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Evaluating Plant-Microbe Associations in Response to Environmental Stressors to Enhance Salt Marsh Restoration

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Abstract

Despite substantial contributions of ecosystem services, mutualistic plant-microbe associations in wetlands are severely threatened by human activities. Therefore, promoting positive plant-microbe associations underpins coastal wetland restoration, where human stressors and climate change challenge successful outcomes. This study examined how salinity stressors influence plant-microbe relationships, where we hypothesized that the presence of marsh microbes would provide a rescue effect by buffering abiotic stressors and yielding higher plant biomass. We used a whole sediment inocula approach and exposed marsh cordgrass (*Sporobolus alterniflorus*) plugs to a factorial experiment with three levels of microbiome addition (microbial inocula, autoclaved microbial inocula, no microbe control) and two levels of salinity (<0.5 psu, 20 psu), replicated ten times. We added marsh-site microbial inocula with autoclaved peat-based greenhouse soil and exposed half the plugs to saltwater (20 psu) and half to freshwater (<0.5 psu). Results revealed that marsh microbial inocula additions during early plant development may ameliorate salinity stressors. Plants treated with microbial inocula and salinity stress exhibited greater aboveground biomass ($P<0.09$) than those under freshwater conditions. With live microbial inocula, the median of aboveground biomass and change in plant height were higher in saltwater compared to freshwater conditions. Change in plant height, belowground biomass, root-to-shoot ratio, soil bacterial diversity H', and evenness J' were similar across microbial and salinity treatments. However, salinity ($P<0.001$) and microbial treatments ($P<0.001$) significantly influenced the bacterial community composition, with distinct assemblages under salinity conditions. This work underscores the need for continued research to develop robust protocols for microbiome stewardship that enhance plant resilience and improve the success of future wetland restoration efforts.

Keywords Soil microbial communities · Salinity stress · Microbiome stewardship · Coastal protection

Introduction

Salt marshes, coastal wetlands, and other coastal ecosystems help mitigate erosion and flooding, protect communities and property from storm surges, efficiently sequester carbon dioxide, and provide habitat for fisheries and many other ecologically important species (Arkema et al., 2013; Duarte et al., 2013; Buchanan et al., 2022). Despite their high ecological and economic importance, these coastal habitats are under threat due to detrimental anthropogenic activities (e.g., land development, dredging) and climate change (e.g., accelerated sea level rise, increased mean global surface temperature, and frequency of heatwaves) (Valiela et al., 2018; Leonardi et al., 2018; Rudershausen et al., 2021; Buchanan et al., 2022; Trivedi et al., 2022). Unfortunately, current restoration efforts are failing to keep pace with current and future losses (Gittman et al., 2019), highlighting the necessity for improved salt marsh restoration techniques (Gittman et al., 2024).

The planting of marsh smooth cordgrass *Sporobolus alterniflorus* (also known as *Spartina alterniflora*) is a common approach to salt marsh restoration efforts, as the plant is a foundation species that can withstand a wide range of

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salinities, inundation periods, and anoxic stress (Gedan & Bertness, 2010). These foundation or habitat-forming species alter their environment, enabling other organisms to establish and persist within an ecosystem (Bruno, 2000). However, when commercially raised nursery plants are transplanted to field restoration sites, they are exposed to abiotic conditions (e.g., salinity and wave energy) and biotic factors (e.g., species interactions) that can induce stress and hinder restoration (Billah et al., 2022). Published studies evaluating the effects of exposing *S. alterniflorus* plants to abiotic stressors prior to establishment at a restoration site are lacking (but *see* Sloey et al., 2025). In addition, the understanding of plant-microbe associations between marsh soil microbiomes and *S. alterniflorus* and their influence on plant growth and survival is limited. Therefore, an examination of the effect of salinity stress and microbial inoculation on marsh plant productivity would further the understanding of ecological mechanisms and aid in restoring coastal wetland ecosystems.

In a broader context, microorganisms can enhance the nutrient acquisition of plants, support growth, and suppress diseases from pathogens (Mariotte et al., 2018; Farrer et al., 2022; Pantigoso et al., 2022; Chepsergon & Moleleki, 2023; Singh et al., 2023). In turn, plants can provide carbon resources and oxygen to root-associated microorganisms (Mariotte et al., 2018; Chepsergon & Moleleki, 2023). Human activities and climate change can and have altered these plant-microbe relationships in ways that promote or suppress plant growth (Cavicchioli et al., 2019; Rojas-Sánchez et al., 2025; Singh et al., 2023; Dini-Andreote, 2025). For example, anthropogenically caused eutrophication and pollution have disrupted carbon, nitrogen, and other nutrient cycles, leading to losses in microbial biodiversity and reduced fungal colonization of plants and microbial respiration (Wei et al., 2013; Cavicchioli et al., 2019; Lin et al., 2020). Global change, such as rising global surface temperatures, heatwaves, droughts, and sea level rise, can directly and indirectly modulate plant physiology, soil composition and chemistry, and microbial community assemblages, often leading to states of stress and dysbiosis (Trivedi et al., 2022; Dini-Andreote, 2025; Zabin et al., 2022). Conversely, the practice of microbiome stewardship, defined as the responsible and conscious management of microbial communities for beneficial conservation and restoration outcomes, can minimize or reverse biodiversity loss, as well as preserve key ecosystem functions and mechanisms (Peixoto et al., 2022; Choudoir et al., 2025). Growing evidence also indicates that microbial communities can enhance plant survivorship and growth in high-stress environments, potentially aiding ecological restoration efforts (McHugh & Dighton, 2004; Mariotte et al., 2018; Li et al., 2021; Rojas-Sánchez et al., 2025; Dini-Andreote, 2025).

