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Planting *Spartina alterniflora* in a salt marsh denuded of vegetation by an oil spill induces a rapid response in the soil microbial community

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ABSTRACT

The need for a comprehensive understanding of coastal wetland restoration strategies became clear following the *Deepwater Horizon* oil spill when quantities of oil reaching the marshes overwhelmed the ecosystem's natural attenuation capacity for several years in the worst-hit areas. Planting and fertilization are common habitat restoration methods in wetlands, but how these treatments impact the native soil microbial community when implemented in formerly oiled salt marshes is not well understood. This research used DNA sequencing to determine how the soil microbial community changed in response to planting, fertilization, and their interaction, and how environmental variables were related to the soil microbial community structure. We studied a salt marsh in northern Barataria Bay, Louisiana, USA that had not revegetated four years after the *Deepwater Horizon* oil spill using factorial combinations of *Spartina alterniflora* transplants and biannual fertilizer applications. Transplants significantly affected the soil microbial community structure during the first 13 months after initiating treatment. A cohort of putative sulfate-reducing bacteria (SRB) were significantly more abundant in the transplanted treatments than in the unplanted treatments after 13 months. The response of the microbial community to fertilizer depended on the presence or absence of transplants during that time. Fertilizer application in the absence of transplants resulted in a proliferation of a *Staphylococcus* taxon after two months, a significant increase in community heterogeneity after 7 months, and a shift in community composition after 13 months, but fertilizer application had no apparent effect on the soil microbial community in combination with *S. alterniflora* transplants. These results suggest that transplanted *S. alterniflora* promotes a rapid shift in the soil microbial community composition with concurrent establishment of a diverse community of SRB and mediates the effect of fertilizer on the soil microbial community in previously oiled salt marsh systems. The rapid response by the microbial community to revegetation suggests that planting *S. alterniflora* will hasten habitat restoration following future oil spills.

1. Introduction

Many coastal marshes face multiple environmental stressors due to human alterations and climate change (Scavia et al., 2002; Syvitski et al., 2009; Newton et al., 2012) that have resulted in a 38% decline in coastal wetlands globally since 1970 (Dixon et al., 2016). The Mississippi River deltaic plain is a prime example of the compounding impacts of multiple stressors that have led to rapid ecosystem loss (Kirwan and Megonigal, 2013; Kemp et al., 2014). These stressors include primarily sediment starvation, sea-level rise, and land subsidence (Blum and Roberts, 2009), but occasional, large releases of crude oil may interact with existing stressors and dramatically accelerate coastal marsh loss due to plant mortality, as was the case following the *Deepwater Horizon* (DWH) oil spill (Mendelssohn et al., 2012; Silliman et al., 2016; Lin et al., 2016).

Following the DWH oil spill, response activities were avoided for all

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but the most heavily oiled marshes (Zengel and Michel, 2013) because such activities are thought to damage the soft soil while enhancing the growth of vegetation very little (DeLaune et al., 1979; Pezeshki et al., 2000). The northern Gulf of Mexico ecosystem, including salt marshes, has a great capacity for biodegradation of oil (Jackson and Pardue, 1999; King et al., 2015). However, the large volume of oil deposited during the DWH spill in the most heavily oiled marshes created a thick layer of oiled wrack (a combination of oil residue and dead plant material) that inhibited tidal flushing and reduced oxygen penetration. These impacts limited the natural weathering of the oil and slowed plant regrowth (Lin and Mendelssohn, 2012; Zengel and Michel, 2013). Remedial action was recommended for sites that were highly impacted (Zengel and Michel, 2013) based on lessons learned from previous spills in which areas that received a thick covering of oil residue as a result of those spills did not recover for several years or decades (Bergen et al., 2000). Raking and cutting was determined to be an effective strategy to remove the mats of wrack and oiled vegetation and was implemented on 11 km of coastal wetlands heavily oiled by the DWH spill (Zengel and Michel, 2013). No further remediation strategies were conducted following debris removal, but the report specified the need for evaluating habitat restoration measures over the longer term (Zengel and Michel, 2013). The importance of developing a treatment that would enhance long-term marsh recovery without causing further damage was highlighted by later research showing that sites that experienced oiling of > 90% of plant stems eroded significantly more than areas that received less oil (Silliman et al., 2016). Therefore, habitat restoration in heavily oiled salt marshes is desirable to reduce the erosion impact from oiling.

The impact of habitat restoration on soil microbial communities should be considered due to the central role played by bacteria and archaea in nutrient cycling and carbon turnover (Harris, 2009). These ecosystem functions are critical in salt marsh environments where denitrification can reduce nutrient pollution (Gardner and White, 2010) and detritus supports a large portion of the ecosystem metabolism (Sobczak et al., 2005). Vegetative plantings and nutrient amendments are potential strategies for increasing vegetation biomass in oil-impacted salt marshes due to the stabilizing effects of vegetation on the shoreline and increased plant growth with fertilization (Shepard et al., 2011; Graham and Mendelssohn, 2016). However, we are unaware of any studies to date that have specifically investigated how the soil microbial community in disturbed, unvegetated salt marshes is impacted by habitat restoration treatments such as planting of vegetation and addition of nutrients. To predict how salt marsh ecosystems will respond to these treatments, it is necessary to first gain an understanding of how soil microbial communities will respond.

Spartina alterniflora, the dominant salt marsh plant species in the northern Gulf of Mexico, can affect the soil microbial community through the release of root exudates and root-derived litter as well as by alteration of the rhizosphere redox potential through the efflux of oxygen from roots. Studies show that *S. alterniflora* often harbors a rhizosphere microbial community distinct from other species in its habitat (Nie et al., 2009; Rietl et al., 2016; Zheng et al., 2017). Sulfate-reducing bacteria (SRB) in particular appear to be promoted by *S. alterniflora* in comparison with other microbial taxa (Zheng et al., 2017), perhaps because of their ability to utilize a wide-range of electron donors arising from plant-derived dissolved organic carbon (Rooney-Varga et al., 1997). The products of fermentative metabolism that occur in the roots during hypoxia (Mendelssohn et al., 1981) and the fatty acids released during root necrosis likely serve as electron donors for sulfate reduction (Hines et al., 1989) and as substrates for the production of microbial biomass carbon (Zheng et al., 2017). Sulfate-reducing prokaryotes are known to occur within four bacterial phyla: Proteobacteria (class Deltaproteobacteria), Firmicutes, Nitrospirae, and Thermodesulfobacteria, and two archaeal phyla: Euryarchaeota and Crenarchaeota. Because sulfate reduction accounts for at least half of decomposition in salt marshes (Howes et al., 1984), consideration of changes to this group of microbes is relevant to the restoration of wetlands with functional characteristics similar to those of undamaged wetlands.

The effect of fertilizer application on the indigenous soil microbial community in terrestrial grasslands is well documented (i.e. Leff et al., 2015), but relatively few studies have assessed the effects of fertilizer applications on wetland soil microbes. This research indicates that nitrogen additions to *S. alterniflora*–dominated salt marshes do not alter the composition of the microbial community (Kearns et al., 2016), but the effects of fertilizer on the microbial community in unvegetated systems are not well understood. Bowen et al. (2009a) found that fertilizer inputs increased bacterial production in a low salt marsh (including mud flats) but not in high marsh zones and that microbial community composition was affected by fertilizer only in zones where filamentous algae were abundant (Bowen et al., 2009b). Fertilizer applied to a fallow rice paddy field did not affect total bacterial abundance assessed by 16S rRNA gene quantities or community composition determined by clone libraries (Liu et al., 2009). Planting vegetation could reduce fertilizer availability to microbes through competition for the nutrients (Inselsbacher et al., 2010), but in the absence of vegetation, fertilization may exert an effect on soil microbes by increasing the availability of nutrients.

