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

RUNNING TITLE: Salt marsh plant-microbiome restoration

Kai A. Davis[1], Mary-Margaret McKinney[2], Rachel K. Gittman[1], Ariane L. Peralta[1,*]

[1]Department of Biology, [2]Integrated Coastal Sciences, East Carolina University, Greenville, North Carolina, USA, 3 Coastal Studies Institute, Wanchese, North Carolina, USA

*Corresponding Author: phone: 252-328-2712, email: peraltaa@ecu.edu

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). Raw amplicon sequence files available at NCBI SRA BioProject ID PRJNA1172654 (https://www.ncbi.nlm.nih.gov/bioproject/1172654).

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*) 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 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.

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

71 the effect of salinity stress and microbial inoculation on marsh plant productivity would further the
72 understanding of ecological mechanisms and aid in restoring coastal wetland ecosystems.
73 In a broader context, microorganisms can enhance the nutrient acquisition of plants,
74 support growth, and suppress diseases from pathogens (Mariotte et al., 2018; Farrer et al., 2022). In
75 turn, plants can provide carbon resources and oxygen to root-associated microorganisms (Mariotte
76 et al., 2018). Human activities and climate change can and have altered these plant-microbe
77 relationships in ways that promoted or suppressed plant growth. For example, anthropogenic-
78 caused eutrophication and pollution have disrupted nutrient cycles and have led to losses in
79 microbial biodiversity (Cavicchioli et al., 2019), while sea level rise and extreme climatic events
80 damage plants and disrupt mutualisms (Zabin et al., 2022). Conversely, the practice of microbiome
81 stewardship — the intentional understanding and manipulation of microbial communities — can
82 lead to increased microbiome diversity, evenness, and homeostasis (Peixoto et al., 2022). Growing
83 evidence has also indicated that microbial communities can increase plant survivorship and
84 growth in high-stress environments, aiding ecological restoration efforts (McHugh & Dighton, 2004;
85 Mariotte et al., 2018).
86 With the urgent need for enhanced restoration techniques, we aim to understand how to
87 promote positive plant-microbe associations to aid in restoring coastal wetland ecosystems where
88 human stressors and climate change challenge restoration outcomes. In this investigation, we
89 evaluated the interactive effects of salinity stress and marsh sediment microbial inoculation on *S.*
90 *alterniflorus* productivity. Conducting a replicated factorial with two levels of salinity and three
91 levels of microbial inocula treatment, we measured the plant height and biomass (aboveground
92 and belowground) of *S. alterniflorus* plugs after a 2-month duration. Additionally, we extracted
93 genomic DNA from the sediment samples to characterize the root-associated microbiomes of the
94 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

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 mid-spring 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 time of planting using a Trimble Virtual Reference Station (VRS; Trimble, Sunnyvale, California, USA), Real Time Kinematic (RTK), Global Positioning System (GPS). Elevations ranged from an average of 0.45 m along the highest row to 0.09 m along the lowest row. Row 4 was approximately along the MHW line at an elevation of

119 0.32 m. Surviving planted plugs in each row were counted every two to three weeks throughout the
120 2023 growing season, and the height of the longest leaf on each plant was recorded to the nearest
121 0.1 cm. Sediment samples were taken, and one plant per row was destructively harvested for
122 biomass measurements two, six, and 16 weeks after planting.

123 We sampled field soil during three dates June 12th, July 13th, and Oct. 5th. We sampled along
124 the transect from row 1 to row 9. At each row, we randomly sampled a plant (above and
125 belowground biomass) and associated soil using a 5 cm diameter PVC corer to 12 cm depth. We
126 stored samples on ice and then at 4 ºC overnight prior to sample processing for downstream
127 microbiome analyses. To separate bulk from root-associated soils, we cut aboveground plant
128 biomass, we shook the sediment from the roots into the plastic bag, and transferred roots and
129 remaining soils into sterile tube and mixed with sterile 1X phosphate buffer solution (1X PBS). After
130 shaking for 5 minutes, we used sterile tweezers to remove roots and transferred cleaned roots into
131 paper bag to dry at 60 °C for 48-hours. We centrifuged the remaining root-associated soils, carefully
132 poured off PBS, and stored these rhizosphere sediments at -20 °C until DNA extractions. After
133 removing the plant from the field sediment sample, we homogenized the sediment sample,
134 transferred a subsample to a sterile WhirlPak bag, and stored the bulk soil sample at -20 °C until
135 DNA extractions.