With the urgent need for enhanced restoration techniques, we aim to understand how to promote positive plant-microbe associations to aid in restoring coastal wetland ecosystems where human stressors and climate change challenge restoration outcomes. In this investigation, we evaluated the interactive effects of salinity stress and marsh sediment microbial inoculation on *S. alterniflorus* productivity. Conducting a replicated factorial experiment with two levels of salinity and three levels of microbial inocula treatment, we measured the change in plant height and biomass (aboveground and belowground) of *S. alterniflorus* plugs after a 2-month duration. Additionally, we extracted genomic DNA from the sediment samples to characterize the soil microbiome of experimentally manipulated plants. As resident marsh microorganisms are well-adapted to a hypersaline environment, we hypothesized that *S. alterniflorus* plugs inoculated with established salt marsh sediment microbial communities would exhibit higher biomass than control plugs when subjected to salt stress.

Methods

Microbiome x Salinity Stress Experimental Design

To test our hypothesis that *S. alterniflorus* plugs inoculated with restoration-area microbial communities would have higher biomass than control plugs under salinity stress, we conducted a factorial experiment with three levels of microbiome addition (*No Addition*, *Autoclaved Inocula*, *Added Inocula*) and two levels of salinity (<0.5 psu, 20 psu), replicated ten times ($n=60$) (Fig. 1). We collected marsh sediment from Hammocks Beach State Park (Swansboro, North Carolina) and purchased *Sporobolus alterniflorus* marsh plugs, from a nearby commercial plant nursery (Garner's Landscaping, Inc., Newport, North Carolina) that routinely grows *S. alterniflorus* plugs from locally collected seeds for salt marsh restoration projects. The commercial marsh plugs were procured in 50-cell propagation trays, with one juvenile plant per 71 cm^3 cell and filled with standard greenhouse mix (Sun Gro ® Fafard peat-based potting mix).

To prepare the microbial treatments, we combined microbial inocula with Fafard 3B potting mix, with 5% of the total volume being marsh sediment and 95% being autoclaved potting mix (following the methods of Lau & Lennon, 2011). Specifically, we combined 10 g of marsh sediment with 190 g of potting mix in an 11.25 cm square pot prior to transplanting the plant plug (25 g average weight). While autoclaving can influence the chemistry of soil organic matter and the accessibility of plant nutrients, it creates a benchmark for sterilization and microbial community establishment. Any variations observed between

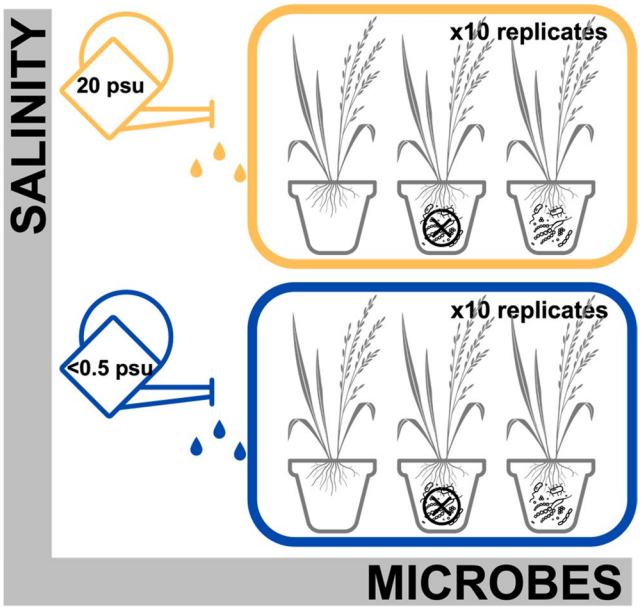


Fig. 1 Experimental design of salinity and marsh sediment microbial inocula treatments. Factorial experiment includes three levels of microbial addition (*No Addition*, *Autoclaved Inocula*, *Added Inocula*) and two levels of salinity (<0.5 psu, 20 psu). Treatments were replicated ten times. Microbial inocula were combined with potting mix and established for four weeks, followed by weekly salt stress treatment, and harvested after eight weeks

microbial treatments can be attributed to the introduction of inocula containing either live or dead soil microorganisms (Lau & Lennon, 2011).

The combination of untreated marsh sediment and autoclaved potting mix represented the *Added Inocula* treatment. For *Autoclaved Inocula*, marsh sediment was autoclaved before being combined, with the purpose of examining whether there were abiotic effects on plant productivity. While the microbial load may not have been completely erased, autoclaving ensured a decreased abundance of resident marsh microorganisms (31 sequence reads for autoclaved field soil compared to >26,000 sequence reads for all other sample types). For *No Addition*, 100% of the volume of the microbial treatment was comprised of autoclaved potting mix. Commercially grown *S. alterniflorus* plugs were obtained on 01 June 2023 and were transferred to 11.25 cm square pots with their respective treatments on 08 June 2023 to begin a four-week acclimation period to their new soil conditions. The plants were kept under laboratory conditions (room temperature at about 25 °C) next to a window for sunlight and were rotated daily to ensure even light exposure.

On 23 June 2023, the plants were transferred from the laboratory to an outside environment in Greenville, North Carolina, ~70 miles northwest of where the plants were purchased. We placed plants in trays that hold 15 pots on outdoor tables, where they received the same sunlight and precipitation conditions across all experimental units. We also began conducting biweekly measurements at this time, recording the maximum plant stem height (in mm) following standard methods (Currin et al., 2008; Gittman et al., 2018; Mendelssohn & Seneca, 1980; Morris & Haskin, 1990) and the number of live stems, with changes in stem height (final – initial) functioning as a proxy for growth. The four-week acclimation period concluded on 09 July 2023, at which point the weekly salinity stress treatment was initiated and continued for four weeks.

