EVALUATING PLANT-MICROBE ASSOCIATIONS IN RESPONSE TO ENVIRONMENTAL STRESSORS TO ENHANCE WETLAND RESTORATION

by

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ABSTRACT - Microorganisms can enhance nutrient acquisition or suppress diseases from pathogens, while plants can provide carbon resources and oxygen to root-associated microbes. However, human activities have altered nutrient cycles and disrupted such mutualisms. Therefore, we need to understand how to promote positive plant-microbe associations to aid in restoring coastal wetland ecosystems where human stressors and climate change (e.g., hurricanes, sea-level rise) challenge restoration outcomes. This study seeks to examine how salinity stressors influence plant-microbe relationships, where we hypothesize that the presence of microbes will buffer salinity stressor effects. We used a whole sediment inocula approach to test this hypothesis. We exposed marsh cordgrass (*Sporobolus alterniflorus*) seedlings to a replicated factorial experiment with three levels of microbiome addition (microbial inocula, autoclaved microbial inocula, no microbe control) and two levels of salinity (0 psu, 20 psu), replicated ten times. We added microbial inocula from the marsh site with autoclaved soilless media and exposed half the seedlings to saltwater (20 psu) and half to freshwater (0 psu). Results revealed that marsh microbial inocula additions during early plant development may ameliorate salinity stressors and could be critical for future restoration efforts. The addition of marsh microbial inocula provided a rescue effect from salinity stress, observed in plant height and aboveground biomass. Belowground biomass, bacterial diversity H', and bacterial evenness J' were similar across microbial and water

treatments (ANOVA, NS); however, the main effects of water (PERMANOVA, R^2=0.100, P<0.001) and microbial (PERMANOVA, R^2=0.086, P<0.001) treatments significantly influenced the bacterial community composition. This work provides evidence that microbial stewardship is essential for buffering against environmental stressors and could promote plant establishment for wetland restoration.

Acknowledgments

I would like to express my sincerest gratitude to my research mentor, Dr. Ariane L. Peralta, for her extensive support and guidance throughout this process. I extend many thanks to the Gittman Lab and Hammocks Beach State Park staff for their field site support. I also thank A. Coughlin, J. Philippe, and the Peralta lab (especially Toby Gallegos, Daphka Joseph, Scott Siebor, Dejah Smith, and Colin Finlay) for microbiome sequencing support, as well as the Department of Biology and East Carolina University. I also thank Dr. Rachel Gittman for serving as an evaluator of this honors thesis. This work was supported by the National Science Foundation and the ECU Undergraduate Research and Creative Activity Award. Without the people and organizations above, this study would not have been possible.

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Introduction

The coastal regions of North Carolina (NC) possess the second largest expanse of coastline protected by various coastal habitats in the continental United States, including marshes, seagrass beds, coastal and kelp forests, as well as oyster and coral reefs (Arkema et al., 2013; Buchanan et al., 2022). Salt marshes, coastal wetlands, and other coastal ecosystems aid in ameliorating erosion and flooding, protect people and property from storm surges, and provide habitat for fisheries and many other species (Arkema et al., 2013; Buchanan et al., 2022). Despite their high ecological and economic importance, these coastal habitats are under threat due to anthropogenic activities (e.g., land development, dredging) and climate change (e.g., accelerated sea level rise, increased frequency of storms) (Valiela et al., 2018; Leonardi et al., 2018; Rudershausen et al., 2021; Buchanan et al., 2022). Unfortunately, current restoration efforts are failing to keep pace with current and future losses, highlighting the necessity for improved salt marsh restoration techniques.

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 foundational species that can withstand a wide range of salinities, inundation periods, and anoxic stress (Gedan & Bertness, 2010). These foundation or habitat-forming species alter their environment, making it possible for other organisms to establish and endure within an ecosystem (Bruno, 2000). However, when commercially raised nursery plants are transferred to field restoration sites, they are exposed to abiotic stressors (e.g., salinity and wave energy) and biotic factors (e.g., species interactions) that can induce stress and hinder restoration (Billah et al., 2022). The effect of exposing *S. alterniflorus* plants to abiotic stressors prior to establishment at a restoration site is unknown. In addition, the understanding of plant-microbe associations between marsh soil microbiomes and *S. alterniflorus* and its 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). In turn, plants can provide carbon resources and oxygen to root-associated microorganisms (Mariotte et al., 2018). Human activities and climate change can and have altered these plant-microbe relationships in ways that promoted or suppressed plant growth. For example, anthropogenic-caused eutrophication and pollution have disrupted nutrient cycles and have led to losses in microbial biodiversity (Cavicchioli et al., 2019), while sea level rise and extreme climatic events damage plants and disrupt mutualisms (Zabin et al., 2022). Conversely, the practice of microbiome stewardship — the intentional understanding and manipulation of microbial communities — can lead to increased microbiome diversity, evenness, and homeostasis (Peixoto et al., 2022). Growing evidence has also indicated that microbial communities can increase plant survivorship and growth in high-stress environments, aiding ecological restoration efforts (McHugh & Dighton, 2004; Mariotte et al., 2018).

