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Microbial Community Response to a Passive Salt Marsh Restoration

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Abstract

In response to current threats to salt marshes, there are increasing efforts to restore these vital coastal ecosystems and promote their resilience to global change drivers. Unfortunately, the economic cost associated with assessing the effectiveness of restoration is prohibitive and more information is needed about the trajectory and timing of restoration outcomes to improve restoration practices. Microbial communities provide essential salt marsh functions so assessing the degree to which microbial communities in restored marshes resemble reference marshes can serve as a proxy indicator for the potential return of microbial function. We studied a recently restored marsh located on Cape Cod, MA, USA, by examining shifts in the microbial community and sediment edaphic properties in three habitats of a degraded oligohaline marsh, both before and after restoration of tidal flooding and in comparison with three nearby *S. alterniflora* reference marshes that never had flow restrictions. We hypothesized that the microbial community would respond rapidly to the restoration and that over time these communities would be indistinguishable from reference marsh communities. We found that soil edaphic characteristics shifted along a trajectory of recovery toward the reference marshes, with increases in salinity and decreases in soil organic matter, percentage of carbon, and percentage of nitrogen. The microbial communities in all three habitats within the restored marsh were different from reference marshes, and both the prokaryotic and fungal communities within *P. australis* and *Typha* sp. habitats became more similar to reference marshes (similarities increasing from an average of 5 to 16% for prokaryotes and 3 to 10% for fungi) during the first 2 years after restoration. In that same time period, by contrast, there was no return of the native marsh vegetation. These results suggest that shifts in microbial community structure occur prior to shifts in marsh vegetation and may facilitate the successful revegetation of restored marshes. Understanding the recovery trajectory of marshes during restoration and the role that microbes play in promoting the long-term sustainability of these habitats is essential; these results suggest that microbial communities respond rapidly and in a positive direction to restoration efforts.

Keywords Fungi · Prokaryotes · 16S rRNA · Internal transcribed spacer region · *Spartina alterniflora* · *Phragmites australis* · *Typha*

Introduction

Salt marshes are important coastal habitats that provide a host of ecosystem services, such as reducing nitrogen pollution, sequestering carbon, and protecting coastal lands from storm surges (Costanza et al. 1989; Valiela and Cole 2002). Salt marshes have very high rates of primary productivity and anoxic sediments that impede decomposition, resulting in carbon sequestration rates that are an order of magnitude higher than terrestrial systems (McLeod et al. 2011). This coastal carbon storage, referred to as "Blue Carbon," underscores the importance of protecting existing coastal wetlands and restoring those that have been degraded with the aim of enhancing carbon storage and promoting other critical ecosystem services.

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Many of the ecosystem services provided by salt marshes are a result of microbial processes. Microbial communities, including prokaryotes and fungi, harbor extensive taxonomic diversity and drive the bulk of biogeochemical cycling in most ecosystems, including salt marshes (Rublee and Dornseif 1978; Falkowski et al. 2008; Bowen et al. 2012; Bowen et al. 2015; Ramirez et al. 2014; Tedersoo et al. 2014; Delgado-Baquerizo et al. 2016; Dini-Andreote et al. 2016a). Microbial cycling of essential elements like carbon, sulfur, and nitrogen, fuels critical processes within salt marshes that either enhance productivity and carbon storage (e.g., nitrogen fixation) or promote organic matter decomposition (e.g., fungal respiration, sulfate reduction, denitrification; Buchan et al. 2003; Howarth and Giblin 1983; Cao et al. 2008). Although work has been done to characterize how specific microbial functions change as a result of restoration (e.g., nitrogen fixation (Piehler et al. 1998, Bernhard et al. 2015), extracellular enzyme production (Duarte et al. 2012), carbon processing (Craft et al. 2003, Santín et al. 2009), among others), those that have examined shifts in the microbial community have done so either via low-resolution DNA fingerprinting techniques (e.g., TRFLP, Bernhard et al. 2012) or via assessment of microbial biomass (Ma et al. 2017). Understanding how, and over what time scales, the microbial community responds to marsh restoration is needed to improve restoration outcomes because shifts in the microbial community could serve as useful indicators for the potential return of microbial function. Although analysis of microbial community structure cannot be explicitly linked to their function, it is reasonable to presume that communities with a similar structure will have similar functions. Thus, comparing microbial community structure in degraded marshes that undergo restoration with intact healthy marshes could provide an indication that the marsh restoration is proceeding along a trajectory toward recovery.

One common mechanism for salt marsh degradation in the northeastern USA is the loss of tidal flow, due largely to historical expansion of roads and railways that channel flow through culverts or tide gates (Warren et al. 2002). This flow restriction decreases saltwater intrusion, which has cascading effects that culminate in altered plant zonation, changes in elevation of the marsh platform, and ultimately, disruption to the underlying geochemistry of the marsh (Portnoy and Giblin 1997; Warren et al. 2002). With decreased salinity, the native, salt-tolerant marsh grasses can be outcompeted by less saltwater-tolerant species such as invasive *Phragmites australis* (Able and Hagan 2003; Rickey and Anderson 2004; Meyerson et al. 2010; Moore et al. 2016). Returning tidal flow to these previously restricted marshes is frequently employed to restore salt marshes, but assessing restoration of essential salt marsh functions is time consuming and expensive (Warren et al. 2002; Roman and Burdick 2012).

In an ideal world, the effectiveness of restoration would be evaluated by monitoring recovery of diversity, return of native vegetation structure, or restoration of ecosystem services (Ruiz-Jaen and Aide 2005; Wortley et al. 2013). However, time and cost constraints often limit the assessment of restoration to an assessment of the aerial extent of restored lands (National Academies of Sciences, Engineering, and Medicine 2017). Response of macro-organisms, particularly the return of native vegetation, is the most common indicator of restoration success; however, it may take decades to establish the full complement of salt marsh plant diversity (Warren et al. 2002). In successful restorations of New England salt marshes, the expansion of native vegetation occurs at rates of approximately 5% per year and other salt marshes showed a return of ecological processes between 5 and 15 years after restoration (Sinicrope et al. 1990; Warren et al. 2002; Craft et al. 2003). Freshwater and upland taxa often die back immediately after exposure to increased seawater inundation, leaving space for native salt marsh vegetation to spread (Smith and Warren 2012). Considering the important role that plant species diversity plays in structuring microbial communities (Hamilton and Frank 2001; Burke et al. 2002; Zak et al. 2003), it remains unclear whether the restoration of sediment microbial communities and their associated ecosystem services will track changes in soil edaphic parameters or changes in marsh vegetation, or follow an independent trajectory.

Microbial community structure and function are frequently altered when microbes are exposed to environmental conditions that deviate from their typical environment (Waldrop and Firestone 2006). Many factors are important in structuring microbial communities in the environment, including temperature (Waldrop and Firestone 2006), salinity (Crump et al. 2004), pH (Lindström et al. 2005), and vegetation type (Burke et al. 2002), with salinity typically considered the most important (Lozupone and Knight 2007). However, the dominant vegetation type can also play a key role in structuring the microbial community (Hamilton and Frank 2001; Burke et al. 2002; Zak et al. 2003). Restoring saltwater flow into degraded oligohaline wetlands, such as those typically found in New England (Warren et al. 2002), alters salinity, changes vegetation structure, and alters sediment biogeochemistry (Portnoy and Giblin 1997), all of which interact to affect microbial community structure. The ecosystem state change that results from the sudden restoration of tidal flow to a degraded oligohaline salt marsh could therefore have important ecosystem consequences.

To evaluate the response of salt marsh microbes to an ecosystem state change induced by restoration of tidal flooding, we sequenced the 16S rRNA gene and the internal transcribed spacer (ITS) region to assess prokaryotic and fungal communities, respectively. We hypothesized that the

change in salinity as a result of restoration would shift the microbial community from a freshwater adapted to a saltwater adapted community over time. Furthermore, we hypothesized that the magnitude of that effect would be mediated by the vegetation in the degraded marsh, such that areas of the degraded marsh dominated by *S. alterniflora* would have a substantially smaller response to restoration than the oligohaline reaches of the restored marsh that are dominated by *P. australis* and *Typha* species.

Methods

Study Location

This study was carried out in four marshes on Cape Cod, MA, USA (Fig. 1), including a restored marsh (Muddy Creek) and three reference marshes (Sparrowhawk marsh, Davis Road marsh, and Crow's Landing marsh), all of which are a part of the Pleasant Bay estuarine complex (Fig. 1b). The Muddy Creek marsh is a degraded, oligohaline marsh located in Harwich, MA, that was restored in the winter/spring of 2016. The original construction of a road and culvert system in the early 1900s restricted tidal exchange with Pleasant Bay, which resulted in degradation of the marsh complex upstream of the culvert (Cape Cod Conservation District 2012). Prior to restoration, Muddy Creek was vegetated by a small amount of the native salt marsh grass *S. alterniflora* in the regions closest to the culvert and large regions dominated by the common marsh invasive, *P. australis*, and an oligohaline wetland plant from the genus *Typha* (Fig. 1c). In the winter/spring of 2016, the culvert was replaced with a spanning bridge constructed over the creek, allowing for the return of nearly full tidal flooding to the degraded marsh (Cape Cod Conservation District 2012). By contrast, the three reference marshes were never degraded due to tidal restrictions and are all dominated by *S. alterniflora*, as is typical of healthy salt marshes in this region of New England (Pennings and Bertness 2001).