We conducted a two-year manipulative field experiment to evaluate the effects of planting *S. alterniflora,* fertilization, and their interaction on the rate of recovery of a DWH oil-impacted coastal marsh in northern Barataria Bay, Louisiana, USA. The study site had minimal regrowth of vegetation in the years following the spill and was therefore well suited to testing restoration strategies. The purpose of the experiment was to test the effects of planting and fertilization on the rate of recovery of salt marsh habitat, meiofauna, and soil microbial communities in what was once a heavily oiled shoreline compared with how recovery would proceed without the treatments. The effects of the treatments were evaluated by comparison to untreated controls on a similar shoreline. We did not prevent the natural recruitment of vegetation into the experimental site in order to compare recovery with treatment to recovery without treatment. Such information is critical for informing decisions on habitat restoration in previously oiled salt marshes (Zengel and Michel, 2013, p. 17).

The research described herein focused on the response of the native soil microbial community to restoration treatments applied to an oil-impacted, unvegetated coastal wetland ecosystem. Our objectives were to determine how the microbial community responded to the planting and fertilization treatments as well as which environmental variables influenced the microbial community structure. We hypothesized that planted plots would rapidly establish a predictable microbial community typically associated with *S. alterniflora* because of the oxidation of the rhizosphere by the roots and input of organic carbon substrates from the roots. We hypothesized, however, that those plant-induced differences would dissipate once vegetative cover had become comparable between the treatments through natural revegetation. We further hypothesized that nutrient additions to the unplanted treatment would result in an increase in copiotrophic (i.e., fast growing) taxa and transition to a eutrophic soil community until naturally recruiting vegetation became abundant enough to compete with the microbes and moderate the impact of fertilizer on the microbial community. In order to test these hypotheses, we collected soil microbial samples biannually over the course of the 2.5-year field experiment.

2. Methods

2.1. Site description and sample collection

The experiment was carried out in a salt marsh in northern Barataria Bay, Louisiana, USA (N 29.44105°N, W 89.93337°W) that was heavily oiled by the DWH spill four years prior to the start of the experiment. Five locations devoid of vegetation were established along the shoreline

approximately 3 *m* from the water's edge and 12–30 *m* from one another over an area spanning approximately 100 m. At each of the five locations, four 2.1 × 2.1 *m* plots were set up and randomly assigned to one of four combinations of transplant and fertilizer treatments. Hence, there were 20 experimental units; four treatments each replicated five times.

Plots were either planted with *S. alterniflora*, or not planted, and fertilized or not fertilized. We designated treatments as N or S (no transplant or *S. alterniflora*, respectively) and fertilizer as – or + (no fertilizer or fertilizer, respectively). The four treatments were therefore: no transplants and no fertilizer (N-); no transplants plus fertilizer (N+); *S. alterniflora* transplants and no fertilizer (S-); and *S. alterniflora* transplants plus fertilizer (S+). Plugs of 3–5 *S. alterniflora* stems up to 90 cm tall were transplanted into the designated plots at 30-cm intervals. Fertilizer treatments consisted of Osmocote Plus with 15% N (8.3% NH_3-N and 6.7% NO_3-N), 8% P_2O_5, and 11% K_2O applied at a rate of 326 kg N ha^{-1} y^{-1}, 76 kg P ha^{-1} y^{-1}, and 198 kg K ha^{-1} y^{-1}. Fertilizer pellets were inserted at a depth of 10 cm below the soil surface to promote access within the root zone. The plots were planted and fertilized in April 2014, and fertilizer was added to the + treatments approximately every six months thereafter, two months prior to each sampling event. Because vegetation was not prevented from recruiting into the plots, treatments that did not receive transplants contained naturally recruited vegetation later in the experiment, primarily *Distichlis spicata*. After 26 months, four plots were lost to shoreline erosion, including one plot from S+, one from N+, and two from N-.

Samples were collected in the spring (June 2014, May 2015, and June 2016), and fall (November of 2014 and 2015) from a 60 × 60 cm quadrat placed at a different position inside each treatment plot at each sampling date. Samples for microbial analysis were collected with a 2-cm–diameter corer from the top 0–10 cm of soil within the quadrat on each sampling trip. Four cores from each quadrat were homogenized a sterile Whirlpak bag to form a composite sample from each plot on each date. The microbial samples were transported to the lab on ice and stored at −80 °C until DNA extraction. Plant, soil, and benthic invertebrate data were collected from the same subplots on each sampling date by collaborators and are described elsewhere (Johnson et al., 2018).

2.2. Environmental factors

Aboveground vegetation biomass was determined by clipping all plants rooted inside the quadrat at the ground surface. Harvested vegetation was sorted into live or dead components by species. Stems were counted for each species and the samples were dried to a constant mass at 60 °C and weighed for biomass measurements (Lin et al., 2016). Live belowground biomass was obtained from the upper 6 cm of 7.62-cm diameter soil cores by washing the soil over a 2-mm mesh sieve, collecting the live roots and rhizomes, and weighing after drying to a constant mass at 60 °C (Fleeger et al., 2019). Soil inorganic nitrogen was extracted by KCl from the surface 15 cm of soil cores collected with a 5-cm diameter, semi-cylinder peat corer and the extracts quantified with a Flow Solutions IV auto-analyzer (OI Analytical, College Station, TX). Soil pH was measured from 10 g of soil from the same 15 cm core with a benchtop pH meter after 1:1 (dry weight/volume) dilution with water and 2 h incubation period (Fleeger et al., 2019). Soil total petroleum hydrocarbon concentrations in each plot were analyzed at the first sampling point from the upper 7.5 cm and ranged from 0.58 to 0.90 mg g^{-1} soil dry weight (Johnson et al., 2018).

2.3. DNA extraction and sequencing data pre-processing

DNA was extracted with a PowerSoil kit (MOBIO, USA) according to the manufacturer's instructions, and the quality and quantity of the DNA was determined by NanoDrop (Lee et al., 2010). Amplification of the V4 region of the 16S rRNA gene by polymerase chain reaction (PCR) and subsequent sequencing of the amplicons were done as described by Wu et al. (2015). Briefly, DNA extracts were subjected to a two-step PCR with unmodified 515F/806R primers in the first step and 515F/806R primers modified with barcoded phasing primers in the second step. Amplicons were bead purified with an Agencourt® AMPure XP kit (Beckman Coulter, Beverly, MA, USA) after the first PCR and used as templates for the second step. PCR for each sample were prepared in triplicate and the corresponding reactions were combined after each step. The final PCR products were checked on a gel for the expected band size and quantified by PicoGreen (Invitrogen). Equimolar amounts of amplicons for each sample were combined, and then the mixture was purified with a QIAquick Gel Extraction Kit (QIAGEN Sciences, Germantown, MD, USA) and re-quantified with NanoDrop. Sequencing was performed on an Illumina MiSeq instrument by the Institute of Environmental Genomics, University of Oklahoma.

Sequences were imported into QIIME2 (Bolyen et al., 2019) and denoised with the DADA2 (Callahan et al., 2016) plugin. An alignment of the resulting amplicon sequence variants (ASVs) was created with mafft (Katoh, 2002) and used to construct a phylogeny with Fasttree2 (Price et al., 2010). Taxonomy was assigned to ASVs using classify-sklearn (Bokulich et al., 2018) with a naïve Bayes taxonomy classifier trained on the region amplified by 515F/806R of the Greengenes 13_8 99% OTUs reference sequences (McDonald et al., 2012). After taxonomy assignment the *phyloseq* package (McMurdie and Holmes, 2013) in R was used to implement diversity calculations and further process the data. Sequences from chloroplasts and mitochondria, and those not identified at the phylum level were removed. Further details related to processing the amplicon sequencing data are provided in the supplementary material.

2.4. Community composition

Microbial community composition was assessed in terms of differences in the multivariate centroids (central location or position) and differences in the dispersion around those centroids (variability) among treatment groups in the space of the chosen dissimilarity measure at each sampling period. Central location effects were tested by permutational analysis of variance (PERMANOVA) with the command *adonis* in the *vegan* package in R (Anderson, 2001; Oksanen et al., 2019). Pairwise comparisons with a Bonferroni correction for multiple testing were conducted for time points where a significant difference in multivariate centroids were found. Tests for differences in multivariate dispersion (PERMDISP), a dissimilarity-based multivariate generalization of Levene's test that calculates an *F* statistic to compare the average distance of samples to the spatial median of their group (Anderson, 2006) were conducted using the *betadisper* function with *vegan* (Oksanen et al., 2019). Pairwise comparisons with a Tukey correction were made where significant differences ($p \leq .05$) in multivariate dispersion were detected. Because PERMDISP does not accommodate factorial designs, treatments were run as single-factor tests. Large within-group differences in multivariate dispersion are known to affect tests of multivariate location such as PERMANOVA (Anderson, 2006), and community dispersion itself can be interpreted as a measure of community composition in terms of group homogeneity (Anderson, 2006).