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 with two levels of salinity and three levels of microbial inocula treatment, we measured the plant height and biomass (aboveground and belowground) of *S. alterniflorus* plantlings after a 2-month duration. Additionally, we extracted genomic DNA from the sediment samples to characterize the root-associated

microbiomes of the laboratory-treated seedlings. We hypothesized that *S. alterniflorus* plantlings inoculated with restoration-area microbial communities would have higher survivorship and growth rates than control seedlings when salt-stressed.

Methods

Experimental Design

To test our hypothesis, we conducted a factorial experiment with three levels of microbiome addition (+ marsh microbes, autoclaved marsh microbes, NO microbes) and two levels of salinity (<0.5 psu, 20 psu), replicated ten times (n=60) **(Figure 1).** Marsh sediment was collected from Hammocks Beach State Park (Swansboro, North Carolina), while the marsh plantlings, *Sporobolus alterniflorus*, were purchased from a commercial plant nursery (Garner's Nursery & Garden Center, Roanoke Rapids, North Carolina). To prepare the microbial treatments, we combined microbial inocula with Fafard 3B potting mix, where 5% of the total volume was marsh sediment and 95% was autoclaved potting mix (following the methods of Lau & Lennon, 2011). 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 microbial treatments can be attributed to the introduction of inocula containing either live or deceased soil microorganisms (Lau & Lennon, 2011).

The combination of untreated marsh sediment and autoclaved potting mix represented the [+ Microbes] treatment. For [- Microbes], marsh sediment was autoclaved before being combined, with the purpose of examining whether there were abiotic effects on plant productivity. For [NO Microbes], 100% of the volume of the microbial treatment was comprised of autoclaved potting mix. The plantlings were then transferred to 4.5" square pots with their respective treatments and allowed to acclimate to the soil conditions for four weeks to prevent environmental shock. This was followed by weekly salt stress treatment for three weeks.

Figure 1.

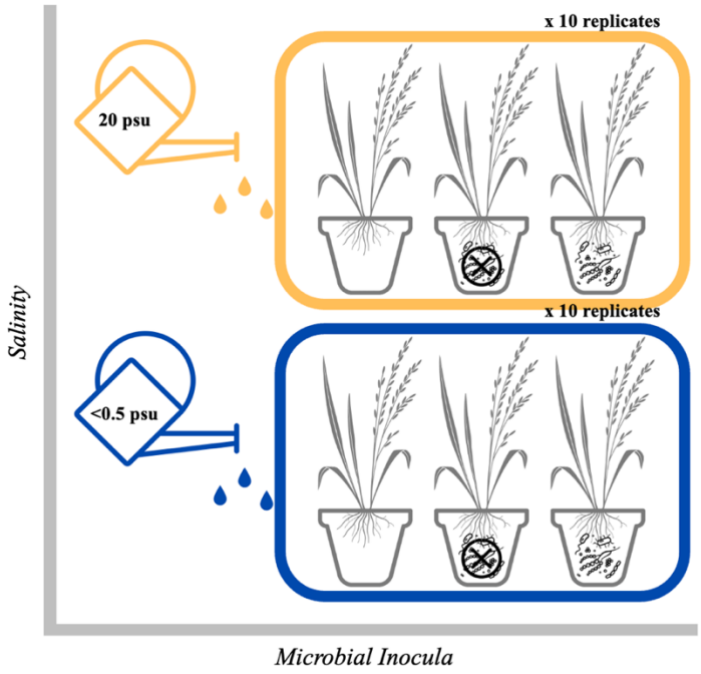

Figure 1: Experimental design of salinity/microbe treatments. Factorial experiment with three levels of microbial addition (+ marsh microbes, autoclaved marsh microbes, and no microbes) and two levels of salinity (<0.5 psu, 20 psu). Treatments were replicated ten times. Microbial inocula were combined with potting mix and established for four weeks, followed by weekly salt stress treatment, and harvested after eight weeks.

For the salinity treatment, we exposed each potted seedling to 15 minutes of 20 psu saltwater or 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, which represented 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 physiology, 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 factors associated with prolonged inundation. After eight weeks, the plants were harvested and processed for plant biomass and microbial sequencing.

Sample Processing

We measured maximum plant height and number of live stems biweekly. At the end of the experiment, after two months, we harvested aboveground biomass and roots, dried the plant biomass samples at 60 °C for 48 hours, and weighed aboveground and root biomass. For soil bacterial characterization and analysis, we combined about 10 g of plant roots and 30 mL of sterilized water into 50 mL centrifuge tubes and shook at 100 RPM for 1 hour. We removed the washed roots and stored samples at -20 °C until DNA extraction and 16S rRNA amplicon sequencing (described below).

