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

RUNNING TITLE: Salt marsh plant-microbiome restoration

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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 examined how salinity stressors influence plant-microbe relationships, where we hypothesize that the presence of microbes will buffer stressor effects. We used a whole sediment inocula approach and exposed marsh cordgrass (*Sporobolus alterniflorus*) plugs to a replicated factorial experiment with three levels of microbiome addition (microbial inocula, autoclaved microbial inocula, no microbe control) and two levels of salinity (<0.5 psu, 20 psu), replicated ten times. We added microbial inocula from the marsh site with autoclaved soilless media 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 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 over time. Aboveground and belowground biomass, bacterial diversity H', and bacterial evenness J' were similar across microbial and water treatments; however, water and microbial treatments significantly influenced the bacterial community composition. This work provides evidence that microbiome stewardship buffers against environmental stressors and could promote plant establishment for coastal wetland restoration.

Keywords: soil microbial communities, salinity stress, microbiome stewardship, coastal protection

INTRODUCTION

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 (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 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* plugs after a 2-month duration. Additionally, we extracted genomic DNA from the sediment samples to characterize the root-associated microbiomes of the laboratory-treated plugs. We hypothesized that *S. alterniflorus* plugs inoculated with restoration-area microbial communities would have higher survivorship and growth rates than control plugs when salt-stressed.

METHODS

Microbiome x Salinity Stress Experimental Design

To test our hypothesis that *S. alterniflorus* plugs inoculated with restoration-area microbial communities would have higher survivorship and growth rates than control plugs when salt-stressed, 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) (Figure 2). Marsh sediment was collected from Hammocks Beach State Park (Swansboro, North Carolina), while the marsh plugs, *Sporobolus alterniflorus*, were purchased from a commercial plant nursery (Garner's Landscaping, Inc., Newport, 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 'Added Inocula' treatment. For the 'Autoclaved Inocula' treatment, marsh sediment was autoclaved before being combined, with the purpose of examining whether there were abiotic effects on plant productivity. For the 'No Addition' treatment, 100% of the volume of the microbial treatment was comprised of autoclaved potting mix. The plugs were transferred to 4.5" square pots with their respective treatments on 08 June and allowed to acclimate to the soil conditions for four weeks under laboratory conditions (room temperature at about 25 °C next to a window for sunlight) . We

118 measured the maximum plant height and number of live stems biweekly, with changes in average
119 stem height functioning as a proxy for growth. The first height was recorded on 23 June, which was
120 the day when the plants were moved from the laboratory to the outside environment. Following
121 acclimation, weekly salt stress treatment began on 09 July and ran for four weeks.
122 For the salinity treatment, we exposed each potted plug to 15 minutes of 20 psu saltwater or
123 freshwater. Saltwater was made with Instant Ocean Sea Salt, with a general ratio of ~300 g Instant
124 Ocean to 14 L of tap water. This established a concentration of ~20 psu, which represented salinity
125 levels at Hammocks Beach State Park, where salinity ranges from 20 to 35 psu (personal
126 communication, park manager). Although the duration of salt exposure in our experimental setup
127 may be shorter compared to natural field conditions experienced by plants, it was anticipated that
128 the deposited salt would gradually accumulate in the soil due to evaporation, mimicking real-world
129 scenarios. This deliberate manipulation allowed us to specifically examine the effects of salt stress
130 on plant physiology, distinct from the stress induced by prolonged inundation, which typically leads
131 to anoxia. Thus, our experimental design ensured a focused investigation into salt stress dynamics
132 without confounding factors associated with prolonged inundation.
133
134 ***Plant and Soil Sample Processing***
135 At the end of the experiment, after eight weeks, we harvested aboveground biomass and
136 roots, dried the plant biomass samples at 60 °C for 48 hours, and weighed aboveground and root
137 biomass. For soil bacterial characterization and analysis, we homogenized the remaining soil,
138 transferred soil into sterile WhirlPak bags, and stored samples at -20 °C until DNA extraction and
139 16S rRNA amplicon sequencing (described below).
140
141 **Field Site Description**

The field study focused on *S. alterniflorus* growth and survival across an elevation gradient. The field site chosen for planting was a small section of sandy, eroded shoreline behind a recently installed offshore sill at Hammocks Beach State Park on Queens Creek in Swansboro, North Carolina. The section of beach contained roughly equal areas above and below the mean high water (MHW) line (Figure 1).

Field Plant and Soil Sampling

Commercially grown *S. alterniflorus* plugs were obtained on 1 June 2023 from a local nursery that routinely grows *S. alterniflorus* plugs from locally collected seeds for salt marsh restoration projects. The number of stems in each cell in the nursery trays was counted and the length of the longest leaf in each cell was measured to 0.1 centimeter (cm). All plants were then trimmed to a total height of 20 cm. Nine rows of plants were installed (n=270): five above MHW (n=142) and four below (n=128), with approximately 7 cm between plugs and 25 cm between rows (Figure 1). The remaining plugs were relocated to the laboratory for salinity and microbial treatments.

The location and elevation of each row were measured at the time of planting using a Trimble Virtual Reference Station (VRS; Trimble, Sunnyvale, California, USA), Real-Time Kinematic (RTK), and Global Positioning System (GPS). Elevations ranged from an average of 0.45 m along the highest row to 0.09 m relative to North American Vertical Datum of 1988 (NAVD88) along the lowest row. Row 4 was approximately along the MHW line at an elevation of 0.32 m NAVD88. Surviving planted plugs in each row were counted every two to three weeks throughout the 2023 growing season, and the height of the longest leaf on each plant was recorded to the nearest 0.1 cm. Sediment samples were taken, and one plant per row was destructively harvested for biomass measurements two, six, and 16 weeks after planting.