136

137 ***Microbiome x Salinity Stress Experimental Design***

138 To test our hypothesis, we conducted a factorial experiment with three levels of microbiome
139 addition (No Addition, Autoclaved Inocula, Added Inocula) and two levels of salinity (<0.5 psu, 20
140 psu), replicated ten times (n=60) (Figure 2). Marsh sediment was collected from Hammocks Beach
141 State Park (Swansboro, North Carolina), while the marsh plugs, *Sporobolus alterniflorus*, were
142 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 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.

For the salinity treatment, we exposed each potted plugs 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

167 without confounding factors associated with prolonged inundation. After eight weeks, the plants
168 were harvested and processed for plant biomass and microbial sequencing.
169
170 ***Plant and Soil Sample Processing***
171 We measured maximum plant height and number of live stems biweekly. At the end of the
172 experiment, after two months, we harvested aboveground biomass and roots, dried the plant
173 biomass samples at 60 °C for 48 hours, and weighed aboveground and root biomass. For soil
174 bacterial characterization and analysis, we homogenized the remaining soil, transferred soil into
175 sterile WhirlPak bags, and stored samples at -20 °C until DNA extraction and 16S rRNA amplicon
176 sequencing (described below).
177
178 ***DNA Extraction and Bacterial Amplicon Sequencing***
179 To characterize the sediment microbiomes of the laboratory-treated plugs, we extracted
180 genomic DNA from sediments using the Qiagen DNeasy PowerSoil Kit, employing a combination of
181 chemical and mechanical methods to lyse the sample following the manufacturers protocol. The
182 isolated DNA was quantified using the Nanodrop spectrophotometer and diluted to a standardized
183 concentration of 10 ng μl^{-1} to ensure uniformity across all samples. This process facilitated
184 equimolar concentrations of DNA for downstream applications. Triplicate Polymerase Chain
185 Reactions (PCR) were subsequently conducted on all extracted samples, serving as an efficient
186 downstream technique for the amplified DNA. To characterize bacterial communities, we used
187 barcoded primers (515FB/806R) initially developed by the Earth Microbiome Project (Caporaso et
188 al. 2012) to target the V4 region of the bacterial 16S subunit of the ribosomal RNA gene (Caporaso
189 et al., 2012; Apprill et al., 2015; Parada et al., 2016). For each sample, three 50 µL PCR libraries
190 were prepared by combining 35.75 µL molecular grade water, 5 µL Amplitaq Gold 360 10x buffer, 5

