RESEARCH ARTICLE

Elucidation of the rhizosphere microbiome linked to *Spartina alterniflora* phenotype in a salt marsh on Skidaway Island, Georgia, USA

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One sentence summary: Smooth cordgrass, *Spartina alterniflora*, rhizosphere microbial community assembly is linked to plant phenotype and shaped by the availability of terminal electron acceptors.

Editor: Gary King
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ABSTRACT

Smooth cordgrass, *Spartina alterniflora*, dominates salt marshes on the east coast of the United States. While the physicochemical cues affecting *S. alterniflora* productivity have been studied intensively, the role of plant–microbe interactions in ecosystem functioning remains poorly understood. Thus, in this study, the effects of *S. alterniflora* phenotype on the composition of archaeal, bacterial, diazotrophic and fungal communities were investigated. Overall, prokaryotic communities were more diverse and bacteria were more abundant in the areas colonized by the tall plant phenotype in comparison to those of short plant phenotype. Diazotrophic methanogens (*Methanomicrobia*) preferentially colonized the area of the short plant phenotype. Putative iron-oxidizing *Zetaproteobacteria* and sulfur-oxidizing *Campylobacteria* were identified as indicator species in the rhizosphere of tall and short plant phenotypes, respectively. Finally, while diazotrophic populations shaped microbial interactions in the areas colonized by the tall plant phenotype, fungal populations filled this role in the areas occupied by the short plant phenotype. The results here demonstrate that *S. alterniflora* phenotype and proximity to the root zone are selective forces dictating microbial community assembly. Results further reveal that reduction–oxidation chemistry is a major factor driving the selection of belowground microbial populations in salt marsh habitats.

Keywords: salt marsh; *Spartina alterniflora*; plant microbiome; plant–microbe interaction; microbial diversity and composition; iron- and sulfur-oxidizing bacteria; indicator species

INTRODUCTION

Salt marshes are highly productive ecosystems in which organic matter accumulates to high concentrations from root production and rhizodeposition (Bertness *et al.* 2008; Hopkinson, Cai and Hu 2012). Salt marshes provide numerous ecosystem services ranging from shoreline erosion protection (Williams, Coles and Primavera 2007) to the maintenance of water quality and prevention of eutrophication (Hopkinson and Giblin 2008). Additionally, a significant portion of commercial fish species in the United States utilize salt marshes as nursery habitats to complete their life cycle (Beck *et al.* 2001). Salt marshes along the Atlantic coast of North America are frequently characterized by extensive stands of smooth cordgrass, *Spartina alterniflora*, distributed in two main phenotypes: (i) a tall form (>1 m) that grows near estuarine tidal creeks and (ii) a short form (<50 cm)

that dominates the high marsh (Valiela, Teal and Deuser 1978; Mendelssohn and Morris 2000).

High rates of carbon fixation and rhizodeposition are among the most notable characteristics of *S. alterniflora*. In comparison with other marsh grasses, *S. alterniflora* exhibits significantly higher carbon fixation rates (Howes, Dacey and Teal 1985; Liao *et al.* 2007), and deposits up to 55% of photosynthetically fixed carbon to the sediment within 24 h (Spivak and Reeve 2015), thereby supporting the development of the dense microbial biomass (Bu *et al.* 2015). Microbial communities in organic-rich salt marsh sediments are regularly exposed to sulfate-rich tidal waters, resulting in rapid rates of anaerobic decomposition through the sulfate reduction pathway, which in turn produces hydrogen sulfide, a potent phytotoxin (Jørgensen 1982; Wasmund, Mussmann and Loy 2017; Jorgensen, Findlay and Pellerin 2019). The interaction of numerous abiotic factors creates stable chemical and physical gradients that impact plant physiology, extending from tidal creeks to the high marsh. The concentration of sulfur and iron forms, redox potential and salinity are among the most critical factors controlling salt marsh plant physiology and zonation (Bertness 1991; Mendelssohn and Morris 2000; Kostka *et al.* 2002; Kostka, Roychoudhury and Van Cappellen 2002; Lamers *et al.* 2013). In addition to nutrient availability, these abiotic factors are likely to affect sediment microbial community composition, activity and interactions with a plant host.

Plant-associated microorganisms (bacteria, archaea and fungi) play an essential role in plant primary production through mediation of nutrient acquisition, regulation of stress hormones and detoxification of phytotoxins (Cytryn and Kolton 2011; Bardgett and van der Putten 2014; Fierer 2017; Rodríguez-Llorente *et al.* 2019). In return, plants actively secrete carbon sources that serve as substrates for microbial growth (Berg and Smalla 2009). Thus, the natural recruitment of surrounding microbial community members is one of the numerous mechanisms that plants have developed to deal with stressors such as nutrient limitation, salinity and the presence of phytotoxins (Dimkpa, Weinand and Asch 2009). The 'rhizosphere effect' on microbial communities is well documented among model and terrestrial plants (Berg and Smalla 2009; Bulgarelli *et al.* 2012; Lundberg *et al.* 2012; Tkacz *et al.* 2015). However, studies linking the microbial community to *S. alterniflora* phenotype in pristine salt marshes are scarce (Ravit, Ehrenfeld and Haggblom 2003; Angermeyer, Crosby and Huber 2016; Rietl *et al.* 2016; Zogg, Travis and Brazeau 2018), and environmental factors that affect the microbial recruitment processes by the salt-tolerant plants poorly understood.

Salt marsh ecosystems harbor dense microbial communities; however, because of the complex ecological interactions between microbial populations, the functional capacity of the overall communities may differ from the linear summary of the functional potential of the individual populations (Faust and Raes 2012; van der Heijden and Hartmann 2016). Network analysis can provide crucial information on microbial community interactions that cannot be obtained by traditional analysis of microbial community composition and calculation of diversity metrics (Deng *et al.* 2012). Moreover, understanding ecological interactions between community members may be used to identify keystone species of wetland ecosystems, which may be used to predict the consequences of environmental perturbations (van der Heijden and Hartmann 2016; Urakawa and Bernhard 2017; Banerjee, Schlaeppi and van der Heijden 2018). Network analyses have been successfully applied to explore the ecological interaction patterns among microbial species in a broad range of soil and wetland ecosystems (Barberán *et al.* 2012; Hartmann *et al.* 2014; Lin *et al.* 2014; Hartman *et al.* 2018).

The overall goal of this work is to elucidate the compositional and structural diversity of the microbial communities that are intimately associated with the roots of *S. alterniflora*, and determine their potential roles in the health and productivity of the plant host. Thus, it was hypothesized that *S. alterniflora* phenotypes recruit distinct rhizosphere and bulk sediment microbial communities, varying in size and diversity, and guided by specific keystone species. To support this hypothesis, a next-generation sequencing approach was applied to investigate the composition of prokaryotic, diazotrophic and fungal communities in the rhizosphere and bulk sediments of tall and short forms of *S. alterniflora* from a pristine salt marsh on Skidaway Island, Georgia. Additionally, quantitative polymerase chain reaction (qPCR) analysis was employed to estimate the absolute abundance of bacterial, archaeal and diazotrophic communities. Finally, the ecological roles of and interactions between microbial community members were inferred from co-occurrence network analysis. The results here demonstrate that *S. alterniflora* phenotype and proximity to the root zone are indeed selective forces that shape microbial community abundance and diversity in salt marsh habitats.

MATERIALS AND METHODS

Site description

This study was conducted at the Saltmarsh Ecosystem Research Facility (SERF) on Skidaway Island in Savannah, Georgia, on 23 May 2017. The SERF marsh experiences daily tides and the air temperatures at this site range from 2°C in the winter to as high as 40°C in the summer, while overlying water temperatures vary between 12 and 25°C. The natural vegetation of the marsh is dominated by a nearly monospecific stand of smooth cordgrass, *S. alterniflora*, and the sediments are intensively bioturbated by fiddler crabs, mainly *Uca pugnax* (Kostka, Roychoudhury and Van Cappellen 2002).

Sample collection and DNA extraction

Three and six individual *S. alterniflora* plants were gently collected from the sediments colonized by tall or short plant phenotypes, respectively. Plant root systems were rinsed with filtered autoclaved saline solution 0.9% NaCl. Approximately, 10 g of roots were separated from rhizomes by tweezers, placed into 50 ml sterile tubes and washed twice in 20 ml of filtered autoclaved saline solution with gentle inversion. Sediment remaining attached to the root system was considered to be rhizosphere. Our sampling method cannot be used to discriminate between endophytes, epiphytes or root tightly associated with microbes; thus, we refer to these combined root-associated communities as 'rhizosphere'. Four bulk sediment cores per site (at 0–20 cm depth) were collected from the same locations using polycarbonate cores (Kostka, Roychoudhury and Van Cappellen 2002). Two independent DNA extractions were performed from each of the rhizosphere and bulk sediments using the DNeasy PowerSoil kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol, and quantified with the Qubit HS Assay kit (Invitrogen, Carlsbad, CA, USA). Finally, 5 ng of the extracted DNA was used to generate small-subunit rRNA (SSU rRNA), nitrogenase subunit H *(nifH)* and nuclear ribosomal internal transcribed spacer region (ITS) amplicons.

Quantitative polymerase chain reaction (qPCR) amplification

Three technical replicates were performed for each of the qPCR assays on a StepOnePlus machine using PowerUp SYBR Green Master Mix (Applied Biosystems, Austin, TX, USA). Bacterial, archaeal and diazotrophic community sizes were estimated based on the abundance of SSU rRNA genes, and *nifH* genes using standard primer sets 331F/518R, Arch787/Arch1059R and PolF/PolR for quantification of bacterial, archaeal and nitrogen-fixing microorganisms, respectively, according to previously described reaction conditions and standard curves (Warren *et al.* 2017; Carrell *et al.* 2019; Kolton *et al.* 2019). The specificity of PCR products was confirmed by melting curve analyses and gel electrophoresis of the amplicons. Bacterial, archaeal SSU rRNA and *nifH* gene copy numbers were calculated based on standard curves and presented as gene copy numbers per gram of wet sediment.

High-throughput libraries preparation and sequencing

The PCR reactions were performed in triplicates, and products were combined before barcoding. Microbial community composition was determined by applying a two-step high-throughput sequencing-based protocol (Green, Venkatramanan and Naqib 2015). The prokaryotic communities were determined by the amplification V4 variable regions of the SSU rRNA gene using the primer set 515F and 806R (Caporaso *et al.* 2011), as previously described (Wilson *et al.* 2016; Kolton *et al.* 2017, 2019). The diversity and composition of diazotrophic communities were assessed by amplification of the *nifH* gene fragment (encoding the nitrogenase reductase subunit) as a molecular marker for nitrogen-fixing microorganisms, using IGK3 and DVV primer set to generate 396 bp PCR products as previously described (Gaby *et al.* 2018; Carrell *et al.* 2019). The analysis of the fungal communities was performed based on the ITS1 region of the fungal rRNA gene using the primer set ITS1F (5′-AAGTCGTAA CAAGGTTTCC) and ITS2 (5′-GCTGCGTTCTTCATCGATGC) (White *et al.* 1990; Smith and Peay 2014). Resulting barcoded amplicon libraries were sequenced on an Illumina MiSeq 2000 platform using a 500-cycle v2 sequencing kit, paired-end 2×250 cycle sequencing mode at the DNA Services Facility at the University of Illinois at Chicago as previously described (Green, Venkatramanan and Naqib 2015). The Illumina-generated SSU rRNA, *nifH* and ITS gene amplicon sequences were deposited in the BioProject database (www.ncbi.nlm.nih.gov/bioproject) under accession PRJNA589133, PRJNA589172 and PRJNA589257, respectively.