For the salinity treatment, we bottom-watered each potted plug for 15 min with either ~20 psu saltwater or ~0.5 psu freshwater. Saltwater was made with Instant Ocean Sea Salt, with a general ratio of ~300 g Instant Ocean to 14 L of tap water. This established a concentration of ~20 psu, approximating salinity levels at Hammocks Beach State Park, where salinity ranges from 20 to 35 psu (personal communication, park manager). Although the duration of salt exposure in our experimental setup may be shorter compared to natural field conditions experienced by plants, it was anticipated that the deposited salt would gradually accumulate in the soil due to evaporation, mimicking real-world scenarios. This deliberate manipulation allowed us to specifically examine the effects of salt stress on plant survival and biomass, distinct from the stress induced by prolonged inundation, which typically leads to anoxia. Thus, our experimental design ensured a focused investigation into salt stress dynamics without confounding stressors associated with prolonged inundation (Wang et al., 2019). Further, we opted for a short-duration, saltwater bottom-watering because it could be easily replicated by nurseries. Top-watering and subsequent freshwater flushing could be prohibitively labor- and equipment-intensive since saltwater would have to be flushed out of watering systems and equipment and inefficient to apply salinity treatments to multiple trays of plants simultaneously.

Plant and Soil Sample Processing

At the end of the experiment, after eight weeks total, we harvested aboveground and belowground biomass, dried the samples at 60 °C for 48 h, and then weighed the discrete biomass samples. For soil bacterial characterization and analysis, we homogenized the remaining soil, transferred soil into sterile WhirlPak bags, and stored samples at -20 °C until DNA extraction and 16 S rRNA amplicon sequencing (described below).

DNA Extraction and Bacterial Amplicon Sequencing

To characterize the sediment microbiomes of the laboratory-treated plugs as well as any community composition

differences, we extracted genomic DNA from sediments using the Qiagen DNeasy PowerSoil Kit, employing a combination of chemical and mechanical methods to lyse the sample following the manufacturer's protocol. The isolated DNA was quantified using the Nanodrop spectrophotometer and diluted to a standardized concentration of 10 ng μl^{-1} to ensure uniformity across all samples. This process facilitated equimolar concentrations of DNA for downstream applications. To characterize bacterial communities, we used barcoded primers (515FB/806R) initially developed by the Earth Microbiome Project (Caporaso et al., 2012) to target the V4 region of the bacterial 16 S subunit of the ribosomal RNA gene (Caporaso et al., 2012; Apprill et al., 2015; Parada et al., 2016). For each sample, three 50 µL PCR libraries were prepared by combining 35.75 µL molecular grade water, 5 µL Amplitaq Gold 360 10x buffer, 5 µL $MgCl_2$ (25 mM), 1 µL dNTPs (40mM total, 10mM each), 0.25 µL Amplitaq Gold 360 polymerase, 1 µL 515 forward barcoded primer (10 µM), 1 µL 806 reverse primer (10 µM), and 1 µL DNA template (10 ng μL^{-1}). Thermocycler conditions for PCR reactions were as follows: initial denaturation (94 °C, 3 min); 30 cycles of 94 °C for 45 s, 50 °C for 30 s, 72 °C for 90 s; final elongation (72 °C, 10 min). The triplicate 50 µL PCR libraries were combined and then cleaned using the Axygen® AxyPrep Magnetic (MAG) Bead Purification Kit (Axygen, Union City, California, USA). Cleaned PCR products were quantified using QuantIT dsDNA BR assay (Thermo Scientific, Waltham, Massachusetts, USA) and diluted to a concentration of 10 ng μL^{-1} before pooling libraries in equimolar concentration of 5 ng μL^{-1}. We sequenced the pooled libraries using the Illumina MiSeq platform using paired-end (2 × 250 bp) reads (Illumina Reagent Kit v2, 500 reaction kit) at the Duke University Center for Genomic and Computational Biology Core Sequencing Facility.

Sequences were processed using the mothur pipeline (v1.48.3) (Schloss et al., 2009; Kozich et al., 2013). We assembled contigs from the paired-end reads, quality trimmed using a moving average quality score (minimum quality score 35), aligned sequences to the SILVA rRNA database (v138.2) (Quast et al., 2013), and removed chimeric sequences using the VSEARCH algorithm (Rognes et al., 2016). We created operational taxonomic units (OTUs) by first splitting sequences based on taxonomic class and then binning into OTUs based on 97% sequence similarity. Taxonomic identity was assigned using the SILVA rRNA database (v138.2) (Quast et al., 2013). We used an OTU-based 3% distance threshold instead of using amplicon sequence variant (ASV) of a single base difference, especially given our high diversity microbial communities based on short-read (250 bp) amplicon sequences, and that similar broadscale microbiome patterns are observed using OTU- or ASV-based approaches (Glassman & Martiny, 2018). In addition, the OTU-based approach limits overestimation of the number of ASVs due to PCR bias and sequencing errors (Schloss, 2021).

Statistical Analyses

All statistical analyses, calculations, and graphical manipulations were run in the R environment (Rv4.5.1; R Studio 2025.05.1 + 513) using the vegan, ade4, and picante packages, and custom functions (Dray et al., 2023; Oksanen et al., 2022). We evaluated the main and interactive effects of inoculation and salinity on *S. alterniflorus* aboveground biomass, belowground biomass, root-to-shoot ratio (defined as the proportion of belowground biomass relative to aboveground biomass), and change in maximum plant stem height (calculated as the difference in maximum plant height measured at the start and end of the experiment). We tested parametric assumptions of normality by visualizing residuals using Q-Q plots and conducting Shapiro-Wilk tests. Since the data violated the normal distribution, we conducted Kruskal-Wallis tests and *post-hoc* Dunn's Test (Holm p-value adjustment, if significance was found) to assess group differences due to treatments. Given the modest sample size, a p-value < 0.05 threshold would substantially increase the risk of Type II error. We used a more permissive cutoff of p-value < 0.10 to identify potentially meaningful associations while minimizing Type II error (Di Stefano, 2003). We report exact p-values and interpret findings meeting p-value < 0.1 as suggestive rather than definitive.

Prior to microbial statistical analyses, we removed low-abundance OTUs that were represented by < 10 sequences across the data set. We also omitted two samples where the total sequences were less than 10,000 reads, which represented issues with the library preparation. We calculated the relative abundance by dividing each OTU by the total number of sequence reads for each sample prior to conducting community composition analyses. Samples were organized by salinity treatment and microbial treatments. Soil bacterial diversity metrics were then computed, including Shannon Diversity (H') and Pielou's Evenness (J') with corresponding ANOVA tests, providing insights into community composition by assessing both species richness and species evenness.