Sample Collection and Processing

This study used a modified Before-After, Control-Impact design by sampling before and after restoration in the degraded marsh and in comparison to reference marshes. We opted for a modified design, such that we used three reference marshes, as opposed to a single reference marsh, to better constrain the range of possible recovery trajectories (Underwood 1992). Samples were collected from the three reference marshes and the restored marsh in June, August, and October of 2015, before the restoration and in June, July, August, and

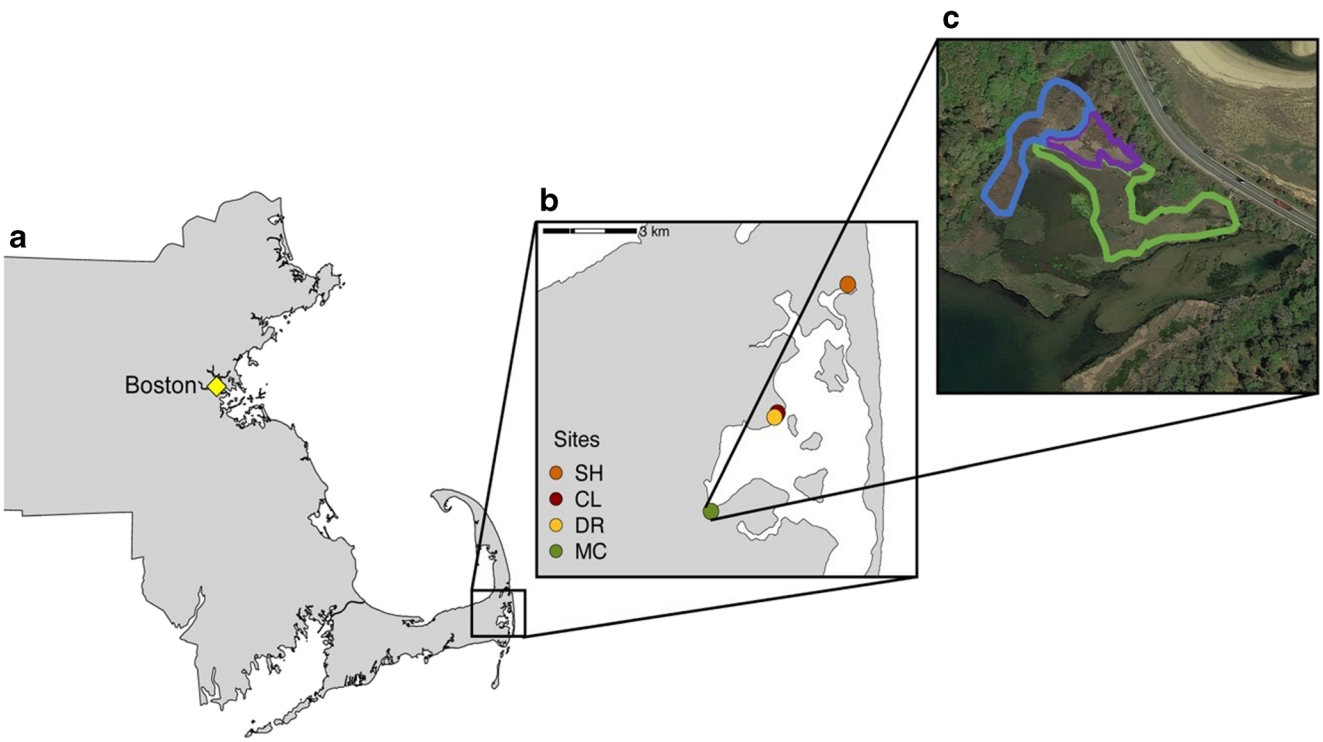

Fig. 1 Map of the field locations for this study. **a** Map of eastern Massachusetts, USA. **b** Location of four Cape Cod, MA, marshes where our study took place. Three of these systems served as reference marshes (Sparrowhawk—SH, Crow's Landing—CL, Davis Road—DR), and the fourth marsh was restored in the winter of 2015 (Muddy Creek—MC). **c** The perimeter of each vegetation zone prior to restoration of the Muddy Creek marsh is outlined in color (green—*S. alterniflora*, purple—*Typha*, blue—*P. australis*)

October after the restoration (2016–2017), with our first sampling in 2016 occurring just 3 months after restoration construction was complete. In each marsh location, multiple sites were selected from within each habitat type. At any given site, we considered our monthly sampling to be independent from sampling in previous months based on the differences in time and space scales (samples were collected haphazardly within each habitat with the specific location randomly selected each month) of our sampling relative to those of the microbial community. In Muddy Creek, we haphazardly selected six sites from the *S. alterniflora* habitat, three sites from the *P. australis* habitat, and three sites from the *Typha* habitat. In each reference marsh, we selected six sites from within the *S. alterniflora* habitat. From each site, we collected and homogenized approximately 25 mL of surface sediment (top 1–2 cm) using a 7.5-cm-diameter core tube at low tide. Sediment was aliquoted into cryovials for preservation of DNA, flash frozen in liquid nitrogen, and stored at − 80 °C until analysis. The remaining sediment was aliquoted into a 15-mL centrifuge tube and stored at − 20 °C for analysis of organic matter content. A subset of surface sediment was analyzed for porosity, determined by changes in mass after drying at 60 °C for 72 h, and for sediment organic matter (SOM) content, measured as loss-on-ignition after combustion at 500 °C for 5 h. A final subset of pre-combusted sediments was acidified with hydrochloric acid and analyzed on a Costech ECS 4010 Elemental Analyzer (Costech Analytical Technologies Inc., Valencia, CA, USA) for percent carbon (%C) and percent nitrogen (%N) content. Porewater was collected at each site using sippers (Neubauer 2013) placed at a depth of 25 cm and salinity was measured using a handheld refractometer.

Nucleic Acid Extraction and Sequence Preparation

DNA from each sample was extracted from approximately 0.25 g of sediment using the Qiagen PowerSoil® DNA Isolation Kit (Qiagen, Valencia, CA, USA) following manufacturer's protocols. The V4 hypervariable 16S rRNA region of the extracted DNA was PCR-amplified in triplicate using a universal 515F forward primer (5′-GTGCCAGCMGCCGCGGTAA-3′) and uniquely barcoded 806R reverse primers (5′-GGAC TACHVGGGTWTCTAAT-3′; Caporaso et al. 2011). Both primers also contained relevant Illumina adaptors (Caporaso et al. 2012). PCR for the 16S rRNA gene was performed on triplicate 25-μL reactions with 10 μL 5Prime Hot Master Mix (Quanta Bio, Beverly, MA, USA), 13.5 μL PCR-grade water, 0.25 μL of 20 μM forward and reverse primers, and 1 μL of DNA template. Samples were amplified with the following cycling conditions: 94 °C for 3 min followed by 35 cycles of 94 °C for 45 s, 50 °C for 60 s, 72 °C for 90 s, and a final cycle of 72 °C for 10 min. The first fungal ITS region was also amplified in triplicate with primers ITS1F and ITS2 (Walters et al. 2015). PCR for the ITS region was performed on triplicate 25-μL reactions with 10 μL 5Prime Hot Master Mix, 13.5 μL PCR-grade water, 0.25 μL of 20 μM forward and reverse primers, and 1 μL of DNA template. Samples were amplified with the following cycling conditions: 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 50 °C for 60 s, 72 °C for 90 s, and a final cycle of 72 °C for 10 min. Triplicate PCR products from both the 16S rRNA gene and the ITS region were separately pooled and gel purified using a Qiagen QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. A second 8-cycle PCR was performed on purified fungal ITS products to attach Illumina adaptors and unique dual indices using the Illumina Nextera Kit (Illumina Inc., San Diego, CA, USA) following the manufacturer's instructions. After indexing, the ITS samples were purified using a Qiagen PCR purification kit (Qiagen, Valencia, CA, USA). Concentrations of purified amplicons for both the 16S rRNA gene and ITS fragments were measured on a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Cambridge, MA, USA) and pooled in equal proportion to be sequenced on an Illumina MiSeq (Illumina, San Diego, CA, USA) using a 500-cycle kit and V2 chemistry.

Data Analysis

To identify abiotic changes due to restoration, we performed linear mixed model regression with the environmental parameter as the response variable, habitat and year as fixed effects, and sampling site within the marsh and sampling date within year as random effects using the *lme4* package (Bates et al. 2015) in R (R Core Team 2017). We performed multiple comparisons of means on the model using the *multcomp* package in R with Bonferroni correction (Hothorn et al. 2008) to determine changes over time in reference and restored marshes.