Bioinformatic sequence analysis

Initially, the forward and reverse amplification primer sequences were removed from the raw fastq files with Cutadapt v.1.8.1 (Martin 2011). Resulting sequences were quality-filtered and assembled into error-corrected amplicon sequence variants (ASVs) using DADA2 v1.10.0 (Callahan *et al.* 2016), and filtered to meet the expected lengths of 251–255 bp for V4 variable regions of the SSU rRNA gene, and 330–360 bp for *nifH* gene fragment. Chimera sequences were removed using the removeBimeraDe-novo function of DADA2. These high-quality sequences were annotated to the SILVA SSU rRNA reference alignment (release 132), UNITE (release 7.2) or *nifH* reference alignment database (Quast *et al.* 2012; Koljalg *et al.* 2013; Gaby *et al.* 2018) using the RDP classifier (Wang *et al.* 2007) as implemented in Mothur v.1.40 software (Schloss *et al.* 2009) with a minimum confidence threshold of 50%. Sequences classified as 'plant', 'chloroplast', 'mitochondria', 'protist' or 'did not match any taxonomic Class' were excluded from the final dataset.

Data analysis in R

Statistical analyses were conducted in R v3.5 (R Development Core Team 2018). Alpha diversity indices were calculated based on the number of unique ASVs (richness) and Shannon community diversity. Randomly selected 22 931, 1208 and 2123 sequences per sample were used to estimates alpha diversity indices for prokaryotic, diazotrophic and fungal communities, respectively. Alpha diversity indices of bulk and rhizosphere microbial communities were calculated for each *S. alterniflora* phenotype and tested for significant statistical differences using a two-sided Wilcoxon test.

The final datasets were filtered to remove ASV that appeared only once in the dataset and/or had <15 counts. After discarding rare ASVs, a total of 9 249 384 SSU rRNA, 149 678 *nifH* and 356 247 ITS sequences were normalized by cumulative sum scaling (CSS; Paulson *et al.* 2013) and used for the compositional and beta diversity analyses. Major variance components of beta diversity were determined using unconstrained principal coordinates analysis (PCoA) of Bray–Curtis distance matrices. The effect of *S. alterniflora* growth forms on community similarity was estimated with a permutational multivariate analysis of variance (PERMANOVA) and BETADISP statistical tests with 1000 permutations. Additionally, pairwise correlations between dissimilarity matrices generated from each of the analyzed genes were assessed by a Mantel test with 1000 permutations. Ordination and statistical analyses were carried out in phyloseq and vegan R packages (Dixon 2003; McMurdie and Holmes 2013).

To identify possible indicator species, we used a correlation-based approach employing the *multipatt* function with 9999 permutations in the Indicspecies package. This analysis integrates two species traits: exclusivity (exclusively present in the habitat) and fidelity (present in all samples of that habitat; De Caceres, Legendre and Moretti 2010). Additionally, a machine learning algorithm, random forests (RF; Breiman 2001), with 1000 trees followed by the Boruta algorithm for feature selection (average z-scores of 1000 runs; Kursa and Rudnicki 2010) was applied to predict specific ASVs that significantly segregated between *S. alterniflora* growth forms. Finally, ASVs that were selected by both approaches were coined 'indicator species'. The relative abundances of the selected ASVs were calculated at the taxonomic class levels, and heatmaps were constructed based on z-score transformed abundances to improve normality and homogeneity of variances.

Network analysis was applied to gain a better understanding of potential interactions of the indicator species. Meta-co-occurrence networks were constructed to visualize interactions between bacterial, fungal and diazotrophic communities associated with tall and short *Spartina* phenotypes using the SpiecEasi R package (https://github.com/zdk123/SpiecEasi). SpiecEasi (Sparse InversE Covariance estimation for Ecological Association and Statistical Inference) infers direct microbial interactions from the community composition data with the Meinshausen–Buhlmann's ('mb') neighborhood selection. This selection method reduces the number of spurious interactions and outperforms other conventional methods used for assessing networks in microbial community datasets (Kurtz *et al.* 2015). The resulting adjacency matrices were converted into network objects, and network properties were estimated using the igraph package (Csardi and Nepusz 2006). Well-connected microbial

taxa with other members in microbial co-occurrence networks potentially have a broad regulatory effect on their ecosystems. We identified keystone species as five ASVs with the highest number of connections ('degree centrality'). Keystone species were calculated separately for each of the *Spartina* phenotypes related networks.

RESULTS

High-throughput sequencing of small subunit ribosomal RNA genes, the fungal ITS1 region of rRNA operons and *nifH* gene fragments yielded a total of 924 938, 356 247 and 161 079 high-quality sequences, respectively. High-quality sequences were evenly distributed across all samples, resulting in 20 632–48 833 sequences per sample (median 34 730) for prokaryotic communities; 2088–45 026 sequences per sample (median 9730) for fungal communities; and 1138–10 782 sequences per sample (median 5413) for diazotrophic communities. Among the high-quality sequences obtained, 2699 prokaryotic, 266 fungal and 690 *nifH* unique ASVs were observed across all samples.

Microbial diversity analysis

Profound differences were observed in the diversity, abundance and composition of microbial communities associated with different *S. alterniflora* phenotypes and distance from the roots (Figs 1–3). Rarefaction curve-based species richness and Shannon diversity analyses support that microbial communities' alpha diversity is significantly affected by plant phenotype. More diverse prokaryotic and diazotrophic communities were established in the area dominated by the tall phenotype of *S. alterniflora*, whereas diversity indices of the bulk sediment did not differ from those observed in the rhizosphere (Fig. 1). Conversely, species richness and Shannon diversity revealed that the fungal community is more diverse in the area dominated by the short plant phenotype. Additionally, a clear diversity gradient from bulk to rhizosphere sediment was exhibited by the fungal community (Fig. 1).

To determine the influence of *S. alterniflora* phenotype and association with the rhizosphere on microbial communities' beta diversity, unconstrained PCoA and PERMANOVA based on the Bray–Curtis distance matrices were performed. These analyses underlined the important role of plant phenotype on the distribution of microbial communities. Accordingly, plant phenotype explained almost 50% of variation in prokaryotic ($R^2 = 0.49$, $P < 0.001$, Fig. 2A; Supplementary Data 1, Supporting Information) and ~35% of variation in diazotrophic ($R^2 = 0.35$, $P < 0.001$, Fig. 3A; Supplementary Data 1, Supporting Information) community composition. Furthermore, the rhizosphere was shown to be secondarily influential, explaining ~15% of the variation in the prokaryotic and diazotrophic communities composition ($R^2 = 0.15$, $P < 0.005$; $R^2 = 0.18$, $P < 0.001$, Fig. 2A, Fig. 3A; Supplementary Data 1, Supporting Information). While prokaryotic communities were selected mainly by plant phenotype, fungal community distribution was primarily determined by the influence of the rhizosphere. The rhizosphere explained nearly 23% of the variation in fungal communities ($R^2 = 0.225$, $P < 0.001$; Figure S1, Supplementary Data 1, Supporting Information), whereas only 8% of fungal community dissimilarity ($R^2 = 0.079$, $P < 0.05$) was attributed to plant phenotype (Figure S1, Supplementary Data 1, Supporting Information). Overall, *S. alterniflora* phenotype and the rhizosphere explained up to 71.1, 65.4 and 40.1% of the variance in the *Spartina*-associated prokaryotic, diazotrophic and fungal communities, respectively.

Since the PERMANOVA test is sensitive to differences in group dispersion, a permutation test for homogeneity of multivariate dispersions (BETADISP, Anderson 2006) was used to evaluate multivariate homogeneity of dispersions for each group. Accordingly, the results of the BETADISP analysis indicated that group dispersion of beta diversity, calculated with Bray–Curtis distances, were not significantly different for the prokaryotic and diazotrophic communities. These dispersion tests suggest that observed differences between microbial communities were primarily driven by true biological differences and not an artifact of within-group dispersion.

Finally, Mantel tests were applied to determine the correlation between the distribution patterns of the prokaryote, diazotrophic and fungal communities. Mantel tests revealed a significant correlation between the prokaryotic, diazotrophic and fungal communities. The distribution of fungal communities was significantly correlated with the distributions of the prokaryotic and diazotrophic communities ($R_{Mantel} = 0.45$, $P < 0.001$; $R_{Mantel} = 0.53$, $P < 0.001$, respectively), although these correlations were weaker than the correlation between the prokaryotic and diazotrophic communities ($R_{Mantel} = 0.89$, $P < 0.001$).

Prokaryotic community analysis

Microbial abundance and taxonomic composition varied with plant phenotype and proximity to the root system. Community size estimation at the domain level indicated contrasting trends in bacterial and archaeal populations. Significantly denser bacterial populations were observed in the sediment of the tall plant phenotype in comparison to the area colonized by the short plants (Fig. 2B; Supplementary Data 2, Supporting Information). In parallel, the relative abundance of the archaeal populations increased as a result of the transition from the tall to the short plant sediment (Fig. 2D; Supplementary Data 2, Supporting Information). The differences in microbial community composition, as revealed by a next-generation sequencing approach, correlated well with absolute abundance as estimated by qPCR of SSU rRNA genes. The abundance of bacterial SSU rRNA genes was nearly 15-fold higher in the bulk sediment of the tall plants (9.44 ± 0.23 gene copy numbers per gram of wet sediment) relative to the bulk sediment of the short plants [8.01 ± 0.11 log(gene copy numbers per gram of wet sediment)] (Fig. 2C). Conversely, the abundance of archaeal SSU rRNA genes was nearly 15-fold higher in the bulk sediment of the short plants [5.15 ± 0.07 log(gene copy numbers per gram of wet sediment)] in comparison to the bulk sediment of the tall plants [3.94 ± 0.53 log(gene copy numbers per gram of wet sediment)] (Fig. 2C).

