem respiration, methane (CH_4) emissions, and EEA data came from Herbert et al. (2018). Means sharing the same letter are not significantly different ($p < 0.05$) according to Tukey's honestly significant difference test for pore-water salinity, sulfide, NH_4^+, and NO_3^- and Ryan-Einot-Gabriel-Welsch multiple range test for pore-water SRP, DOC, ecosystem respiration, CH_4 emissions, and EEA.

Treatment	Pore-water Salinity	Pore-water Sulfide (mg L^{-1})	Pore-water NH_4^+ (μg N L^{-1})	Pore-water NO_3^- (μg N L^{-1})	Pore-water SRP (μg P L^{-1})	Pore-water DOC‡ (mg L^{-1})	Soil surface temperature (°C)	Ecosystem respiration (g C m^{-2} d^{-1})	CH_4 emissions† (g C m^{-2} d^{-1})	ΣC-acquiring EEA (nmoles g dry soil^{-1} h^{-1})
Control	0.65(0.12)^{b}	0.07(0.01)^{b}	4.59(2.25)^{b}	11.56(1.89)^{a}	12.61(1.77)^{b}	8.25(0.86)^{a}	20.67(0.62)^{b}	7.60(1.05)^{b}	−0.08(0.61)^{a}	329.55(27.57)^{b}
Fresh	0.55(0.05)^{b}	0.15(0.06)^{b}	2.62(0.81)^{b}	10.52(0.82)^{a}	16.28(5.00)^{b}	9.93(1.63)^{a}	19.90(0.48)^{b}	5.26(0.75)^{b}	−1.04(1.22)^{a}	330.15(40.20)^{b}
Pulse	2.31(0.35)^{a}	1.62(0.73)^{a}	13.97(9.97)^{b}	15.93(2.82)^{a}	25.44(5.08)^{b}	8.74(0.65)^{a}	20.88(0.51)^{b}	7.06(1.01)^{b}	0.49(0.18)^{a}	483.01(52.26)^{c}
Press	1.57(0.36)^{a,b}	1.29(0.53)^{a}	545.75(188.74)^{a}	15.33(4.21)^{a}	94.28(34.33)^{a}	10.07(1.62)^{a}	25.56(0.77)^{a}	4.89(1.28)^{a}	0.08(0.24)^{a}	178.61(86.89)^{a}

*EEA data were collected in March 2015 (Herbert et al. 2018).
†CH_4 emissions were significantly lower in Press plots compared to other treatments in October 2014, March 2015, October 2015, March 2016, and June 2016 (Herbert et al. 2018).
‡DOC was significantly less in Press plots compared to other treatments in April 2014, March 2015, and March 2016 (Herbert et al. 2018).

temperature among treatments. Tukey's honestly significant difference (HSD) test was used to separate means at α = 0.05. SPSS was used to conduct the statistical analyses (version 25, IBM Corp., 2017).

Microbial characterization

We characterized bacterial and archaeal composition using 16S rRNA amplicon sequencing. After extracting DNA from each sample using a MoBio PowerSoil DNA extraction kit (Carlsbad, California), we amplified the V4 region of the 16S rRNA gene using 5PRIME HotMasterMix and 515F and 806R primers with customized Illumina sequencing adapters and unique sample barcodes following conditions described in detail elsewhere (Daum 2017). Amplicons were then pooled at approximately equal molar concentrations after quantification using a Roche LightCycler 480 real-time PCR instrument. The pooled sample was then sequenced on the MiSeq sequencing platform with a v3 600 Reagent kit following a 2 × 300 indexed run recipe at the Joint Genome Institute in Walnut Creek, California. Raw sequences were processed using the iTagger v. 2.2 pipeline (https://bitbucket.org/berkeleylab/jgi_itagger/src/itagger2/) and USEARCH (v. 9.2). Briefly, paired end reads were merged and quality-filtered using expected error filtering. The resulting sequences were then clustered into operational taxonomic units (OTUs) at 97% similarity using the UPARSE algorithm (Edgar 2013). Finally, OTUs were classified using the Ribosomal Database Project reference. The final OTU table and metadata can be found in the Zenodo archive (Wisnoski and Lennon 2020), and raw sequence data are available at the NCBI Sequence Read Archive (BioProject PRJNA611801).