166 We sampled field soil on three dates:12 June, 13 July, and 5 October 2023. We sampled
167 along the transect from row 1 to row 9. At each row, we randomly sampled a plant (above and
168 belowground biomass) and associated soil using a 5 cm diameter PVC corer to 12 cm depth. We
169 stored samples on ice and then at 4 ºC overnight prior to sample processing for downstream
170 microbiome analyses. To separate bulk from root-associated soils, we cut aboveground plant
171 biomass, shook the sediment from the roots into a plastic bag, and then transferred roots and
172 remaining soils into a sterile tube and mixed with sterile 1X phosphate buffer solution (1X PBS).
173 After shaking for 5 minutes, we used sterile tweezers to remove roots and transferred cleaned roots
174 into a paper bag to dry at 60 °C for 48 hours. We centrifuged the remaining root-associated soils,
175 carefully poured off PBS, and stored these rhizosphere sediments at -20 °C until DNA extractions.
176 After removing the plant from the field sediment sample, we homogenized the sediment sample,
177 transferred a subsample to a sterile WhirlPak bag, and stored the bulk soil sample at -20 °C until
178 DNA extractions.
179
180 ***DNA Extraction and Bacterial Amplicon Sequencing***
181 To characterize the sediment microbiomes of the laboratory-treated plugs as well as any
182 community composition differences between rhizosphere and bulk field soil samples, we extracted
183 genomic DNA from sediments using the Qiagen DNeasy PowerSoil Kit, employing a combination of
184 chemical and mechanical methods to lyse the sample following the manufacturer's protocol. The
185 isolated DNA was quantified using the Nanodrop spectrophotometer and diluted to a standardized
186 concentration of 10 ng μl^{-1} to ensure uniformity across all samples. This process facilitated
187 equimolar concentrations of DNA for downstream applications. Triplicate Polymerase Chain
188 Reactions (PCR) were subsequently conducted on all extracted samples, serving as an efficient
189 downstream technique for the amplified DNA. To characterize bacterial communities, we used

190 barcoded primers (515FB/806R) initially developed by the Earth Microbiome Project (Caporaso et
191 al. 2012) to target the V4 region of the bacterial 16S subunit of the ribosomal RNA gene (Caporaso
192 et al., 2012; Apprill et al., 2015; Parada et al., 2016). For each sample, three 50 µL PCR libraries
193 were prepared by combining 35.75 µL molecular grade water, 5 µL Amplitaq Gold 360 10x buffer, 5
194 µL $MgCl_2$ (25 mM), 1 µL dNTPs (40mM total, 10mM each), 0.25 µL Amplitaq Gold 360 polymerase, 1
195 µL 515 forward barcoded primer (10 µM), 1 µL 806 reverse primer (10 µM), and 1 µL DNA template
196 (10 ng μL^{-1}). Thermocycler conditions for PCR reactions were as follows: initial denaturation (94 °C,
197 3 minutes); 30 cycles of 94 °C for 45 seconds, 50 °C for 30 seconds, 72 °C for 90 seconds; final
198 elongation (72 °C, 10 minutes). The triplicate 50 µL PCR libraries were combined and then cleaned
199 using the Axygen® AxyPrep Magnetic (MAG) Bead Purification Kit (Axygen, Union City, California,
200 USA). Cleaned PCR products were quantified using QuantIT dsDNA BR assay (Thermo Scientific,
201 Waltham, Massachusetts, USA) and diluted to a concentration of 10 ng μL^{-1} before pooling libraries
202 in equimolar concentration of 5 ng μL^{-1}. We sequenced the pooled libraries using the Illumina
203 MiSeq platform using paired-end (2×250 bp) reads (Illumina Reagent Kit v2, 500 reaction kit) at the
204 Duke University Center for Genomic and Computational Biology Core Sequencing Facility.
205 Sequences were processed using the mothur (v1.42.0) (Schloss et al. 2009) MiSeq pipeline
206 (Kozich et al., 2013). We assembled contigs from the paired-end reads, quality trimmed using a
207 moving average quality score (minimum quality score 35), aligned sequences to the SILVA rRNA
208 database (v132) (Quast et al. 2013), and removed chimeric sequences using the VSEARCH
209 algorithm (Rognes et al. 2016). We created operational taxonomic units (OTUs) by first splitting
210 sequences based on taxonomic class and then binning into OTUs based on 97% sequence
211 similarity. Taxonomic identity was assigned using the SILVA rRNA database (v132) (Quast et al.
212 2013). We used an OTU-based 3% distance threshold instead of using amplicon sequence variant
213 (ASV) of a single base difference, especially given our high diversity microbial communities based

214 on short-read (250 bp) amplicon sequences, and that similar broadscale microbiome patterns are
215 observed using OTU- or ASV-based approaches (Glassman and Martiny 2018). In addition, the OTU-
216 based approach limits overestimating the number of ASVs due to PCR bias and sequencing errors
217 (Schloss 2021).
218
219 ***Statistical Analysis***
220 We evaluated the interactive effects of inoculation and salinity on *S. alterniflorus*
221 aboveground biomass, belowground biomass, root-to-shoot ratio, and maximum plant height using
222 a series of generalized linear models with inoculation and salinity treatment as fixed effects. All
223 statistical analyses, calculations, and graphical manipulations were run in the R environment (R
224 version 4.4.1, 2024; R Studio version 2024.09.0+375, 2024) using the vegan, ade4, picante
225 packages, and custom functions (Dray et al., 2023; Oksanen et al., 2022).
226 Prior to microbial statistical analyses, we removed low abundance OTUs that were <10
227 sequences across the data set. We also omitted two samples where the total sequences were less
228 than 10,000 reads, which represented issues with the library preparation. Samples were organized
229 by salinity treatment and microbial treatments. Several soil bacterial diversity metrics were then
230 computed, including Shannon Diversity (H') and Pielou's Evenness (J') with corresponding ANOVA
231 tests. These metrics provide insights into community composition by assessing both species
232 richness and species evenness. Species richness refers to the number of different species present
233 in a community, while species evenness examines the distribution of individuals among those
234 species (Morris et al., 2014). Shannon Diversity (H') measures the uncertainty in identifying
235 individual organisms within a species. In highly diverse and evenly distributed systems, there is
236 more significant uncertainty regarding the identity of unknown individuals, whereas in less diverse
237 systems, it is easier to predict the identity of unknown individuals, leading to lower uncertainty

238 (Morris et al., 2014). Pielou's evenness index (J'), also known as the Shannon evenness index,
239 complements measures of species richness by providing insights into the distribution of individuals
240 among different species. The index ranges from 0 to 1, with 0 indicating complete unevenness (i.e.,
241 one species dominates the community) and 1 indicating perfect evenness (i.e., all species are
242 equally abundant) (Chao & Ricotta, 2019). Pielou's Index standardizes the Shannon H' to account
243 for differences in species richness, allowing for comparisons of evenness across communities with
244 varying species numbers. For water and microbial response variables, we performed Tukey's
245 Honest Significant Difference (HSD) post-hoc analysis of the ANOVA models using the emmeans
246 package in R. We compared estimated marginal means (EMMs) to conduct pairwise comparisons
247 between treatment groups (Lenth 2022).
248 We examined the microbial community variation explained by inoculation and salinity
249 treatment using a permutational multivariate analysis of variance (PERMANOVA). Principal
250 Coordinates Analysis (PCoA) graphs were generated to visualize the multivariate relationships
251 between samples based on their microbial community compositions, allowing for exploration of
252 how the varying experimental factors influenced the overall microbial diversity and structure.
253 To analyze prominent indicator species within the microbial communities, we used the
254 package 'indicspecies'. Indicator species — selected based on their ability to reflect environmental
255 conditions — indicate changes in the environment and predict diversity patterns (De Cáceres et al.,
256 2010). The indicspecies package facilitates this analysis by examining the relationship between
257 species occurrence or abundance and site classification into groups representing habitat types or
258 disturbance states (De Cáceres et al., 2010).
259
260 **RESULTS**
261 ***Evidence for Marsh Microbiomes Buffering Plants from Salinity Stress***