We evaluated the extent to which inoculation and salinity treatment explained variation in bacterial community composition using permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis dissimilarity. We also conducted a multivariate homogeneity of group dispersions using the betadisper

function in the vegan package (Table S5). We found that group dispersions according to main effects and the interaction of inoculation and salinity were similar. Therefore, significant PERMANOVA results are attributed to treatment differences and not differences in group dispersions. Next, Principal Coordinates Analysis (PCoA), based on Bray-Curtis dissimilarities, was used to visualize relationships among samples according to bacterial community composition.

To identify prominent indicator species within the bacterial communities, we used the package 'indicspecies'. Indicator species, which are selected based on their ability to reflect treatment groups, can indicate changes in the environment and predict diversity patterns (De Cáceres et al., 2010). The indicspecies package facilitates this analysis by examining the relationship between species occurrence or relative abundance and site classification into groups representing habitat types or disturbance states (De Cáceres et al., 2010).

Results

Marsh Microbial Addition and Salinity Stress Influences Plants to Varying Degrees

The effects of salinity (<0.5 psu and 20 psu) and microbial treatments (*No Addition*, *Autoclaved Inocula*, *Added Inocula*) produced mixed results for *S. alterniflorus* height and biomass. The change in maximum plant stem height (final height – initial height) over the course of the experiment was similar across salinity and microbial treatments (Kruskal-Wallis, $C^2(5)=3.54$, $P=0.617$) (Fig. 2A). In contrast, aboveground biomass was significantly influenced by the interaction of microbial and salinity treatments (Kruskal-Wallis, $C^2(5)=11.24$, $P=0.047$). Pairwise comparisons revealed a difference in biomass between the *No Addition + Freshwater* and *Autoclaved Inocula + Saltwater* groups (Table 1; Fig. 2B). The *S. alterniflorus* plants in the freshwater control had more aboveground biomass than plants in the saltwater autoclaved inocula treatment ($Z=2.92$, $P=0.003$, adj $P=0.052$) (Table 1). A similar pattern was observed between *Autoclaved Inocula + Saltwater* and *Added Inocula + Saltwater* treatments, where plants

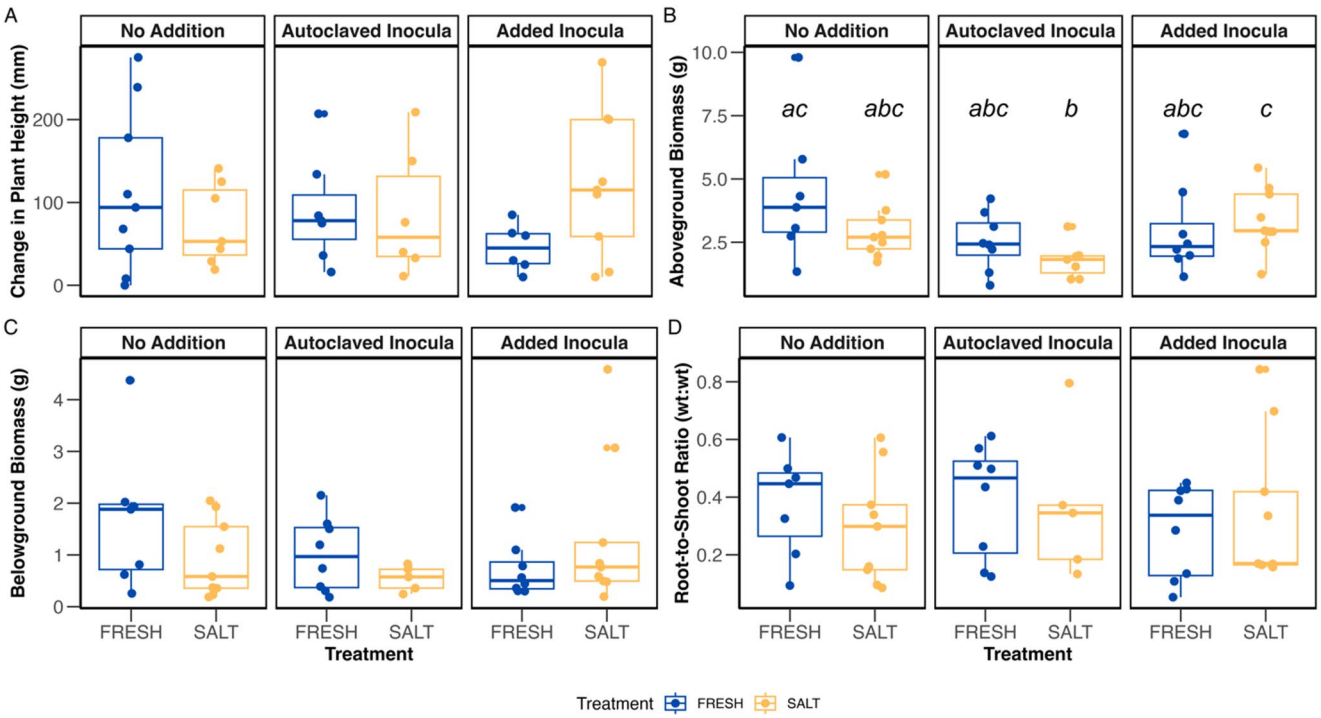


Fig. 2 Boxplots depicting the change in plant height (in mm) (**A**), aboveground biomass (**B**), belowground biomass (**C**), and root-to-shoot ratio (**D**) on the y-axes, in response to marsh soil microbes (*No Addition*, *Autoclaved Inocula*, *Added Inocula*) and salinity stress (<0.5 psu FRESH, 20 psu SALT) treatments on the x-axes. Color represents salinity stress (orange) and freshwater control (blue), while symbols represent individual data points. The boxplot is a visual representation of 5 key summary statistics: the median, the 25% and 75% percentiles, and the whiskers, which represent the feasible range of the data as determined by 1.5 × the interquartile range. Change in plant height, belowground biomass, and root:shoot ratio (defined as the proportion of belowground biomass relative to aboveground biomass) results were not significant (NS). For aboveground biomass (**B**), different letters above boxplots indicate significant difference at adjusted $P<0.10$ based on Dunn's test pairwise comparisons (see also Table 1)