To investigate prokaryotic and fungal community composition, we analyzed sequence data using QIIME2 (version 2018.2, Caporaso et al. 2010) and R. In QIIME2, we demultiplexed 6,043,999 16S rRNA gene sequences and 11,229,682 ITS sequences using the DADA2 plugin (Callahan et al. 2016) with chimera removal to infer amplicon sequence variants (ASVs). ASVs are a high-resolution classification for microbes analogous to the traditional use of species (Callahan et al. 2017). ASVs occurring only once were removed from downstream analyses. Taxonomy of the 16S rRNA gene sequences was assigned using the Greengenes 16S rRNA gene sequence database (version 13–8; McDonald et al. 2012) and ASVs designated as chloroplasts or mitochondria were removed. We used VSEARCH (Rognes et al. 2016) to assign fungal taxonomy using the UNITE database (Nilsson et al. 2018) and we assigned taxonomy if the percent identity was greater than 60%. All 16S rRNA and ITS sequence data are publicly available via NCBI's Sequence Read Archive under accession number: PRJNA602775.

To identify whether patterns in community structure were more strongly determined by habitat, location (reference vs. restored marshes), or time (year) within our samples, we performed multiple network analyses utilizing the *igraph* package in R (Csardi and Nepusz 2006). Separately, we used the prokaryotic and fungal community data to build correlation matrices using the Spearman's rank correlation. Specifically, we measured the correlation between the prokaryotic and fungal communities of each sample pair, with habitat, location, and time as sample factors. We then used these correlation matrices to create weighted and undirected graphs, where the weight of the edges or links between samples represented the correlation in abundance for shared prokaryotes or fungi. We used a community detection algorithm based on the leading eigenvector centrality (Newman 2006) to document the degree of compartmentalization by identifying distinct clusters (modules) of samples in the network (i.e., groups of samples that were more connected to each other than to others). Additionally, we used Monte Carlo simulations to determine whether the degree of compartmentalization or modularity observed in the network was significant. Specifically, we generated 999 random networks by shuffling the correlations within each sample and then computed modularity. We then calculated the *p* value as the proportion of random networks whose modularity was greater than or equal to that observed in the original (non-shuffled) network. Finally, to determine whether distinct modules identified through the network analysis were associated with differences between habitat, location, and time we conducted a χ^2 test to determine the association between these three factors and their assigned module obtained via the network community detection algorithm. We then calculated the classification rate for habitat, location, and time to determine the proportion of time each factor correctly classified samples into a module. To determine the relative influence of habitat, location, and time on microbial community composition, eigen centrality scores were also computed for all nodes in the network using the *igraph* package in R (Csardi and Nepusz 2006). A high eigen centrality score indicates nodes that are highly connected to many other important nodes in the network. Significant differences in eigen centrality were determined via Kruskal-Wallis test and post hoc pairwise comparisons with sequential Bonferroni correction were performed to determine the differences between levels within each factor.

We used ASV tables derived for both prokaryotes and fungi to calculate how taxonomic diversity of the microbial community changed over time. We imported the quality controlled ASV tables derived from QIIME2 into the R package *phyloseq* (McMurdie and Holmes 2013) and rarefied both tables to the lowest sequencing depth (~ 9600 for the prokaryotic community, ~ 1500 for the fungal community). We calculated Shannon diversity on the rarefied datasets using *phyloseq* and visualized the data using *ggplot2* (Wickham 2016).

We tested differences in alpha diversity among restored and reference marshes via linear models. We tested for deviation from normality using a Shapiro-Wilk normality test and examined differences among habitat, year, and the interaction between habitat and year, using linear models via the 'lm' function in R. Residuals of the model were inspected and tested for deviation and homoscedasticity using Levine's test. Due to the heteroscedasticity in the linear model results, we also used mixed effects models, via the *lme4* package in R, to improve model fit. We included habitat, sampling year, and the interaction between habitat and year as fixed effects and month of sampling and sampling site within each marsh as random effects. We evaluated the mixed effects and linear models using Akaike's information criterion (AIC) according to the AIC function in R v3.6.1. We visually inspected the residuals of each mixed effects model and report the result of the best model according to the lowest AIC value.

We calculated beta diversity for both prokaryotic and fungal communities with QIIME2 using Bray-Curtis dissimilarities calculated from a rarefied ASV matrix. To visualize beta diversity in the restored and reference marshes, we performed principal coordinate analysis (PCoA) of the Bray Curtis dissimilarity values using the *vegan* package (Oksanen et al. 2018) in R. Significant differences in community composition for each habitat in the restored marsh compared with all three reference marshes was performed using PERMANOVA (Anderson 2017) with 10,000 permutations. To further examine the effect of restoration on different habitats within the restored marsh over time, we compared the similarity (1-Bray Curtis dissimilarity) between reference marshes and *S. alterniflora*, *P. australis*, and *Typha* habitats separately with a one-way ANOVA in R (R Core Team 2017) and tested pairwise comparisons with a Tukey HSD test. To assess which environmental variables correlated with shifts in the prokaryotic and fungal communities in the *P. australis* and *Typha* habitats, we performed canonical correspondence analysis (CCA) using *vegan*. A model containing all environmental variables was run using the "envfit" function to identify significant environmental parameters and parameters were removed if the variance inflation factor was greater than 10. We also used random forest modeling to determine which taxa within the sediments of each habitat were most important in classifying prokaryotic and fungal communities before and after restoration. To test whether the microbial community is predictive of restoration status, we employed independent random forest classification models using the R package *randomForest* (Liaw and Wiener 2002) within each habitat. We built a model that classified samples based on pre (2015) and post (2016 and 2017) restoration using 100 trees and all default settings on the full ASV dataset. We used the R package *ggplot2* to visualize the 10 most discriminatory features (ASVs) of the model based on the GINI index.

Results

Environmental Parameters

Sediment and pore water characteristics of the *P. australis* and *Typha* habitats showed patterns that were initially divergent from reference marshes but that moved in a trajectory toward reference marshes in each year after restoration, whereas the *S. alterniflora* marsh in the restored site was not significantly different from reference marshes (Fig. 2, Table 1, Table S1). For example, pore water salinity was significantly lower in *P. australis* and *Typha* habitats of the restored marsh prior to restoration; however, pore water salinity in the *S. alterniflora* habitat within the restored marsh did not differ from the reference marshes either before or after restoration. After restoration, there was a significant increase in pore water salinity in both the *P. australis* and *Typha* habitats, such that 2 years after restoration, salinity in these habitats of the restored marsh was no longer significantly different than the reference marshes (Fig. 2a). Similar patterns were observed for other parameters, with sediment organic matter, %C, and %N all being higher in the *Typha* and *P. australis* habitats prior to restoration and decreasing to more closely mirror the reference marshes within 2 years of the restoration occurring.

Microbial Community Structure

To determine whether habitat, location, or time could be used to infer prokaryotic and fungal community composition, we performed a network analysis across all three factors (Fig. 3;