191 μL $MgCl_2$ (25 mM), 1 μL dNTPs (40mM total, 10mM each), 0.25 μL Amplitaq Gold 360 polymerase, 1
192 μL 515 forward barcoded primer (10 μM), 1 μL 806 reverse primer (10 μM), and 1 μL DNA template
193 (10 ng μL^{-1}). Thermocycler conditions for PCR reactions were as follows: initial denaturation (94 °C,
194 3 minutes); 30 cycles of 94 °C for 45 seconds, 50 °C for 30 seconds, 72 °C for 90 seconds; final
195 elongation (72 °C, 10 minutes). The triplicate 50 μL PCR libraries were combined and then cleaned
196 using the Axygen® AxyPrep Magnetic (MAG) Bead Purification Kit (Axygen, Union City, California,
197 USA). Cleaned PCR products were quantified using QuantIT dsDNA BR assay (Thermo Scientific,
198 Waltham, Massachusetts, USA) and diluted to a concentration of 10 ng μL^{-1} before pooling libraries
199 in equimolar concentration of 5 ng μL^{-1}. We sequenced the pooled libraries using the Illumina
200 MiSeq platform using paired-end (2×300 bp) reads (Illumina Reagent Kit v2, 500 reaction kit) at the
201 Duke University Center for Genomic and Computational Biology Core Sequencing Facility.
202 Sequences were processed using the mothur (v1.42.0) (Schloss et al. 2009) MiSeq pipeline
203 (Kozich et al., 2013). We assembled contigs from the paired end reads, quality trimmed using a
204 moving average quality score (minimum quality score 35), aligned sequences to the SILVA rRNA
205 database (v132) (Quast et al. 2013), and removed chimeric sequences using the VSEARCH
206 algorithm (Rognes et al. 2016). We created operational taxonomic units (OTUs) by first splitting
207 sequences based on taxonomic class and then binning into OTUs based on 97% sequence
208 similarity. Taxonomic identity was assigned using the SILVA rRNA database (v132) (Quast et al.
209 2013). We used an OTU-based 3% distance threshold instead of using amplicon sequence variant
210 (ASV) of a single base difference, especially given our high diversity microbial communities based
211 on short-read (250 bp) amplicon sequences and that similar broadscale microbiome patterns are
212 observed using OTU- or ASV-based approaches (Glassman and Martiny 2018). In addition, the OTU-
213 based approach limits overestimating the number of ASVs due to PCR bias and sequencing errors
214 (Schloss 2021).

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 4.4.1, 2024; R Studio version 2024.09.0+375, 2024) using the vegan, ade4, picante packages, and custom functions (Dray et al., 2023; Oksanen et al., 2022). Prior to statistical analyses, we low abundance OTUs that were <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. Samples were organized by salinity treatment and microbial treatments. Several soil bacterial diversity metrics were then computed, including Shannon Diversity (H') and Pielou's Evenness (J'). 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

239 Index standardizes the Shannon H' to account for differences in species richness, allowing for
240 comparisons of evenness across communities with varying species numbers.
241 We examined the microbial community variation explained by inoculation and salinity
242 treatment using a permutational multivariate analysis of variance (PERMANOVA). Principal
243 Coordinates Analysis (PCoA) graphs were generated to visualize the multivariate relationships
244 between samples based on their microbial community compositions, allowing for exploration of
245 how the varying experimental factors influenced the overall microbial diversity and structure.
246 To analyze prominent indicator species within the microbial communities, we used the
247 package 'indicspecies'. Indicator species — selected based on their ability to reflect environmental
248 conditions — indicate changes in the environment and predict diversity patterns (De Cáceres et al.,
249 2010). The indicspecies package facilitates this analysis by examining the relationship between
250 species occurrence or abundance and site classification into groups representing habitat types or
251 disturbance states (De Cáceres et al., 2010).
252
253 **RESULTS**
254 ***Evidence for Marsh Microbiomes Buffering Plants from Salinity Stress***
255 Saltwater stressors and autoclaved microbial addition 'Autoclaved Inocula' tended to
256 decrease the rate of plant growth (ANOVA, $F_{2,260}$=3.184, P=0.043, Figure 3). For 'Added Inocula'
257 compared to 'Autoclaved Inocula', we observed an increase in plant height over time, especially
258 under salt stress treatment (Figure 3). Results also revealed that for the 'Autoclaved Inocula' and
259 'No Addition', freshwater treatments consistently showed increased plant growth compared to
260 saltwater. However, in the 'Added Inocula' treatments, this trend was reversed, with the saltwater
261 treatment resulting in greater median plant height compared to its freshwater counterpart. This
262 may hint at salt marsh microbial addition providing a rescue effect from salinity stress.