Microbiome Analyses

To characterize the sediment microbiomes of the laboratory-treated seedlings, we extracted genomic DNA from sediments using the Qiagen DNeasy PowerSoil Kit, employing a combination of chemical and mechanical methods to lyse the sample (See **Figure 2** for workflow). Initially, the sample was homogenized by adding lysis buffer to a Powerbead Tube containing ~0.25g of the sample, followed by mechanical homogenization using a bead-tube adapter. The crude lysate underwent purification via multiple centrifugation steps to remove impurities. The purified lysate was then mixed with DNA binding solution and passed through a silica spin filter membrane, followed by a two-step washing procedure with ethanol to ensure membrane cleanliness (DNeasy PowerSoil Pro Kits). The silica-bound DNA was eluted using a 10 mM Tris elution buffer.

The isolated DNA was quantified using the Nanodrop spectrophotometer and diluted to a standardized concentration of 10 ng/µl to ensure uniformity across all samples. This process facilitated equimolar concentrations of DNA for downstream applications. Triplicate Polymerase Chain Reactions (PCR) were subsequently conducted on all extracted samples, serving as an efficient downstream technique for the amplified DNA. 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 16S subunit of the ribosomal RNA gene (Caporaso et al., 2012; Apprill et al., 2015; Parada et al., 2016). Purified PCR products were combined in equimolar concentrations (5 ng/µl) and underwent paired-end (2×300 bp) sequencing (Illumina MiSeq platform) at the Duke University Center for Genomic and Computational Biology Core Sequencing Facility.

Sequences were processed using the mothur pipeline (Kozich et al., 2013). We aligned sequences to the Silva Database and removed chimeric sequences using the VSEARCH algorithm (Rognes et al., 2016). Operational taxonomic units (OTUs) were binned to 97% sequence similarity prior to evaluating community patterns.

Figure 2.

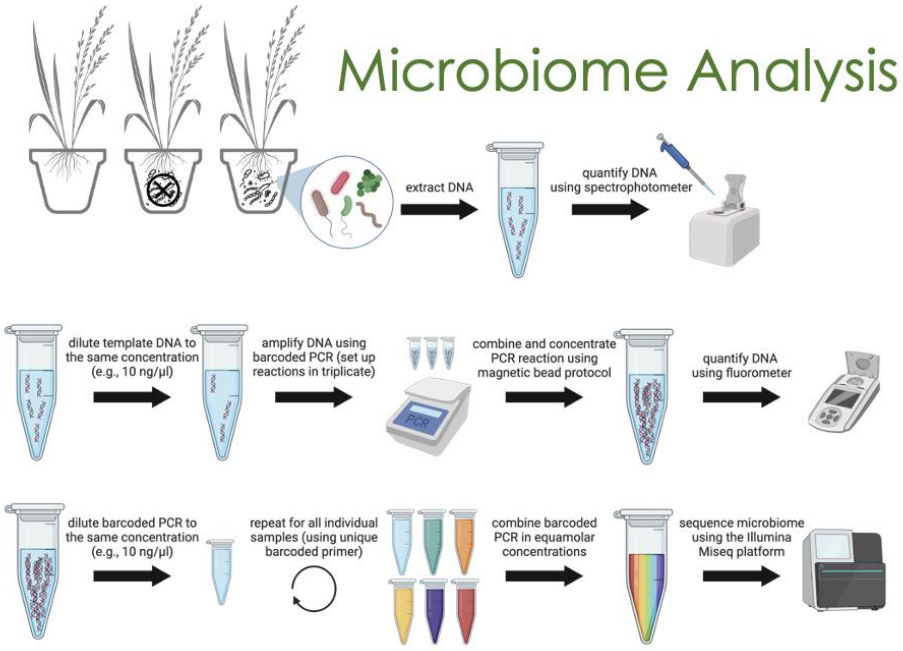

Figure 2. Workflow of DNA extraction and 16S rRNA gene amplicon sequencing library preparation of sediment microbiomes.

Statistical Analysis

We evaluated the interactive effects of inoculation and salinity on *S. alterniflorus* aboveground biomass, belowground biomass, root-to-shoot ratio, and maximum plant height using a series of generalized linear models with inoculation and salinity treatment as fixed effects. All statistical analyses, calculations, and graphical manipulations were run in the R environment (R version 4.3.1, 2023; R Studio version 2023.06.1+524, 2023) using the vegan, ade4, picante packages, and custom functions (Dray et al., 2023; Oksanen et al., 2022). Samples were organized by salinity treatment (colored blue for freshwater and orange for saltwater) and microbial treatments. Several soil bacterial diversity metrics were then computed, including OTU richness, Shannon Diversity (H'), and Pielou's Evenness. These metrics provide insights into community composition by assessing both species richness and species evenness. Species richness refers to

the number of different species present in a community, while species evenness examines the distribution of individuals among those species (Morris et al., 2014). Shannon Diversity (H') measures the uncertainty in identifying individual organisms within a species. In highly diverse and evenly distributed systems, there is more significant uncertainty regarding the identity of unknown individuals, whereas in less diverse systems, it is easier to predict the identity of unknown individuals, leading to lower uncertainty (Morris et al., 2014). Pielou's evenness index (J), also known as the Shannon evenness index, complements measures of species richness by providing insights into the distribution of individuals among different species. The index ranges from 0 to 1, with 0 indicating complete unevenness (i.e., one species dominates the community) and 1 indicating perfect evenness (i.e., all species are equally abundant) (Chao & Ricotta, 2019). Pielou's Index standardizes the Shannon H' to account for differences in species richness, allowing for comparisons of evenness across communities with varying species numbers.