The majority of retrieved bacterial sequences were affiliated with the phyla *Proteobacteria*, *Chloroflexi*, *Bacteroidetes*, *Acidobacteria* and *Epsilonbacteraeota*, which comprised up to 69 and 80% of bacterial sequences associated with short and tall plants, respectively (Fig. 2B; Supplementary Data 2, Supporting Information). Overall, *Proteobacteria* and *Chloroflexi* represented the dominant phyla. Furthermore, the relative abundance of *Proteobacteria* was significantly higher among the tall plants (51.7 ± 4%, $P < 0.001$) relative to the short plants (28.2 ± 5.8%). In contrast, *Chloroflexi* were approximately twice as high ($P < 0.001$) in the area colonized by short plants with a relative abundance of 26 ± 3.6% compared to around only 12.5% in the area colonized by tall plant phenotype (Fig. 2B; Supplementary Data 2, Supporting Information). Moreover, members of
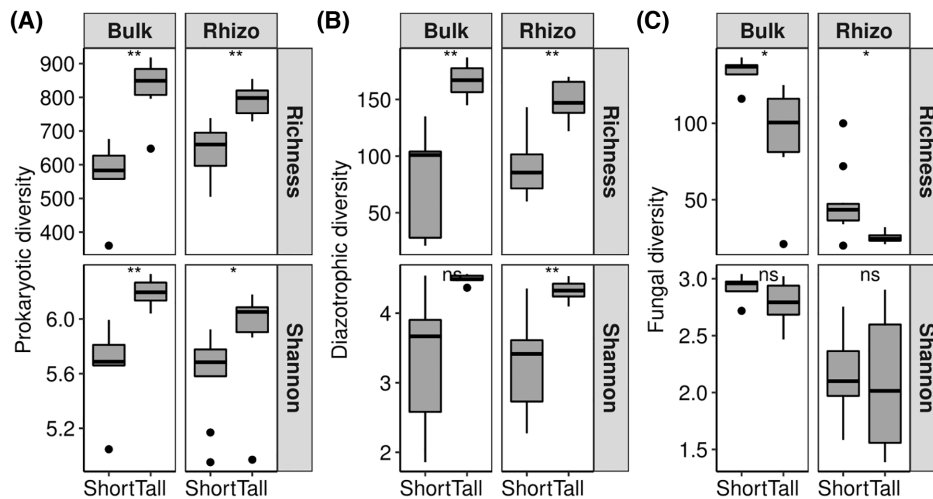
Figure 1. Alpha diversity indices of the microbial communities associated with tall and short phenotypes of *S. alterniflora* from Skidaway Island, Georgia, USA. **(A)** Prokaryotic community; **(B)** diazotrophic community; and **(C)** fungal community. Alpha diversity indices were calculated based on a randomly selected 22 931, 1208 and 2123 sequences per sample for prokaryotic, diazotrophic and fungal communities, respectively. The 'Bulk' and 'Rhizo' indicate bulk and rhizosphere sediments, respectively. 'Short' and 'Tall' define the *S. alterniflora* phenotype. The asterisks indicate statistically significant differences between observations based on a Wilcoxon signed-rank test. *$P < 0.05$, **$P < 0.01$ and 'ns' indicates no significant differences.

the *Epsilonbacteraeota* were specifically enriched in the rhizosphere sediments of both plant phenotypes (Fig. 2B; Supplementary Data 2, Supporting Information). The archaeal community was distributed across six phyla and contributed up to 16% of the total SSU rRNA gene sequences (Fig. 2D; Supplementary Data 2, Supporting Information). Generally, the relative abundance of archaeal phyla changed according to the plant growth form, with the vast majority of archaeal phyla showing a higher relative abundance in the area of short plants. An exception was the *Thaumarcheota*, which showed a significantly higher relative abundance ($P < 0.001$) in the area occupied by tall plants (Fig. 2D; Supplementary Data 2, Supporting Information).

Since iron and sulfur biogeochemical cycles are closely associated and play an important role in the carbon and the nitrogen cycles of salt marshes, we screened our sequence data to investigate potential sulfate-reducing and iron/sulfur-oxidizing prokaryotes. In general, a significantly higher relative abundance of these functional groups was observed in the rhizosphere sediments in comparison to the bulk sediments, regardless of plant phenotype (Fig. 4A). Collectively, sulfate-reducing bacteria (SRB) accounted for ~12.5% of the prokaryotic community detected at the Skidaway salt marsh and showed a higher relative abundance in the areas colonized by the short plant phenotype (Fig. 4A; Supplementary Data 3, Supporting Information). Further, the dominant SRB families detected in our sequence database were *Desulfarculaceae*, *Desulfobacteraceae* and *Desulfobulbaceae*, which collectively contributed up to 9% of the prokaryotic communities (Supplementary Data 3, Supporting Information). Nevertheless, different niche preferences were observed for each SRB family. While the relative abundance of the *Desulfarculaceae* decreased ~2-fold from the short to the tall phenotype, an opposing trend was observed for the *Desulfobacteraceae* (Fig. 4D). The relative abundance of the *Desulfobulbaceae* increased almost 10-fold from $0.45 \pm 0.02\%$ in the rhizosphere of short plants up to $4.76 \pm 0.32\%$ in the rhizosphere of tall plants (Fig. 4D).

Plant phenotype did not appear to influence the relative abundance of either putative sulfur-oxidizing bacteria (SOB) or

putative iron-oxidizing bacteria (FeOB) in bulk sediments. However, a significant rhizosphere effect was observed, as SOB and FeOB were, in general, more abundant in the rhizosphere in comparison to the bulk sediments at the genus level (Fig. 4A). Whereas the relative abundance of SOB in the rhizosphere of tall plants ($4.3 \pm 0.96\%$) was significantly lower ($6 \pm 0.21\%$) than that in the rhizosphere of short plants (Fig. 4A; Supplementary Data 3, Supporting Information), the relative abundance of FeOB in the rhizosphere of tall plants ($0.9 \pm 0.1\%$) was significantly higher than that in the rhizosphere of short plants ($0.3 \pm 0.03\%$) (Fig. 4A; Supplementary Data 3, Supporting Information). Dominant SOB genera were taxonomically affiliated with the *Campylobacterales* and *Desulfovibrionales* orders (Fig. 4C). While SOB genera *Sulfurovum* and *Sulfurimonas* were significantly lower in the rhizosphere of tall plants in comparison with the rhizosphere of short plants, the relative abundance of *Arcobacter* and *Desulfovibrio* genera were substantially higher in the rhizosphere of the tall plants relative to the rhizosphere of short plants (Fig. 4C; Supplementary Data 3, Supporting Information). Members of the genus *Mariprofundus*, taxonomically affiliated with *Zetaproteobacteria*, were dominant FeOB at the Skidaway salt marsh. Moreover, *Mariprofundus* was exclusively detected in the rhizosphere sediments. Finally, a significantly higher relative abundance of *Mariprofundus* was present in the rhizosphere of the tall plants ($0.55 \pm 0.07\%$) in comparison to the rhizosphere of the short phenotype ($0.17 \pm 0.04\%$) (Fig. 4B; Supplementary Data 3, Supporting Information).

Diazotrophic community analysis

Diazotrophic communities showed similar trends in agreement with overall prokaryotic communities. Using a qPCR approach, a significantly higher abundance of putative diazotrophs was observed in rhizosphere sediments in comparison to the bulk sediments; however, no significant differences in the absolute abundances of putative diazotrophs were observed between short and tall plant phenotypes (Fig. 3B). The relative abundance of putative bacterial diazotrophs in bulk sediments of short plants comprised $83.1 \pm 4.1\%$ and increased up to $97.5 \pm 1\%$
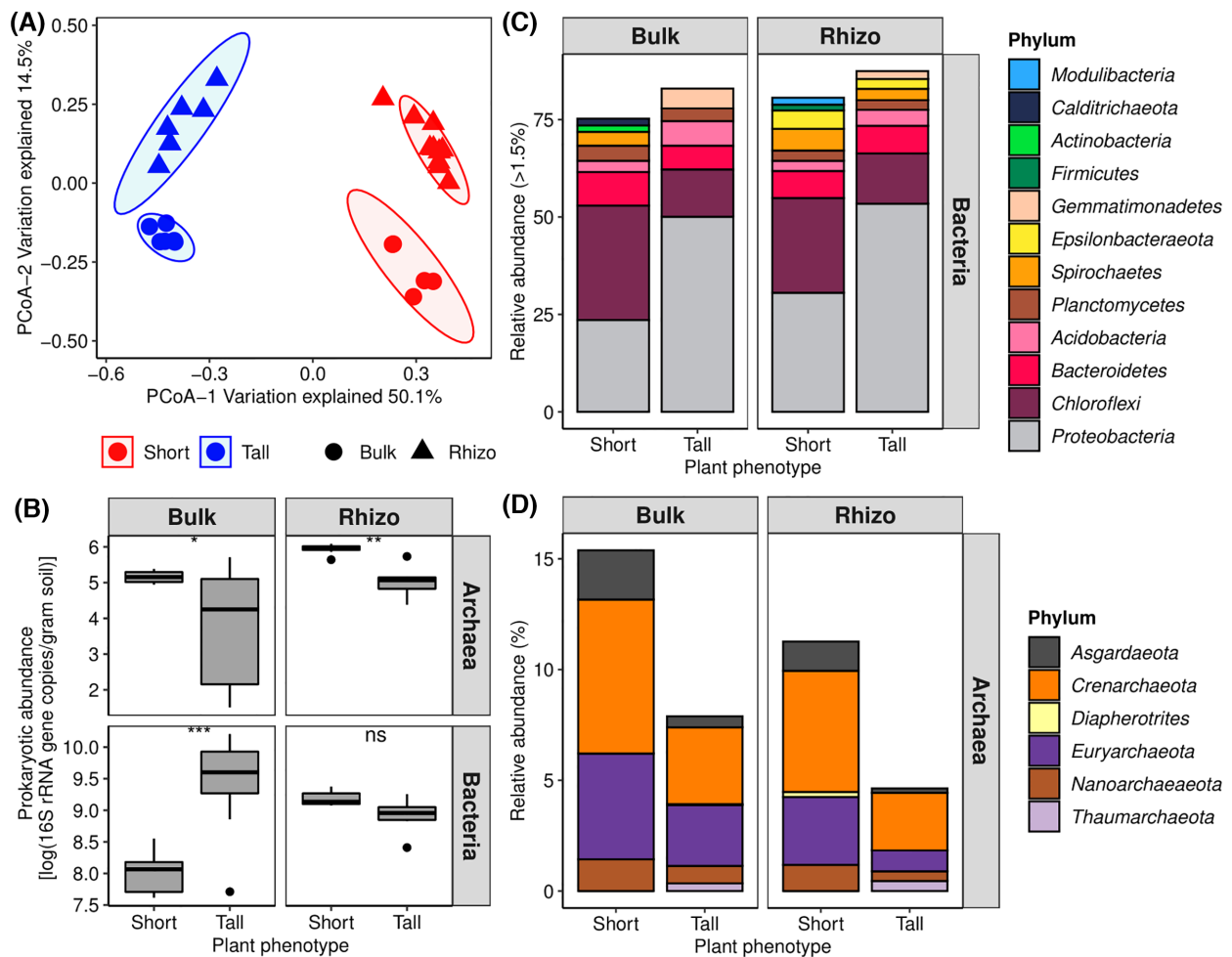
Figure 2. Beta diversity and composition of prokaryotic communities associated with tall and short phenotypes of *S. alterniflora* from Skidaway Island, Georgia, USA. **(A)** PCoA of prokaryotic community compositions based on the Bray–Curtis distance matrices calculated from CSS normalization of the final data; **(B)** bacterial and archaeal absolute abundance determined by qPCR of SSU rRNA genes with domain level primers; **(C)** relative abundance of the dominant bacterial phyla; **(D)** relative abundance of the most abundant archaeal phyla. The 'Bulk' and 'Rhizo' indicate bulk and rhizosphere sediments, respectively. 'Short' and 'Tall' define the *S. alterniflora* phenotype. Diversity, composition and absolute abundances of the prokaryotic communities were assessed from the same samples collected from bulk and rhizosphere sediments of the tall and short forms of *S. alterniflora*. Triplicate samples were used to quantify the abundances of prokaryotes. The asterisks indicate statistically significant differences between observations based on a Wilcoxon signed-rank test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and 'ns' indicates no significant differences.