Analysis of variance revealed that microbial inocula manipulation (ANOVA, $F_{2,192}$=4.681, P=0.010) and date (i.e., time) (ANOVA, $F_{3,192}$=4.468, P=0.004) significantly influenced changes in plant stem height, while water treatments were insignificant (ANOVA, $F_{1,192}$=2.481, P=0.117) (Table 1). When comparing plants treated with salt water, the change in stem heights over time of plants with autoclaved restoration-area marsh inocula (Autoclaved Inocula) was less than plants with added microbial inocula (Added Inocula) (Figure 3, Table 2) (EMMs, estimate=−72.76 ± 24.9, t(192)=−2.922, P=0.044). Similarly, 'Added Inocula' and freshwater treatment yielded more productive growth than 'Autoclaved Inocula' and saltwater treatment (EMMs, estimate= 77.79 ± 24.9, t(192)=3.124, P=0.0248) (Figure 3, Table 2). Results also revealed that for 'Autoclaved Inocula' and 'No Addition' treatments, freshwater consistently showed increased plant growth compared to saltwater. However, in the 'Added Inocula' treatment, this trend was reversed, with the saltwater treatment resulting in near-equal or greater median plant height over time compared to its freshwater counterpart. This may hint at salt marsh microbial addition providing a rescue effect from salinity stress.

We observed a similar (but weaker) trend with aboveground plant biomass measurements, where 'Added Inocula' treatment may have buffered salinity stress and resulted in greater aboveground biomass when compared to freshwater treatment (Figure 4a, Table 3). While not statistically significant (ANOVA, $F_{2,21}$ = 0.656, P = 0.526), we did observe that the aboveground biomass tended to be greater under 'Added Inocula' in saltwater conditions (Figure 4a). When looking at belowground biomass (Figure 4b, Table 4), we observed similar root biomass across the microbial and water treatments (ANOVA, $F_{2,31}$ = 1.496, P = 0.240), which could represent that the allocation of the plant's carbon was more directed at aboveground biomass rather than belowground.

286 ***Salinity and Microbiome Addition Influenced Bacterial Composition More than Diversity***

287 The experimental treatments of salinity and marsh microbial inocula additions resulted in
288 an average bacterial Shannon Diversity H' of 6.448 ± 0.083 (Figure 5a) and Pielou's Evenness J' of
289 0.850 ± 0.0057 (Figure 5b). Soil bacterial diversity metrics were similar across water (ANOVA, $F_{1,48}$ =
290 0.040, P = 0.842) and microbial treatments (ANOVA, $F_{2,48}$ = 1.789, P = 0.178) (Table 6). Likewise,
291 evenness metrics were similar across treatments of water (ANOVA, $F_{1,48}$ = 0.041, P = 0.840) and
292 microbial addition (ANOVA, $F_{2,48}$ = 1.861, P = 0.167) (Table 7).

293 Conversely, microbial and water treatments significantly modified bacterial community
294 composition (Figure 6, Table 5). The main effects of water (PERMANOVA, R^2=0.1002, P<0.001) and
295 microbial (PERMANOVA, R^2=0.0863, P<0.001) treatments significantly influenced bacterial
296 community composition (Table 5). We observed distinct bacterial community composition
297 associated with water treatment, while there was more overlap in bacterial communities across
298 microbial treatments for salt compared to freshwater treatments (Figure 6).

299

300 ***Context-Dependent Responses of Bacterial Indicator Taxa Across Treatments***

301 To further examine bacterial associations with microbial and salinity treatments, we used
302 indicator species analysis to identify a subset of bacterial taxa representing each experimental
303 root-associated microbiome in salt and freshwater treatments (Table S1). There were 48 OTUs
304 significantly associated with freshwater conditions across all inocula treatments from phyla
305 *Proteobacteria*, *Acidobacteria, Actinobacteria*, *Verrucomicrobia*, *Planctomycetes*, and
306 *Gemmatimonadetes*; and 37 OTUs were associated with all saltwater treatments from phyla
307 *Proteobacteria*, *Bacteroidetes*, *Acidobacteria*, and *Verrucomicrobia*. In freshwater treatments,
308 prominent OTUs represented orders *Myxococcales*, *Burkholderiales*, and *Gp1*, the family
309 *Desulfuromonadaceae*, and the genus *Dongia*. In contrast, in the saltwater treatments, OTUs

310 represented taxa from the genus *Opitutus*, family *Rhodospirillaceae*, and order *Myxococcales*
311 (Table S1).
312 For the microbial treatments, there were 30 OTUs were significantly associated with the 'No
313 Addition' control treatment from phyla *Proteobacteria, Planctomycetes*, *Bacteroidetes*,
314 *Acidobacteria*, *Verrucomicrobia*, *Actinobacteria*, *Gemmatimonadetes*; 8 OTUs were associated
315 with 'Autoclaved Inocula' treatment from phyla *Proteobacteria*, *Verrucomicrobia*, *Actinobacteria*;
316 and 22 OTUs were associated with the Added Inocula' treatment from phyla *Proteobacteria*,
317 *Verrucomicrobia*, *Acidobacteria, Actinobacteria*, and *WPS-1* (Table S2). The top OTUs for the 'No
318 Addition' treatments represented taxa from the families *Rhizomicrobium*, *Plantomycetaceae*,
319 *Sinobacteraceae*, and *Chitnophagaceae*; for 'Autoclaved Inocula' treatment indicator species
320 represented families *Myxococcales* and *Rhodospirillales* and orders *Myxococcales* and
321 *Rhodospirillales*; and for the 'Added Inocula' treatment, top indicator species represented genera
322 *Geobacter*, *Prosthecobacter* and family *Gp1* (Table S2).
323 Finally, when characterizing differences in field soil samples, indicator species analysis
324 revealed 22 OTUs significantly associated with bulk plant soil from phyla *Proteobacteria* and
325 *Bacteroidetes*; 26 OTUs were associated with rhizosphere soil from phyla *Proteobacteria*,
326 *Chloroflexi*, *Firmicutes*, and two unidentified phyla (Table S3). Noteworthy OTUs from bulk soil
327 were represented by taxa from the order *Chromatiales*, families *Desulfobulbaceae*,
328 *Rhodobacteraceae*, and *Desulfobacteraceae,* and the genus *Robertkochia*. Rhizosphere-
329 associated OTUs were represented by the families *Methylococcaceae* and *Sinobacteraceae*
330 (*Solimonadaceae*) and the order *Bacillales*.
331
332 ***Plant-Associated Marsh Microbiomes and Elevation Influenced Community Composition***

In support of the short-term abiotic and biotic effects on bacterial communities and plant growth, the characterization of sediment microbiomes at a newly planted marsh restoration site revealed that plant associations (bulk vs. rhizosphere) and shoreline location (below and above mean high waterline) influenced bacterial community composition over time (Figure 7).