We examined the microbial community variation explained by inoculation and salinity treatment using a permutational multivariate analysis of variance (PERMANOVA). Principal Coordinates Analysis (PCoA) graphs were generated to visualize the multivariate relationships between samples based on their microbial community compositions, allowing for exploration of how the varying experimental factors influenced the overall microbial diversity and structure.

To analyze prominent indicator species within the microbial communities, we used the package 'indicspecies'. Indicator species — selected based on their ability to reflect environmental conditions — 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 abundance and site classification into groups representing habitat types or disturbance states (De Cáceres et al., 2010).

Results

Plant Height and Biomass

Saltwater stressors and autoclaved microbial addition [-Microbes] tended to decrease the rate of plant growth, which was observed in higher median values calculated for saltwater (blue) compared to freshwater (orange) boxplots (ANOVA, $F_{2,260}$=3.184, P=0.043, **Figure 3**). For [+Microbes] compared to [-Microbes] over time, we observed an increase in plant height, especially under salt stress treatment in orange **(Figure 3)**. A notable observation is that for the [-Microbes] and [NO Microbes], freshwater treatments consistently showed increased plant growth compared to saltwater. However, in the [+Microbes] treatments, this trend was reversed, with the saltwater treatment resulting in greater median plant height compared to its freshwater counterpart. This may hint at salt marsh microbial addition [+ Microbes] providing a rescue effect from salinity stress.

Figure 3.

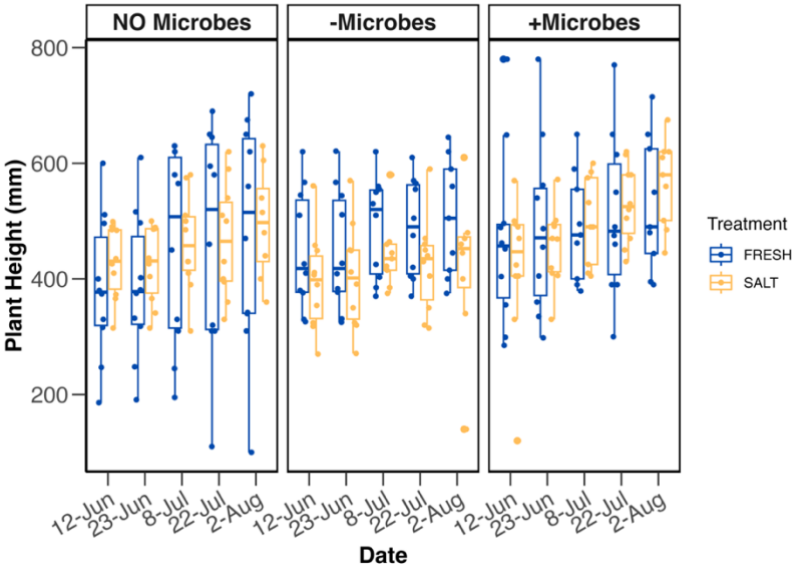

Figure 3. Boxplots depicting plant height (in mm) on the y-axis over time on the x-axis in response to marsh soil microbes and salinity stress treatments. Color represents salinity stress (orange) and freshwater control (blue). The boxplot is a visual representation of 5 key summary statistics: the median, the 25% and 75% percentiles, and the whiskers, which represent the feasible range of the data as determined by 1.5 × the interquartile range. Symbols represent individual data points.

A similar trend was observed with aboveground plant biomass measurements, where [+Microbes] treatment buffered salinity stress and resulted in more growth when compared to freshwater treatment (**Figure 4**). Although not statistically significant (ANOVA, $F_{2,45} = 0.662$, $P = 0.521$), the reversal trend was still exhibited; aboveground biomass tended to be higher under [+Microbes] in salt compared to freshwater conditions. When looking at belowground biomass (**Figure 5**), we observed similar root biomass across the microbial and water treatments (ANOVA, $F_{2,45} = 1.458$, $P = 0.243$). This was interesting as it shows that the allocation of the plant's carbon was more directed at aboveground biomass rather than belowground.

Figure 4.