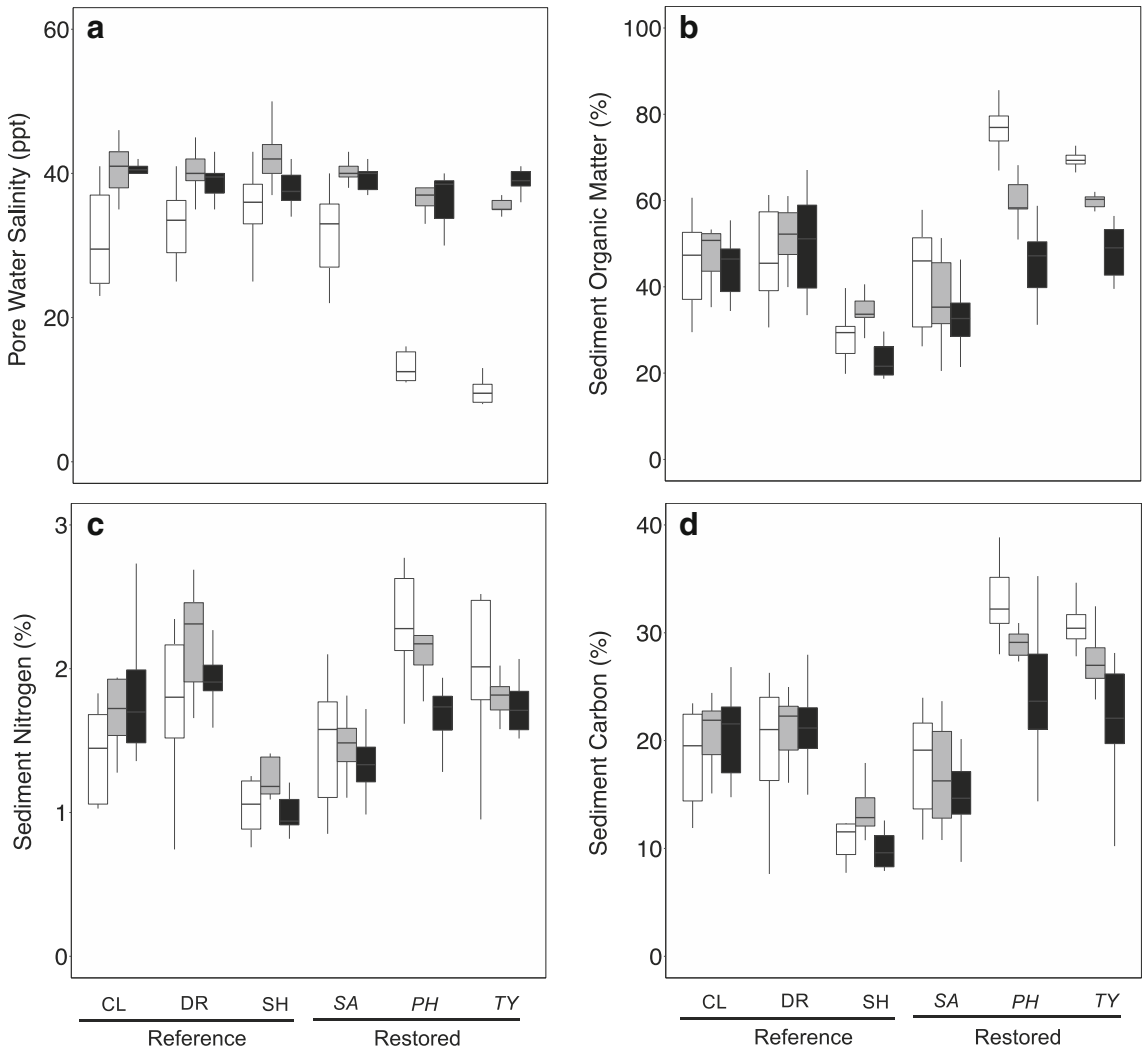

Fig. 2 Change in environmental parameters in reference (Crow's Landing—CL, Davis Road—DR, Sparrowhawk—SH) and restored marshes (SA—*S. alterniflora*, PH—*P. australis*, and TY—*Typha*; 2015—open bars, 2016—gray bars, 2017—black bars). **a** Salinity, **b** sediment organic matter, **c** %N in sediment, and **d** %C in sediment. In the restored marsh, years 2016 and 2017 represent 1-year and 2-year post-restoration, respectively, while no manipulation occurred across all 3 years within reference marshes. Results of the linear mixed effects models are provided in Table 1, and results from the multiple comparisons post hoc tests are provided in Table S1

Table 1 Results from linear mixed model regression with the environmental parameter as the response variable, habitat and year as fixed effects, and sampling site and sampling date as random effects. Habitats were represented as the three habitats within the restored marsh (*P. australis*, *Typha*, and *S. alterniflora*) and the *S. alterniflora* of the reference marshes

Effect	Test	Salinity	SOM	%C	%N
Habitat	*F*	37.09	115.40	28.72	89.00
	df	3	3	3	3
	p	< 0.001	< 0.001	< 0.001	< 0.001
Year	*F*	198.20	91.40	9.94	28.23
	df	2	2	2	2
	p	< 0.001	< 0.001	< 0.001	< 0.001
Habitat × year	*F*	30.50	18.43	5.64	7.83
	df	6	6	6	6
	p	< 0.001	< 0.001	< 0.001	< 0.001

Table 2). Modules derived from the network analysis represent clusters of samples that are more similar to each other than they are with samples in other modules. Prokaryotic communities resolved into four modules (Fig. 3a, b), with the modularity in this network being significantly larger than observed in 999 randomly shuffled networks obtained via Monte Carlo simulations. The network modules were significantly associated with habitat and with location, but not with time (Fig. S1; Table 2). Additionally, habitat was the best predictor of network modularity (correctly classifying 62% of the samples), followed by location (59%), and time (37%). Modules labeled 1–3 were samples exclusively derived from the *S. alterniflora* habitat in both reference and restored marshes, with module 3 containing samples almost exclusively from the reference marshes (module 3: reference marsh—98%). Modules one and two were more evenly spread across samples collected from the *S. alterniflora* habitat from both reference and restored marshes. Module 4 almost exclusively contained samples from the restored marsh *P. australis* and *Typha* habitats (module 4: *P. australis*—54%, and *Typha*—44%). These results are consistent with the eigen centrality scores, which demonstrate that *S. alterniflora* had a significantly higher eigen centrality and was therefore more well connected in the network than *Typha* or *P. australis* (Fig. S2A). Similarly, the restored site had significantly lower eigen centrality scores than the reference marshes (Fig. S2C), while the eigen centrality values for time showed no consistent pattern (Fig. S2B).

Fungal communities resolved into five modules (Fig. 3c, d), with the modularity in this network also being significantly larger than observed in 999 random networks obtained via Monte Carlo simulations (p value = 0.001). Although the network modules were significantly associated with habitat, location, and time (Table 2, Fig. S1B), there was considerable variation in the predictive power of each of the factors. Habitat was the best predictor of network modularity (correctly classifying 51% of the samples), followed by location (47%) and time (36%). In the fungal network, module 5 consisted entirely of samples taken from the *S. alterniflora* habitat in 2015, prior to restoration. Module 1 consisted mostly of reference marsh *S. alterniflora* (70%) or post-restoration *S. alterniflora* samples (module 1: *S. alterniflora* 2016–8% and *S. alterniflora* 2017–18%). Samples in the remaining modules (modules 2–4) were derived from a mixture of all habitats and years. Consistent with these results, the eigen centrality scores for the fungal community indicated small differences in centrality by habitat, and no difference in centrality as a result of time or treatment (Fig. S3).

Restoration Effects on Microbial Diversity

Within the prokaryotic community, PERMANOVA analysis indicated that there were significant differences in beta diversity between the restored marsh *S. alterniflora*, *P. australis*, and *Typha* habitats compared with the reference marshes (Fig. 4, Table 3). There were also significant differences by year within the restored marsh *S. alterniflora*, *P. australis*, and *Typha* habitats, though there was no effect of year within the reference marshes. Within all three habitats, the prokaryotic community in the restored marsh samples were clearly differentiated from the reference marshes along the primary axis of a principal coordinates analysis (Fig. 4) and there was additional differentiation by year along the secondary axis, although those distinctions were strongest in the *P. australis* and *Typha* habitats (Fig. 4a, b). Further, we compared the similarity in prokaryotic communities between the reference marshes and each habitat in the restored marsh and determined there was a significant increase in the similarity to the reference marsh with time since restoration in the *P. australis* habitat and the *Typha* habitat, but not in the *S. alterniflora* habitat.

In the fungal community (Fig. 5, Table 3), there were significant differences between the restored marsh *S. alterniflora*, *P. australis*, and *Typha* habitats compared with the reference marshes. There were also significant effects of year in the *S. alterniflora*, *P. australis*, and *Typha* habitats of the restored marsh, but not the reference marshes. Within all three habitats, the restored marsh differentiated from the reference marshes along the primary axis and differentiated by year along the secondary axis. Further, as with the prokaryotic community, the similarity among fungal communities of the reference marshes and *P. australis* and *Typha* increased with time. In contrast to the prokaryotic community, the similarity in the fungal community between restored and reference marshes also increased in the *S. alterniflora* habitat.

Canonical correspondence analysis results (Fig. S4) indicated that SOM and salinity were the most important factors explaining the shift in prokaryotic community of