Before beginning the analyses, several steps were taken to reduce the influence of rare species. For each sampling date, any ASV that was observed at < 3 times in 3 samples was removed, then those that with a relative abundance < 0.01% were also removed. Sampling depth was normalized by rarefaction, random sampling without replacement until a common sequence number is reached, to mitigate the effect of library size. The depth was selected by identifying for each sampling date the lowest number of reads per sample > 7000 after the above filtering steps. In November 2015, two samples with fewer than 7000 reads were discarded (one sample from N+ and N-, respectively). Abundance counts were $\log_{10}(x + 1)$ normalized to stabilize variance, and then

distance matrices were constructed from the resulting ASV tables using the weighted UniFrac dissimilarity measure, a coefficient of phylogenetic relatedness weighted for abundance (Lozupone and Knight, 2005).

2.5. Species richness

Species richness was calculated on rarefied ASVs without rare taxa removed. Sampling depth was normalized by rarefaction to 10,000 reads per sample prior to testing and samples with < 10,000 reads were removed (one sample from N- at 13 months). Tests for the effects of planting, fertilizer application, and time on alpha diversity were done using a linear mixed-effects model with the function *lmer* in the lmerTest package (Bates et al., 2015; Kuznetsova et al., 2017) using Satterthwaite's method for denominator degrees of freedom. We used the inverse Simpson metric as a response variable to represent alpha diversity based on its interpretability and use in the literature (Zhou et al., 2002). The metric was square root transformed so model residuals did not differ from normality determined by a Shapiro-Wilks test. Specific pairwise contrasts of interest were selected a priori, and a Tukey adjustment was applied to correct for multiple testing.

2.6. Differential abundance analysis

A generalized linear model was fitted to each taxon following a negative binomial distribution using the DESeq2 package (Love et al., 2014) to test for differences in the abundance of taxa between treatments on sampling dates where significant differences in community composition ($p \leq .05$) were indicated by PERMANOVA or PERMDISP. The unrarefied ASV table was filtered by the following steps in order to reduce low-count and noisy ASVs, to improve interpretability of the differentially abundant taxa, and approximate operational taxonomic units (OTUs). ASV's that were observed at least 3 times in 3 samples over the entire dataset were retained and cophenetically agglomerated by phylogeny at a distance of h = 0.05, the approximate genus level (Yang et al., 2016) using the command *tip-glom* with agglomerative clustering in phyloseq (McMurdie and Holmes, 2013). Those ASVs are referred to here as OTUs or taxa. After filtering and phylogenetic agglomeration, 1321 taxa remained. Those taxa were tested for differential abundance between the four treatments at each sampling time using DESeq2 (Love et al., 2014). Shrunken log-fold change (LFC) estimates were produced by the "ashr" method (Stephens, 2017), and *p*-values were adjusted for multiple testing by the Benjamini-Hochberg method. Filtering *p*-values based on Cook's distance was disabled because large variations in taxa counts between replicates in this type of field study are not unreasonable. Taxa whose abundance differed between treatments at $p \leq .05$ and whose absolute value of $\log_2$-fold change (LFC) > 0.5 were considered significantly differentially abundant between treatments. Such taxa with a mean abundance > 10 are presented here.

Non-metric multidimensional scaling (nMDS) biplots were constructed in *vegan* (Oksanen et al., 2019) from the pairwise UniFrac distances used for beta diversity tests, significant taxa, and environmental variables. The explanatory environmental variables were scaled and fit to the nMDS plots using the *envfit* function. Those with significant correlations ($p \leq .1$) to the community composition ordination were plotted with ggplot2 (Wickham, 2016). To determine which environmental variables were associated with differentially abundant taxa, the Spearman correlation between the relative abundance of those taxa and environmental variables that were significant by the *envfit* test were calculated for sampling periods in which a difference in community structure was detected. In the case of highly correlated variables (e.g., *S. alterniflora* stems and biomass), only the more influential variable as indicated by *envfit* was tested to increase power. The probability values for the correlation tests were adjusted by the Holm (1979) method to control the type I error rate at 0.05.

Table 1
The results of PERMANOVA of treatment effects on the UniFrac dissimilarity of the soil microbial community.

	DF	SS	R^2	F	Pr(> F)
2 months (June 2014)					
Plant	1	0.070	0.223	5.938	0.000
Fertilizer	1	0.025	0.080	2.120	0.046
Plant:Fertilizer	1	0.030	0.095	2.527	0.022
Residual	16	0.188	0.602		
Total	19	0.312	1.000		
7 months (November 2014)					
Plant	1	0.044	0.124	2.829	0.000
Fertilizer	1	0.031	0.085	1.943	0.026
Plant:Fertilizer	1	0.034	0.093	2.132	0.014
Residual	16	0.251	0.699		
Total	19	0.360	1.000		
13 months (May 2015)					
Plant	1	0.136	0.322	9.646	0.000
Fertilizer	1	0.029	0.067	2.018	0.047
Plant:Fertilizer	1	0.032	0.076	2.265	0.032
Residual	16	0.226	0.535		
Total	19	0.423	1.000		
19 months (November 2015)					
Plant	1	0.019	0.080	1.314	0.202
Fertilizer	1	0.017	0.070	1.151	0.299
Plant:Fertilizer	1	0.015	0.060	0.987	0.428
Residual	13	0.192	0.790		
Total	16	0.243	1.000		
26 months (June 2016)					
Plant	1	0.025	0.067	0.985	0.414
Fertilizer	1	0.018	0.048	0.705	0.647
Plant:Fertilizer	1	0.023	0.064	0.935	0.447
Residual	12	0.299	0.821		
Total	15	0.364	1.000		

Treatment plots were either planted with *Spartina alterniflora*, or not planted, and fertilized or not fertilized.

3. Results

3.1. Community composition

Significant differences in community composition in terms of the positions of the multivariate centroids between treatments were detected at two months, seven months, and 13 months after initiation of the experiment (June 2014–May 2015; Table 1). The effect of the interaction between planting and fertilization on the positions of the centroids was significant for each of those time points. A significant difference in the dispersion around the treatment centroids was found only at the seven-month time point (Supplementary Table 1). The results at each time point are described in further detail below.

At two months, the effect of the interaction between planting and fertilization on community composition in terms of the positions of the centroids was significant (PERMANOVA $p = .022$; Table 1). Pairwise testing indicated significant differences in community composition between N- and both S+ and S- treatments, and between N+ and S− (Table 2). No effect of fertilizer application on community composition was detected within the planting treatments (between N+ and N- or between S+ and S-; Table 2). Microbial community composition was significantly related to *S. alterniflora* biomass and stem density, *D. spicata* biomass and stem density, total biomass, and soil nitrate concentration (Table 3). At this point, *S. alterniflora* biomass was closely related to total biomass as very little natural vegetation recruitment into the unplanted plots had occurred. *S. alterniflora* biomass explained > 50% of the variation in the microbial communities' dissimilarity (Table 3). nMDS ordination showed that the soil microbial communities in N+ and N− treatments separated from each other along a gradient of soil nitrate concentration (Fig. 1A), which explained 36% of the variation in the ordination (Table 3).

Table 2
Pairwise PERMANOVA of the UniFrac dissimilarity of the soil microbial community between each level of treatment at time points when significant interactions were detected.