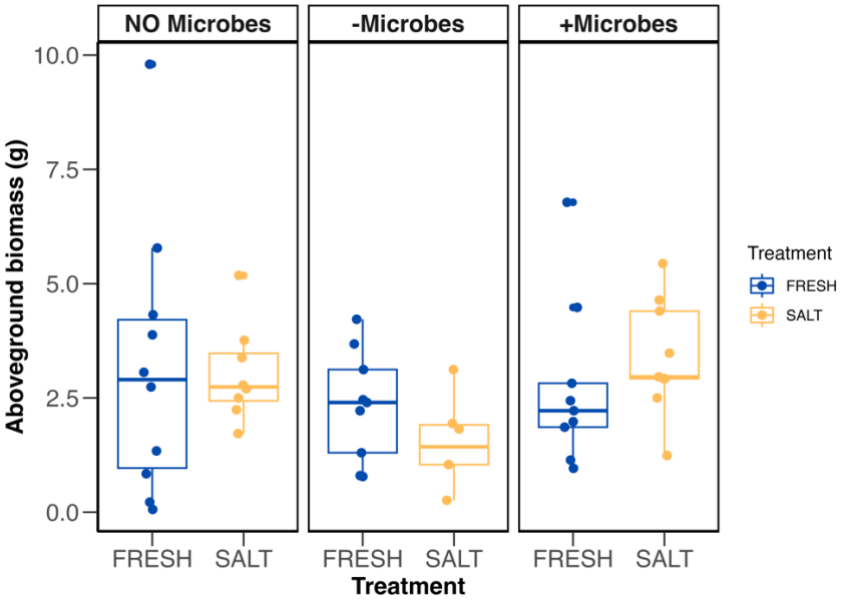

Figure 4. Boxplots depicting aboveground biomass (in g) on the y-axis in response to marsh soil microbes and salinity stress treatments on the x-axis. Color represents salinity stress (orange) and freshwater control (blue). The boxplot is a visual representation of 5 key summary statistics: the median, the 25% and 75% percentiles, and the whiskers, which represent the feasible range of the data as determined by 1.5 × the interquartile range. Symbols represent individual data points.

Figure 5.

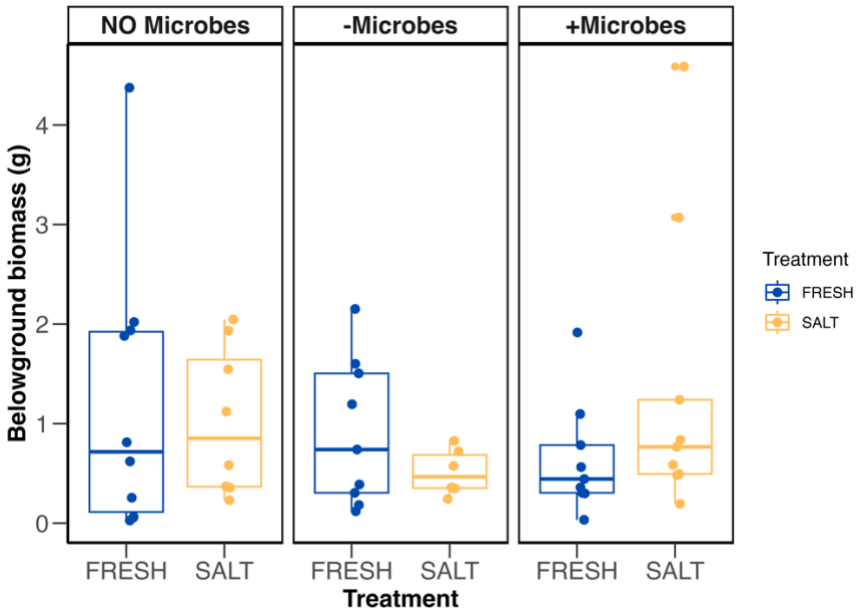

Figure 5. Boxplots depicting dried belowground biomass (in g) on the y-axis in response to marsh soil microbes and salinity stress treatments on the x-axis. Color represents salinity stress (orange) and freshwater control (blue). The boxplot is a visual representation of 5 key summary statistics: the 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. Symbols represent individual data points.

Microbial Community Diversity and Evenness

The experimental treatments of salinity and marsh microbial inocula additions resulted in an average bacterial Shannon Diversity H' of 6.448 ± 0.083 and Pielou's Evenness J' of 0.850 ± 0.0057. There were no significant differences in bacterial diversity and evenness detected among treatments (ANOVA, inocula: NS, water: NS). Interestingly, the pattern with [+Microbes * Saltwater] was present in both Shannon Diversity **(Figure 6)** and Pielou's Evenness **(Figure 7)** indices, with greater medians measured in the saltwater compared to freshwater treatments.