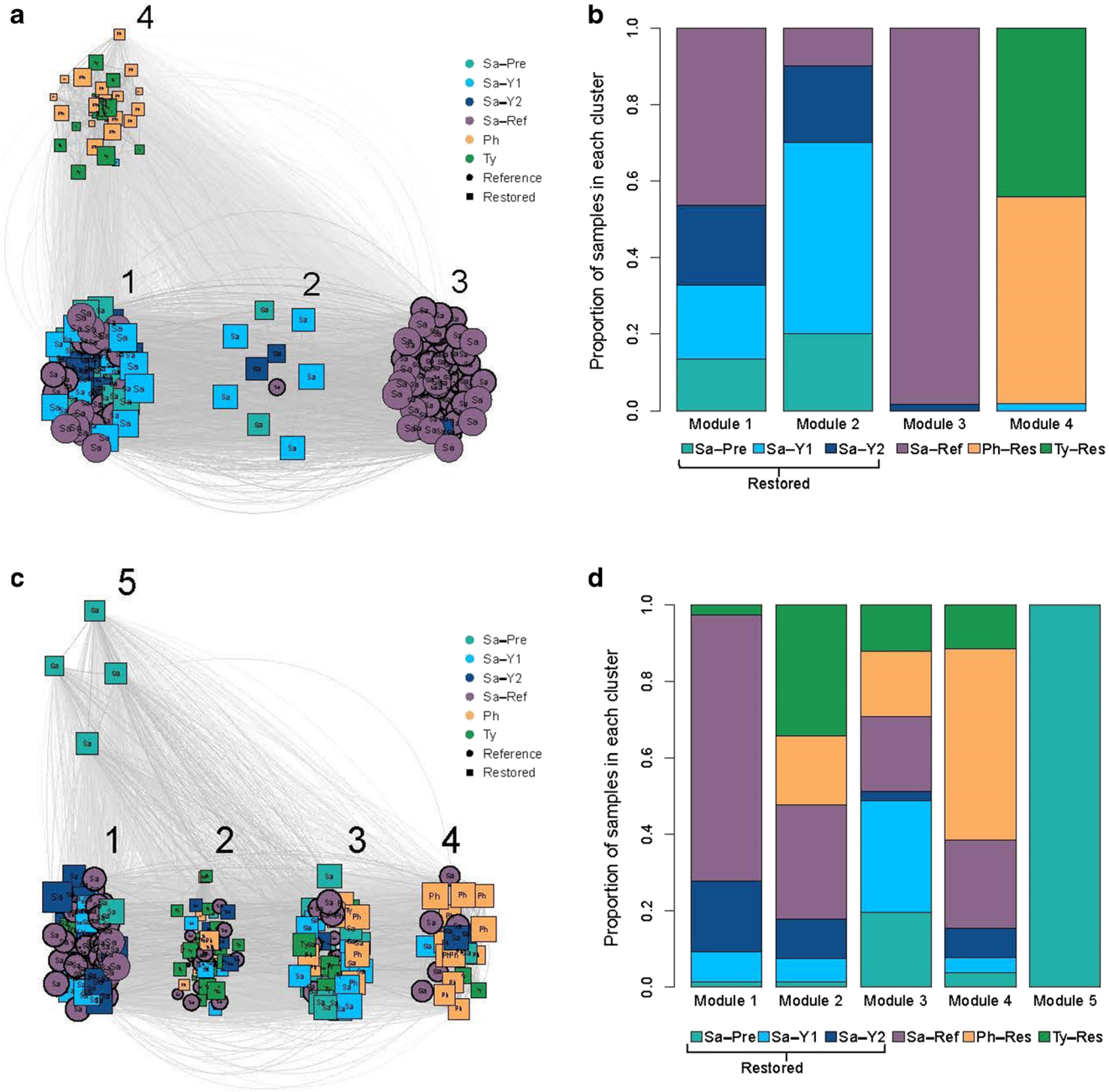

Fig. 3 Network-derived community structure of the prokaryotic (**a**, **b**) and fungal (**c**, **d**) communities in reference (circles) and restored (squares) marshes. **a** Prokaryotic communities resolve into four modules. **b** Stacked bar plot indicating the proportional representation of samples in each module depicted in **a**. **c** Fungal communities resolve into five clusters. **d** Stacked bar plot indicating the proportional representation of samples in each module depicted in **c**. Sa = *S. alterniflora*, pre = prior to restoration, Y1 = 1 year after restoration, Y2 = 2 years after restoration, Ty = *Typha* Sp., Ph = *P. australis*

P. australis ($F = 3.15$, $p = 0.001$) and *Typha* ($F = 3.42$, $p = 0.001$). In the restored *S. alterniflora* prokaryotic community, the strongest correlation was with SOM and %C in the sediments ($F = 2.27$, $p = 0.001$; Fig. S4). Similarly, the fungal community structure (Fig. S5) was strongly influenced by SOM, %N, and the C/N ratio in both *P. australis* and *Typha* habitats ($F = 2.88$, $p = 0.001$; $F = 2.40$, $p = 0.001$, respectively). None of the measured environmental parameters were significant predictors of the *S. alterniflora* fungal community.

We calculated Shannon Diversity estimates for each habitat in each year and assessed model fit using AIC scores (Fig. S6, Table S2). A linear model proved to be the best model to describe the alpha diversity of the

Table 2 Chi-squared test results and classification rates for network analysis. χ^2 indicates whether network modules depicted in Fig. 3 were significantly associated with vegetation type or time. Classification percentage indicates the proportion of time each factor correctly classified samples into the relevant module

Factor	Df	χ^2	*p* value	Classification
Prokaryotes				
Vegetation type	6	185.99	2.2×10^{-16}*	61.78%
Reference vs. restored	3	116.79	2.2×10^{-16}*	59.16%
Year	6	5.09	0.53	37.17%
Fungi				
Vegetation type	6	75.74	3.5×10^{-13}*	51.40%
Reference vs. restored	3	43.55	7.59×10^{-6}*	46.73%
Year	6	26.45	8.7×10^{-4}*	35.51%

prokaryotic community, with an AIC of 39.0 compared with 83.8 for the mixed effects model; however, the linear model also had significant deviations from homoscedasticity ($p = 0.019$) and therefore estimates should be interpreted with caution. Alpha diversity of the prokaryotic community was significantly lower 2 years after restoration in the *P. australis* and *Typha* habitat of the restored marsh. We observed similar homoscedastic deviations in the fungal alpha diversity but, similarly, found that the linear model offered the best fit according to AIC (Fig. S6, Table S2). However, except for a small increase in fungal diversity in the *S. alterniflora* habitat of the restored marsh 2 years post-restoration, there were no significant differences in fungal diversity through time or as a result of restoration.

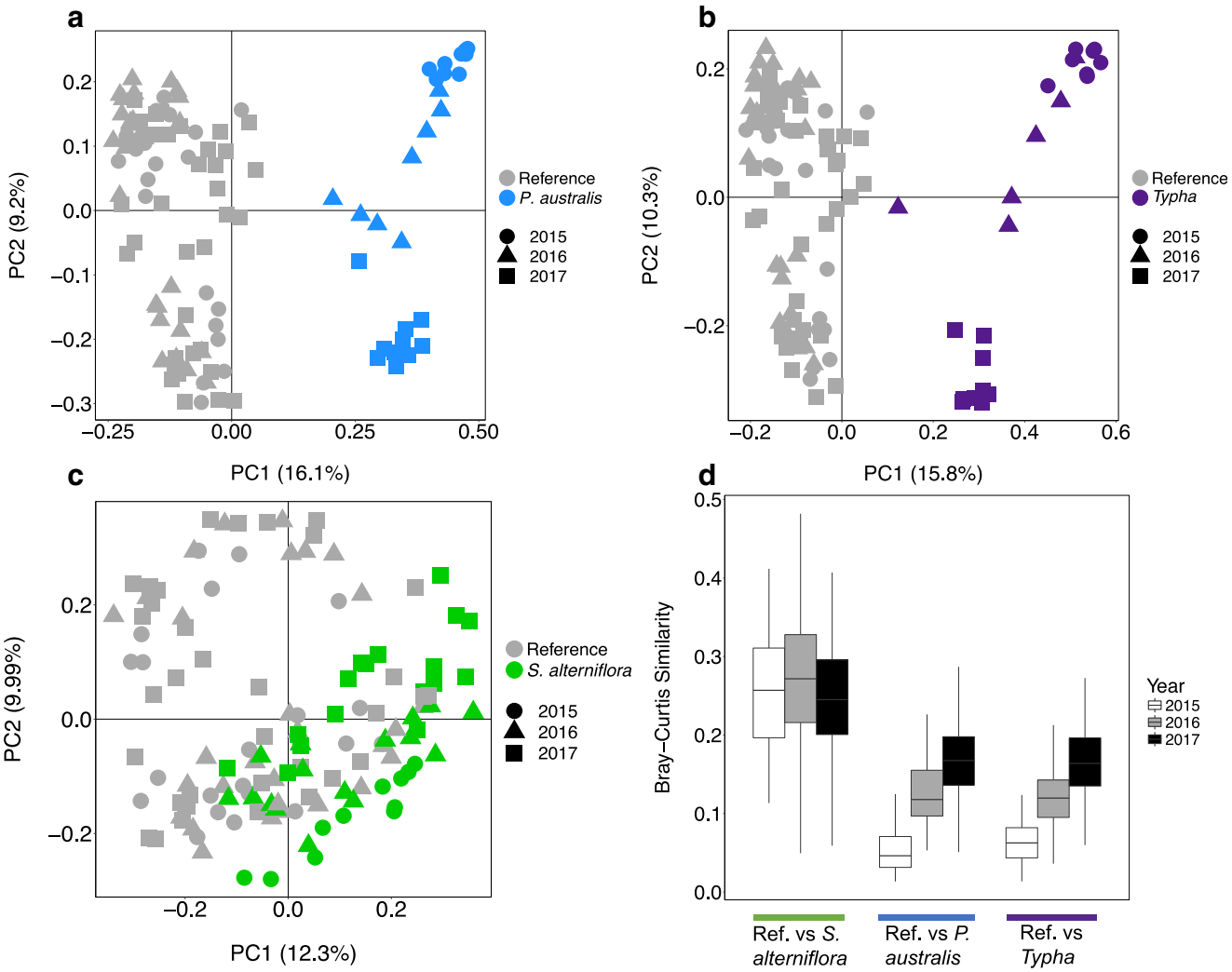

Fig. 4 Principal coordinates analysis constructed using Bray-Curtis dissimilarity of the prokaryotic community for each habitat in the restored marsh compared to reference marshes: **a** *P. australis*, **b** *Typha*, and **c** *S. alterniflora*. **d** Change in similarity to reference marshes before (2015) and after (2016 and 2017) restoration in each of the restored marsh habitats. There was no significant difference over time between the reference marshes and *S. alterniflora* restoration site (ANOVA; $F = 0.06$, df = 2, $p = 0.81$), but similarity between the reference and restored *P. australis* (ANOVA; $F = 591.2$, df = 2, $p < 0.001$) and *Typha* habitats (ANOVA; $F = 427.3$, df = 2, $p < 0.001$; Fig. 5d) increased with time