Contrast	SS	F	R^2	p.adj
2 months (June 2014)				
Not planted Unfertilized vs Not planted Fertilized	0.043	3.42	0.299	0.287
Not planted Unfertilized vs Spartina Unfertilized	0.035	4.077	0.338	0.048
Not planted Unfertilized vs Spartina Fertilized	0.026	2.399	0.231	0.053
Not planted Fertilized vs Spartina Unfertilized	0.069	5.423	0.404	0.047
Not planted Fertilized vs Spartina Fertilized	0.064	4.323	0.351	0.103
Spartina Unfertilized vs Spartina Fertilized	0.012	1.061	0.117	1
7 months (November 2014)				
Not planted Unfertilized vs Not planted Fertilized	0.035	1.591	0.166	1
Not planted Unfertilized vs Spartina Unfertilized	0.039	3.731	0.318	0.043
Not planted Unfertilized vs Spartina Fertilized	0.03	3.108	0.28	0.052
Not planted Fertilized vs Spartina Unfertilized	0.045	2.064	0.205	0.301
Not planted Fertilized vs Spartina Fertilized	0.039	1.848	0.188	0.563
Spartina Unfertilized vs Spartina Fertilized	0.029	3.091	0.279	0.251
13 months (May 2015)				
Not planted Unfertilized vs Not planted Fertilized	0.045	3.238	0.288	0.051
Not planted Unfertilized vs Spartina Unfertilized	0.081	5.923	0.425	0.046
Not planted Unfertilized vs Spartina Fertilized	0.076	5.441	0.405	0.044
Not planted Fertilized vs Spartina Unfertilized	0.089	6.213	0.437	0.047
Not planted Fertilized vs Spartina Fertilized	0.087	5.985	0.428	0.056
Spartina Unfertilized vs Spartina Fertilized	0.015	1.068	0.118	1

P-values were adjusted by a Bonferroni correction to control the type I error rate in multiple tests.

At seven months, the interaction of planting and fertilization on community composition in terms of the positions of the centroids was significant (Table 1, PERMANOVA $p = .014$), and there was a significant difference in community composition in terms of variability between treatments ($p = .007$, Supplementary Table 1). Post hoc tests of revealed that the position of the N− centroid was significantly different from both S− and S+, but no differences in position between the other treatments were detected (Table 2). Pairwise contrasts of dispersion indicated that the N+ treatment had significantly greater variability than the other treatments (Fig. 2 and Supplementary Table 2). A substantial quantity of *D. spicata* biomass recruited into the unplanted plots (averaging 264.3 and 377.3 g m^{-2} in N− and N+, respectively; Supplementary Table 3) and was the sole environmental variable of those we measured significantly associated with microbial community composition at that time point (Table 3).

The interaction of planting and fertilization had a significant effect on the positions of the group centroids at 13 months ($p = .032$), but transplants explained a substantially greater portion of the variance in community composition than fertilizer (32% vs 7%, Table 1). Pairwise comparisons indicated there was no difference in community composition between S- and S+, but the community compositions of all the other treatments were significantly different from each other (Table 2). Microbial communities in the transplanted treatments were clearly separated from the unplanted treatments along a gradient of increasing dead biomass and *S. alterniflora* biomass, and those that did not receive transplants separated toward increasing *D. spicata* stems (Fig. 1C). Meanwhile, microbial communities in the N− and N+ treatments separated along the soil ammonium concentration and, to a lesser extent, pH gradients. Dead aboveground biomass was most strongly related to microbial community composition, followed by soil ammonium concentration (Table 3).

No significant differences in community composition were detected among treatments at 19 or 26 months. Belowground biomass was moderately related to community structure at 19 months, and at 26 months *D. spicata* biomass, total biomass, and total dead biomass were significantly related to the microbial community structure in the plots ($P < .1$, Table 3). However, these differences were not attributable to the treatments we applied because the effects of the treatments on community structure were not significant at these time points. Moreover, samples collected at 19 and 26 months corresponded with the phase in the marsh's recovery after the unplanted treatments were naturally revegetated. The results at these time points no longer reflect a comparison between the microbial communities in vegetated and unvegetated marsh soils, but are indicative of a convergence in the soil microbial community compositions between the planted and unplanted treatments following natural vegetation recovery.

3.2. Microbial abundances

We determined which taxa (i.e., the OTUs resulting from agglomerating ASVs at the approximate genus level) were differentially abundant between treatments at time points where significant differences in community composition were detected (Supplementary Table 5). At two months, a *Staphylococcus* taxon was greater in N+ than all other treatments, accounting for the substantial increase in phylum Firmicutes (Fig. 3). No taxa differed significantly between S− and S+. A taxon within Desulfobulbaceae was significantly more abundant in both S- and S+ than N-. A taxon from the family Sinobacteraceae, a lineage containing primarily bacterial strains isolated from polluted environments (Zhou et al., 2008), was significantly greater in the N- treatment than S-.

At seven months, November 2014, the N+ treatment had significantly less differentially abundant taxa from the order Bacteroidales, the family Ectothiorhodospiraceae, and the Deltaproteobacteria order NB1-j than all other treatments, and more of a *Dyella* taxon. Several differentially abundant taxa within the Gammaproteobacteria family HTCC2089, which consists of marine oligotrophs (Cho and Giovannoni, 2004), were greater in the *S. alterniflora* treatments than in the unplanted treatments, regardless of

Table 3
The correlation of environmental variables to nMDS ordinations of the UniFrac dissimilarity of the soil microbial community at five sampling times.

	2 months		7 months		13 months		19 months		26 months	
	R^2	P-val.	R^2	P-val.	R^2	P-val.	R^2	P-val.	R^2	P-val.
Spartina stems	0.528	0.002	0.139	0.297	0.166	0.213	0.158	0.261	0.203	0.259
Spartina biomass	0.549	0.002	0.062	0.604	0.412	0.012	0.129	0.376	0.089	0.584
Distichlis stems	0.380	0.017	0.478	0.007	0.249	0.089	0.150	0.255	0.519	0.012
Distichlis biomass	0.416	0.015	0.513	0.005	0.192	0.168	0.117	0.386	0.605	0.003
Total biomass	0.525	0.004	0.019	0.851	0.392	0.013	0.048	0.713	0.445	0.028
Total dead biomass	0.175	0.181	0.028	0.761	0.556	0.001	0.080	0.571	0.523	0.007
Belowground biomass	0.288	0.066	0.041	0.704	0.128	0.305	0.270	0.095	0.154	0.348
pH	0.202	0.149	0.054	0.571	0.238	0.092	0.254	0.117	0.274	0.138
Nitrate	0.360	0.021	0.171	0.204	0.121	0.327	0.086	0.519	0.208	0.254
Ammonium	0.228	0.118	0.033	0.742	0.464	0.008	0.091	0.482	0.267	0.161

The stress values for the ordinations were < 0.15.

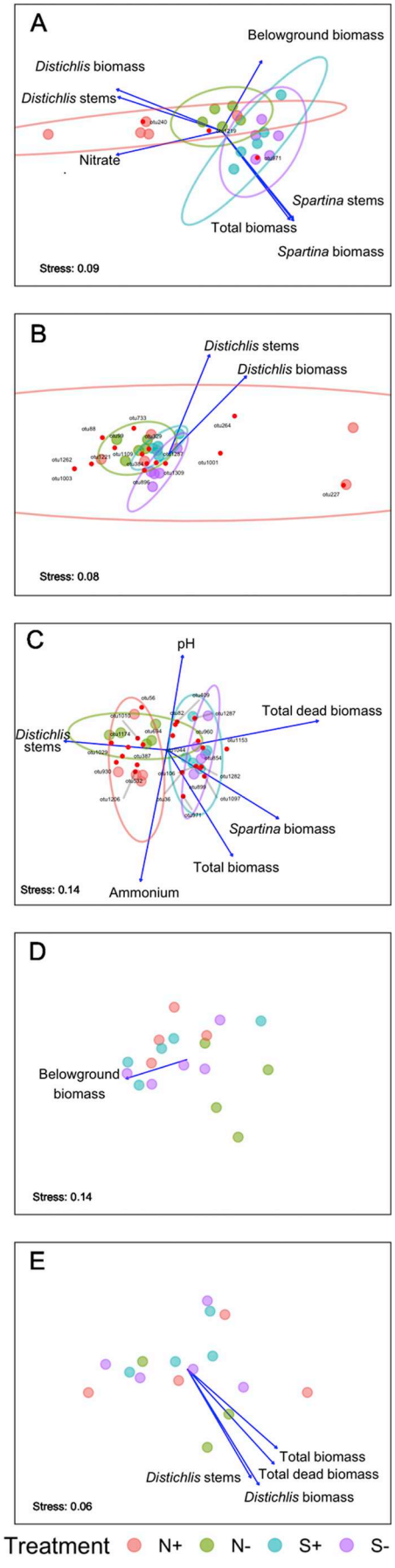

Fig. 1. nMDS bi-plots of the UniFrac dissimilarity of soil microbial communities. Environmental variables significantly correlated to the ordination (blue arrows), and differentially abundant OTUs (red points). The treatments include: no transplants plus fertilizer (N+, pink); no transplants and no fertilizer (N−, green); Spartina alterniflora transplants plus fertilizer (S+, blue); and S. alterniflora transplants and no fertilizer (S−, purple). Each plot represents a sampling date after initiating the treatments: (A) 2 months, (B) 7 months, (C) 13 months, (D) 19 months, and (E) 26 months. Ellipses represent 95% confidence intervals. No difference in community composition was detected between the treatments at 19 or 26 months, so confidence intervals are not shown and differentially abundant OTUs between treatments were not tested. Only OTUs with means > 20 are plotted in C to reduce crowding. Taxonomic identification and the results of differential abundance tests can be found in Supplementary Table 6. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