in the rhizosphere of tall plants. Conversely, the relative abundance of putative archaeal diazotrophs increased from $2.45 \pm 1\%$ in the rhizosphere of tall plants to $16.9 \pm 4.1\%$ in the bulk sediment of short plants (Fig. 3C; Supplementary Data 4, Supporting Information). Approximately 84.3% of the diazotrophic community was distributed across three dominant classes, including *Gammaproteobacteria, Deltaproteobacteria* and *Clostridia* (Fig. 3C; Supplementary Data 4, Supporting Information). Specifically, members of the *Teredinibacter* genus within the *Cellvibrionaceae*, well known for their cellulolytic capabilities, were most abundant among diazotrophic populations (Fig. 5A; Supplementary Data 5, Supporting Information). The dominant genera *Teredinibacter* ($13.7 \pm 2\%$) and *Tolumonas* ($9.9 \pm 1.6\%$) collectively contributed up to 25% of the putative diazotrophic community in the rhizosphere of tall plants. Populations with documented capabilities to mediate the iron and/or sulfur cycles contributed $17.2 \pm 1.7\%$ and $17.9 \pm 1\%$ of the diazotrophic community in the area colonized by tall or short plants, respectively. More specifically, putative diazotrophic iron-oxidizing bacteria, taxonomically affiliated with autotrophic *Sideroxydans* ($1.6 \pm 0.46\%$) and heterotrophic *Bradyrhizobium* ($2 \pm 0.27\%$)

genera, were significantly enriched in the rhizosphere of tall plants (Fig. 5B; Supplementary Data 5, Supporting Information). In contrast, putative diazotrophic sulfur-oxidizers, taxonomically belonging to *Thioalkalispira* genus ($6.9 \pm 0.6\%$), were significantly enriched in the rhizosphere of short plants (Fig. 5B; Supplementary Data 5, Supporting Information). A comparative analysis of prokaryotic and diazotrophic communities revealed the potential for methanogenic archaea to mediate nitrogen input in the area colonized by short plants. While putative diazotrophic methanogens, taxonomically affiliated with *Methanomicrobiaceae*, comprised only $0.0 \pm 0.0\%$ and $0.1 \pm 0.09\%$ of overall prokaryotic communities in the bulk sediments and the rhizosphere, respectively, their relative abundance comprised up to $15.5 \pm 3.5\%$ and $8.5 \pm 1.2\%$ of diazotrophs (Fig. 5C; Supplementary Data 4, Supporting Information).

Fungal community analysis

In contrast to prokaryotic and diazotrophic communities, which appear to be shaped by plant phenotype as well as distance from the root system, fungal populations uniformly colonized bulk
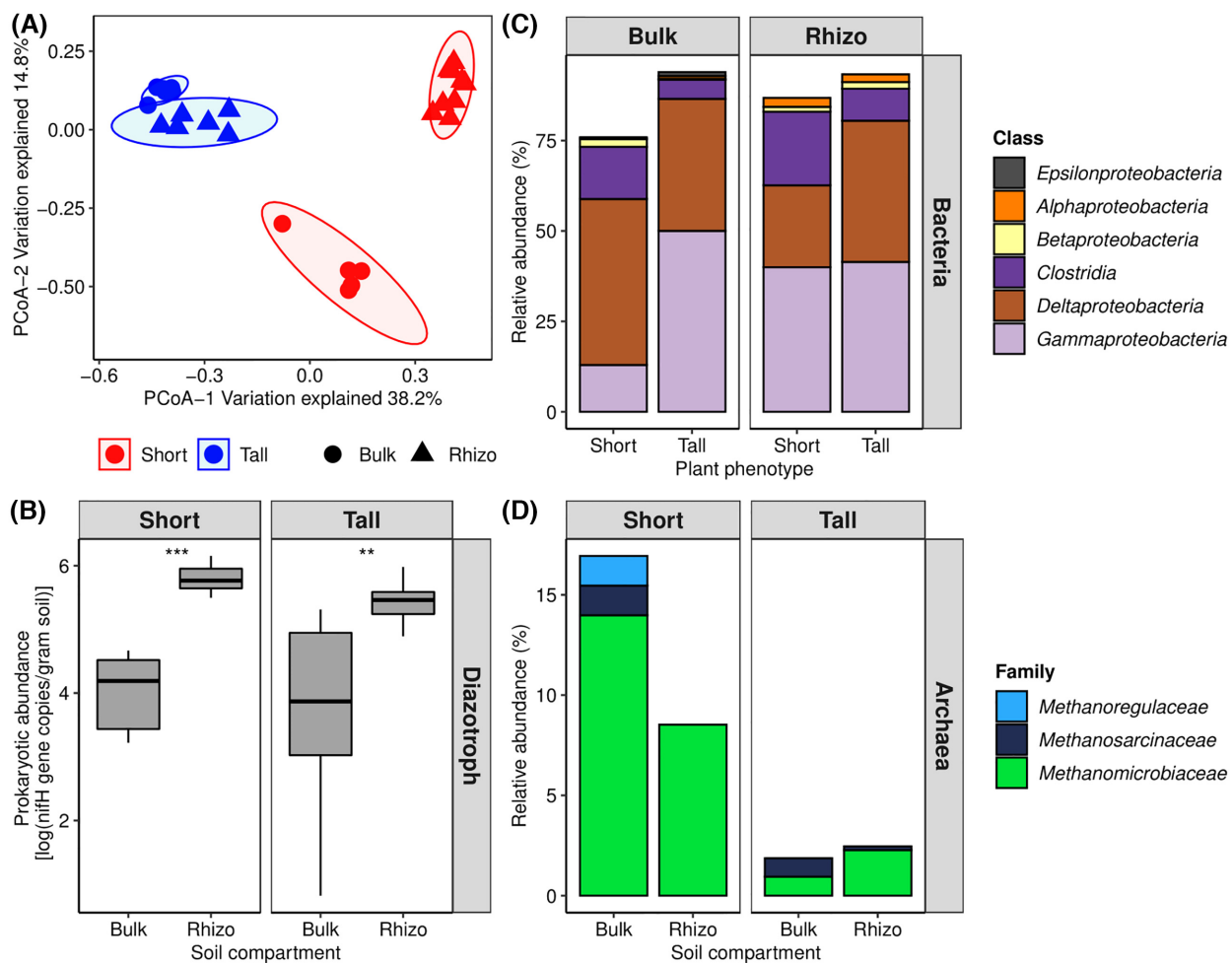
Figure 3. Beta diversity and composition of diazotrophic communities associated with tall and short phenotypes of *S. alterniflora* from Skidaway Island, Georgia, USA. **(A)** PCoA of diazotrophic community compositions based on the Bray–Curtis distance matrices calculated from CSS normalization of the final data; **(B)** diazotrophic absolute abundance determined by qPCR of the *nifH* gene genes; **(C)** relative abundance of the dominant bacterial classes; **(D)** relative abundance of the most abundant archaeal families. The 'Bulk' and 'Rhizo' indicate bulk and rhizosphere sediments, respectively. 'Short' and 'Tall' define the *S. alterniflora* phenotype. Diversity, composition and absolute abundances of the diazotrophic communities were assessed from the same samples collected from bulk and rhizosphere sediments of the tall and short forms of *S. alterniflora*. Triplicate samples were used to quantify the abundances of diazotrophs with the PolF/PolR *nifH* primer set. The asterisks indicate statistically significant differences between observations based on a Wilcoxon signed-rank test. $**P < 0.01$ and $***P < 0.001$.

and rhizosphere sediments of the *Spartina* plants. Beta diversity (Figure S1, Supporting Information) and taxonomic analyses of fungal communities suggest that *Spartina* phenotype and the distance from the root had a limited effect on the fungal community. Moreover, the observed distribution of the fungal community was mainly effected by changes in abundance within the community at lower taxonomic levels, most likely at the ASV level. Overall, fungal community composition remained stable, and no trends were observed across plant phenotype and/or distance from the roots. Members of the *Ascomycota* (69.9 ± 1.1%) and *Basidiomycota* (29.3 ± 0.8%) phyla accounted for nearly 100% of fungal sequences and their relative abundance did not appear to be influenced by plant phenotype and/or by the distance from the plant roots. Although we were unable to detect the significant differences in the relative abundances of fungal populations at the genus, family, class and phylum levels, members of the *Venturiales* order developed significantly denser populations in the rhizosphere sediments relative to the bulk sediments, regardless of plant phenotype. The relative abundance of the *Venturiales* in the rhizosphere sediments of the short and tall plants were 3.5 ± 0.4% and 3.7 ± 1.1%, respectively. In contrast,

their relative abundance in the bulk sediments were 1.2 ± 0.2% and 1.2 ± 0.4% for the short and tall plants, respectively (Figure S2, Supporting Information). On the other hand, the *Tremellales* order tends to establish a higher population in the bulk sediments. In the area colonized by the short plant phenotype, the relative abundance of the *Tremellales* were 18.1 ± 0.9% and 12.1 ± 0.9% in the bulk and rhizosphere sediments, respectively. Additionally, the relative abundance of the *Tremellales* in the area colonized by taller plants were 22.6 ± 0.9% and 16.6 ± 0.9% in the bulk and rhizosphere sediments, respectively (Figure S2, Supporting Information).