Table 3 Results of PERMANOVA analysis indicating significant differences in prokaryotic and fungal communities between restored and reference marshes within each habitat type of the restored marsh

Factor	Df	*F*	R^2	*p* value
Prokaryotes: by habitat				
Spartina alterniflora	1	10.5	0.07	< 0.01
Phragmites australis	1	22.3	0.15	< 0.01
Typha spp.	1	20.48	0.14	< 0.01
Prokaryotes: by year				
Spartina alterniflora	2	5.91	0.08	< 0.01
Phragmites australis	2	5.49	0.07	< 0.01
Typha spp.	2	5.81	0.07	< 0.01
Fungi: by habitat				
Spartina alterniflora	1	17.04	0.12	< 0.01
Phragmites australis	1	9.77	0.07	< 0.01
Typha spp.	1	15.77	0.11	< 0.01
Fungi: by year				
Spartina alterniflora	2	3.32	0.05	< 0.01
Phragmites australis	2	2.84	0.05	< 0.01
Typha spp.	2	2.62	0.04	< 0.01

Taxa Responsible for Observed Differences After Restoration

Lastly, we used random forest modeling of the most abundant prokaryotic (Fig. 6, Table S3) and fungal (Fig. 7, Table S4) ASVs to determine which taxa were important in explaining the differences before and after restoration in each habitat. For the prokaryotic model, in all cases the ASVs important in discriminating pre- and post-restoration were relatively low in abundance compared with the overall prokaryotic community. The taxonomic assignment and associated DNA sequence for the prokaryotic ASVs are provided in Table S3. The prokaryotic model had out-of-box error estimates of 24% for *P. australis*, 31% for *Typha*, and 25% for *S. alterniflora.* This model had mixed performance in correctly classifying samples into pre- and post-restoration (Fig. 6). For all three habitat types, the models correctly classified all but one sample collected after restoration but largely misclassified all the pre-restoration samples, likely resulting from shifts in the prokaryotic community between 2016 and 2017 that were included together in the model. Several ASVs, including ASV 3 (from the Acidobacterial class Holophagae; Table S3), ASV 6 (from the Gammaproteobacterial class) and ASV 9 (most closely related to the Chromatiales genus *Thiococcus*) persisted before restoration and one year after restoration but were largely eliminated two years after restoration in the *P. australis* habitat. Similarly, in the *Typha* habitat ASV 12 (from the Alphaproteobacterial genus *Rhodomicrobium*) and ASV 18 (from the Alphaproteobacterial genus *Pseudolabrys*) persisted before and one year after restoration, but was absent two years after restoration (Fig. 6). Differences before and after restoration in the *S. alterniflora* habitat were even more subtle. ASV 21 (from the Actinobacterial class Thermoleophilia) was abundant in a number of pre-restoration samples, and found in far fewer post-restoration samples and ASV 26 (a different ASV in the class Thermoleophilia) persisted in 2016 but was largely absent from samples in 2017. The rest of the taxa identified by the *S. alterniflora* model were of varying abundances through the time course of our sampling.

Random forest modeling did a better job discriminating samples based on the fungal community structure (Fig. 7) with out of box error estimates of 12.5% for *P. australis,* 15.15% for *Typha*, and 12.9% for *S. alterniflora* fungal communities. The random forest model generated from the *P. australis* fungal data correctly classified the post-restoration samples 100% of the time, but was only 50% accurate in classifying the pre-restoration samples. Similarly, for the *Typha* model, only one sample was misclassified from post-restoration, but only 44% of samples from before restoration were correctly classified based on their fungal taxonomy. The *S. alterniflora* random forest model of the fungal community was able to predict post restoration samples with 91% accuracy, but was not nearly as accurate (26%) at predicting samples prior to restoration.

In contrast to the prokaryotic taxa, taxa recognized as differentiating the fungal community before and after restoration often accounted for a large proportion of all fungal sequences (Fig. 7, Table S4). For example, in the *P, australis* habitat prior to restoration three different fungal ASVs accounted for between 25 and 90% of all sequences in some samples (ASV 1, ASV 2, and ASV 7). As a result of poor representation of salt marsh fungal taxa in public databases, we were not able to assign meaningful taxonomy to many of these ASVs, though we were able to identify ASV 7 as a member of the Rozellomycota class. The taxonomic assignment (when available) and associated DNA sequence for the fungal ASVs are provided in Table S4. After restoration, the fungal community in the *P. australis* habitat became dominated by ASV 3, an unidentified fungus that often accounted for more than 50% of all sequences in the first year after restoration, but decreased in abundance in the second year (Fig. 7). The fungal taxa in the *Typha* habitat that were differentiated as a result of restoration accounted for a much lower proportion of the total number of sequences, compared with the other habitats (Fig. 7). This shift was dominated by three ASVs (ASV 11, ASV 15, and ASV 16), which all decreased in relative abundance after restoration. ASV 11 and ASV 15 are both members of the *Pleosporales* family and ASV 16 could not be identified. The patterns in the fungal ASVs that were affected by restoration in the *S. alterniflora* marsh are less clear than in the other two habitats (Fig. 7).