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* plugs 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 'Added Inocula' at early plant development stages may ameliorate salinity stress (as demonstrated

357 by the reversal trend in median plant heights, Figure 3). Conversely, autoclaved microbial media
358 and salinity stress treatment were shown to decrease the rate of growth in plant stem height (Table
359 2). While intuitive, this provides evidence that the sterilization of belowground community
360 influences and the addition of nonessential or less-utilized abiotic materials can hinder plant
361 productivity. These findings are in accordance with a similar study, where greenhouse-raised *S.*
362 *alterniflora* that were exposed to saltwater (10 ppt salinity) and microbial consortium (a selection of
363 plant growth-promoting rhizobacteria) experienced the highest rates of growth when compared to
364 other treatments (Bledsoe & Boopathy, 2016).

365 Aboveground biomass results further supported evidence that microbial inocula additions
366 during early plant development may be critical for establishing stress-tolerant plugs. While not
367 statistically significant (Tables 3 and 4), we observed similar patterns for the belowground biomass.
368 The allocation of carbon to aboveground biomass more than belowground is of interest as it may
369 hint at efficient primary productivity and carbon sequestration but less carbon storage and root
370 establishment. As salt marsh roots indirectly modulate soil dynamics and facilitate resilience to
371 abiotic stress (Feagin et al., 2009), additional studies are necessary to determine the consequence
372 of gradual stressors (e.g., sea-level rise, tidal forces) on the success of plant establishment during
373 marsh restoration.

374 When analyzing patterns in bacterial diversity, average bacterial diversity H' and evenness J'
375 were similar across microbial and water treatments. The 'Autoclaved Inocula' treatment tended to
376 decrease the ranges of H' and J', while greater ranges existed in the 'Added Inocula' treatment.
377 Additionally, marsh microbial inocula under salinity stress tended to exhibit greater medians in H'
378 and J' than that of freshwater, in support of the reversal trend. Throughout contemporary literature,
379 it is well-accepted that increases in microbial biodiversity and evenness are associated with
380 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 water treatments significantly influenced community composition, with distinct bacterial communities forming in the salt and freshwater groups (Table 1, Figure 6). This may indicate the strength of the selective pressure of environmental conditions in forming community structure and should be considered when applying microbial inocula for restoration. Further, our results suggest that the elevation gradient and associated abiotic factors found *in situ* may play roles in varying the bacterial community composition along the shoreline (Figure 7). The separation along the primary coordinate axis indicates that shoreline elevation and related environmental factors significantly influence bacterial community structure. Likewise, distinct clustering patterns between bulk soil and rhizosphere samples (Figure 7) suggest that the root environment exerts selective pressures, fostering unique bacterial assemblages compared to the surrounding bulk soil (REFs).

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 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 add 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. One such overlapping bioindicator was between saltwater treatments and rhizosphere soil samples, with and OUT from

the nitrogen-fixing family *Desulfuromonadaceae* (Greene, 2014). However, in general, microbial bioindicators were unique among water and microbial addition treatments, as well as bulk vs. rhizosphere samples, further providing evidence that plant-microbe interactions and environmental conditions uniquely structure soil communities. It is important to note that due to bacteria's high biodiversity, many remain unculturable and unidentified (Stewart, 2012), which represents 23 bacterial taxa that could not be resolved past the phylum or kingdom level.

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 distinct bacterial assemblages when under the influence of salinity treatments. The pre-treatment of salt conditions and sediment microbes could help grasses buffer from abiotic and biotic field stressors after marsh grass plugs are transplanted and exposed to stressful environmental conditions. This work provides evidence that microbial stewardship is essential for buffering against ecological 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 and 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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STATEMENTS AND DECLARATIONS

The authors declare no competing interests.

TABLES

Table 1. Summary ANOVA comparing changes in stem height (in mm) due to main effects (water treatment, microbial inocula treatment, date) and the interaction water × microbial treatments × date. Bold text indicates significant differences (P ≤ 0.05).

Effect	*DF*	Sum of Sqs	Mean Sq	*F-value*	*P-value*
Microbe TRT	2	100859	50429	4.6807	**0.0104**
Water TRT	1	26730	26730	2.4810	0.1169
Date	3	144419	48140	4.4682	**0.0047**
Water TRT: Microbe TRT	2	18190	9095	0.8442	0.4315
Microbe TRT: Date	6	15666	2611	0.2423	0.9619
Water TRT: Date	3	3457	1152	0.1069	0.9560
Microbe TRT: Water TRT: Date	6	31374	5229	0.4853	0.8188
Residuals	192	2068598	10774		

Table 2. Summary of estimated marginal means (EMMs) contrasting isolated experimental manipulation effects on plant stem height. Results are averaged over time. P value adjustment: tukey method for comparing a family of 6 estimates. Bold text indicates significant differences (P ≤ 0.05).