fertilizer application. Conversely, a differentially abundant taxon from the order Solirubrobacterales, a clade that has been isolated from Antarctica and is likely resistant to strong UV (Pulschen et al., 2017), was greater in the unplanted treatments than in the *S. alterniflora* treatments. The S- treatment had significantly less of a taxon from the family JTB36 in the *Deltaproteobacteria* than all other treatments. A differentially abundant taxon from the family Rhodobacteraceae, a family found in high abundances in biofilms (Elifantz et al., 2013) and in association with algae (Dogs et al., 2017), was less abundant in N- than all other treatments.

The greatest number of differentially abundant taxa occurred at 13 months, and the treatments with the greatest number of differentially abundant taxa between them were the N- and S+ treatments with 57. All of the putative sulfur/sulfate-reducing bacteria (SRB) were more abundant in the *S. alterniflora* treatments than in the unplanted treatments, with the one exception of a *Desulfobacca* taxa that was more abundant in N- than S+ (Fig. 4). There was also variation within the putative SRB in the unplanted treatments based on fertilizer application; SRB from the genera *Desulfococcus* and *Desulfosarcina*, as well as a Syntrophobacteraceae sp. were significantly more abundant in the N- treatment, whereas those within the Desulfobulbaceae, Desulfarculaceae, and Desulfuromonadales were significantly more abundant in the N+. A highly abundant Deltaproteobacteria taxa from the order MBNT15 was significantly more abundant in the N− treatment than in all other treatments. The proliferation of this taxa accounted for the similar relative abundances of Deltaproteobacteria among the N− treatment and the planted treatments; the N+ treatment, in which the taxa did not proliferate, had relatively less Deltaproteobacteria at this time point (Fig. 3). Another highly abundant taxon from the order MND1 within the Betaproteobacteria was significantly more abundant in the unplanted treatments than in the *S. alterniflora* treatments, regardless of fertilizer application. Several taxa within the phylum Chloroflexi were differentially abundant among the N− treatment and all other treatments; only one taxon from this phylum was differentially abundant between the N+ and S+ treatments, and none were differentially abundant between the N+ and S- or S+ and S- treatments. All but one of the differentially abundant taxa within the class Gammaproteobacteria were significantly more abundant in the S+ and S−. treatments than in the N+ treatment. A taxon within the archaea order pGrfC26 as well as an Ignavibacteriaceae taxon were significantly less abundant in the S+ treatment than all other treatments; they accounted for two of only four taxa that were differentially abundant between S− and S+.

We investigated the relationship between differentially abundant taxa and the environmental variables measured that were significantly related to the microbial community structure. No taxa were correlated with environmental variables at two or seven months, but at 13 months, several taxa were significantly correlated with total dead vegetation biomass after adjustment for multiple testing (Fig. 5). A taxon within Thermodesulfovibrionaceae (otu293; $p = .007$, $\rho = 0.801$),

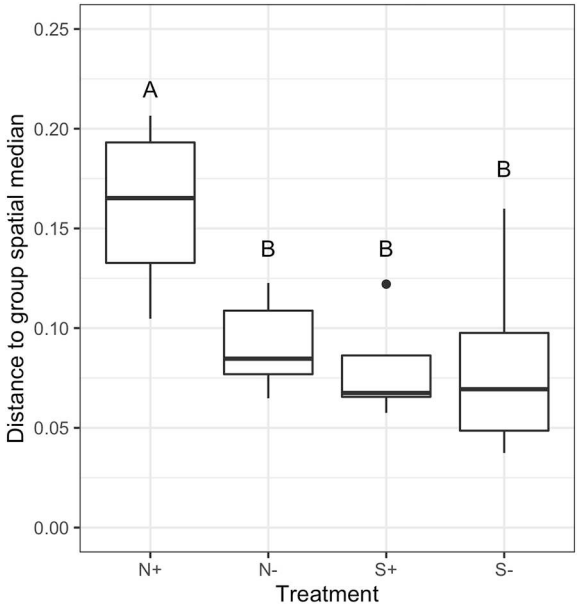

Fig. 2. The average distance, in the space of the UniFrac dissimilarity of microbial community composition, of samples to the spatial median of their treatment group seven months after initiating treatments. The treatments include: no transplants plus fertilizer (N+); no transplants and no fertilizer (N−); *Spartina alterniflora* transplants plus fertilizer (S+); and *S. alterniflora* transplants and no fertilizer (S−). Letters indicate significant differences between treatments from pairwise contrasts.

Desulfuromonadaceae (otu856; $p = .016$, $\rho = 0.781$), and an unidentified Gammaproteobacteria taxon (otu1212; $p = .006$, $\rho = 0.806$) were significantly and positively correlated with total dead biomass. A Gammaproteobacteria taxon in the order HTCC2089 (otu1308; $p = .017$, $\rho = -0.779$) was significantly negatively correlated with total vegetation dead biomass.

3.2.1. Alpha diversity

There was a significant interaction of planting, fertilizer, and sampling date on alpha diversity ($F_{4,71} = 5.33$, $p = .001$; Supplementary Table 4). Diversity was not different at any point between the S+ and S- treatments, nor did diversity in either change significantly over the course of the experiment (Fig. 6, Supplementary Table 4). No significant differences in alpha diversity were observed until 13 months after initiation, May 2015, at which time diversity in the N+ treatment was significantly higher than all other treatments at that time point (vs N−: $t = 4.630$, $p = .001$; vs S+: $t = 4.070$, $p = .008$; vs S-: $t = 3.677$, $p = .032$) as well as compared to the first observation at 2 months ($t = -6.012$, $p < .001$). Aside from the increase in diversity in the N + treatment, none of the other treatments differed from each other or changed significantly over time. Diversity tended to decline in the N+ treatment from 13 months to 19 months so and no differences were observed among the treatments at that time point. After 26 months, N+ had significantly greater diversity than at 2 months ($t = -5.174$, $p < .001$), and diversity in both N− and N+ was significantly greater than in S− ($t = 4.498$, $p = .002$, and $t = 4.431$, $p = .002$).