Indicator species analysis

An indicator species analysis was applied to identify populations of prokaryotic, diazotrophic and fungal communities, whose occurrences varied significantly with plant phenotype and the distance from the root system. Additionally, we confirmed the selection by applying a machine learning approach with a random forest algorithm. The combined
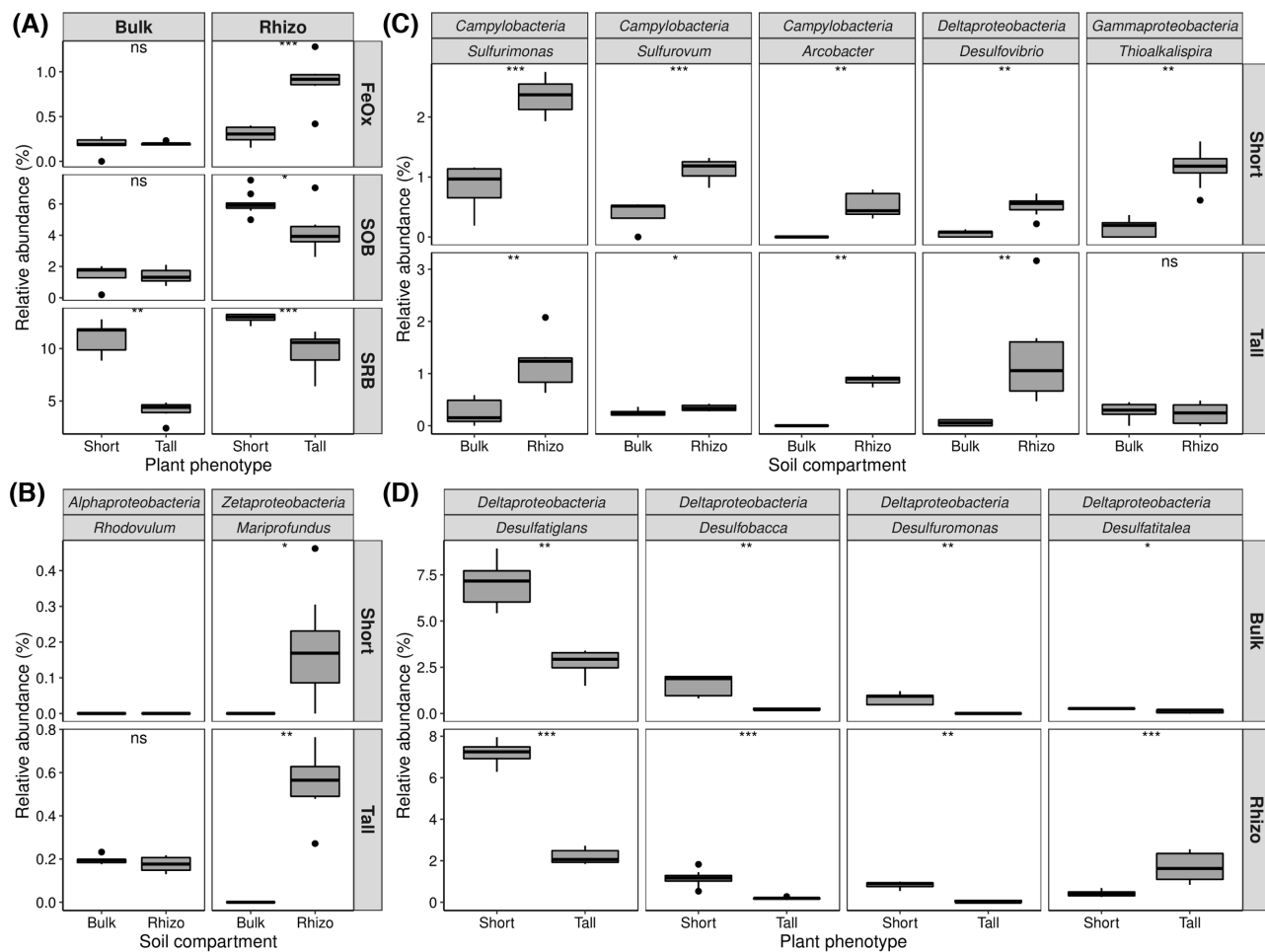
Figure 4. Taxonomic composition of iron and sulfur-cycling communities associated with tall and short phenotypes of *S. alterniflora* from Skidaway Island, Georgia, USA. Prokaryotic SSU rRNA gene amplicon libraries were annotated to the SILVA SSU rRNA reference alignment (release 132), and putative iron and sulfur metabolizing bacteria were extracted. **(A)** The overall distribution of the iron and sulfur metabolizing bacteria: iron-oxidizing bacteria—FeOx; sulfide-oxidizing bacteria—SOB; sulfur/sulfate-reducing bacteria—SRB, **(B)** dominant putative iron-oxidizing bacteria, **(C)** dominant putative sulfide-oxidizing bacteria and **(D)** dominant sulfur/sulfate-reducing bacteria. The 'Bulk' and 'Rhizo' indicate bulk and rhizosphere sediments, respectively. 'Short' and 'Tall' define the *S. alterniflora* phenotype. The asterisks indicate statistically significant differences between observations based on a Wilcoxon signed-rank test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and 'ns' indicates no significant differences.

approach revealed 175 prokaryotic, 70 diazotrophic and 15 fungal unique indicator species. Accordingly, the 175 prokaryotic indicator species were distributed across 35 classes of 24 phyla, representing $14.2 \pm 0.94\%$ and $21 \pm 1.34\%$ of the prokaryotic community in the area colonized by the tall and short plant phenotypes, respectively. Moreover, cluster analysis of indicator species at the class level revealed distinct groups specifically associated with plant phenotypes and distance from the root system (Fig. 6). While heatmap groups 1 and 2 were explicitly associated with the tall plant phenotype, groups 3 and 4 were indicators of the short phenotype. Additionally, members of clusters 1 and 3 separate according to distance from the root system (Fig. 6). Therefore, in corroboration of taxonomic analysis, *Zetaproteobacteria* and *Campylobacteria* were indicator species for iron and sulfur oxidation in the rhizosphere sediments of the tall and the short plant phenotypes, respectively (Fig. 6). Finally, the indicator species belonging to archaeal classes *Bathyarchaeia*, *Thermoplasmata* and *Woesearchaeia* were exclusively observed in the bulk sediments of short plant phenotypes (Fig. 6). Indicator diazotroph species were distributed across six classes of four phyla, representing a relative abundance of $32.5 \pm 2.8\%$ and

$28.4 \pm 1.1\%$ of diazotrophs in bulk and rhizosphere sediments, respectively. Diazotrophic indicator species predominantly clustered according to plant phenotype and distance from the root surface (Figure S2, Supporting Information). Moreover, in agreement with prokaryotic indicator species, methanogenic archaea *Methanomicrobia* with the potential for diazotrophy were explicitly observed in the bulk sediment of the short plant phenotype (Figure S2, Supporting Information).

Characteristics of microbial networks

The ecological roles and interactions between indicator species were inferred from co-occurrence network analysis. Accordingly, we constructed and characterized separate microbial co-occurrence networks for the tall and the short plant phenotypes. The tall plant network contained 177 nodes (106 prokaryotic, 59 diazotrophic and 12 fungal unique indicator species) connected by 514 edges, while the short plant network contained 147 nodes (106 prokaryotic, 28 diazotrophic and 13 fungal unique indicator species) connected by 491 edges. Moreover, the topological characterization of the microbiome networks revealed that the
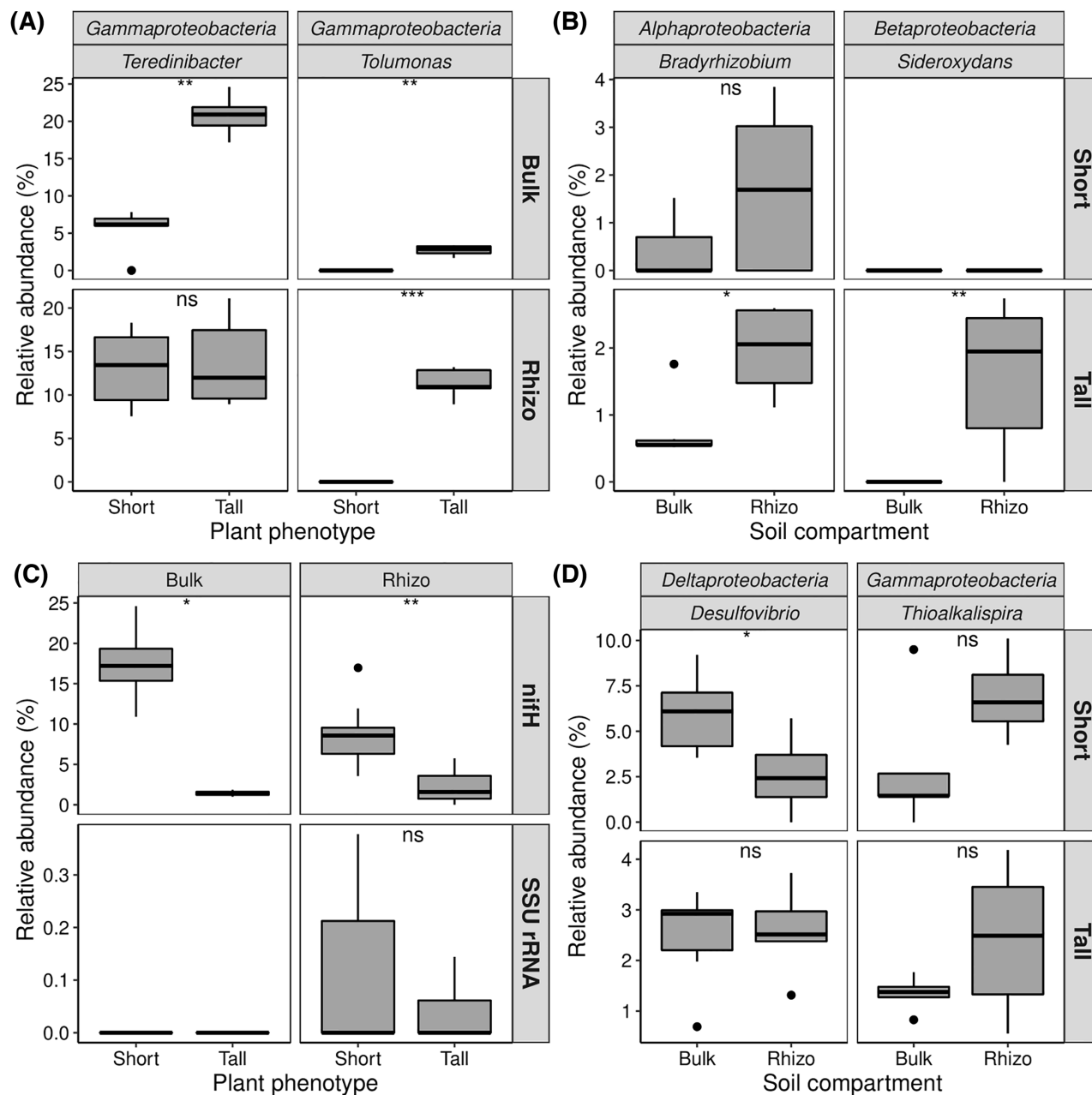
Figure 5. Taxonomic composition of diazotrophic communities associated with tall and short phenotypes of *S. alterniflora* from Skidaway Island, Georgia, USA. **(A)** Dominant diazotrophic genera; **(B)** dominant putative iron-oxidizing diazotrophic bacteria; **(C)** relative abundance of methanogenic *Methanomicrobiaceae* in the prokaryotic and diazotrophic communities; and **(D)** dominant putative sulfide-oxidizing diazotrophic bacteria. The 'Bulk' and 'Rhizo' indicate bulk and rhizosphere sediments, respectively. 'Short' and 'Tall' define the *S. alterniflora* phenotype. The asterisks indicate statistically significant differences between observations based on a Wilcoxon signed-rank test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and 'ns' indicates no significant differences.

area colonized by the short plant phenotype had a greater network density (0.046), node betweenness centrality (0.213) and lower mean distance (3.336) in comparison to the area colonized by the tall plant phenotype (Table 1). Additionally, while microbial interactions in the tall plants were mainly regulated by diazotrophic populations (Fig. 7A), microbial assembly associated with the short plant phenotype was governed by fungal populations (Fig. 7B).