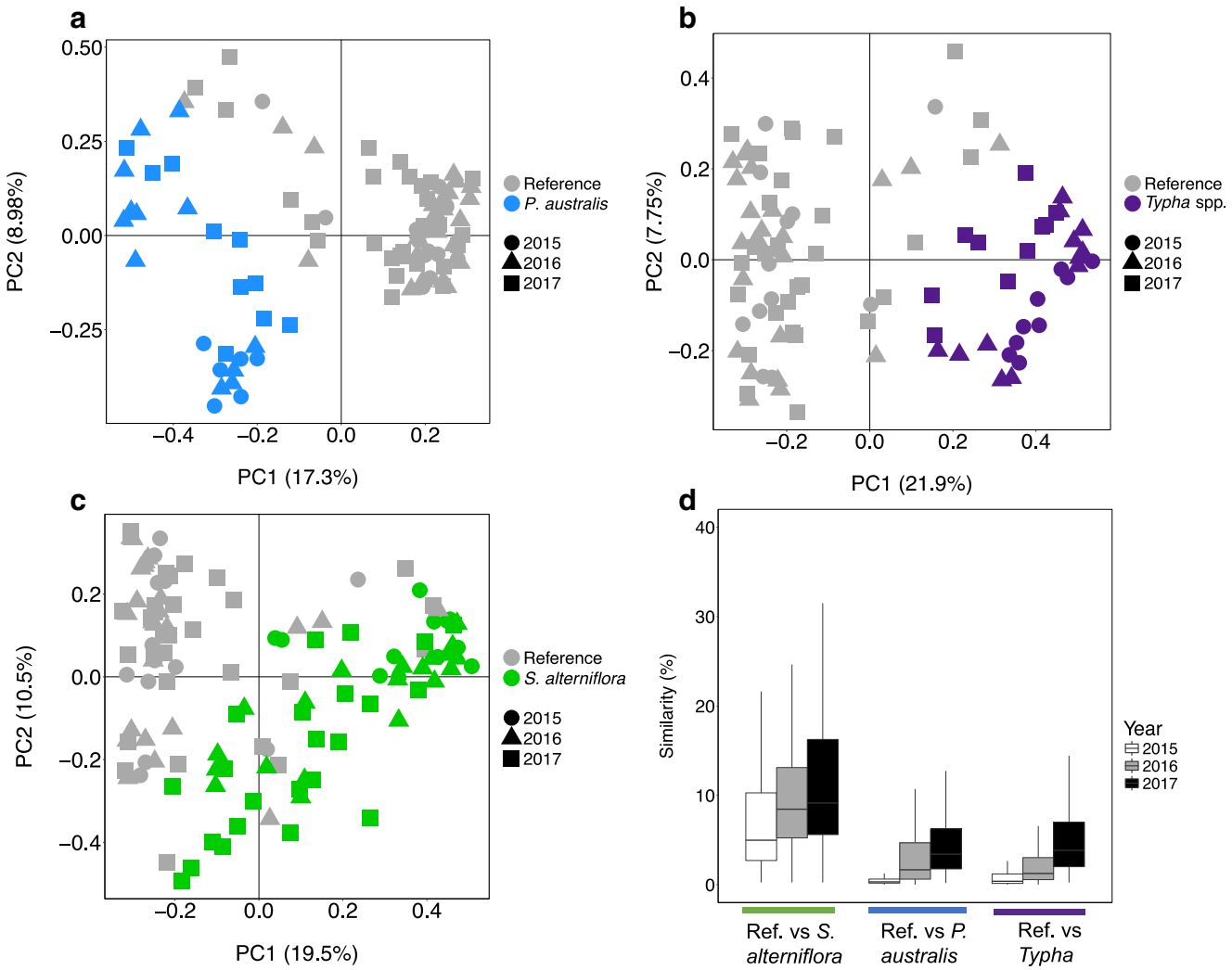

Fig. 5 Principal coordinate analysis constructed using Bray-Curtis dissimilarity of the fungal community for each habitat in the restored marsh compared with reference marshes: **a** *P. australis*, **b** *Typha*, and **c** *S. alterniflora*. **d** Change in similarity to reference marshes before (2015) and after (2016 and 2017) restoration in each of the restored marsh habitats. There were significant increases in similarity between the restored habitats and reference marshes over time in all three habitats (*P. australis*: $F = 39.78$, df = 2, $p < 0.001$, *Typha*: $F = 47.57$, df = 2, $p < 0.001$, and *S. alterniflora*: $F = 15.94$, df = 2, $p < 0.001$)

Discussion

We examined changes in the sediment microbial communities of three habitats within a degraded marsh before the removal of a tidal restriction that impeded saltwater flow and for two years after the restriction was removed and compared these changes to three nearby reference marshes. Changes in salinity can exhibit strong control over microbial community structure in a number of habitats (Lozupone and Knight 2007; Hartman et al. 2008; Fierer and Lennon 2011); however, the direct effect on the microbial community of a state change from low to high salinity in a vegetated marsh system has not been examined. We hypothesized that removal of the tidal restriction would significantly alter the microbial communities after restoration as a result of changes to the salinity regime, and that, over time, these communities would come to resemble communities in the reference marshes. We observed dramatic shifts in both the prokaryotic (Fig. 4) and fungal (Fig. 5) communities as a consequence of the return of seawater via the restoration. For both the fungal and prokaryotic communities, there were significant differences among the three habitat types, but each year after restoration, the microbial communities in the *P. australis* and *Typha* habitats increased in their similarity to reference marshes (Figs. 4d and 5d). Further, by the second year after restoration, the prokaryotic community in these habitats had dramatically lower Shannon diversity than reference marshes, likely resulting from a loss of taxa that are outcompeted under saline conditions. These results suggest that examining sediment microbial communities may provide an early assessment of restoration

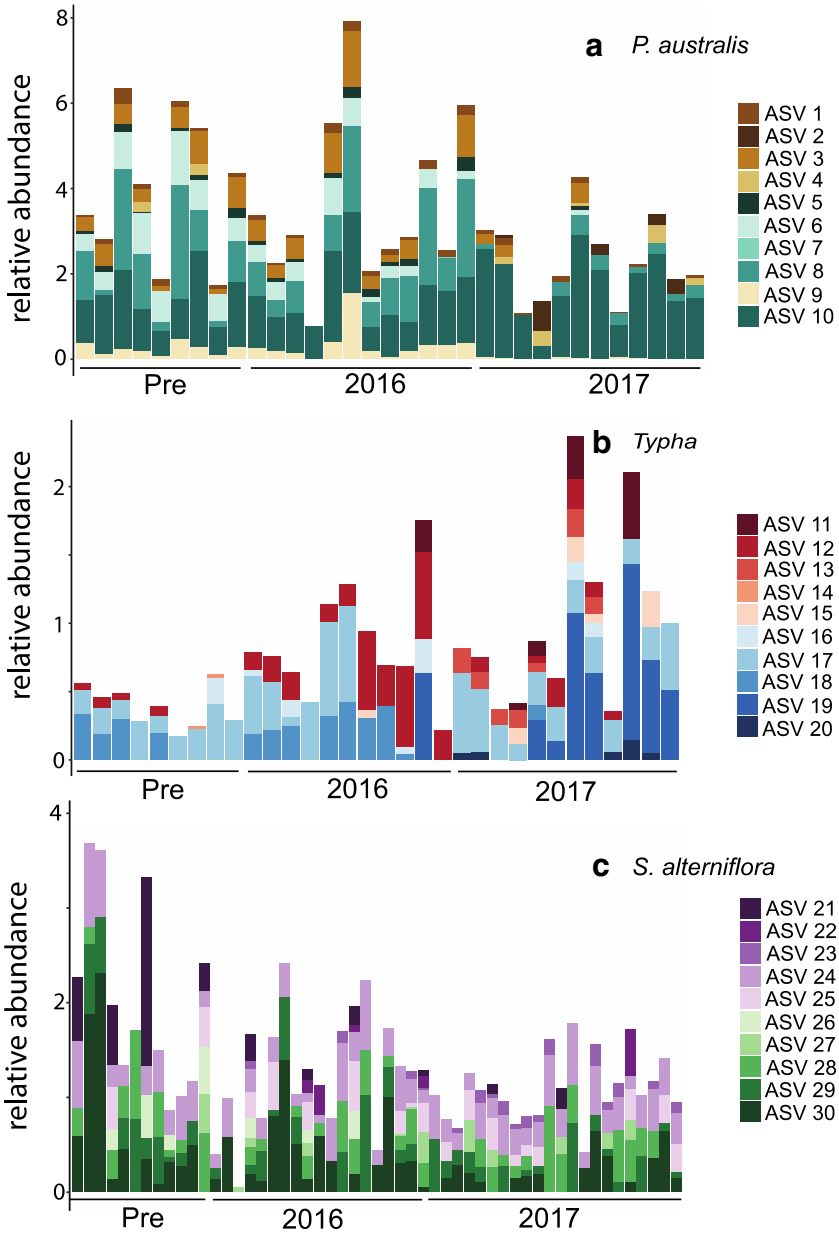

Fig. 6 Random forest model predictions of prokaryotic amplicon sequence variants (ASVs) that significantly differentiate before (2015) and after (2016 and 2017) restoration in the **a** *P. australis*, **b** *Typha*, and **c** *S. alterniflora* habitats. Closest taxonomic assignments and sequence information are provided in Table S3

effectiveness, and annual monitoring of the shift toward reference marshes in the microbial community could be used to determine the rate of recovery of the system.

Two years after the restoration was complete, despite increases in salinity and decreases in soil carbon and nitrogen stocks (Fig. 2), there was no evidence of replacement of the *P. australis* and *Typha* sp. by native halotolerant marsh vegetation. Rather, the increase in salinity after restoration resulted in dieback of both *P. australis* and *Typha* but with no recovery of native vegetation in the first 2 years after restoration. This is not surprising, however, as restoration of full vegetation in New England marshes can take decades (Warren et al. 2002). Plant species composition (Zak et al. 2003; Barberán et al. 2015; Prober et al. 2015), and even plant lineages within a species (Bowen et al. 2017), can be important in structuring microbial communities. If the plant species cover in the degraded marsh was the primary factor influencing microbial community structure, then we would not expect to see a shift in the microbes until colonization by new halotolerant vegetation. However, our results indicate a dramatic rapid shift in the microbial community that preceded shifts in the vegetation and suggest that the salinity shift is the proximal driver of microbial community change in this system. Future work should focus on disentangling whether this shift in the microbial community facilitates the restoration of native vegetation, or whether the change in edaphic characteristics independently shifts both the plant and microbial communities over different timescales. There is evidence for the facilitation of