Diversity analysis

We compared patterns of α- and β-diversity among treatments to assess microbial responses to seawater intrusion. We performed rarefaction on the community data, subsampling communities to 143,105 reads, and then relativized OTU abundances to the total number of reads per sample. We characterized within-sample (α) diversity as the effective number of OTUs by taking the exponential of Shannon's index (i.e., Hill number with degree = 1), which improves comparisons among groups (Jost 2006). We used ANOVA to compare differences in α-diversity among treatments, followed by Tukey's HSD test to generate confidence intervals for group differences. To explain differences in community structure among treatments (i.e., β-diversity), we first transformed OTU relative abundances with the Hellinger transformation (appropriate for ordinations, Legendre and Gallagher 2001), then used permutational multivariate ANOVA (PERMANOVA) to determine the significance of the treatment effects. We implemented pairwise PERMANOVA with Benjamini-Hochberg corrected p-values using the "RVAideMemoire" R package (v. 0.9-77, Hervé 2020). We used redundancy analysis (RDA) to quantify the importance of individual environmental variables (pore-water chemistry, soil surface temperature) for explaining community differences among treatments.

We also analyzed the responses of two key functional groups. First, we classified potential sulfate reducers as a subset of 16S rRNA sequences belonging to the following orders in the δ-Proteobacteria: Desulfuromonadales, Desulfarculales,