Contrast	*estimate*	SE	DF	*T-ratio*	*P-value*
CONTROL FRESH - Microbe_neg FRESH	-3.31	24.0	192	-0.138	1.0000
CONTROL FRESH - Microbe_pos FRESH	-33.72	24.5	192	-1.378	0.7400
CONTROL FRESH - CONTROL SALT	12.52	24.7	192	0.508	0.9958
CONTROL FRESH - Microbe_neg SALT	44.06	24.9	192	1.770	0.4878
CONTROL FRESH - Microbe_pos SALT	-28.69	24.5	192	-1.173	0.8493
Microbe_neg FRESH - Microbe_pos FRESH	-30.41	24.0	192	-1.267	0.8026
Microbe_neg FRESH - CONTROL SALT	15.83	24.2	192	0.654	0.9865
Microbe_neg FRESH - Microbe_neg SALT	47.37	24.4	192	1.938	0.3823
Microbe_neg FRESH - Microbe_pos SALT	-25.38	24.0	192	-1.058	0.8974
Microbe_pos FRESH - CONTROL SALT	46.24	24.7	192	1.876	0.4204
Microbe_pos FRESH - Microbe_neg SALT	77.79	24.9	192	3.124	**0.0248**
Microbe_pos FRESH - Microbe_pos SALT	5.03	24.5	192	0.206	0.9999
CONTROL SALT - Microbe_neg SALT	31.54	25.1	192	1.257	0.8076
CONTROL SALT - Microbe_pos SALT	-41.22	24.7	192	-1.672	0.5522
Microbe_neg SALT - Microbe_pos SALT	-72.76	24.9	192	-2.922	**0.0444**

Table 3. Summary ANOVA comparing changes in aboveground biomass due to main effects (water treatment, microbial inocula treatment) and the interaction water × microbial treatments. Bold text indicates significant differences (P ≤ 0.05).

Effect	*DF*	Sum of Sqs	Mean Sq	*F-value*	*P-value*
Microbe TRT	2	15.199	7.5994	2.6148	0.08926
Water TRT	1	2.458	2.4581	0.8458	0.36485
Water TRT: Microbe TRT	2	3.811	1.9054	0.6556	0.52618
Residual	31	90.097	2.9064		

Table 4. Summary ANOVA comparing changes in belowground biomass due to main effects (water treatment, microbial inocula treatment) and the interaction water × microbial treatments. Bold text indicates significant differences (P ≤ 0.05).

Effect	*DF*	Sum of Sqs	Mean Sq	*F-value*	*P-value*
Microbe TRT	2	2.803	1.40151	1.3003	0.2869
Water TRT	1	0.036	0.03587	0.0333	0.8564
Water TRT: Microbe TRT	2	3.225	1.61240	1.4960	0.2398
Residual	31	33.413	1.07782		

Table 5. Summary PERMANOVA comparing microbial community composition due to main effects (water treatment, microbial inocula treatment) and the interaction water × microbial treatments. Bold text indicates significant differences (P ≤ 0.05).

Effect	*DF*	Sum of Sqs	R^2	*F-value*	*P-value*
Water TRT	1	0.4953	0.1002	6.2325	**<0.001**
Microbe TRT	2	0.4267	0.0863	2.6845	**<0.001**
Water TRT: Microbe TRT	2	0.2062	0.0417	1.2973	0.1399
Residual	48	3.8147	0.7718		
Total	53	4.9429	1.0000		

Table 6. Summary ANOVA comparing changes in Shannon Diversity H' due to main effects (water treatment, microbial inocula treatment) and the interaction water × microbial treatments. Bold text indicates significant differences (P ≤ 0.05).

Effect	*DF*	Sum of Sqs	Mean Sq	*F-value*	*P-value*
Water TRT	1	0.000	0.00033	0.005	0.947
Microbe TRT	2	0.254	0.12716	1.769	0.181
Water TRT: Microbe TRT	2	0.184	0.09182	1.278	0.288
Residual	48	3.450	0.07187		

Table 7. Summary ANOVA comparing changes in Pielou's Evenness J' due to main effects (water treatment, microbial inocula treatment) and the interaction water × microbial treatments. Bold text indicates significant differences (P ≤ 0.05).

Effect	*DF*	Sum of Sqs	Mean Sq	*F-value*	*P-value*
Water TRT	1	0.000002	0.0000016	0.005	0.946
Microbe TRT	2	0.001336	0.0006682	1.936	0.155
Water TRT: Microbe TRT	2	0.000738	0.0003688	1.068	0.352
Residual	48	0.016569	0.0003452		

FIGURES

Figure 1.

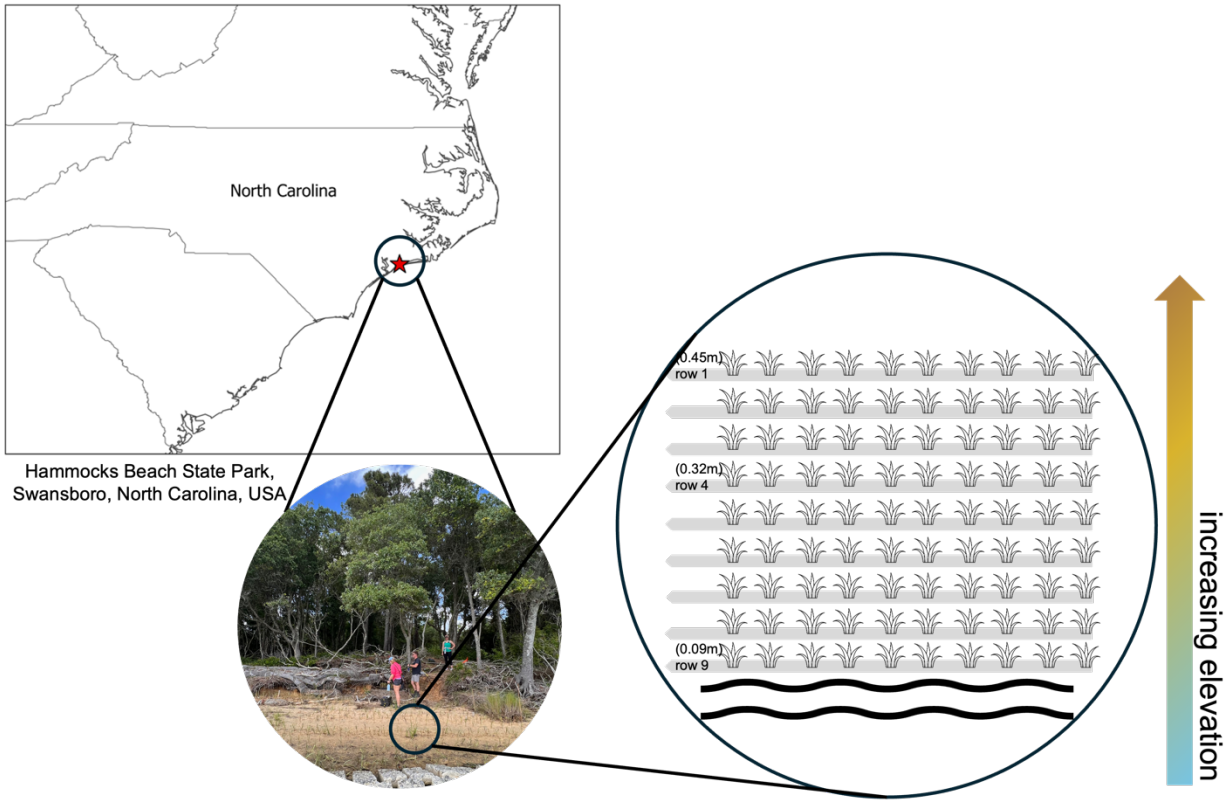
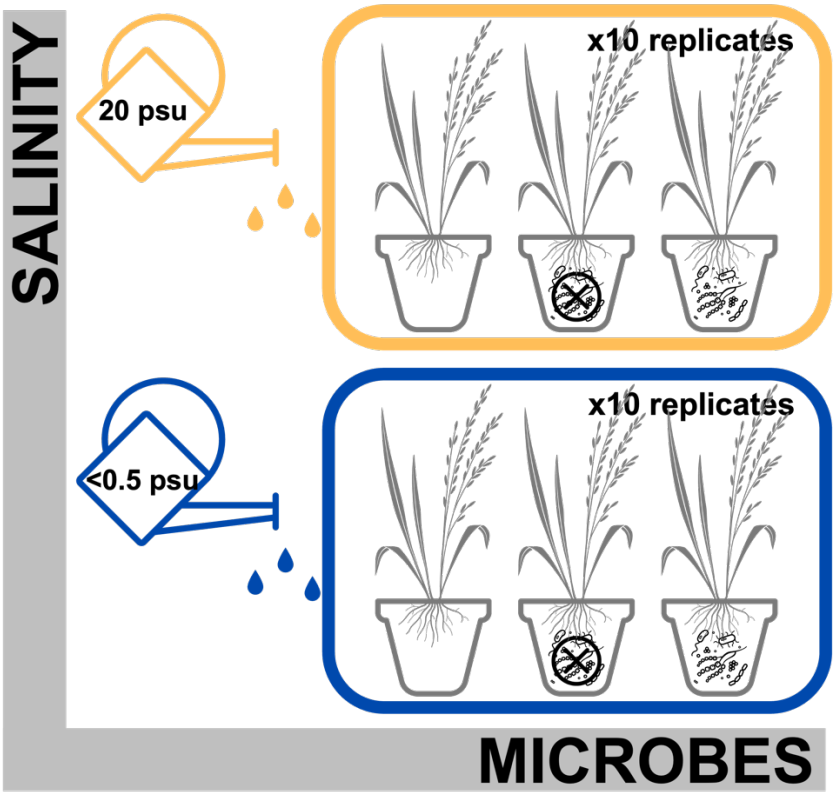