Figure 6

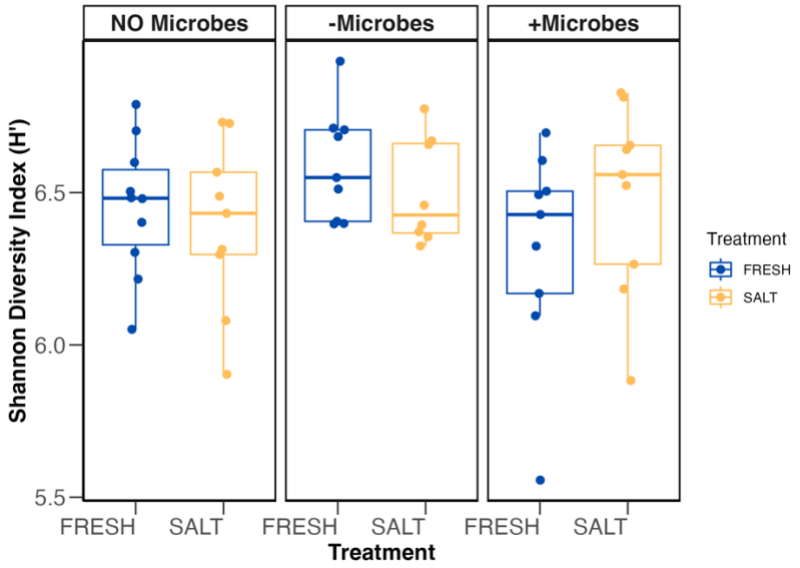

Figure 6. Boxplots for bacterial diversity representing Shannon Diversity Index (H') associated with microbial (+ marsh microbes, autoclaved marsh microbes, NO microbes) and salinity (<0.5 psu, 20 psu) treatments. Colors indicate salinity treatment (fresh in blue, salt in orange). Symbols represent individual data points.

Figure 7.

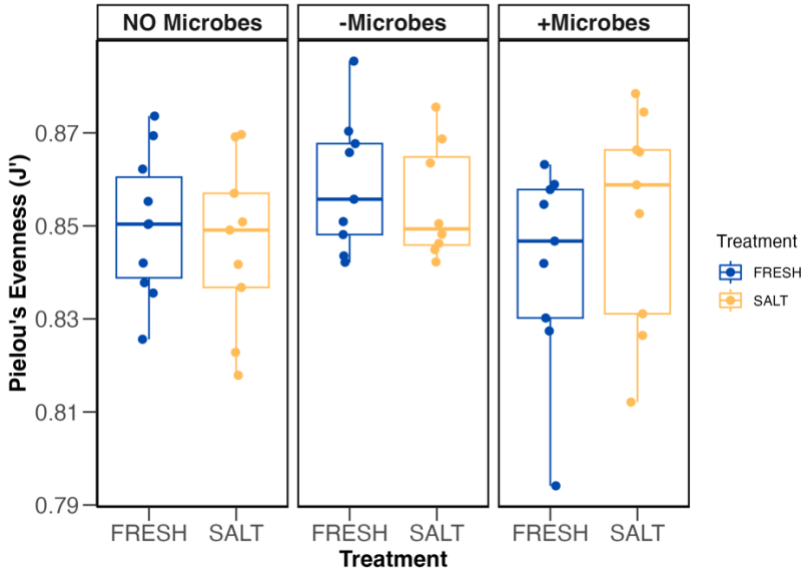

Figure 7. Boxplots for bacterial diversity representing Pielou's Evenness (J') associated with microbial (+ marsh microbes, autoclaved marsh microbes, NO microbes) and salinity (<0.5 psu, 20 psu) treatments. Colors indicate salinity treatment (fresh in blue, salt in orange). Symbols represent individual data points.

Microbial and water treatments influenced bacterial community composition **(Figure 8, Table 1).** The main effects of water (PERMANOVA, R^2=0.100, P<0.001) and microbial (PERMANOVA, R^2=0.086, P<0.001) treatments significantly influenced bacterial community composition **(Table 1)**. Along PCoA axis 2, we observed distinct bacterial community composition associated with water treatment, while there was more overlap in bacterial communities across microbial treatments for salt compared to freshwater treatments **(Figure 8)**.

Figure 8.