Table 1 Non-parametric *post-hoc* Dunn's test for pairwise comparisons of aboveground biomass across microbial and salinity treatments. *P*-value adjustment: Holm method. Note: Kruskal-Wallis test statistic: $C^2(5)=11.24$, $P=0.047$

Comparison	Z Statistic	*P*-value	Adjusted *P*-value
No Addition Fresh – No Addition Salt	1.18	0.240	1.00
No Addition Fresh – Autoclaved Inocula Fresh	1.68	0.092	1.00
No Addition Salt – Autoclaved Inocula Fresh	0.573	0.566	1.00
No Addition Fresh – Autoclaved Inocula Salt	2.92	0.003	**0.052**
No Addition Salt – Autoclaved Inocula Salt	1.92	0.055	0.709
Autoclaved Inocula Fresh – Autoclaved Inocula Salt	1.33	0.182	1.00
No Addition Fresh – Added Inocula Fresh	1.49	0.135	1.00
No Addition Salt – Added Inocula Fresh	0.372	0.710	1.00
Autoclaved Inocula Fresh – Added Inocula Fresh	-0.196	0.844	0.844
Autoclaved Inocula Salt– Added Inocula Fresh	-1.52	0.128	1.00
No Addition Fresh – Added Inocula Salt	0.372	0.710	1.00
No Addition Salt – Added Inocula Salt	-0.859	0.390	1.00
Autoclaved Inocula Fresh – Added Inocula Salt	-1.41	0.159	1.00
Autoclaved Inocula Salt – Added Inocula Salt	-2.73	0.006	**0.090**
Added Inocula Fresh – Added Inocula Salt	-1.20	0.228	1.00

with salt marsh microorganisms had more aboveground biomass relative to plants with autoclaved inocula (Z = -2.73, $P=0.006$; adj $P=0.090$) (Table 1). No other pairwise comparisons were statistically significant for aboveground biomass analysis. Next, belowground biomass was similar across microbial and salinity manipulations (Kruskal-Wallis, $C^2(5)=4.61$, $P=0.465$) (Fig. 2C). Finally, the root-to-shoot ratios (defined as the proportion of belowground biomass relative to aboveground biomass) were also similar across treatments (Kruskal-Wallis; $C^2(5)=2.65$, $P=0.754$) (Fig. 2D).

Salinity and Microbial Addition Influenced Bacterial Composition More Than Diversity

Salinity and microbial treatments did not influence bacterial diversity. The experimental treatments of salinity and marsh microbial inocula additions resulted in an average bacterial Shannon Diversity H' of 6.456±0.264 (Fig. 3A) and Pielou's Evenness J' of 0.850±0.018 (Fig. 3B). Soil bacterial diversity metrics were similar across salinity (ANOVA, $F_{1,48}=0.035$, $P=0.853$) and microbial treatments (ANOVA, $F_{2,48}=1.53$, $P=0.227$) (Table S3). Likewise, evenness metrics were similar across treatments of salinity (ANOVA, $F_{1,48}=0.048$, $P=0.827$) and microbial inocula addition (ANOVA, $F_{2,48}=1.72$, $P=0.190$) (Table S4).

Conversely, microbial and salinity treatments significantly modified bacterial community composition (Table 2). The main effects of salinity (PERMANOVA, $R^2=0.1007$, $F=6.245$, $P<0.001$) and microbial inocula (PERMANOVA, $R^2=0.0853$, $F=2.644$, $P<0.001$) treatments significantly influenced bacterial community composition (Table 2). There was no significant interaction effect of salinity and microbial treatment on bacterial community structure (PERMANOVA, $R^2=0.0396$, $F=1.228$, $P=0.182$).

To visualize the effect of treatments on bacterial community composition, we used Principal Coordinates Analysis (PCoA) based on a Bray-Curtis dissimilarity matrix (Fig. 3C and D). We observed distinct bacterial community composition according to saltwater or freshwater exposure along the second principal coordinate axis (PCoA axis 2, accounting for 11.7% of variance). A more nuanced separation by microbial inocula was observed along the first principal coordinate axis (PCoA axis 1, accounting for 21.9% of variance). Within both salinity groups, communities showed a consistent gradient along PCoA axis 1. *Autoclaved Inocula* treatment resulted in the most distinct communities, particularly under freshwater conditions, where it was most separated from the other groups.

Context-Dependent Responses of Bacterial Indicator Taxa Across Treatments

To further examine bacterial associations with microbial and salinity treatments, we employed indicator species analysis to identify a subset of bacterial taxa that represent the interaction and main effects of microbial and salinity treatments (Table S1). There were 46 OTUs significantly associated with freshwater conditions across all inocula treatments from phyla *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Verrucomicrobia*, *Planctomycetes*, and *Gemmatimonadetes*; and 37 OTUs were associated with all saltwater treatments from phyla *Proteobacteria*, *Bacteroidetes*, *Acidobacteria*, *Verrucomicrobia*, and *Spirochaetes*. In freshwater treatments, prominent OTUs represented orders *Myxococcales*, *Caulobacterales*, *Rhizobiales*, and *Rhodospirillales*, and the genus *Opitutus*. In contrast, in saltwater treatments, top OTUs represented taxa from the orders *Myxococcales*, *Burkholderiales*, and *Sphingobacteriales*, family *Desulfuromonadaceae*, and genus *Dongia* (Table S1).