4. Discussion

The purpose of the study described herein was to test the effects of planting, fertilization, and their interaction on the recovery of a marsh shoreline that had been denuded by heavily oiling four years prior. We compared the effects of these treatments to untreated controls in order to produce results that could support recommendations for the restoration of salt marsh habitat impacted by future oil spills. The microbial responses in the first 13 months were largely the result of the planting and fertilization treatments we applied and represent important information for a critical period of time during which recovery of the benthic community was occurring (Johnson et al., 2018). The natural recruitment of vegetation with time corresponded with the convergence of the microbial community compositions between the planted and unplanted treatments in the period of 13 to 26 months. Although the establishment of vegetation in the unplanted treatments during this later period precludes making comparisons between

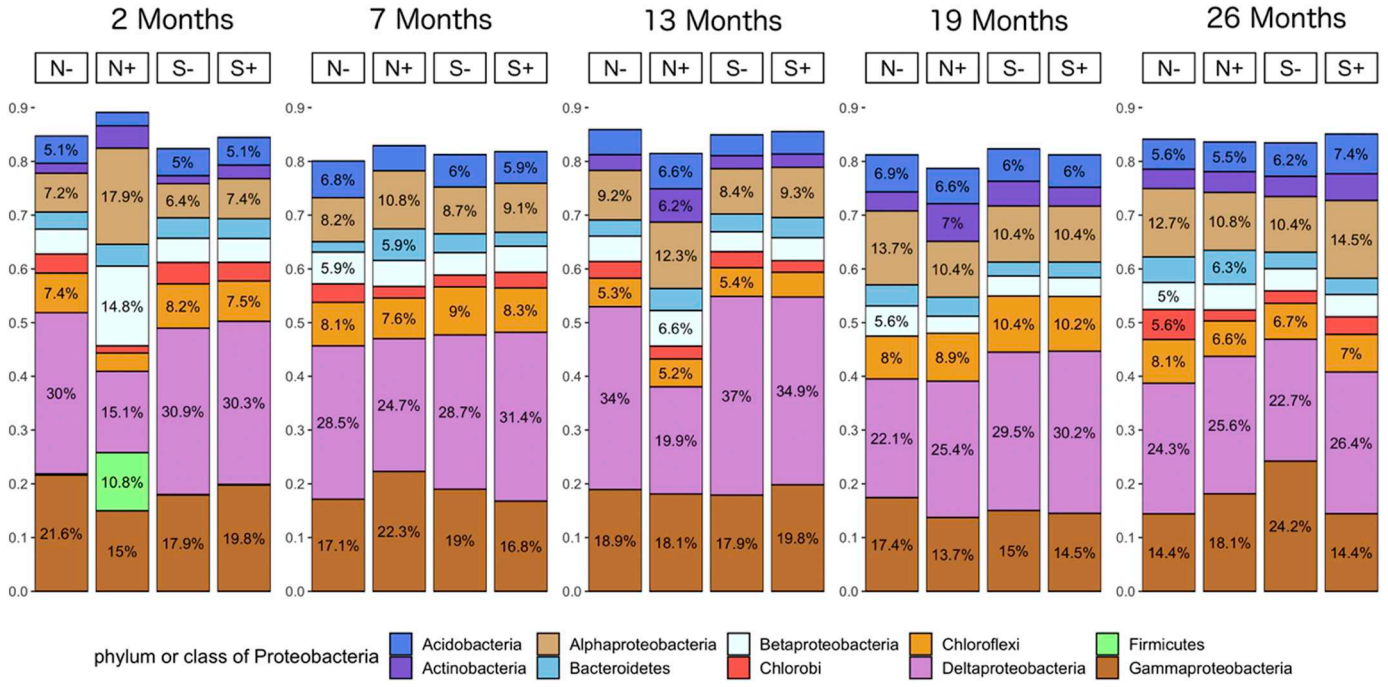

Fig. 3. The mean relative abundances of the top 4 phyla (Proteobacteria, the most dominant abundant phyla, is shown by class) in each treatment at five sampling time points after initiating the experiment. The treatments include: no transplants and no fertilizer (N−); no transplants plus fertilizer (N+); *Spartina alterniflora* transplants and no fertilizer (S−); and *S. alterniflora* transplants plus fertilizer (S+). Phyla with relative abundance values < 5% are omitted.

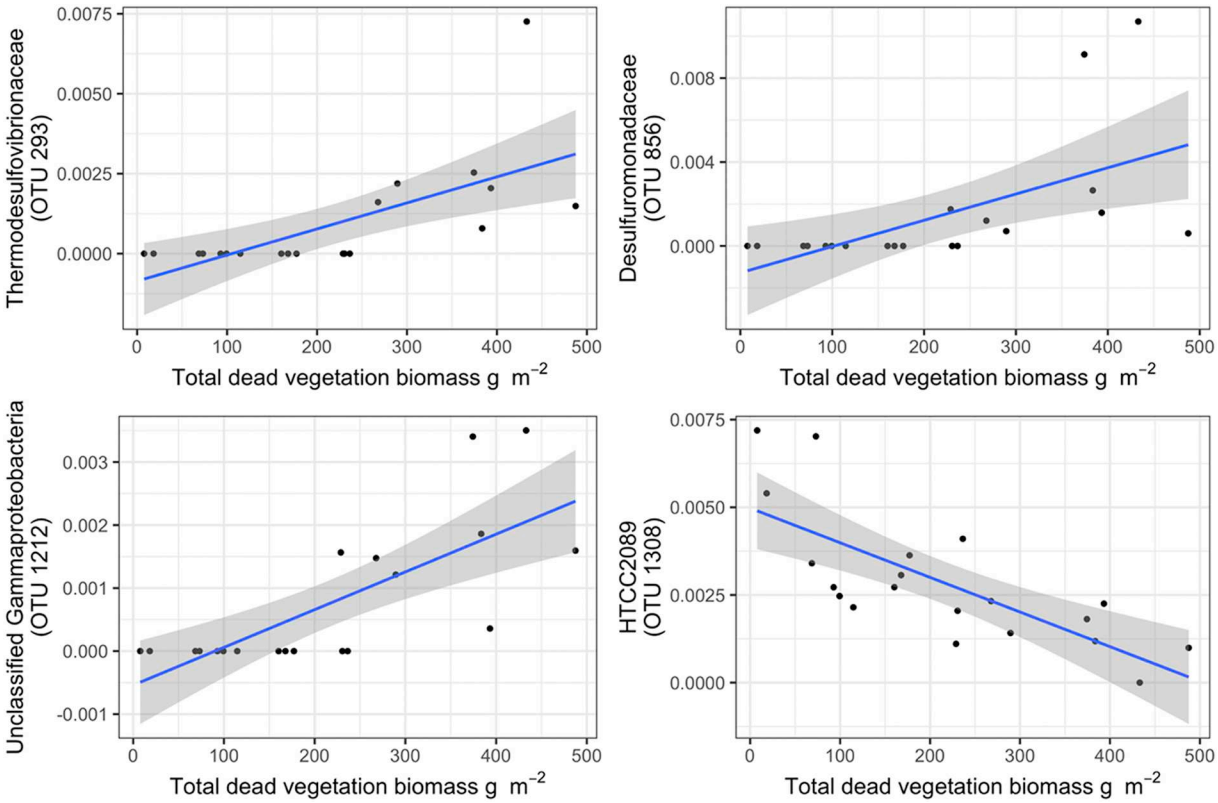

Fig. 4. The relative abundance of taxa plotted with environmental variables they were significantly correlated to 13 months after initiating the experiment. The lowest assigned taxonomy for the taxa are shown.

vegetated and unvegetated systems, the convergence of the microbial communities under vegetated conditions supports our result that vegetation has a significant effect on microbial community composition in oil-impacted salt marshes denuded of vegetation.

4.1. *S. alterniflora* influence on microbial diversity

In the present study, we found that *S. alterniflora* transplants strongly affected microbial community composition, structure, and diversity for 13 months after initiating treatment. The *S. alterniflora* rhizosphere is a hotspot for carbon and nutrient cycling compared to unvegetated soils; it supports greater numbers of SRB (Zheng et al., 2017) and substantially greater denitrification (Hinshaw et al., 2017), and it hosts larger numbers of methanogens (Franklin et al., 1988). Differences in community composition among the treatments were greatest at 13 months, at which time the treatments that received *S. alterniflora* transplants had a microbiome that included significantly greater abundances of a cohort of putative SRB than the unplanted