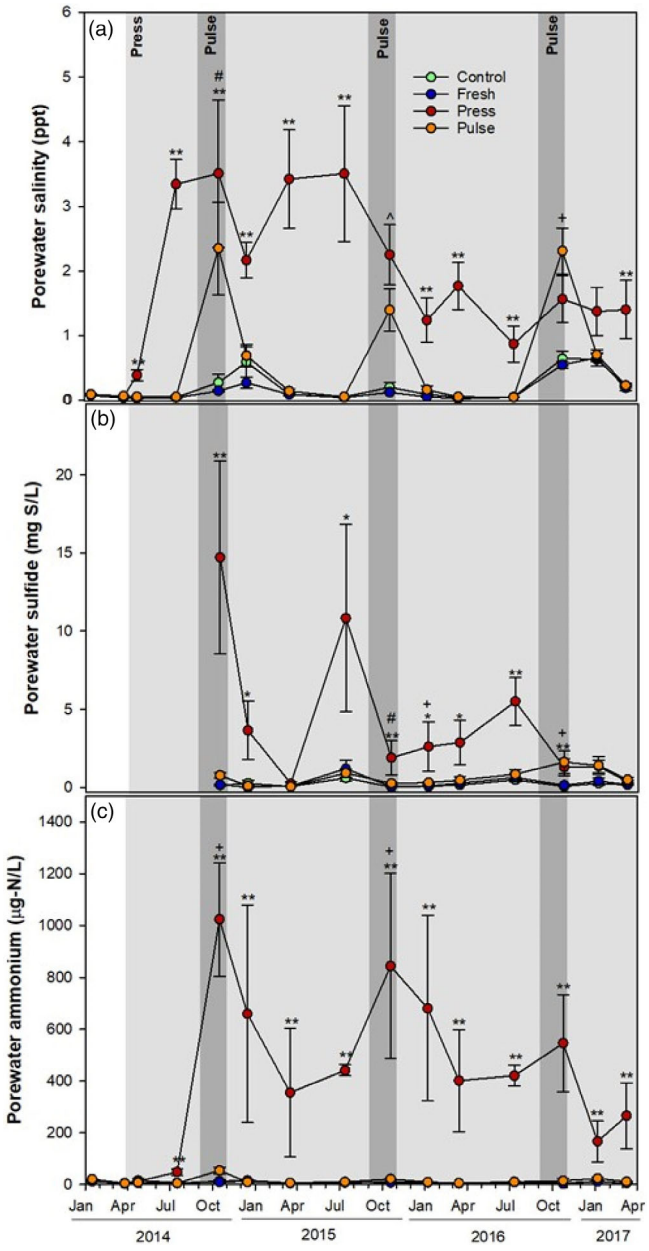

Fig. 3. Pore-water (**a**) salinity, (**b**) sulfide, and (**c**) ammonium concentrations (means ± SE) of treatments over time. Light gray shading indicates duration of press treatment and darker gray shaded bars indicate the timing and duration of the pulse treatment. Microbial samples were collected in October 2016. ** = press > other treatments ($p < 0.05$); * = press > than other treatments ($p < 0.10$); # = press > other treatments except pulse ($p < 0.05$); + = pulse > other treatments except press ($p < 0.05$); ^ = press and pulse > other treatments ($p < 0.05$). Figure modified from Widney et al. (2019).

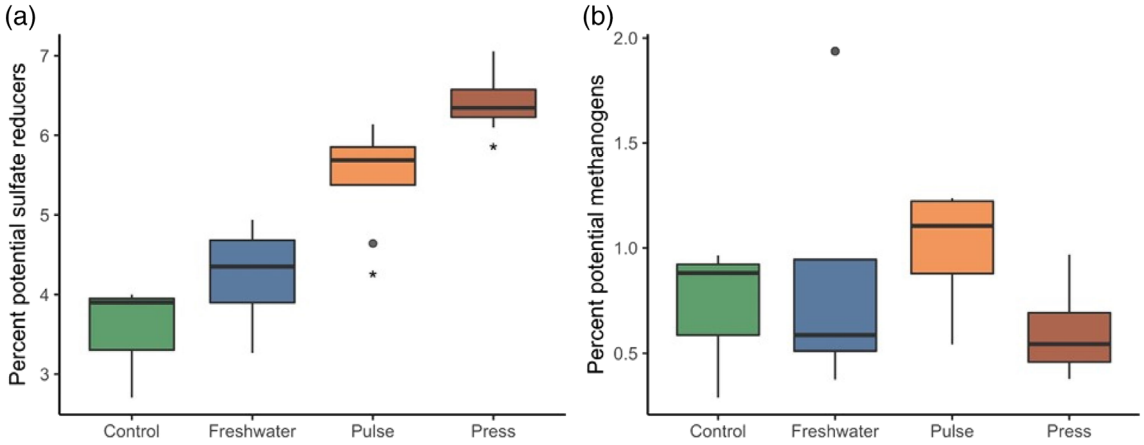

Fig. 4. (**a**) Potential sulfate reducer abundance and (**b**) potential methanogen abundance based on the total DNA community. * indicates press and pulse are different from control ($p < 0.01$) and fresh ($p < 0.01$, press) ($p < 0.10$, pulse).

Desulfobacterales, Desulfovibrionales, Desulfurellales, while recognizing that this may be an incomplete estimate of sulfate reducers. Second, we classified potential methanogens based on the summed relative abundances of archaeal sequences belonging to the following orders: Methanobacteriales, Methanomicrobiales, Methanosarcinales, and Methanocellales. We used ANOVA and Tukey's method to determine the significance and effects of experimental treatments on the relative abundances of these taxonomic groups in the communities. All analyses were completed using the R environment (v. 3.6.0) and the vegan R package (Oksanen et al. 2019).