For the microbial treatments, 28 OTUs were significantly associated with the *No Addition* control treatment

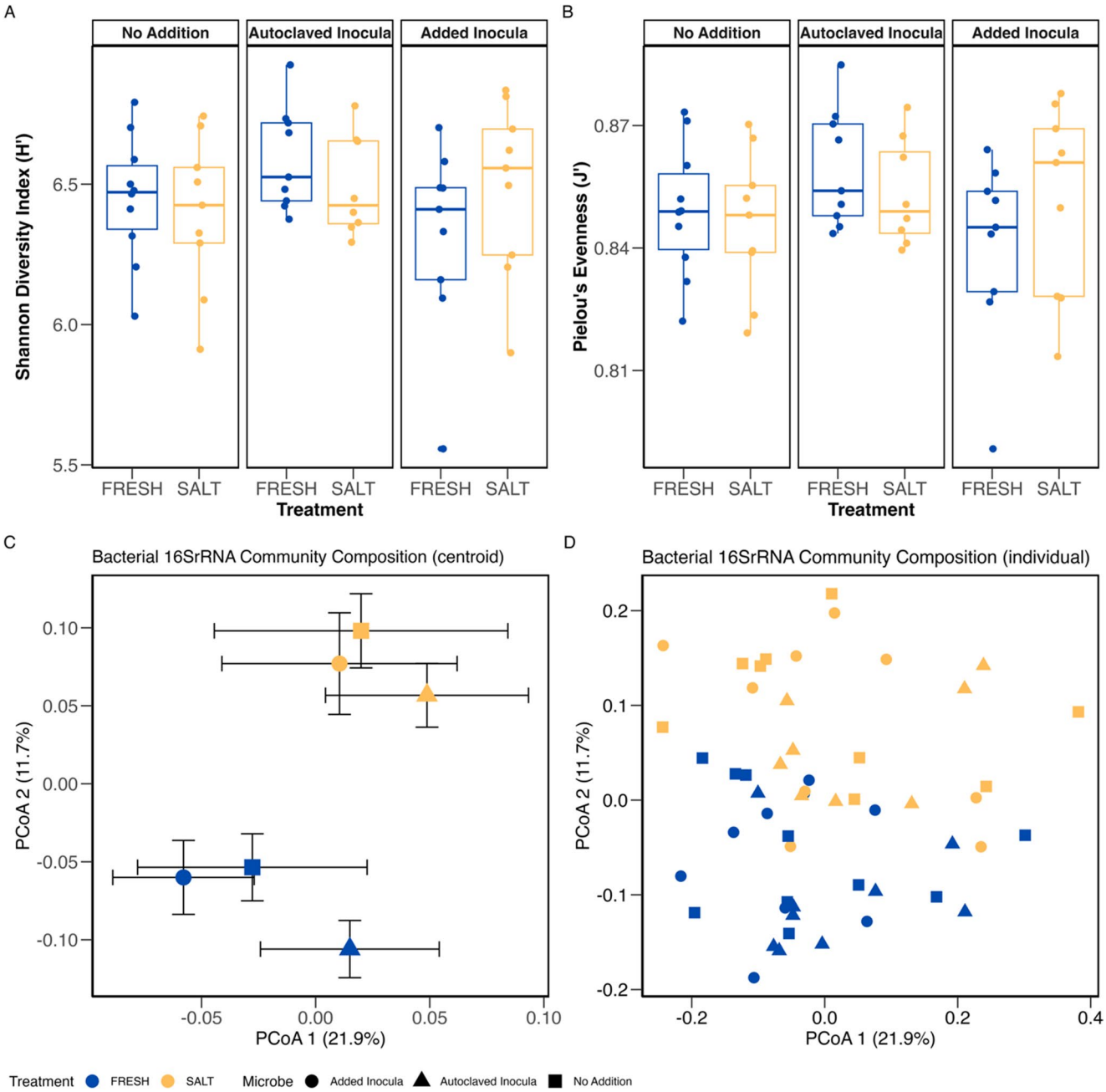


Fig. 3 Boxplots for bacterial diversity representing Shannon Diversity Index (H') (**A**) and Pielou's Evenness (J') (**B**) associated with microbial and salinity treatments. The average Shannon Diversity H' equaled 6.456 ± 0.264, and Pielou's Evenness J' equaled 0.850 ± 0.018, with no significant differences found across treatments. Also included are ordination plots based on a principal coordinates analysis depicting bacterial community composition according to salinity and microbial treatments, representing the average centroid across treatments (**C**) and individual samples (**D**). Main effects of salinity (PERMANOVA: $R^2 = 0.1007$, $P < 0.001$) and microbial (PERMANOVA: $R^2 = 0.0853$, $P < 0.001$) treatments significantly influenced bacterial community composition. No significant effect of salinity × microbial treatment on community structure (PERMANOVA: $R^2 = 0.0396$, $P = 0.182$) (See Table 2). Symbols are colored according to salinity treatment (orange = saltwater (SALT), blue = freshwater (FRESH)) and shaped by microbial treatment (circles = *Autoclaved Inocula*, triangles = *Added Inocula*, squares = *No Addition*)

from phyla *Proteobacteria*, *Planctomycetes*, *Bacteroidetes*, *Acidobacteria*, *Verrucomicrobia*, *and Gemmatimonadetes*; 8 OTUs were associated with *Autoclaved Inocula* from phyla *Proteobacteria* and *Verrucomicrobia*; and 24 OTUs were associated with *Added Inocula* from phyla *Proteobacteria*, *Verrucomicrobia*, *Acidobacteria*, and *Actinobacteria* (Table S2). The top OTUs for the *No Addition* treatments represented taxa from the order *Myxococcales*, families

Table 2 Summary PERMANOVA results comparing bacterial community composition (based on the Bray-Curtis dissimilarity matrix) across main effects (salinity treatment, microbial inocula treatment) and the interaction of salinity × microbial treatments. Bold text indicates significant differences ($P \leq 0.05$)

Effect	DF	Sum of Sqs	R^2	F-value	*P*-value
Salinity TRT	1	0.4931	0.1007	6.245	**< 0.001**
Microbe TRT	2	0.4176	0.0853	2.644	**< 0.001**
Salinity TRT × Microbe TRT	2	0.1940	0.0396	1.228	0.182
Residual	48	3.7900	0.7743		
Total	53	4.8946	1.0000		

Plantomycetaceae, *Chitnophagaceae*, and genera *Rhizomicrobium* and *Opitutus*. For *Autoclaved Inocula*, indicator species represented bacterial taxa in the orders *Myxococcales* and *Rhodospirillales*, taxa in the family *Comamonadaceae*, and taxa in the genus *Hyphomicrobium*. Finally, top indicator species for *Added Inocula* represented taxa in the genera *Geobacter*, *Prosthecobacter*, *Phenylobacterium*, and *Opitutus*, and taxa in the class *Gp1* (Acidobacteria) (Table S2).