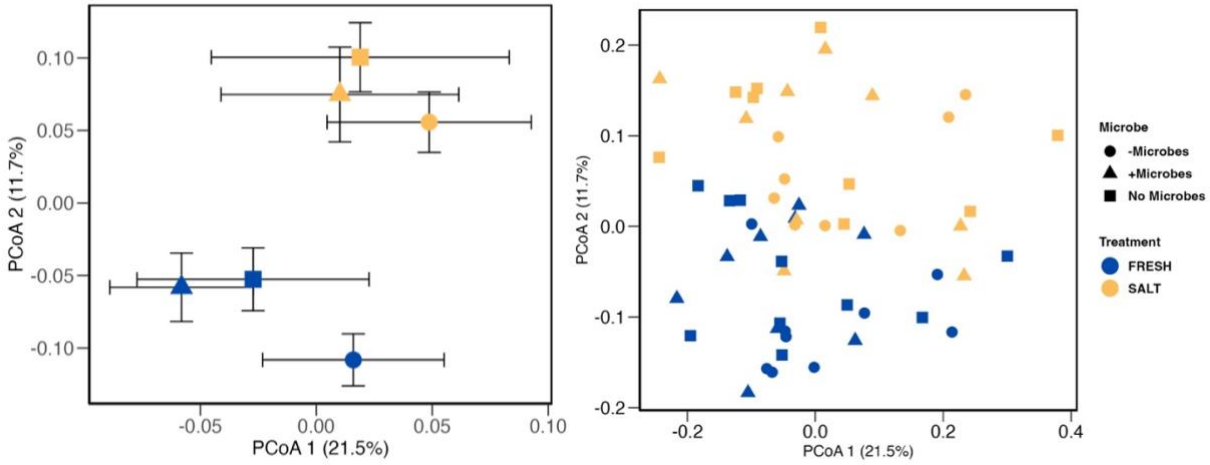

Figure 8. Grouped (left) and individual (right) ordination based on a principal coordinates analysis depicting bacterial community composition according to salinity and microbial treatments. Symbols are colored according to salinity treatment (orange = saltwater, blue = freshwater) and shaped by microbial treatment (circles = [-Microbes], triangles = [+Microbes], squares = [NO Microbes]).

Table 1. PERMANVOVA test of the interactive effects of salinity and microbial treatments on bacterial operation taxonomic units (OTUs). Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' '.

	DF	**Sum Of Sqs**	R^2	***F***	***p*-Value**	**Significance**
Water TRT	1	0.4953	0.10021	6.2325	9.999e-05	***
Microbe TRT	2	0.4267	0.08632	2.6845	0.0002	***
Water TRT: Microbe TRT	2	0.2062	0.04172	1.2973	0.1290	
Residual	48	3.8147	0.77175			
Total	53	4.9429	1.00000			

Bacterial Indicator Operational Taxonomic Units

To further examine bacterial associations with microbial and salinity treatments, we used indicator species analysis to identify a subset of bacterial taxa representing each experimental root-associated microbiome in salt and freshwater treatments **(Table 2, Table 3)**. Out of 826 identifiable taxa at greater than 1% relative abundance in the data set, 13 OTUs were strongly associated with freshwater, and 12 OTUs were associated with saltwater treatments. In freshwater treatments, prominent OTUs (**Table 2**) included the following: *Bacteroidetes*, *Polyangiaceae*, *Verrucomicrobia Subdivision 3*, an unclassified Bacteria, *Verrucomicrobia Subdivision 3*, *Bacteroidetes*, *Bacteroidetes*, *Proteobacteria*, an unclassified Bacteria, *Veillonellaceae* ($p < 0.001$), *Bacteroidetes* ($p < 0.01$), *Cytophagales* ($p < 0.05$), and *Bacteroidetes* ($p < 0.001$).

Table 2. Bacterial taxa (OTUs) representing the unique taxa associated with the freshwater treatment according to indicator species analysis. This summary represents the top bacterial taxa associated with each treatment type.

OTUs (Genus or higher)	**Indicator value**	***p*-Value**
Bacteroidetes	0.999	0.001 ***
Polyangiaceae	0.992	0.001 ***
Verrucomicrobia sDiv3	0.981	0.001 ***
Bacteria unclassified	0.964	0.001 ***
Verrucomicrobia sDiv3	0.963	0.001 ***
Bacteroidetes	0.959	0.001 ***
Bacteroidetes	0.930	0.001 ***
Proteobacteria	0.907	0.001 ***
Bacteria unclassified	0.902	0.001 ***
Veillonellaceae	0.816	0.001 ***
Bacteroidetes	0.816	0.004 **
Cytophagales	0.709	0.023 *
Bacteroidetes	0.598	0.001 ***

Associations were calculated using species indicator analyses (Indicator value) in R. Significance levels: $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$.

Table 3. Bacterial taxa (OTUs) representing the unique taxa associated with the saltwater treatment according to indicator species analysis. This summary represents the top bacterial taxa associated with each treatment type.

OTUs (Genus or higher)	Indicator value	*p*-Value
Cyanobacteria	0.995	0.001 ***
Chitinophagaceae	0.989	0.001 ***
Saprospiraceae	0.976	0.001 ***
Bacteria unclassified	0.974	0.001 ***
Gammaproteobacteria	0.966	0.001 ***
Cyanobacteria	0.952	0.001 ***
Bacteria unclassified	0.945	0.001 ***
Legionella	0.942	0.001 ***
Gammaproteobacteria	0.886	0.001 ***
Bacteria unclassified	0.875	0.001 ***
Acidobacteria Gp10	0.783	0.048 *
Myxococcales	0.731	0.001 ***

Associations were calculated using species indicator analyses (Indicator value) in R. Significance levels: $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$.

The 12 bacterial indicator OTUs representing salinity-treated microbiomes (**Table 3**) included the following: *Cyanobacteria*, *Chitinophagaceae*, *Saprospiraceae,* an unclassified Bacteria, *Gammaproteobacteria*, *Cyanobacteria*, an unclassified Bacteria, *Legionella*, *Gammaproteobacteria*, an unclassified Bacteria ($p < 0.001$), *Acidobacteria Gp10* ($p < 0.05$), and *Myxococcales* ($p < 0.001$).