Keystone taxa appear to play a vital role in interspecies interactions and ecosystem functioning (Banerjee, Schlaeppi and van

der Heijden 2018). Moreover, their removal from the ecosystem may result in a drastic shift in the ability of the microbial community to support ecosystem functions (van der Heijden and Hartmann 2016). Here, we define keystone taxa as the five most connected indicator species (Table 2; Supplementary Data 6, Supporting Information). The central location of keystone taxa in the network and their taxonomic identity indicate that *Proteobacteria*-affiliated members of diazotrophic genera *Teredinibacter* and *Tolumonas* are functionally redundant and/or influence broad processes such as organic matter decomposition
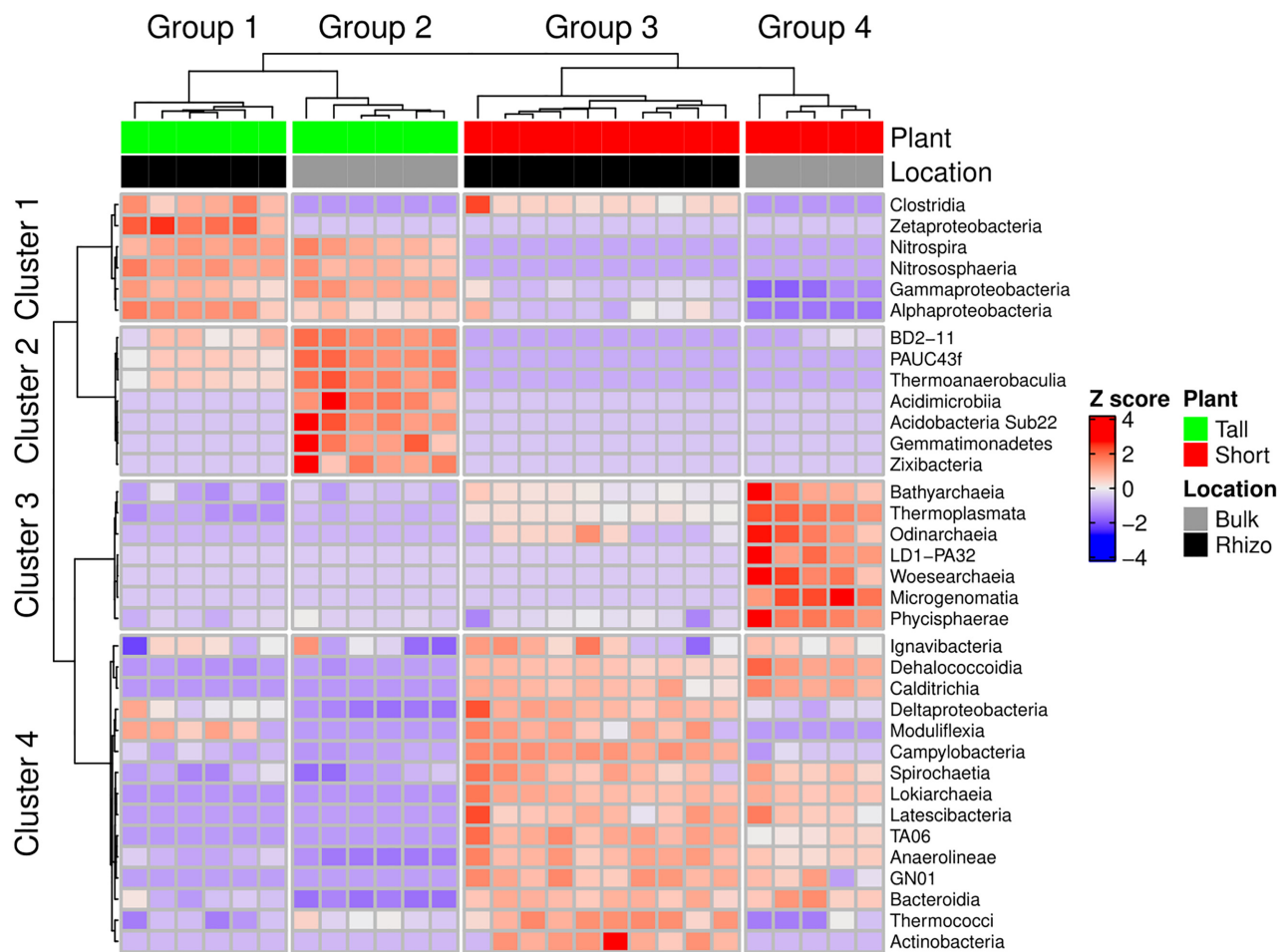
Figure 6. The relative abundance of prokaryotic indicator species represented according to *S. alterniflora* phenotypes and distance to the root zone on Skidaway Island, Georgia, USA. Hierarchical clustering heatmap showing the correlation in abundance variation of prokaryotic indicator species. An indicator species analysis was applied to identify microbial populations, whose occurrences varied significantly between the plant phenotypes and with distance from the root system; analysis was confirmed using a machine learning approach with a random forest algorithm. This combined approach revealed 175 prokaryotic indicator species. The relative abundances of the indicator species were calculated at the taxonomic class levels, and heatmaps were constructed based on z-score transformed abundances to improve normality and homogeneity of variances. The 'Bulk' and 'Rhizo' indicate bulk and rhizosphere sediments, respectively. 'Short' and 'Tall' define the *S. alterniflora* phenotype.

Table 1. Properties of bulk and rhizosphere microbial meta co-occurrence networks.

	[a]Bacteria		[a]Archaea		[a]Fungi	Interactions		Network density	Mean distance	Centrality		
	16S	NifH	16S	NifH	ITS	Positive	Negative			Closeness	Betweenness	Degree
Tall	100	58	6	1	12	229	61	0.033	4.084	0.022	0.115	0.240
Short	92	27	14	1	13	193	58	0.046	3.336	0.019	0.213	0.297

[a]Number of indicator species in the network.

(Table 2). Additionally, network analysis underlines the importance of the *Crenarchaeta*-affiliated versatile *Bathyarchaeota* in the area colonized by the short plant phenotype.

DISCUSSION

While a wealth of information is available on the structure and functioning of microbial communities in salt marsh sediments associated with *S. alterniflora* (Bowen *et al.* 2011, 2012; Kearns

et al. 2016), relatively few studies have focused on the role of plant phenotype or the rhizosphere. Using a DNA fingerprinting approach, Bowen *et al.* (2009) observed that bacterial composition in a New England salt marsh did not differ between *Spartina* growth forms early in the growing season (May–June), whereas a significant difference was observed later in the year (July–September). Differences in bacterial communities between growth forms were attributed to local environmental parameters such as benthic primary production and the source of organic carbon. Studies conducted in *Spartina*-dominated marsh
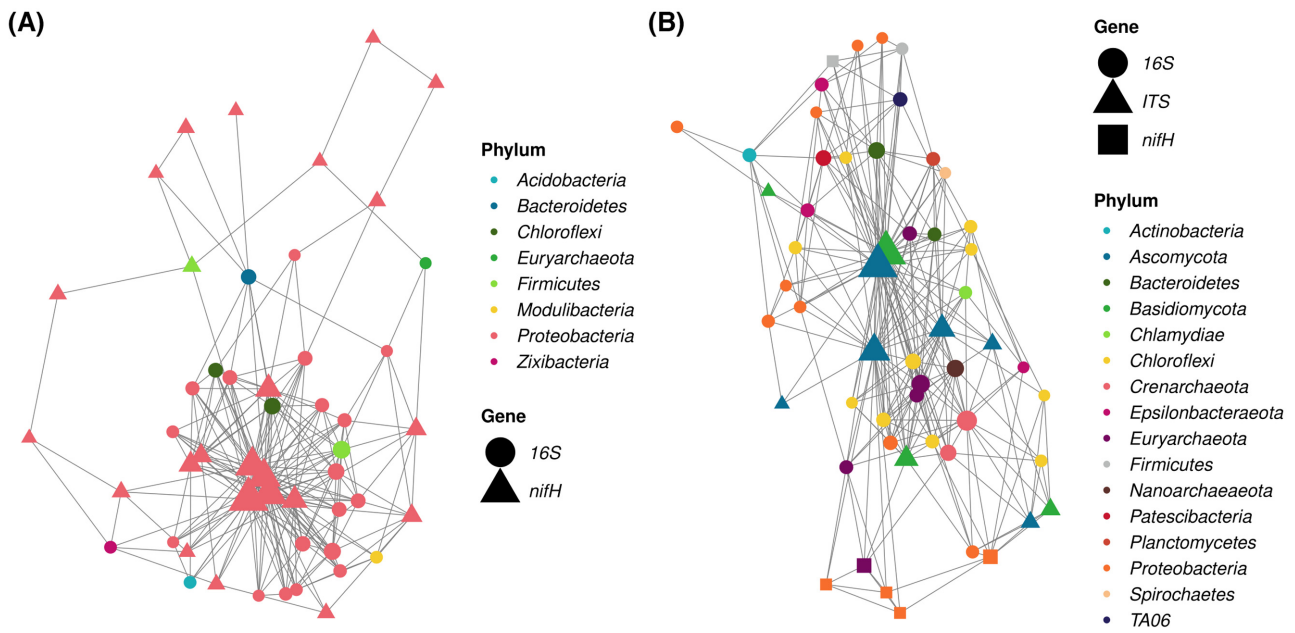
Figure 7. Microbiome networks. SpiecEasi network maps of the 175 prokaryotic, 70 diazotrophic and 15 fungal unique indicator species at the ASV level from: **(A)** the tall *S. alterniflora* and **(B)** short *S. alterniflora* forms. ASVs are color coded based on their taxonomic affiliation. Only indicator species with >7 interactions are presented.

Table 2. Identity and relative abundance of the keystone microbes.

Plant	Sequence ID	Gene	Class	Family	Genus	Number interactions	% of the interaction	Relative abundance (%)
Tall	*NifH.ASV_374*	*NifH*	*Gammaproteobacteria*	*Gammaproteobacteria sp.*	*Gammaproteobacteria.sp*	48	16.6	0.32 (0.1)
Tall	NifH.ASV_174	*NifH*	*Gammaproteobacteria*	*Cellvibrionaceae*	*Teredinibacter*	38	13.1	0.37 (0.11)
Tall	NifH.ASV_284	*NifH*	*Gammaproteobacteria*	*Cellvibrionaceae*	*Teredinibacter*	35	12.1	0.36 (0.11)
Tall	NifH.ASV_200	*NifH*	*Gammaproteobacteria*	*Aeromonadaceae*	*Tolumonas*	33	11.4	0.34 (0.11)
Tall	NifH.ASV_274	*NifH*	*Deltaproteobacteria*	*Syntrophobacteraceae*	*Syntrophobacter*	21	7.2	0.44 (0.14)
Short	ITS.ASV_1	ITS	*Leotiomycetes*	*Leotiomycetes*	*Leotiomycetes*	50	19.9	4.74 (0.54)
Short	ITS.ASV_29	ITS	*Tremellomycetes*	*Bulleribasidiaceae*	*Vishniacozyma*	49	19.5	0.31 (0.12)
Short	ITS.ASV_31	ITS	*Leotiomycetes*	*Leotiomycetes*	*Leotiomycetes*	32	12.7	0.31(0.12)
Short	ITS.ASV_124	ITS	*Leotiomycetes*	*Leotiomycetes*	*Leotiomycetes*	22	8.8	0.24 (0.09)
Short	16S.ASV_715	16S	*Bathyarchaeia*	*Bathyarchaeia*	*Bathyarchaeia*	22	8.8	0.08 (0.03)

areas in Maine, USA (Zogg, Travis and Brazeau 2018) and in China (Lin *et al.* 2019) corroborated our results. Based on beta diversity analysis of SSU rRNA gene amplicons, these studies concluded that bacterial communities were distinct between *Spartina* growth forms, and a rhizosphere effect was observed. Zogg, Travis and Brazeau (2018) further linked plant genotype to bacterial community composition and suggested that root characteristics along with redox conditions in the rhizosphere could explain the differences observed between plant growth forms. In contrast to our results, rhizosphere alpha diversity did not differ significantly between tall and short plant phenotypes in previous work (Zogg, Travis and Brazeau 2018; Lin *et al.* 2019), and a higher diversity was observed in the rhizosphere in comparison to the bulk sediments (Zogg, Travis and Brazeau 2018). Although absolute abundance was not quantified in previous studies of bulk sediments associated with *Spartina* growth forms, higher bacterial abundance was observed in the rhizosphere of tall plants in comparison to short plants (Lin *et al.*

2019). Overall, our results confirm strong plant–microbe associations in the *Spartina* root zone by revealing contrasts in the community composition and abundance of archaea along with bacteria across gradients in plant growth form. Further, niche diversification in the tall plants appears to favor bacteria, whereas the short plant phenotype selects for archaea and fungi.