Results

Microbial composition

We observed differential effects of seawater intrusion on microbial communities in the treatments: Press inputs of seawater reduced α-diversity by 25% relative to the control treatment (ANOVA, $F_{3,11} = 4.98$, $p = 0.017$) whereas pulse additions had no effect on α-diversity (Tukey HSD, $p = 0.441$, Fig. 1). Seawater additions also affected microbial community composition (PERMANOVA, $F_{3,11} = 4.43$, $p = 0.001$, $r^2 = 0.55$), with significantly different ($p < 0.05$) community composition detected between each treatment except between control and fresh plots (Fig. 2). Composition in the pulse treatments, although different from other treatments, was more similar to control and fresh treatments than the press treatment (Fig. 2). A permutation test ($n = 999$) revealed that the overall RDA model was significant (df = 6, Variance = 0.19, $F = 2.03$, $p = 0.004$), and that RDA1 was significant ($p = 0.003$) but the RDA2 axis was not significant. Microbial composition in the pulse treatment was associated with higher pore-water salinity and sulfides than control and fresh treatments (Fig. 2; Table 1). The press treatment was associated with higher NH_4^+, DRP, and soil surface temperature (Fig. 2), which were a consequence of the continuous seawater additions (Widney et al. 2019). Continuous (press) salinity also led to persistent changes in pore-water chemistry, including increased salinity, ammonium, and sulfides (Herbert et al. 2018) whereas the pulse treatment exhibited only transient increases in pore-water salinity and sulfides that declined to background levels once dosing ceased (Fig. 3) (Widney et al. 2019). Soil surface temperature also increased in the press plots as macrophytic vegetation died and more light reached the soil surface (Table 1, Fig. S2).

Seawater manipulations also affected microbial functional groups. Relative abundance of potential sulfate reducers was nearly double in the press plots (6.5%, Tukey HSD, $p = 0.0004$) than in the control treatment (3.5%) (ANOVA, $F_{3,11} = 15.34$, $p = 0.003$, Fig. 4a) and 30% greater compared to the fresh treatment (4.5%) (Tukey HSD, $p = 0.0019$). Abundance of sulfate reducers also was enriched in the pulse treatment (5.5%) compared to the control treatment (Tukey HSD, $p = 0.0078$) and was marginally greater compared to the fresh treatment (Tukey HSD, $p = 0.054$) (Fig. 4a). Relative abundance of sulfate reducing bacteria (SRB) did not differ between press and pulse treatments (Tukey HSD, $p = 0.23$). Contrary to our prediction, relative abundance of potential methanogens, which ranged from 0.5 to 1.1%, did not differ among treatments (ANOVA, $F_{3,11} = 0.494$, $p = 0.694$) (Fig. 4b).

Discussion

We observed reduced microbial diversity (Fig. 1), altered microbial composition (Fig. 2), and increased relative abundance of SRB (Fig. 4a) in response to 2.5 yr of continuous (press) seawater intrusion in a TFM. However, episodic (pulse) seawater intrusion had different effects on TFM microbial communities. Pulse plots had greater abundance of SRB than the control and fresh treatments (Fig. 4a) but no difference in α-diversity (Fig. 1). In addition, the community composition of pulse plots diverged from control and fresh treatments, but it exhibited a weaker response to salinity (and shifted in a

different compositional direction) than the press treatment (Fig. 2). Therefore, our long-term field experiment reveals the importance of the timescale of seawater intrusion, with TFM microbial communities responding differently to episodic and continuous seawater intrusion.

Seawater intrusion may have influenced microbial communities by modifying pore-water chemistry. Both press and pulse treatments exhibited elevated pore-water sulfide concentrations (Table 1; Fig. 3), which is consistent with the observed increase in SRB. While the press treatment had consistently higher pore-water sulfide, pore-water sulfide in the pulse treatment was elevated only during the 2-month dosing window (Fig. 3). Osmotic stress from seawater additions may have also contributed to the decrease in microbial diversity and altered community composition in the press treatment. Salt stress has been shown to alter microbial community structure in freshwater systems, potentially favoring those that can tolerate higher salinities (Van Gray et al. 2020).

Microbial community response to seawater intrusion also depends on nutrient concentration. In wetland sediments exposed to both increasing salinity and nutrients (N, P), increased salinity alone increased bacterial diversity, whereas increased salinity in combination with N or N + P decreased diversity (Jackson and Vallaire 2009). In our experiment, press treatments resulted in not only elevated salinity but also higher pore-water inorganic N (ammonium, nitrate) concentrations compared to the other treatments (Table 1; Fig. 3) (Widney et al. 2019). While the pulse plots experienced transient increases in salinity, pore-water N did not increase as it did in the press plots (Widney et al. 2019). Thus, the combination of salinity and elevated N could have led to decreased diversity in the press plots.