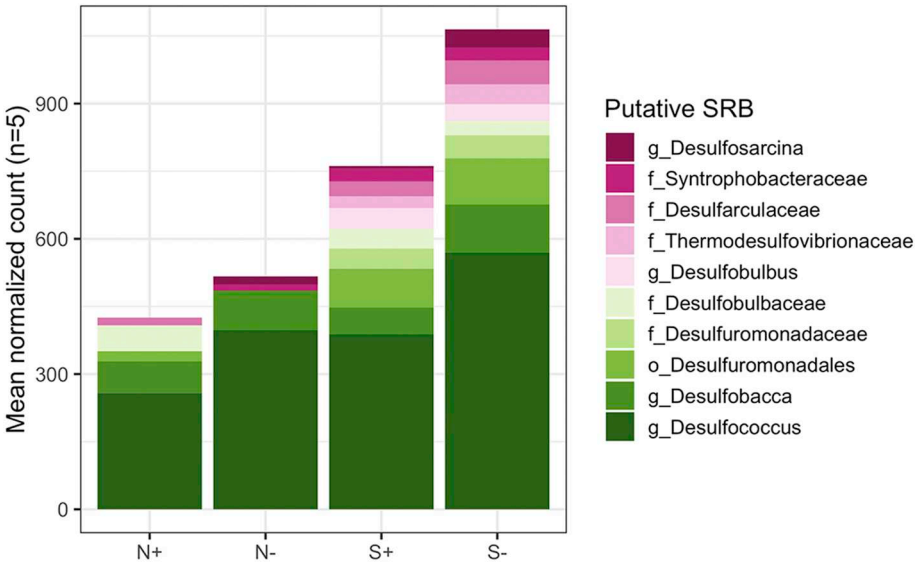

Fig. 5. The abundance of putative sulfur/sulfate-reducing bacteria (SRB) that were differentially abundant 13 months after initiating the treatments. Counts were normalized to account for sequencing depth by the DESeq2 method. The lowest assigned taxonomy for an OTU is given.

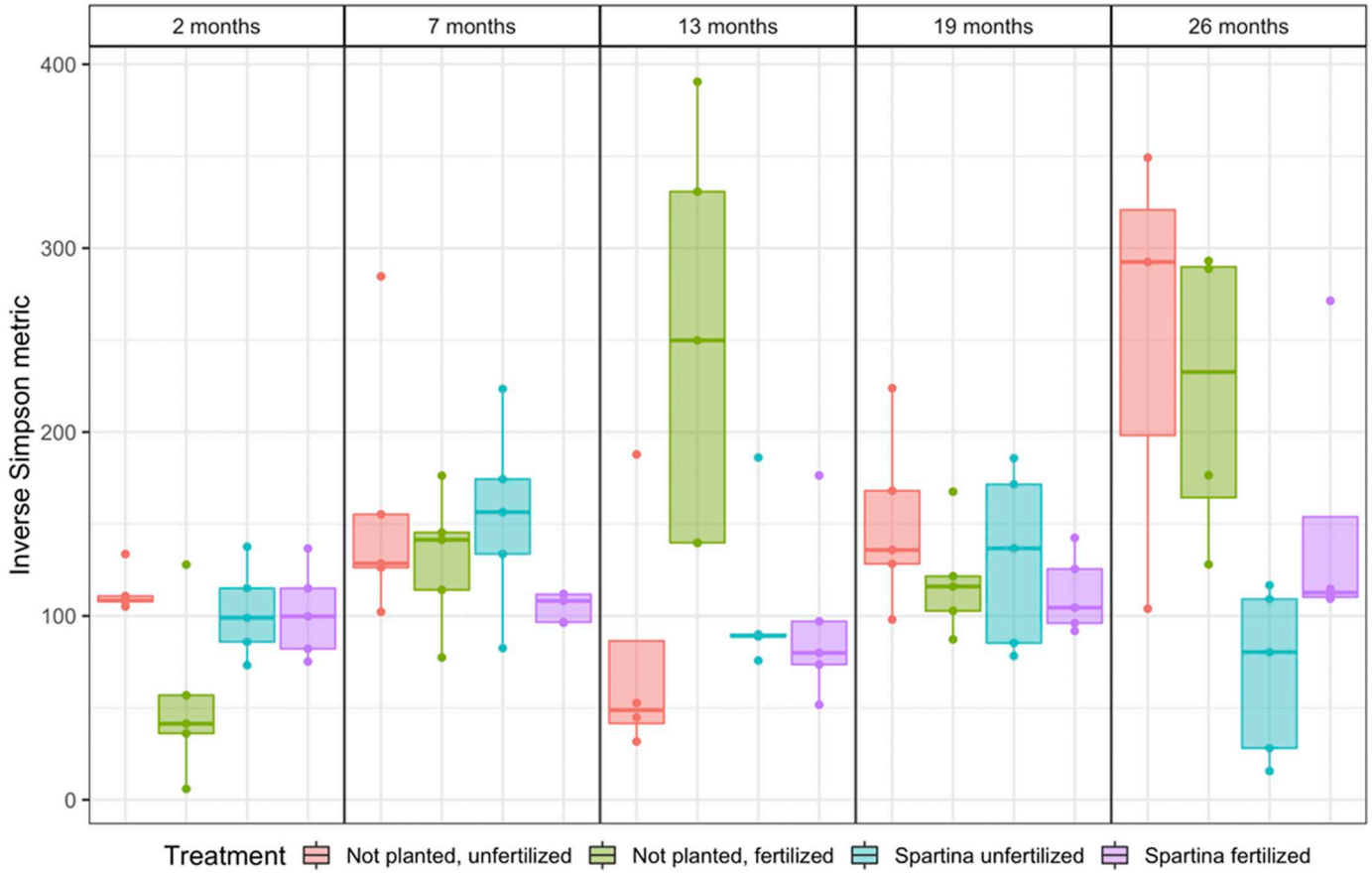

Fig. 6. Alpha diversity within treatments over the course of the experiment. The treatments include: no transplants and no fertilizer (N−); no transplants plus fertilizer (N+); *Spartina alterniflora* transplants and no fertilizer (S−); and *S. alterniflora* transplants plus fertilizer (S+). Bars indicate the median, boxes extend from the first to the third quartile, and whiskers extend to the largest or smallest value at most 1.5*IQR (inter quartile range).

treatments.

The effect of *S. alterniflora* on SRB is of particular interest because at least 50% of decomposition in salt marshes proceeds via sulfate reduction (Howes et al., 1984). *Spartina alterniflora* is well known to harbor a diversity of SRB (Rooney-Varga et al., 1997; Llobet-Brossa et al., 2002; Bahr et al., 2005; Zheng et al., 2017). The availability of carbon compounds controls sulfate reduction activity in salt marshes (Bahr et al., 2005), and thus plants play a role in regulating SRB. Sulfate reduction rates are greatest during periods of aboveground plant growth, when carbohydrates are mobilized in the rhizome and leak into the rhizosphere (Hines et al., 1989). Roots also regulate microbial activity in the surrounding environment, though ethanol and malate produced during anaerobic metabolism (Mendelssohn et al., 1981) that can be used directly by many SRB. The significantly greater abundances of a diverse group of SRB that we identified in the transplanted treatments relative to the unplanted treatments suggests that the *S. alterniflora*-mediated soil environment better supports this metabolic pathway. The abundances of two of the differentially abundant putative SRB, Thermodesulfovibrionaceae and Desulfuromonadaceae, were significantly and positively correlated with dead vegetation biomass. When aboveground tissue senesces, nutrients and carbohydrates are translocated from above- to belowground tissues. During this process, some leaches out into the soil and thereby provides substrates for SRB. The availability of the complex mixtures of carbon compounds that support the metabolism of SRB was likely increased by transplanting *S. alterniflora* in this study.

It is also notable that the *S. alterniflora* treatments consisted of a greater diversity of Gammaproteobacteria than the N+ treatment and a greater diversity of Chloroflexi than the N− treatment at 13 months. These groups are commonly reported in salt marsh rhizospheres (e.g. Lv et al., 2016; Rietl et al., 2016; Engel et al., 2017) and therefore may be integral to ecosystem functional processes. However, the diversity within these groups beyond the phylum or class level is frequently not well described in the literature on surveys of microbial communities in wetland ecosystems. In the present study, the detection of treatment effects on microbial abundances at finer taxonomic levels points to the importance of considering changes beyond the phylum or class level. For example, Deltaproteobacteria was dominated by a single taxon (MBNT15) in the N− treatment at 13 months when abundances of most putative SRB, primarily members of Deltaproteobacteria, were significantly lower than in the *S. alterniflora* treatments.