Although differences in the diversity and composition of bacterial communities have been established across *Spartina* growth forms in salt marsh sediments, phylogenetic analysis has generally been limited and comparable studies are not available for fungal or archaeal communities. Unlike prokaryotes, fungal communities did not appear to be structured by *Spartina* growth form as trends were not observed in beta diversity analysis (Figure S1, Supporting Information). In contrast, higher alpha diversity was observed for fungal communities in the bulk sediment in comparison to the rhizosphere (Fig. 1). This observation is surprising since most fungi are strict aerobes, dependent upon oxygen supply for growth, and *Spartina* roots release oxygen into

the rhizosphere (Sundby *et al.* 1998; Holmer, Gribsholt and Kristensen 2002; Koop-Jakobsen *et al.* 2018). Although our sequencing efforts were insufficient to recover anaerobic fungal communities, the obligate anaerobic fungi taxonomically affiliated with *Neocallimastigomycota* were observed in the bulk sediments of *Spartina*-dominated salt marshes of North Carolina, USA (Picard 2017). Members of the *Neocallimastigomycota* were considered as major contributors to the degradation of the recalcitrant plant polymers within the digestive tracts of many herbivores, including humans and mammals (Selinger, Forsberg and Cheng 1996; Ljungdahl 2008; Mar Rodríguez *et al.* 2015) and prone to frequent horizontal transfer of genes encoding polysaccharide degrading enzymes (Gruninger *et al.* 2014). Additionally, *Neocallimastigomycota* lack mitochondria and supply their energy needs by the NADH oxidation, leading to formation of bioavailable hydrogen (Gruninger *et al.* 2014), which may support symbiotic relationships with chemoautotrophic prokaryotes (Ivarsson *et al.* 2016). Higher bacterial diversity in the rhizosphere may be interpreted according to the 'resource island effect', whereby a plentiful supply of oxygen and labile carbon substrates produced by roots helps bacteria to combat a dynamic and stressful intertidal zone (Zogg, Travis and Brazeau 2018). Prokaryotes may outcompete fungi for these same substrates.

Although archaea were previously studied in Louisiana salt marsh sediments (Rietl *et al.* 2016), relatively few samples were analyzed, and neither plant phenotype nor distance from the root system was addressed. Here, we show that archaea are preferentially colonizing the area dominated by the short form of *S. alterniflora*. The majority of archaeal groups detected (*Bathyarchaeota*, *Euryarchaeota*, *Asgardarchaeota*, *Crenarchaeota*) are known for anaerobic metabolism and for colonizing anoxic habitats. For example, the *Euryarchaeota* are predominated by known methanogens, which are mostly strict anaerobes (Lyu *et al.* 2018), and the *Bathyarchaeota* are believed to carry out acetogenesis and dissimilatory nitrogen/sulfur reduction along with methane metabolism in anoxic environments (Zhou *et al.* 2018). Thus, our results indicate that the strongly reducing and sulfidic conditions found in short *Spartina* zones select for anaerobic archaea that are better adapted to these extreme conditions. More research is needed to define the role of archaea in salt marsh ecosystem function.

Microbial populations mediating biogeochemical cycles in *Spartina*-dominated marshes

Previous research has revealed the dynamic interplay between the growth/physiology of macrophyte plants, macrofaunal bioturbation and microbially mediated processes that regulate biogeochemical cycles of carbon, sulfur, iron and nitrogen in salt marsh sediments. In the *Spartina*-dominated Skidaway marsh, sediments are rich in iron minerals and bathed in sulfate-rich seawater. Thus, most organic matter decomposition occurs under anaerobic conditions through microbial respiration processes such as sulfate and iron(III) reduction, which produce sulfide and soluble Fe, respectively (Kostka *et al.* 2002; Kostka, Roychoudhury and Van Cappellen 2002; Gribsholt, Kostka and Kristensen 2003). Sediment anoxia and sulfide accumulation, in particular, limit nitrogen uptake and growth by *Spartina* (Mendelssohn and Morris 2000). Sulfide is toxic to plants and inhibits the microbial nitrogen cycle (nitrification, denitrification) in sediments (Dollhopf *et al.* 2005; Lamers *et al.* 2013). Through catalysis of sulfide and iron oxidation, microbial processes consume toxic sulfur and regulate redox poise in the root

zone to benefit plants. These are just a few examples of how microbial processes may benefit plant health and productivity in salt marsh ecosystems.

A number of biological and geochemical parameters have been invoked to explain the contrasting microbial communities observed in the root zone of tall vs short *Spartina* plants, including plant productivity, oxygen concentration, root exudation and nitrogen limitation (Bowen *et al.* 2009; Zogg, Travis and Brazeau 2018; Lin *et al.* 2019). This study leverages extensive biogeochemical characterization of the same salt marsh sites at Skidaway Island. Our results point to reduction–oxidation chemistry, and the recycling of electron acceptors by multiple processes, as the major factors driving the selection of microbial populations in the root zone of *Spartina*. Substantial evidence collected from Georgia salt marshes indicates that biogeochemical cycles are enhanced in tall *Spartina* areas by a combination of tidal inundation, plant physiology and macrofaunal bioturbation (Kostka *et al.* 2002; Kostka, Roychoudhury and Van Cappellen 2002; Gribsholt, Kostka and Kristensen 2003; Hyun, Smith and Kostka 2007). In addition, similar relationships have been observed in the root zone of other intertidal plants (Hyun *et al.* 2009). In areas occupied by tall *Spartina* near large tidal creeks, chemical exchange, and especially oxygen supply, is enhanced by these processes resulting in the reoxidation of respiration products [sulfide, Fe(II), ammonium] and recycling of electron acceptors [oxygen, Fe(III), sulfate, nitrate]. Microbial activity is stimulated in parallel with plant productivity in the tall *Spartina*, resulting in higher rates of microbial respiration (Kostka, Roychoudhury and Van Cappellen 2002; Hyun, Smith and Kostka 2007). Conversely, the short *Spartina* root zone is highly reducing, with dissolved sulfide accumulating to high levels, and rates of microbial respiration are diminished along with plant production. The microbial nitrogen cycle, in particular, was shown to be inhibited in short *Spartina* zones, resulting in a lower abundance of nitrifying and denitrifying bacteria (Dollhopf *et al.* 2005).

In general, the prevalence of microbial processes inferred from the distribution of microbial groups paralleled with previous biogeochemical measurements. The Fe cycle, along with the nitrogen cycle, is impeded in the short *Spartina*, while microbial sulfate reduction and sulfur oxidation predominate. In agreement, both sulfate-reducing and sulfur-oxidizing microbial groups showed a higher relative abundance in short *Spartina* zones.

Nearly all taxa of known SRB showed a higher relative abundance in the short *Spartina* (Fig. 4; Supplementary Data 3, Supporting Information), where sulfate reduction was shown to be the predominant terminal electron-accepting process coupled to organic matter decomposition (Kostka, Roychoudhury and Van Cappellen 2002; Hyun, Smith and Kostka 2007). The most dominant members of the SRB in the rhizosphere of *Spartina* were affiliated with the genus *Desulfatiglans* of the *Desulfobacteraceae* family (Fig. 4D), previously known as *Desulfobacterium* (Suzuki *et al.* 2014). Metabolically diverse members of *Desulfobacteraceae* are chemoorganoheterotrophs that use sulfate as a terminal electron acceptor to oxidize numerous carbon sources such as ethanol, acetate, butyrate, pyruvate and aromatic compounds completely to CO_2 (Galushko and Kuever 2019). *Desulfatiglans* accounted for up to 8% of the total bacterial reads and contributed up to 65% of the SRB community (Fig. 4D). Members of the *Desulfobacteraceae* family frequently dominate the SRB community in the salt marshes of the eastern coasts of the US and China (Hines *et al.* 1999; Klepac-Ceraj *et al.* 2004; Bahr *et al.* 2005; Lin *et al.* 2019). Collectively, these observations suggest that the *Desulfatiglans* group is comprised of generalist taxa that

are adapted to a wide range of climatic conditions and maintain mutualistic interactions with *S. alterniflora* (Hines *et al.* 1999).

Past research in the Skidaway marsh demonstrated that the rhizosphere of tall *Spartina* plants represents a hotspot of Fe cycling, where Fe minerals are enriched and microbial Fe(III) respiration is closely coupled to Fe(II) oxidation (Gribsholt, Kostka and Kristensen 2003). Roots of *Spartina* plants act as efficient conduits of oxygen to the interior of the sediment because their well-developed aerenchyma system allows oxygen to diffuse from leaves to roots (Sundby *et al.* 1998; Holmer, Gribsholt and Kristensen 2002; Koop-Jakobsen *et al.* 2018). Oxygen supplied by roots and enhanced macrofaunal bioturbation associated with tall *Spartina* recycle reduced Fe, slowing down the turnover of S and impeding sulfate reduction (Gribsholt, Kostka and Kristensen 2003). The distribution of known SOB and Fe(II)-oxidizing bacteria (FeOB) agreed with biogeochemical evidence. Known FeOB showed three to four times higher relative abundance in the rhizosphere of the tall *Spartina* plants in comparison to the bulk sediment and the short *Spartina* (Fig. 4; Supplementary Data 3, Supporting Information). Interestingly, the *Thaumarchaeota*, which contains abundant nitrifying members, was the only archaeal group shown to be much more abundant in the tall *Spartina* rhizosphere and sediments (Fig. 2D; Supplementary Data 2, Supporting Information). Conversely, SOB, though present in the tall *Spartina*, had a higher relative abundance in the short *Spartina* rhizosphere (Fig. 4).

In previous studies employing a cultivation-based approach, iron plaques in the rhizosphere of wetland plants were shown to be colonized by a dense microbial community (Emerson, Weiss and Megonigal 1999), with FeOB estimated to account for up to 1% of the community (Weiss *et al.* 2003). However, few studies have applied culture-independent methods to quantify and to assess the ecophysiological properties of FeOB in salt marshes. *Mariprofundus* of the *Zetaproteobacteria* and *Sideroxydans* of the *Betaproteobacteria* were shown to predominate among FeOB communities associated with the *Spartina* rhizosphere (Fig. 3A and B), and a higher relative abundance was associated with the tall plant phenotype (Fig. 3A). Similarly, *Mariprofundus* was detected in association with *Spartina* plants exposed to restoration practices that enhance chemical exchange, thereby increasing redox potential (Thomas *et al.* 2019). *Sideroxydans* and *Mariprofundus* are microaerophilic chemolithoautotrophic bacteria that use iron as a sole energy source and are incapable of heterotrophic growth or oxidation of reduced S compounds common to the rhizosphere (Emerson *et al.* 2007, 2013; Weiss *et al.* 2007; Emerson, Fleming and McBeth 2010). These observations suggest that FeOB are uniquely adapted to Fe oxidation in the rhizosphere of salt marsh plants. Genomic and physiological analyses of FeOB revealed the potential for diazotrophy in *Sideroxydans* ES-1 and some strains of *Mariprofundus* (Kato *et al.* 2015). The presence of diazotrophic FeOB demonstrates a potential link between Fe oxidation, nitrogen and carbon fixation in *Spartina*-dominated salt marshes.