Discussion

As understanding of microbial ecology continues to advance, the roles of belowground microbial communities in coastal wetland restoration have attracted growing interest (Birnbaum & Trevathan-Tackett, 2023). Many empirical studies have demonstrated the reliance of plants on soil microbiomes in tolerating environmental stresses, including salinity, inundation, drought, and limited nutrient availability (reviewed in Farrer et al., 2022; Rodriguez et al., 2008). Furthermore, the adaptive and evolutionary responses of plants to changing environmental conditions have been shown to rely heavily on the context of microbial community assemblage, with plants exhibiting increased fitness when associated with stress-adapted microbiomes (Lau & Lennon, 2012). Presently, gaps in knowledge hinder the success of complete ecosystem restoration projects (Duarte et al., 2015); therefore, the foundational knowledge gained from biological conservation and species restoration studies is of utmost relevance and importance. In our investigation, the interactive effects of microbial inocula additions and salinity treatment on *S. alterniflorus* height, biomass, and bulk soil microbiomes produced various results. We hypothesized that inoculating *S. alterniflorus* plugs with salt marsh microbial communities would buffer salt stress and promote plant biomass; a subset of our results supports this hypothesis.

The data revealed consistent trends among treatment groups. For the *No Addition* and *Autoclaved Inocula* treatments, median biomass and change in plant height were higher under freshwater conditions compared to saltwater. This pattern reversed for the *Added Inocula* treatment, which showed higher medians under saltwater conditions (Fig. 2A–C). Despite these patterns in the descriptive statistics, statistical analyses indicated no significant effect of microbial and salinity treatments on changes in plant height and belowground biomass. This null result, particularly for bioinoculation experiments, is not uncommon across both laboratory and field settings and can be attributed to several factors (reviewed in Compant et al., 2019). Possible explanations include, but are not limited to, insufficient establishment of microbial communities due to the short 8-week duration of our design; absent vital satellite taxa (i.e., rare taxa) in the given dosage of microbial inocula; and/or the multifactorial nature of salinity stress limiting the rescue effect of inocula (e.g., alleviating osmotic stress, but not ion toxicity), resulting in a plant growth response too subtle to detect statistically without more precise physiological metrics and/or larger sample size. Recent evidence has also indicated that endophytic bacteria (those within the plant tissue) provide greater benefits to host health when under stress than rhizome-associated bacteria (Malik & Arora, 2022; Peng et al., 2023). Because plants were not inundated during the experiment, the microbial responses observed here likely reflect shifts among root-associated taxa adapted to oxic to suboxic conditions, including facultative anaerobes, rather than obligate anaerobes characteristic of fully anoxic sediments. Although inundation and the resulting anoxic conditions are key drivers of microbial processes in many salt marsh systems, evaluating these dynamics was beyond the scope of the present study. In all, the high variability observed in plant biomass responses within treatment groups likely overwhelmed the signal of any potential treatment effect, reducing the statistical power of our experiment. To further investigate whether marsh microbial inoculation ameliorates abiotic stress, we recommend future experiments with greater statistical power (increased sample sizes) to address variability in responses, as well as more enhanced physiological stress markers (e.g., proline accumulation, leaf ion concentration responses to stress) (Hayat et al., 2012; Azizi et al., 2017).

Aboveground biomass results provided more nuance and clarity on the interactive effects of salinity and microbial additions. Compared to the freshwater control, *Autoclaved Inocula* under salinity stress experienced a greatly inhibited biomass (Table 1; Fig. 2B). These results provide evidence that the sterilization of belowground microbial communities and the addition of chemically inert soil components (i.e., sand) can hinder plant productivity, possibly inducing nutrient limitations for the host plants and nutrient cycling microbes. Comparatively, plants under salinity stress that

were inoculated with marsh microorganisms exhibited greater aboveground biomass, further supporting a growing body of research that microbial inocula additions during early plant development may be critical for establishing resilience to environmental stressors (Trivedi et al., 2021; Peng et al., 2023; Shade, 2023) For example, greenhouse-raised *S. alterniflorus* that were exposed to saltwater (10 ppt salinity) and microbial consortium (a selection of plant growth-promoting rhizobacteria) experienced the highest rates of growth when compared to other treatments (Bledsoe & Boopathy, 2016).

Belowground biomass and root-to-shoot ratios were similar across treatment types (Fig. 2C and D), yielding further insight into the plants' stress response strategy and possible limitations of our methodology. The short duration of our experiment may have been insufficient to elicit measurable changes in biomass allocation. Longer-term morphological changes, like root system restructuring, are often not as prioritized as immediate physiological adjustments (i.e., shoot growth) (Poorter et al., 2012). Additionally, the physical constraints of the pot sizes may have limited root expansion and shoot growth, possibly masking treatment effects. Despite this, some studies have suggested that optimal growth levels can be reached over a broad range of carbon allocation patterns (Sugiura & Tateno, 2011). Future work should investigate how pre-exposing *S. alterniflorus* to salinity stress and marsh microbiomes influences long-term productivity after transplantation to field conditions. In a natural, unrestricted setting, the evaluation of carbon allocation between belowground and aboveground biomass would be more ecologically relevant than in the restrictive environment of a pot.