Figure 1. Location of field marsh sediment and plant sampling at Hammocks Beach State Park, Swansboro, North Carolina, USA. *Sporobolus alterniflorus* transplants were planted along an elevation gradient. Row 1 represented the highest elevation and row 9 represented the lowest elevation. At three time points (12 June 2023, 13 July 2023, 5 October 2023), one plant sample and associated bulk soil were collected and processed to examine bulk and rhizosphere microbiomes. Marsh sediment from this field site was used as the microbial inocula treatment in the salinity x microbe experiment.

715 **Figure 2.**



716

717 **Figure 2.** Experimental design of salinity/microbe treatments. Factorial experiment with three levels

718 of microbial addition (No Addition, Autoclaved Inocula, Added Inocula) and two levels of salinity

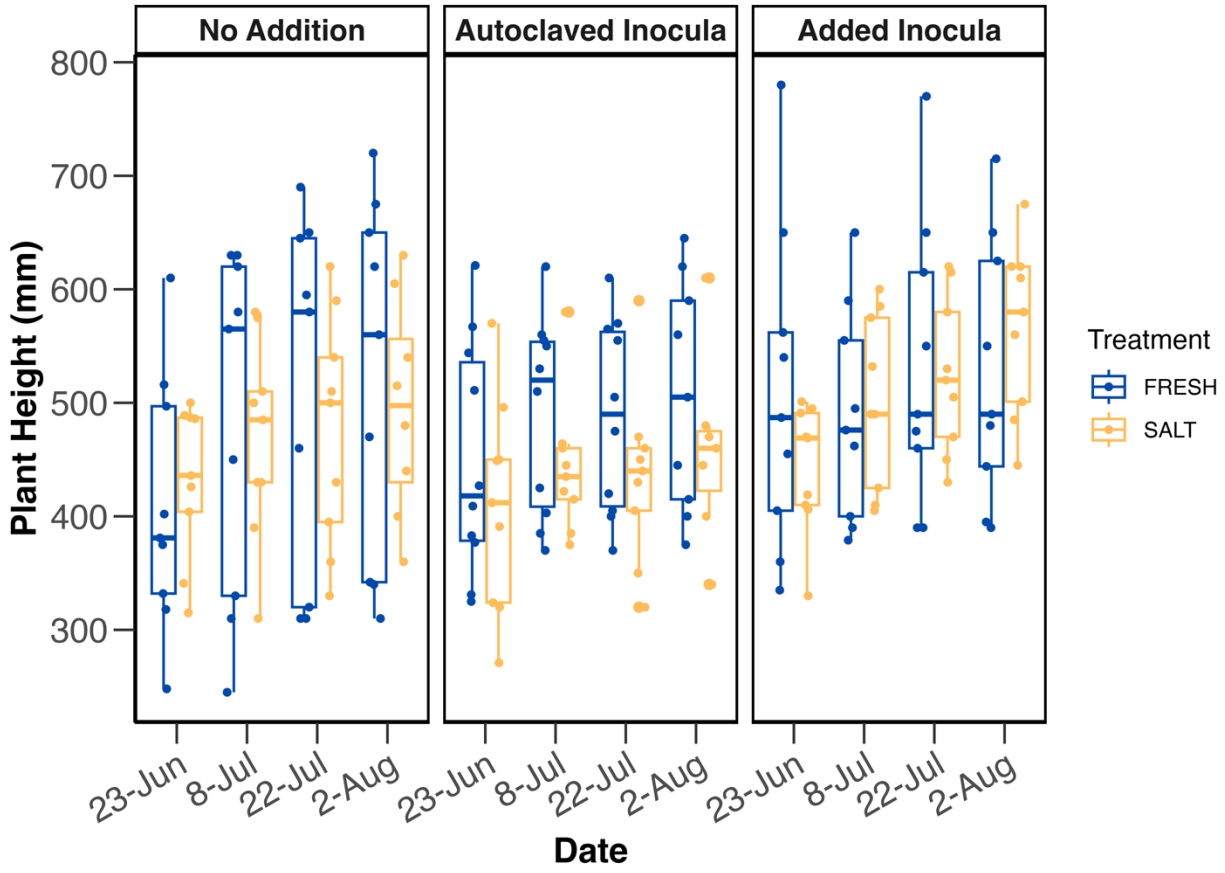
719 (<0.5 psu, 20 psu). Treatments were replicated ten times. Microbial inocula were combined with

720 potting mix and established for four weeks, followed by weekly salt stress treatment, and harvested

721 after eight weeks.