The dominant SOB observed, members of the *Campylobacteraceae* in the *Epsilonbacteraeota*, were significantly enriched in the rhizosphere of short plants (Fig. 4C). Members of the *Campylobacterales* support growth from the oxidation of reduced sulfur compounds coupled with the reduction of nitrate or oxygen (Takai *et al.* 2005; Campbell *et al.* 2006). In agreement with our observations, members of *Epsilonbacteraeota* were enriched in *Spartina* root-associated microbial communities in salt marshes of China (Hong *et al.* 2015; Huang *et al.* 2016; Lin *et al.* 2019) and Louisiana, USA (Rietl *et al.* 2016). The enrichment of *Epsilonbacteraeota* such as *Sulfurimonas*, *Sulfurovum* and *Arcobacter* on the root surfaces of

S. alterniflora suggests that the rhizosphere is a preferable niche for these microbial groups, and supports the hypothesis that plant recruitment of these particular bacterial populations can help overcome abiotic stresses in coastal environments through sulfide detoxification and sulfate assimilation. Previous work on the roots of *Spartina* (Mcclung and Patriquin 1980; Mcclung, Patriquin and Davis 1983), seagrass rhizomes (Elliott, Spear and Wyllie-Echeverria 2006) and the roots of rice seedlings (Joshi and Hollis 1977) implicated SOB in the detoxification of sulfides. Sulfur isotopic composition in *Spartina* and seagrasses point to a significant portion of S uptake derived from microbial sulfide oxidation occurring within the plant tissues (Carlson and Forrest 1982; Frederiksen *et al.* 2006; Holmer *et al.* 2009; Holmer and Kendrick 2013). In contrast, the chemical oxidation of sulfide is estimated to be approximately four orders of magnitude slower than biological (Jørgensen and Revsbech 1983; Millero 1986). These observations underline a crucial role for biological sulfur oxidation in sulfide detoxification and the supply of sulfur to the plants. Thus, the recruitment and maintenance of SOB in the rhizosphere appears essential for *Spartina* plant survival.

Diazotrophic prokaryotes associated with *Spartina*

The health and primary productivity of *Spartina* salt marsh ecosystems rely on the activity of nitrogen-fixing bacteria or diazotrophs. The presence and activity of diazotrophs are particularly important in young or restored marshes where rates of biological nitrogen fixation are sufficient to support plant growth (DeLaune, Feijtel and Patrick 1989; White and Howes 1994; Currin, Joye and Paerl 1996; Tyler, Mastronicola and McGlathery 2003). Diazotrophic community composition and rates of the nitrogen fixation in the salt marsh vary seasonally and with the distance from the root surface, and correlate with plant morphology (Fig. 2A; Gamble *et al.* 2010; Davis *et al.* 2011). In comparison with bulk sediment, the rhizosphere sediments frequently harbor more active diazotrophic communities (Patriquin 1978; Yoch and Whiting 1986). Moreover, diazotrophic activity is tightly associated with plant photosynthetic capacity (Whiting, Gandy and Yoch 1986) and bacterial sulfate reduction rates (Gandy and Yoch 1988).

Although we did not quantify diazotrophic activities, qPCR-based estimates indicated that the rhizosphere harbors a significantly more abundant diazotrophic community than bulk sediments (Fig. 3C). In contrast to past work (Bagwell *et al.* 1998; Piceno, Noble and Lovell 1999; Bagwell and Lovell 2000; Lovell *et al.* 2000, 2001, 2008; Gamble *et al.* 2010; Davis *et al.* 2011), we observed that both *Spartina* growth form and proximity to the root zone impacted the diversity of diazotrophic communities, with higher alpha diversity associated with the rhizosphere. This inconsistency is likely due to the low resolution of previously employed molecular methods. Further, we provide a detailed compositional analysis of diazotrophs in a pristine *Spartina*-dominated salt marsh. In agreement with previous field studies (Lovell *et al.* 2008; Gamble *et al.* 2010; Davis *et al.* 2011), *Spartina* diazotrophic communities were primarily composed of members of the *Gammaproteobacteria*, *Deltaproteobacteria* and *Clostridia* (Fig. 3B) that together comprised ∼85% of the overall sequence abundance. Surprisingly, methanogenic archaea affiliated with the *Methanomicrobiaceae* along with *Teredinibacter* within the *Cellvibrionaceae* were the most abundant diazotrophic groups detected (Fig. 5B). Members of the *Teredinibacter* genus (Distel *et al.* 2002) have been linked to the degradation of complex polysaccharides in studies of decaying *S. alterniflora* in marshes on the Chesapeake Bay or Sapelo Island (Andrykovitch and Marx

1988; Gonzalez *et al.* 1997). *Teredinibacter* genomes contain genes that are primarily specialized in the degradation of terrestrial polysaccharides (Yang *et al.* 2009), and the group includes facultative endosymbionts with a rare ability to simultaneously use cellulose as a sole carbon source and fix nitrogen (Distel *et al.* 2002; Yang *et al.* 2009). The combination of cellulolytic and diazotrophic capacities is rare and was reported for several strictly anaerobic bacteria, such as *Clostridium hungatei* (Monserrate, Leschine and Canale-Parola 2001). The high relative abundance of the *Teredinibacter* and its unique abilities to digest cellulose and fix nitrogen indicate that this genus might play an important role in the coupling of the carbon and nitrogen cycles.

While accounting for <0.1% of prokaryotic communities, methanogens of the *Methanomicrobiaceae* comprised up to nearly 20% of diazotrophs (Fig. 5C). In support of our observation, a significant enrichment of *Methanomicrobia* was observed as a result of *Spartina* invasion in salt marshes of the Yangtze River estuary (Huang *et al.* 2016), and diazotrophic methanogens were also reported in wetlands of the Florida Everglades (Mehta, Butterfield and Baross 2003; Bae *et al.* 2018). Since methanogens are believed to be outcompeted by SRB in saline sediments (Oremland, Marsh and Polcin 1982), these high abundances are unexpected. Nevertheless, the availability of substrates might reduce the competition and support the coexistence of two functional guilds (Schubauer and Hopkinson 1984; Peng *et al.* 2011). To overcome salinity stress, *S. alterniflora* accumulates high concentrations of the methylated amines and releases them to the salt marsh sediment (Cavalieri 1983; Dacey and Wakeham 1986; Wang and Lee 1994), and methylated compounds are considered to be non-competitive substrates for methanogens since sulfate reducers do not utilize these compounds. Finally, it was estimated that up to 90% of the total methane production in salt marsh sediments could be originating from methylated compounds (Oremland, Marsh and Polcin 1982; King, Klug and Lovley 1983). Thus, methylotrophic methanogenesis is likely a substantial terminal electron-accepting process in salt marshes (Zeleke *et al.* 2013; Kelley *et al.* 2014; Yuan *et al.* 2016), whereby methanogens compete successfully with SRB. Although the role of methanogenic nitrogen fixation in the nitrogen budget is not clear, these results warrant further study.

Sedimentary microorganisms play a crucial role in nutrient cycles and ecosystem productivity (Bardgett and van der Putten 2014; Fierer 2017), and often respond quickly to changes in environmental conditions. Thus, variations in microbial communities and/or specific populations may be used as an indicator of the wetland ecosystem status (Urakawa and Bernhard 2017). Co-occurrence network analysis was applied to understand the ecological interactions of the microbial community and to reveal populations that can be used as biological indicators for the Skidaway Island salt marsh. The topological characterization of interaction networks revealed that keystone diazotrophic populations shape the microbial network in the area of the tall plant phenotype (Fig. 7A). Keystone species commonly play a disproportional important role in ecosystem functioning relative to their abundance (Power *et al.* 1996; Banerjee, Schlaeppi and van der Heijden 2018). We identify diazotrophic strains affiliated with *Teredinibacter*, *Tolumonas* and *Syntrophobacter* genera as a keystone species of the area colonized by the tall plant phenotype (Fig. 7A, Table 2; Supplementary Data 6, Supporting Information). The highly similar gammaproteobacterial *nifH* sequences were previously detected in the *Spartina* rhizosphere in South Carolina and Maryland salt marshes (Supplementary Data 6, Supporting Information; Gamble *et al.* 2010; Berthrong *et al.* 2014). Moreover, the positive contribution of

keystone diazotrophic populations to plant productivity has also been observed in dune grasslands (Van Der Heijden *et al.* 2006). Although the relative abundances of these strains in the *nifH* sequence data were <0.4% and were undetectable in the SSU dataset, their central location in the network emphasizes their important role in salt marsh nutrient cycles. Low abundant taxa have been identified as keystone species in contaminated soils and wheat rhizosphere environments (Chao *et al.* 2016; Hartman *et al.* 2018). Interestingly, microbial assemblages of the short plant phenotype were governed by fungal and archaeal populations (Fig. 7B, Table 2; Supplementary Data 6, Supporting Information), which were characterized by low relative abundances. Selected keystone microbial species were affiliated with fungal *Leotiomycetes* and archaeal *Bathyarchaeia* classes (Table 2). *Leotiomycetes* comprise an ecologically diverse class that includes mycorrhizas, plant endophytes, plant pathogens and saprophytes (Johnston *et al.* 2019). Members of the *Bathyarchaeota* were recently shown to colonize coastal and intertidal sediments (Zhou *et al.* 2018). Moreover, genomic analyses indicate that *Crenarchaeota*-affiliated *Bathyachaeota* are generalists with a versatile metabolism and ability to utilize a variety of substrates, including dead plant material, aromatic compounds and methylated substrates (Zhou *et al.* 2018), and potentially interact with many members from the surrounding microbial community. Thus, it is not surprising to find them among keystone species. Although the relative abundance of the *Bathyarchaeota* keystone taxa in the area of the short plants was 0.076 ± 0.03% (Table 2), the overall abundance of *Bathyarchaeota* was 5.9 ± 0.34%, thus emphasizing the potential role of *Bathyarchaeota* as ecosystem engineer of the Skidaway Island salt marsh. Although co-occurrence network analysis is a useful tool to assess community structure and to reveal potential interactions among community members, it does not necessarily describe the direct physiological interactions between community members (Faust and Raes 2012). Thus, future experiments are required to interpret the ecophysiological role of the identified keystone species.

CONCLUSIONS

Our study shows that distinct microbial communities are associated with *S. alterniflora* phenotype and proximity to the root zone in salt marsh habitats. A strong effect of plant phenotype on microbial diversity and abundance was observed. Generally, areas colonized by the tall plant phenotype hosted more diverse and abundant microorganisms in comparison to areas colonized by the short plant phenotype. Redox chemistry, driven by a combination of tidal inundation, plant physiology and macrofaunal bioturbation, acts as a strong selective force in shaping microbial community structure. The rhizosphere, where oxygen is supplied by roots, serves as a hotspot of iron and sulfur oxidation processes, with *Zetaproteobacterial* iron oxidizers preferentially colonizing the rhizosphere of the tall plants, while a higher relative abundance of *Epsilonbacteraeota*-affiliated sulfur oxidizers was detected in the rhizosphere of the short plant phenotype. Finally, different community organizations were dictated by plant phenotype. While diazotrophic microorganisms appear to shape ecological interactions in the area colonized by the tall plant phenotype, the fungal community fills this role in the area occupied by the short *Spartina* phenotype. A better understanding of salt marsh microbial ecology and plant–microbe interactions will facilitate the development of future coastal restoration programs with the potential to simultaneously maximize

plant productivity while reinforcing the shoreline and maximizing carbon sequestration.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

AUTHORS' CONTRIBUTIONS

MK and JEK conceived the study; JEK collected samples from the field; and MK performed the experiment, the data analyses. MK and JEK wrote the manuscript. JEK and JLR provided valuable insight and ideas during numerous sessions of discussion. All authors provided critical comments on the manuscript and gave their final approval for publication.

FUNDING

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