In addition to the direct effects of seawater on vegetation, sulfide also likely contributed to reduced productivity and plant death in the press plots (Solohin et al. 2020) as hydrogen sulfide is toxic to vegetation (Lamers et al. 2013). Due to vegetation loss from the press treatments, soil surface temperatures were 5–10°C higher relative to other treatments (Table 1; Fig. S2), which could have implications for microbial community structure. Increased temperature is associated with higher microbial activity and respiration, but effects on microbial composition in response to soil warming are more variable (Hendershot et al. 2017). However, because negative or unimodal relationships between diversity and temperature are possible outcomes (Hendershot et al. 2017), elevated temperature may also be a contributing factor to diversity declines in press plots.

The decline in microbial diversity and composition in the press treatment may be linked to carbon limitation. Vegetation is essential to ecosystem C cycles as it provides the fuel to sustain microbial processes and the effects of seawater intrusion on vegetation can have a cascading effect on these heterotrophs. Press plots had considerably less above- (Fig. S1) and below-ground biomass, root production (Solohin et al. 2020), pore-water DOC, net ecosystem exchange, and CH_4 emissions than other treatments (Herbert et al. 2018). Ecosystem respiration and extracellular enzyme activity (EEA) also was lower in the press treatment (Table 1), suggesting that the microbial community was carbon limited. Neubauer et al. (2013) reported that long-term (3.5 yr) seawater exposure in a TFM also resulted in lower soil CO_2 emissions and EEA which they also attributed to salinity-induced changes in availability of labile soil organic carbon. There was no effect of the pulse treatment on ecosystem respiration, CH_4 emissions, or EEA (Table 1) (Herbert et al. 2018).

In contrast to our expectation that methanogen abundance would decrease in response to increased salinity, we observed no differences among treatments (Fig. 4b). Herbert et al. (2018), however, reported lower CH_4 emissions from press plots than in other treatments in previous October (2014, 2015) sampling events, though rates were low compared to summer months. The passage of Hurricane Matthew on 07–08 October 2016 and its associated storm surge exposed the marsh to surface waters with elevated salinities that increased to 5 on 06 October and reached 20 on 07 and 08 October. By 09 October, surface water salinity decreased to < 1. Pore-water salinity in control and fresh plots when we sampled 2 weeks later was slightly elevated (< 1) (Widney et al. 2019) but was well below the threshold (18) at which methanogenesis is significantly depressed (Poffenbarger et al. 2011). Pore-water sulfide concentrations in control and fresh plots in October 2016 (0.07–0.15 mg/L) also did not differ from previous October (2014, 2015) sampling events (0.03–0.52 mg/L), suggesting that the stormwater pulse did not increase sulfate reduction and likely did not suppress methanogenesis.

Finally, microbial community structure may have been affected by interactions among taxa that were introduced via the seawater additions and those in the fresh river water that naturally flooded the area, a phenomenon known as "community coalescence" (Rillig et al. 2015). The resulting community composition may have been affected by differences in environmental conditions between the seawater and river water and by the temporal dynamics of mixing (Rillig et al. 2015). For example, pulse seawater additions modified the environment for ~ 2 months (Fig. 3), but this treatment may have been insufficient to overcome the larger and more frequent additions of fresh river water and the accompanying freshwater microorganisms that were introduced by twice daily tidal fluctuations.

In conclusion, continuous seawater additions in the press plots reduced microbial diversity that was linked to reduced C inputs from macrophytes and diminished extracellular enzyme activity and soil respiration. Microbial diversity and C cycling, however, were not affected in the pulse treatment, indicating that TFM microbial communities may persist in the face of storms and other episodic events. Our findings suggest that chronic additions of seawater from sea level rise or freshwater diversions upstream will reduce the macrophyte inputs

of C and alter microbial community structure, potentially modifying rates of C cycling associated with microorganisms in TFMs.

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