Salinity stress and microbial inocula additions reshaped bacterial community composition without altering overall diversity or evenness. When analyzing patterns in bacterial diversity, average alpha diversity metrics Shannon diversity H' and Pielou's evenness J' were similar across microbial and salinity treatments (Table S3 & S4). *Autoclaved Inocula* treatment tended to decrease the ranges of Shannon diversity (H') and evenness (J'), while greater ranges existed in the *Added Inocula* (Fig. 3A and B). Additionally, marsh microbial inocula under salinity stress tended to exhibit greater medians in Shannon diversity (H') and evenness (J') than those of freshwater, in support of the reversal trend. Past studies have shown that increases in microbial biodiversity and evenness are associated with increased resistance to plant pathogens, improved soil health and ecosystem functions, increased plant growth and survivorship, and many other positive benefits (see Banerjee & van der Heijden, 2023; Birnbaum & Trevathan-Tackett, 2023; Farrer et al., 2022; Graham & Knelman, 2023; Lau & Lennon, 2012). Despite similar bacterial diversity, microbial and salinity treatments significantly influenced community composition, with distinct bacterial communities forming in the salt and freshwater groups (Table 2; Fig. 3C and D). This may indicate the strength of the selective filtering of environmental conditions in microbial community assembly, rather than acting as forces that uniformly suppressed or enhanced microbial richness and evenness. As the composition of microorganisms mattered more than their sheer diversity in the context of specific environmental filters, this should be highly considered when applying microbial inocula for restoration.

As microbial sampling techniques and high-throughput sequencing continue to improve, increasing evidence has highlighted the utility of microbial communities as indicator species (Liang et al., 2023; Ma et al., 2022; Urakawa & Bernhard, 2017). Microorganisms can function as rapid and sensitive bioindicators and exhibit patterns of spatial distribution (e.g., distinct rhizosphere and bulk soil communities) (Urakawa & Bernhard, 2017; Wang et al., 2016); our findings contribute to this growing body of literature. While indicator species analysis revealed dominant microbial assemblages from phyla *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Verrucomicrobia,* phyla known to be relatively abundant and ubiquitous in soil (Janssen, 2006), there were clear distinctions in OTUs with little overlap between treatments (Table S1 & S2). Seven OTUs from the family *Opitutaceae* (phylum *Verrucomicrobia*) overlapped in *Added Inocula*, saltwater, and freshwater treatments, which have been found in a wide variety of environments but have been associated with nutrient cycling in low-oxygen areas (Jayakumar et al., 2017). At the phylum/class level, many indicator OTUs from *Added Inocula* and saltwater were classified into *Verrucomicrobia*, *Acidobacteria GP1*, and *Alphaproteobacteria*, all of which are acknowledged to contain well-known halotolerant and/or oligotrophic bacteria (Kielak et al., 2016; Zhang et al., 2024). Out of ten OTUs classified into *Bacteroidetes*, 70% were found exclusively in saltwater treatments, supporting evidence of halo-tolerance (Canfora et al., 2014), while interestingly, the remaining three were found in the *No Addition* group. In general, however, microbial bioindicators were unique among salinity and microbial addition treatments, further providing evidence that plant-microbe interactions and environmental conditions uniquely structure soil communities (Li et al., 2021; Zhang et al., 2024). It is important to note that the majority of environmental microbes remain unculturable and unidentified (Stewart, 2012), which represent 20 bacterial taxa that could not be resolved past the phylum or kingdom levels. Moreover, microorganisms in nature often live in complex, synergistic communities, with many functions and interactions that remain challenging to resolve using current approaches (Widder et al., 2016).

The patterns in bacterial community composition revealed support for strong environmental filtering due to salinity exposure, even under short (15-minute) weekly pulses. Bacterial community composition was similar among microbial treatments but revealed distinct bacterial assemblages when under the influence of salinity treatments. As such, the pretreatment of saline conditions and resident microbial inocula may offer rescue effects from abiotic and biotic field stressors after marsh grass plugs are transplanted and exposed to environmental conditions. This work supports that microbial stewardship is essential for buffering against ecological stressors and could promote successful plant establishment for wetland restoration. Additionally, our work supports the theory of "habitat-adapted symbioses," highlighting the facultative mutualisms between marsh plants and their symbiotic microorganisms in tolerating high-stress environments (Rodriguez et al., 2008). With many stressors projected to intensify in the near future (e.g., extreme climatic events, sea level rise, droughts and heatwaves, compounding stress from invasive species, anthropogenic change, etc.), marshes will need to adapt to survive, and their associated microorganisms will undergo even more rapid evolutionary changes (Zabin et al., 2022; Billah et al., 2022; Lau & Lennon, 2012; Mueller et al., 2020). By incorporating microbial inocula into salt marsh restoration practices, success could be better leveraged.

Within a broader scope, coastal wetlands and salt marshes are among the most productive ecosystems on Earth and provide numerous ecological and economic services. Salt marshes support fisheries and habitats for biodiversity, filter contaminants, sequester carbon, buffer storm surges and protect shorelines from erosion, support tourism, and facilitate many other functions (Arkema et al., 2013; Buchanan et al., 2022). The preservation and restoration of these habitats are thus vital, aligning with the principles of the One Health framework, which emphasizes how the health of humans, wildlife, and the environment is inextricably linked (Trinh et al., 2018; Peixoto et al., 2022; Banerjee & van der Heijden, 2023). By evaluating the interactions between *Sporobolus alterniflorus*, marsh sediment microbiomes, and environmental stressors, this study provides insight into ecological processes and may aid in salt marsh restoration efforts.

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Declarations

Competing interests The authors declare no competing interests.

Open Research statement All code and data used in this study are in a public GitHub repository (https://github.com/PeraltaLab/2023_PSM_SMM_microbiomes; https://doi.org/10.5281/zenodo.20090256). Raw amplicon sequence files available at NCBI SRA BioProject ID PRJNA1172654 (https://www.ncbi.nlm.nih.gov/bioproject/1172654).

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