Meanwhile, species indicator analysis of microbial treatments revealed three indicator OTUs with NO Microbes [*Acidobacteria*, *Alphaproteobacteria* ($p < 0.01$), and *Myxococcales* ($p < 0.001$)], two associated with – Microbes [*Aeromonas* ($p < 0.05$), and *Cytophagales* ($p < 0.01$)], and one with + Microbes [*Bacteroidetes* ($p < 0.01$)] **(Table 4)**.

Table 4. Bacterial taxa (OTUs) representing the unique taxa associated with the microbial addition treatment according to indicator species analysis. This summary represents the top bacterial taxa associated with each treatment type.

NO Microbes		
OTUs (Genus or higher)	**Indicator value**	***p*-Value**
Acidobacteria	0.866	0.002 **
Alphaproteobacteria	0.859	0.003 **
Myxococcales	0.740	0.001 ***
- Microbes		
Aeromonas	0.874	0.024 *
Cytophagales	0.779	0.003 **
+ Microbes		
Bacteroidetes	0.642	0.003 **

Associations were calculated using species indicator analyses (Indicator value) in R. Significance levels: $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$.

Discussion

While understanding the underpinnings of microbial ecology is still in its relative infancy compared to more contemporary scientific disciplines, the roles of belowground microbial communities in coastal wetland restoration have been of great 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 root-associated microbiomes produced various results. We hypothesized that inoculating *S. alterniflorus* plantlings with salt marsh microbial communities would buffer salt stress and promote plant growth and survivorship; a subset of our results supports this hypothesis.

Upon analyzing plant height data, we concluded that the addition of marsh microbes [+Microbes] at early plant development stages may ameliorate salinity stress (as demonstrated by the reversal trend in median plant heights). Conversely, autoclaved microbial media [-Microbes] and salinity stress treatment were shown to decrease plant growth in aboveground biomass, belowground biomass, and plant height. While intuitive, this confirms that the sterilization of belowground community influences and the addition of nonessential or less-utilized abiotic

materials can hinder plant productivity. These findings are in accordance with a similar study, where greenhouse-raised *S. alterniflora* 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).

Aboveground biomass results further supported evidence that microbial inocula additions during early plant development may be critical for establishing stress-tolerant seedlings. While not statistically significant, belowground biomass displayed a consistent pattern as well. The allocation of carbon to aboveground biomass more than belowground is of interest as it may hint at efficient primary productivity and carbon sequestration but less carbon storage and root establishment. Additional studies are necessary to determine the consequence of increasing wave energy stressors on the success of plant establishment during marsh restoration.

When analyzing patterns in bacterial diversity, average bacterial diversity H' and evenness J' were similar across microbial and water treatments. The [-Microbes] inocula treatment tended to decrease the ranges of H' and J', while greater ranges existed in the [+Microbes] treatment. Additionally, marsh microbial inocula under salinity stress tended to exhibit greater medians in H' and J' than that of freshwater, in support of the reversal trend. Throughout contemporary literature, it is well-accepted 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). If the addition of marsh microbial inocula and their adapted environmental conditions can better predict microbial community assembly, then marsh wetland restoration techniques may have another avenue of improvement and success.

The patterns in bacterial community composition revealed support for strong environmental filtering due to salinity exposure, even under short (15-minute) weekly pulses. The bacterial community composition was similar among microbial treatments but revealed significant dissimilarities in structure when under the influence of salinity treatments. This observation was further described by indicator species analysis, where salinity treatments associated with 13 and freshwater treatments associated with 12 unique indicator OTUs. Many indicator OTUs remain unidentified, some even up to the domain level, necessitating additional analyses of indicator bacterial taxa classification and function to improve understanding of the differences in the microbial community assemblage between treatments.

Future Directions

This investigation serves as a pilot study for large-scale marsh restoration research. Future studies could implement factorial experiments with wave energy and microbial inoculation *in situ*. This would more greatly establish an understanding of the dynamics of marsh microbial treatments under the influence of natural abiotic stressors (i.e., wave energy and salinity). Another avenue of exploration would be understanding root development under similar treatment effects. In this study, marsh plants were housed in pots that may have restricted roots from properly establishing. Additionally, in our study, variation was observed that may be masking treatment effects. Increasing the number of replicates in future studies could help resolve trends in plant and bacterial response to water and microbial treatments.

Significance

The pre-treatment of salt conditions and sediment microbes could help grasses buffer from abiotic and biotic field stressors after marsh grass seedlings are transplanted and exposed to stressful environmental conditions. This work provides evidence that microbial stewardship is important for buffering against environmental stressors and could promote plant establishment for wetland restoration. Additionally, this 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, 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 & protect shorelines from erosion, provide tourism, and facilitate many other functions (Arkema et al., 2013; Buchanan et al., 2022). Therefore, preserving and restoring such ecological habitats are vital to humans, wildlife, and the environment, emphasizing the concept of One Health (Peixoto et al., 2022). By evaluating the interactions between *Sporobolus alterniflorus*, marsh sediment microbiomes, and environmental stressors, this study provides insight into ecological dynamics and may aid in salt marsh restoration efforts considering climate and anthropogenic change.

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