722

723 **Figure 3.**



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726 **Figure 3.** Boxplots depicting plant height (in mm) on the y-axis over time on the x-axis in response to

727 marsh soil microbes and salinity stress treatments. Color represents salinity stress (orange) and

728 freshwater control (blue). The boxplot is a visual representation of 5 key summary statistics: the

729 median, the 25% and 75% percentiles, and the whiskers, which represent the feasible range of the

730 data as determined by 1.5 × the interquartile range. Symbols represent individual data points.

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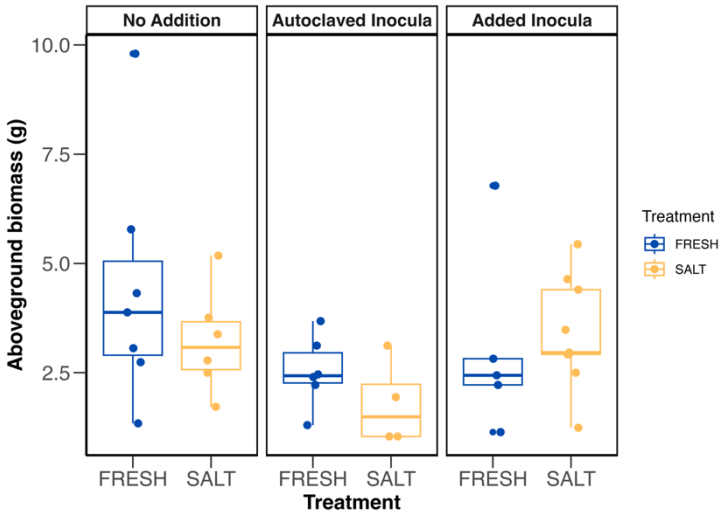
Figure 4.

(a)



(b)

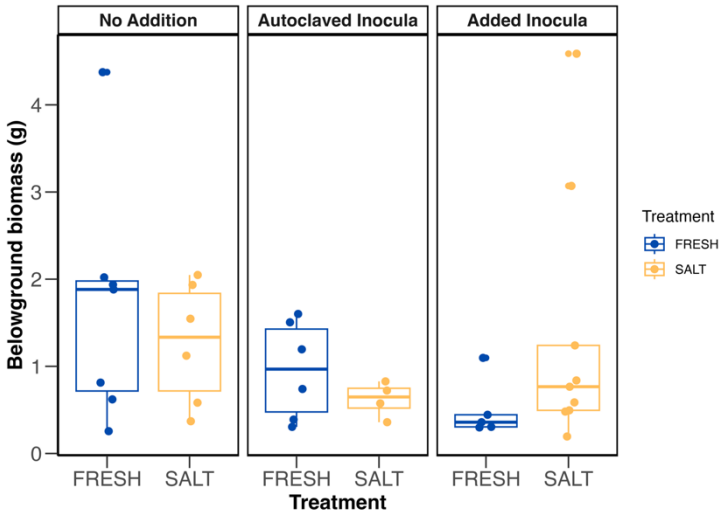
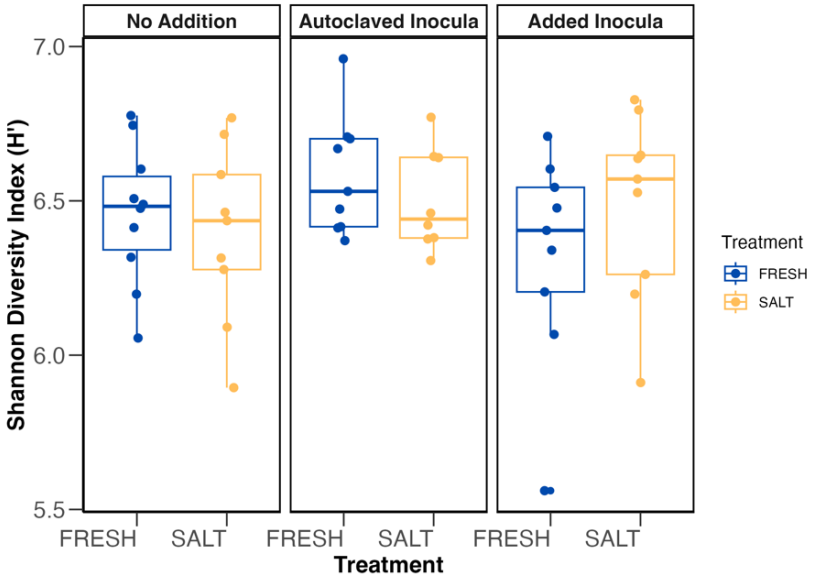


Figure 4. Boxplots depicting aboveground (a) and belowground root (b) biomass (in g) on the y-axis in response to microbial (No Addition, Autoclaved Inocula, Added Inocula) and salinity (<0.5 psu, 20 psu) treatments. Color represents salinity stress (orange) and freshwater control (blue). The boxplot 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.

Figure 5.

(a)



(b)