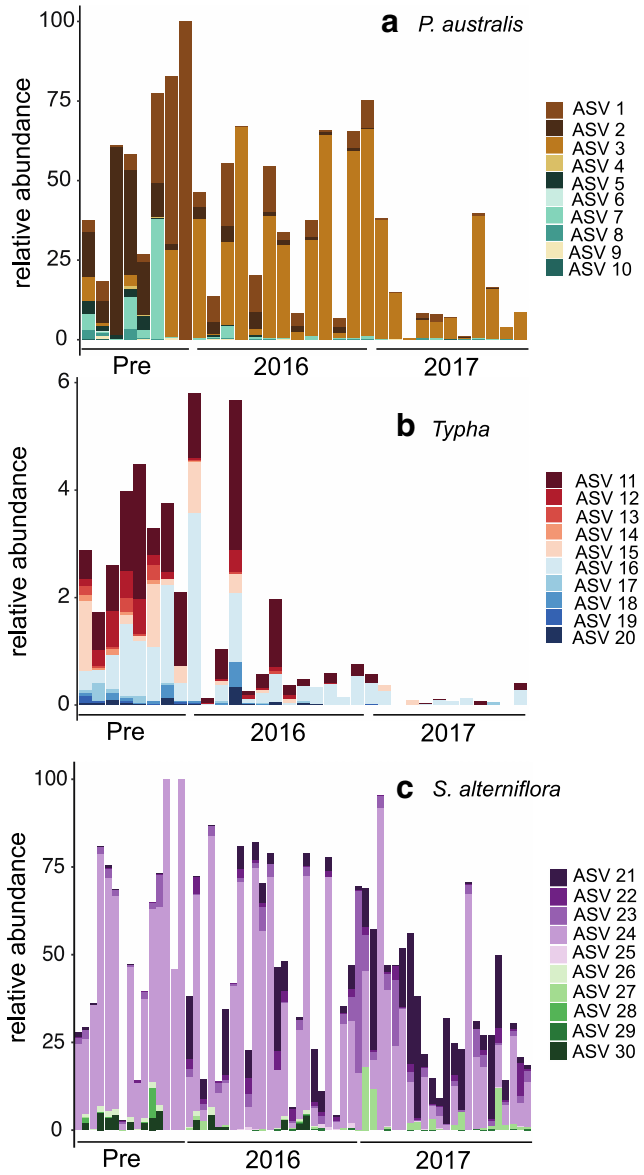

Fig. 7 Random forest model predictions of fungal amplicon sequence variants (ASVs) that significantly differentiate before (2015) and after (2016 and 2017) restoration in the **a** *P. australis*, **b** *Typha*, and **c** *S. alterniflora* habitats. Closest taxonomic assignments and sequence information are provided in Table S4

native vegetation in other grassland habitats (Daleo et al. 2008; de León et al. 2016) and better understanding whether microbial communities play a facilitative role in restoration success will provide valuable information for restoration best practices.

The goal of the restoration was to return saltwater flow to the degraded marsh and we did observe an increase in tidal flooding on the marsh platform after restoration, which altered the characteristics of the sediment and increased the salinity of the pore water, especially in the previously oligohaline reaches of the marsh (Fig. 2). Although restoration of this marsh took place in the winter of 2016, salinity at all sites, including our reference sites, was considerably lower in 2015 compared with the two subsequent years, likely a result of the considerably higher monthly rainfall in 2015 compared with the monthly rainfall in 2016 and 2017 (USGS 2018). Regardless of the year-to-year variability in the reference marshes, which ranged from 25 to 40 ppt, prior to restoration the pore water salinity in the *P. australis* and *Typha* habitats was dramatically lower (10–12 ppt) than the pore water salinity in the *S. alterniflora* habitat of the degraded marsh or in any of the reference marshes. After restoration, additional environmental changes, including decreases in SOM and %C and %N of the sediments in *P. australis* and *Typha* habitats of the degraded marsh, moved consistently in the direction of the reference marshes, further suggesting a trajectory toward recovery in these previously degraded systems, a trajectory that is consistent with similar restorations throughout New England (Sinicrope et al. 1990; Warren et al. 2002; Smith et al. 2009).

The modularity of the microbial community, as indicated by our network analysis (Fig. 3), is consistent with the edaphic shifts induced by the salinity state change. Both the prokaryotic and fungal networks predicted significant modularity in their respective communities. The prokaryotic network suggested that a single module captured the correlations predicted by the *P. australis* and *Typha* habitats (module 4, Fig. 3a) while the remaining modules were a mixture of samples from both reference and restored *S. alterniflora* habitats. Thus, the significant changes that occurred in the environmental data after restoration in the *P. australis* and *Typha* habitats (Fig. 2) also translated into shifts in the prokaryotic communities of those habitats. These shifts were stronger than those observed in the restored marsh *S. alterniflora* habitat, where samples were distributed across several modules. The predictive power of the fungal network was lower than for the prokaryotic network suggesting that habitat and restoration status may be less important in structuring fungal communities in this system.

Following restoration, shifts in specific microbial taxa resulted in changes in microbial community structure, particularly in the *P. australis* and *Typha* habitats. Changes in the sediments within *P. australis* that resulted from restoration led to a decrease in the number of taxa belonging to the Gemmatimonadetes phylum (ASV 5). These taxa are highly abundant phototrophs in soil and are found in estuarine environments, but are sensitive to high salinity and are typically found in low abundance in marine sediments (Zeng et al. 2016). Despite increases in salinity as a result of the restoration, there was a notable decrease in ASV 7 (family Desulfobacteraceae), which are known to be sulfate reducers, and a slight increase in ASV 10 (family Anaerolineaceae), which can decompose alkanes to provide acetate for acetoclastic methanogens (Liang et al. 2015) and are reported to be abundant in *P. australis* stands (Yao et al. 2019).

Among the taxa that discriminated the community in the *Typha* habitat after restoration, there was an increase in

marine-associated taxa such as those belonging to the family Alteromonadaceae (ASV 16; Ivanova et al. 2004). There was also a decrease in root-associated taxa and nitrogen fixing bacteria belonging to the order Rhizobiales (ASV 12; Erlacher et al. 2015), which were abundant one year after restoration, but decreased dramatically two years after restoration. Similarly, ASV 18 decreased one year after restoration. This ASV is also from the order Rhizobiales, and it is most closely related to the *Pseudolabrys* genus, which contains taxa that can grow on hydrogen and reduced sulfur compounds and are most common in freshwater systems (Oren 2014). Methanotrophs from the order Methylococcales were only sporadically abundant, but were not present in the *Typha* marsh prior to restoration.

Although there were no differences in the sediment characteristics we measured, including salinity, between *S. alterniflora* in the restored marsh and the reference marshes, we did see a difference in the prokaryotic communities. The differences in the prokaryotic community in the native vegetation between the restored and reference marshes could be a result of variables we did not measure, including standing stock biomass, inundation time, and additional fine-scale differences in the types of carbon or redox status of the system (Fierer 2017). Results of the random forest model indicated that the prokaryotes important to *S. alterniflora* prior to restoration included bacteria from the phylum Ignavibacteria (ASV 30), a facultative anaerobe (Liu et al. 2012), and two ASVs from the Actinobacteria (order Solirubrobacterales). Members of the Solirubrobacterales were enhanced in heavily disturbed soils associated with industrialized agriculture (Shange et al. 2012) so their decrease in the *S. alterniflora* habitat could portend a positive response to restoration. After restoration the abundance of anoxygenic, sulfur-oxidizing phototrophs closely related to *Halochromatium* (Pjevac et al. 2015), which were also important in the rhizosphere of a *Salicornia europaea* marsh (Yamamoto et al. 2018), increased in *S. alterniflora* sediments. Sulfur oxidizing phototrophs are common in aquatic sediments where hydrogen sulfide is available (Van Gemerden and Mas 1995). Thus, the increase in these phototrophs could suggest a greater availability of hydrogen sulfide in the *S. alterniflora* portion of the restored marsh after restoration.

Differences in the fungal community were also evident across all habitats within the restored marsh compared with reference marshes. The influence of SOM, C/N, and %N were all more important in explaining the structure of the fungal communities than the influence of salinity (Fig. S5). This is consistent with other studies on fungal communities in salt marsh ecosystems that observed a shift in the fungal community over a successional gradient that was correlated with differences in SOM (Dini-Andreote et al. 2016b) as well as the quality of carbon as noted in other grassland studies (Hartmann et al. 2009; Millard and Singh 2010). Fungi are critically important decomposers of organic matter and are often structured by vegetation type and changes in carbon substrates (Newell 2001; Broeckling et al. 2008; Chen et al. 2017). A change in the amount of organic matter, and the die-off of *P. australis* and *Typha* from increased salinity, likely played a role in altering the fungal community within these sediments (McHugh and Dighton 2004). The lack of closely related fungal taxa in reference databases make it impossible, at this time, to infer taxonomy for most fungi that shifted as a result of restoration.

In conclusion, the return of saltwater flow into a degraded oligohaline marsh rapidly altered both the soil edaphic characteristics and the bacterial and fungal communities present in those sediments. In particular, the environmental parameters found in *P. australis* and *Typha* habitats became more similar to reference marshes over time, which is mirrored in both prokaryotic and fungal communities that also increased in similarity to the reference marshes. Restoration success is typically evaluated based on the return of native vegetation, a process that can often take years to occur (Warren et al. 2002; Ruiz-Jaen and Aide 2005; Wortley et al. 2013). The influence of restoration on the microbial community, however, was apparent only a few months after the removal of tidal restrictions, even without any replacement of the vegetation. This suggests that the microbial community in restored marsh sediments recovers faster than plant communities and could therefore be sentinel indicators for tracking the success of marsh restoration. The extent to which these microbial shifts result in changes in the environment that facilitate the re-establishment of native vegetation requires additional research but these results suggest that microbes could be important partners in restoration success.

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