Over the course of the experiment, the microbial community composition in the treatments was consistently and significantly structured by vegetation biomass. It has been suggested that vegetation plays an important role in modifying the physical conditions in salt marshes as well as the soil chemistry (Whitcraft and Levin, 2007). Consistent with this hypothesis, the significantly greater abundances of a taxon within the extremophilic family Solirubrobacterales in the unplanted treatments compared to the *S. alterniflora* treatments at 7 months, perhaps indicated an influence of UV on the soil microbial community. Alpha diversity increased in the unplanted treatments over time but remained remarkably consistent in the *S. alterniflora* treatments over the course of the study. We hypothesize that the growth of *D. spicata* along with *S. alterniflora* in the unplanted plots later in the experiment may have driven the increase in species richness, whereas transplanted treatments maintained specific populations of bacteria and archaea suited to the *S. alterniflora* rhizosphere. Plants dramatically alter the rhizosphere through enrichment with diverse carbon compounds (Brüggemann

et al., 2011; Paterson and Sim, 2013) and differences in root morphology, which can select for specific microorganisms (Berg and Smalla, 2009; Zogg et al., 2018). Two months after initiating the experiment, we observed a significant shift in the soil microbial communities in treatments that received *S. alterniflora* transplants relative to unplanted treatments. At the same time point, collaborators determined that the density of total macroinfauna, primarily consisting of the polychaete *Capitella capitata,* was significantly greater in the transplanted compared to the unplanted treatments (Johnson et al., 2018). It is possible that greater density of macroinfauna in the *S. alterniflora* treatments contributed to the rapid shift in the soil microbial communities in transplanted relative to unplanted treatments in this study. Benthic invertebrates modify the soil environment and the soil microbial community through bioturbation and grazing (Hunting et al., 2012). Together, these results suggest that *S. alterniflora* plays an important role, directly and indirectly, in the establishment of the soil microbial community.

The rapid response of the microbial community to the planting of *S. alterniflora* shares some similarities with the recovery of the benthic invertebrate community following oiling from the DWH spill. Invertebrate recovery in marshes that were heavy oiled (resulting in near complete plant mortality, Lin and Mendelssohn, 2012) was strongly related to the recovery of *S. alterniflora* (Deis et al., 2017; Fleeger et al., 2015, 2018, 2019). For example, some infauna in oiled marshes denuded of vegetation recovered to densities equivalent to unoiled reference sites in about a year after the regrowth of *S. alterniflora* began (Fleeger et al., 2015). These responses to revegetation are similar to those described in this paper and suggest possible associations between the microbial community and the recovery from oiling by the broader salt marsh community. For example, microbes and invertebrates may both directly respond to factors associated with root and rhizome growth that affect soil structure; however, it is also possible that enhancement of SRB associated with revegetation increases carbon and nutrient cycling that aids in the recovery of invertebrates. The rapid response of the microbial community to planting and the possible contribution of microbes to enhancing the recovery of the broader salt marsh community suggest that revegetation is an ecologically important step in recovery after oil spills. The influence of vegetation on the microbial community documented herein supports the recommendation made by Fleeger et al. (2019) that planting *S. alterniflora* could be beneficial for habitat restoration after future oil spills.

4.2. Different effect of fertilizer in the absence of transplants

Fertilizer affected the soil microbial community differently when *S. alterniflora* was planted compared to fertilizer applied in the absence of transplants, but the response over the course of the study was not simply a clear increase in the abundance of copiotrophs as we hypothesized. However, the observed effect of fertilizer on community structure in unplanted treatment but not in the transplanted treatment during the first 13 months of the experiment supports our hypothesis that fertilizer would have a greater impact in the absence of transplants. Fertilizer applied to the unplanted treatment resulted in a bloom of *Staphylococcus* at two months, an increase in community dispersion after seven months, and a directional shift in community composition along a gradient of increasing soil ammonium concentration at 13 months that led to a significant difference in the microbial community composition between the N- and N+ treatments in terms of their multivariate position. The growth of *Staphylococcus* at two months supports our hypothesis that nutrient additions in the absence of plants would promote the growth of copiotrophic taxa, but the responses at seven months and 13 months suggest a different type of response to the nutrient additions.

The increase in microbial community dispersion (i.e., the development of a wider variety of phylogenetically distinct taxa) at seven months in the N+ treatment compared to the other treatments may represent a response of the microbial community to fertilization. Such heterogeneity has been widely reported in animal microbiomes in response to stress (reviewed in Zaneveld et al., 2017). The N+ treatment at seven months appeared to follow such a stress-response pattern. However, the increased dispersion observed in this treatment could have been driven by increased variation among replicates due to the stochasticity of vegetation recruitment enhanced by fertilizer application. Indeed, the replicates in the N+ treatment with the greatest distance from the spatial group median had the greatest *D. spicata* biomass (Fig. 1B). Therefore, additional experiments with the capacity to control for stochastic recruitment of vegetation are needed to determine if fertilizer application in the absence of transplants induces microbial community dispersion in salt marsh soils. This response deserves further consideration because the hypothesis that microbial communities respond to perturbations with increased variation rather than steady-state shifts has implications for understanding how biogeochemical functions in salt marshes will respond to stressors.

A prior study on the effects of fertilizer on salt marshes by Bowen et al. (2011) found that fertilization at a range of nutrient loading rates from 0.6–7.46 g N m^{-2} $week^{-1}$ in a vegetated ecosystem had no effect on the microbial community structure or diversity. The lowest application rate tested by Bowen et al. (2011) was comparable to that of the present study, and the absence of an effect of fertilizer under vegetated conditions is consistent with our finding of no significant difference in community structure between fertilized and unfertilized *S. alterniflora* treatments at any time point. Because plants are stronger competitors for N fertilizer than soil microbes (Inselsbacher et al., 2010), fertilizer application is expected to have a stronger effect on an unvegetated than a vegetated system. A recent study has indicated that long-term fertilizer application to a wetland altered the active microbial community structure represented by RNA, but not the total community structure represented by DNA (Kearns et al., 2016). We did not conduct RNA sequencing in the present study but based on Kearns et al. (2016), we would expect that the effect of fertilizer on the active community as indicated by RNA transcript abundances would be significant. The research presented here adds to the understanding of the work by Bowen et al. (2011) and Kearns et al. (2016) by demonstrating that fertilizer effects the total microbial community when applied to denuded salt marsh soils, such as may be the case during restoration of habitat in an oil-impacted wetland.

5. Conclusion

In this study, we evaluated how factorial combinations of planting and fertilization, potential habitat restoration strategies for promoting ecosystem function in salt marshes denuded of vegetation by a prior oiling event, affected the soil microbial community diversity, structure, and taxa abundances at five time points over 26 months. We found that *S. alterniflora* transplants rapidly and significantly altered the soil microbial community composition, and that fertilizer had a significant effect on the soil microbial community structure in the absence of transplants during the first 13 months after initiating the treatments. Differentially abundant taxa after 13 months in treatments that received *S. alterniflora* transplants included more diverse cohorts of several groups common to salt marsh rhizosphere soils than those left unplanted after 13 months, including sulfur/sulfate-reducing bacteria as well as Gammaproteobacteria and Chloroflexi. These results suggest that planting accelerated the transition of the soil microbial community from an unvegetated to a vegetated type. The effect of fertilizer on the soil microbial community structure in unplanted treatments but not in treatments that received *S. alterniflora* transplants supports previous work showing that the total microbial community structure in vegetated salt marshes is resistant to fertilizer effects. We observed no effect of transplants or fertilizer treatment at 19 or 26 months, corresponding with the time when natural recruitment of vegetation closed the gap in vegetation biomass between unplanted and planted treatments. This

work indicates that planting *S. alterniflora* can rapidly induce changes in the indigenous soil microbial community in a salt marsh denuded by a prior oiling event, and broadens the understanding of how fertilizer affects salt marsh microbial communities. In accordance with previously published findings that planting *S. alterniflora* promotes recovery of benthic infauna (Johnson et al., 2018; Fleeger et al., 2015, 2019), the rapid response of the soil microbial community to revegetation with *S. alterniflora* supports the idea that planting can improve restoration of salt marsh habitat and ecosystem function following future oil spills.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoleng.2020.105815.

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