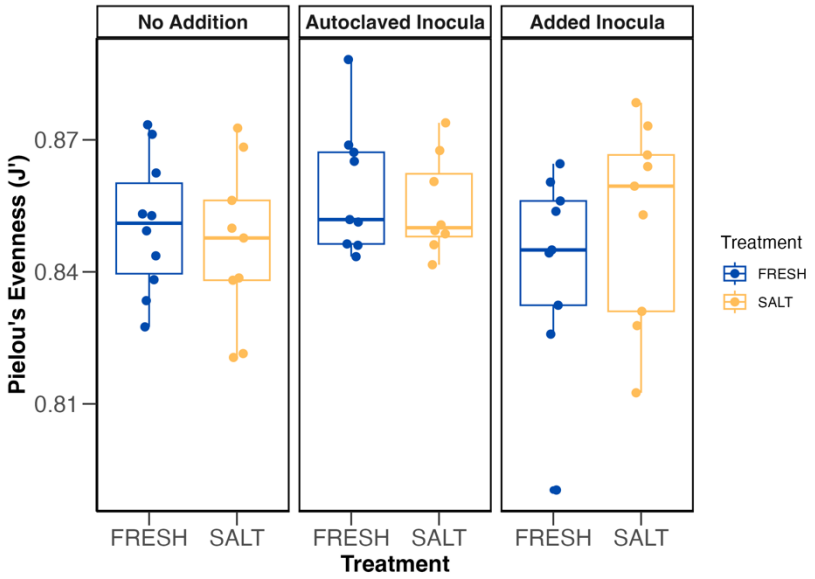
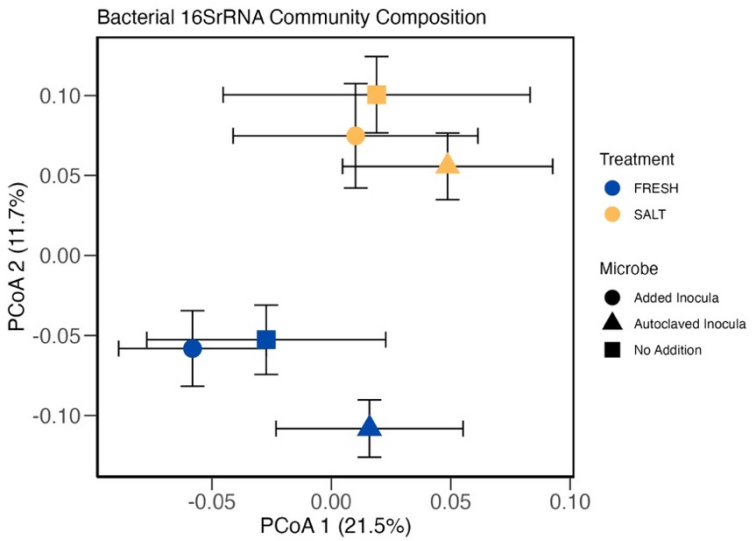


Figure 5. Boxplots for bacterial diversity representing Shannon Diversity Index (H') (a) and Pielou's Evenness (J') (b) associated with microbial (No Addition, Autoclaved Inocula, Added Inocula) and salinity (<0.5 psu, 20 psu) treatments. Colors indicate salinity treatment (fresh in blue, salt in orange). Symbols represent individual data points.

Figure 6.

(a)



(b)

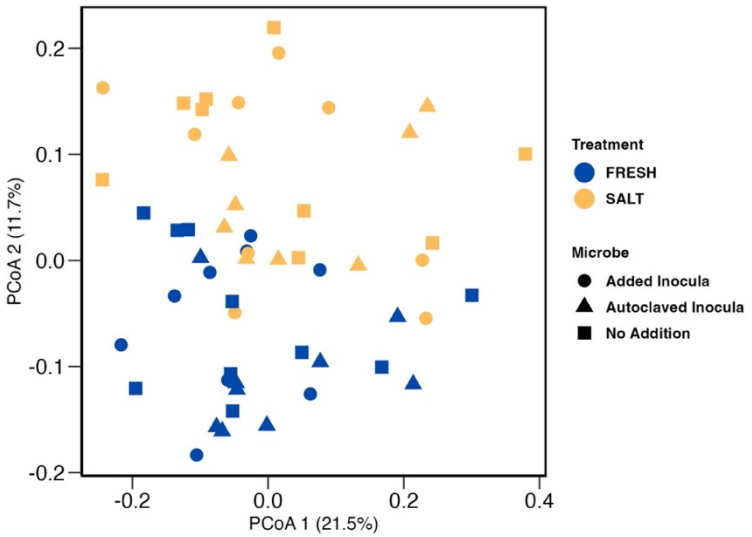
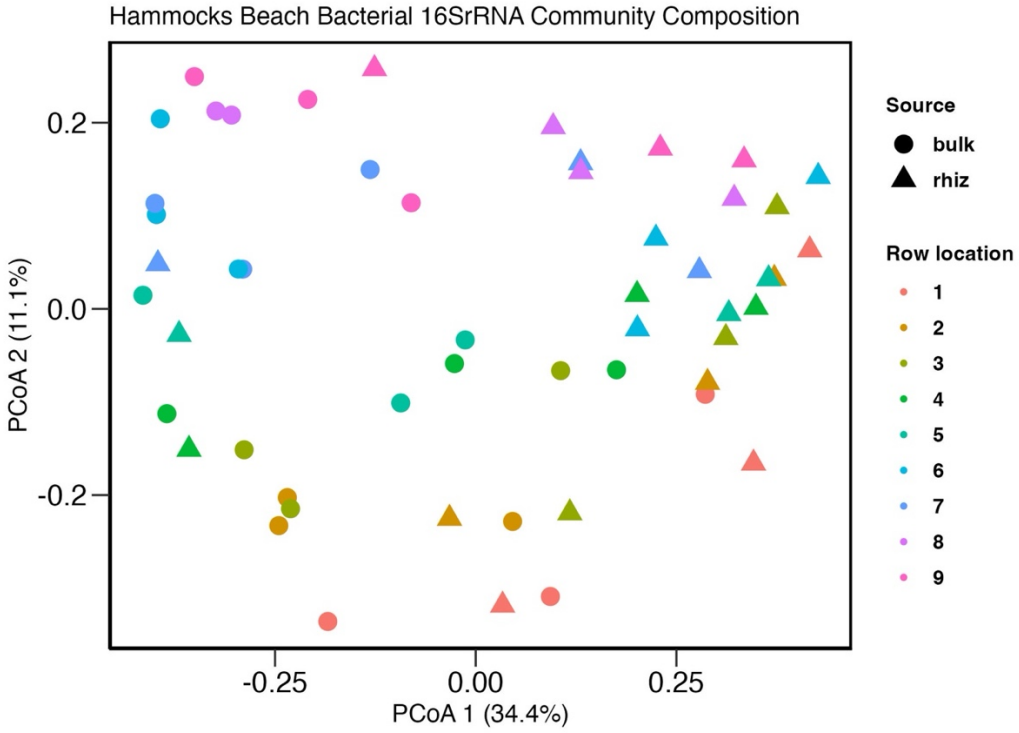


Figure 6. Ordination based on a principal coordinates analysis depicting bacterial community composition according to salinity and microbial treatments representing average centroid across treatments (a) and individual samples (b). Symbols are colored according to salinity treatment (orange = saltwater, blue = freshwater) and shaped by microbial treatment (circles = Autoclaved Inocula, triangles = Added Inocula, squares = No Addition).

806 **Figure 7.**



807

808 **Figure 7.** Ordination based on a principal coordinates analysis depicting bacterial community

809 composition according to salinity gradient from landward (row 1) to seaward (row 9) and sample

810 source (bulk soil = circles, root-associated rhizosphere = triangles). Symbols are colored according

811 to row planted. Row 4 was located at approximately the mean high-water line.

812