263 A similar (but weaker) trend was observed with aboveground plant biomass measurements,
264 where 'Added Inocula' treatment buffered salinity stress and resulted in more growth when
265 compared to freshwater treatment (Figure 4a). Although not statistically significant (ANOVA, $F_{2,45}$ =
266 0.662, P = 0.521), the reversal trend was still exhibited; aboveground biomass tended to be higher
267 under 'Added Inocula' in salt compared to freshwater conditions (Figure 4a). When looking at
268 belowground biomass (Figure 5), we observed similar root biomass across the microbial and water
269 treatments (ANOVA, $F_{2,45}$ = 1.458, P = 0.243) (Figure 4b), which could represent that the allocation of
270 the plant's carbon was more directed at aboveground biomass rather than belowground.
271
272 ***Salinity and Microbiome Addition Influenced Bacterial Composition More than Diversity***
273 The experimental treatments of salinity and marsh microbial inocula additions resulted in
274 an average bacterial Shannon Diversity H' of 6.448 ± 0.083 and Pielou's Evenness J' of 0.850 ±
275 0.0057. Soil bacterial diversity and evenness metrics were similar among treatments (ANOVA,
276 inocula: NS, water: NS). The pattern of greater medians measured in the saltwater compared to
277 freshwater treatments was observed for both Shannon Diversity (Figure 5a) and Pielou's Evenness
278 (Figure 5b) indices.
279 Microbial and water treatments influenced bacterial community composition (Figure 6,
280 Table 1). The main effects of water (PERMANOVA, R^2=0.1002, P<0.001) and microbial
281 (PERMANOVA, R^2=0.0863, P<0.001) treatments significantly influenced bacterial community
282 composition (Table 1). We observed distinct bacterial community composition associated with
283 water treatment, while there was more overlap in bacterial communities across microbial
284 treatments for salt compared to freshwater treatments (Figure 6). In support of the short-term
285 abiotic and biotic effects on bacterial communities and plant growth, the characterization of
286 sediment microbiomes at a newly planted marsh restoration site revealed that plant associations

287 (bulk vs. rhizosphere) and shoreline location (below and above mean high waterline) influenced
288 bacterial community composition over time (Figure S1).
289
290 ***Bacterial Indicator Operational Taxonomic Units Differed Across Treatments***
291 To further examine bacterial associations with microbial and salinity treatments, we used
292 indicator species analysis to identify a subset of bacterial taxa representing each experimental
293 root-associated microbiome in salt and freshwater treatments (Table S1). There were 48 OTUs were
294 significantly associated with the freshwater treatment from phyla *Proteobacteria*, *Verrucomicrobia*,
295 *Plantomycetes*, *Acidobacteria*, *Acidobacteria*, and *Gemmatimonadetes*; and 37 OTUs were
296 associated with saltwater treatments from phyla *Proteobacteria*, *Bacteroidetes*, *Acidobacteria*, and
297 *Verrucomicrobia*. In freshwater treatments, prominent OTUs included orders *Myxococcales*,
298 *Burkholderiales*, and *Gp1*, family *Desulfuromonadaceae*, and genus *Dongia*, while in the saltwater
299 treatments, OTUs represented taxa from the genus *Opitutus*, family *Rhodospirillaceae*, and order
300 *Myxococcales* (Table S1).
301 For the microbial treatments, there were 30 OTUs were significantly associated with the 'No
302 Addition' control treatment from phyla *Proteobacteria, Planctomycetes*, *Bacteroidetes*,
303 *Acidobacteria*, *Verrucomicrobia*, *Actinobacteria*, *Gemmatimonadetes*, 8 OTUs were associated
304 with 'Autoclaved Inocula' treatment from phyla *Proteobacteria*, *Verrucomicrobia*, *Actinobacteria*,
305 and 22 OTUs were associated with the Added Inocula' treatment from phyla *Proteobacteria*,
306 *Verrucomicrobia*, *Acidobacteria, Actinobacteria*, and *WPS-1* (Table S2). The top OTUs for the 'No
307 Addition' treatments represented taxa from the families *Rhizomicrobium*, *Plantomycetaceae*,
308 *Sinobacteraceae*, and *Chitnophagaceae*; for 'Autoclaved Inocula' treatment indicator species
309 represented families *Myxococcales* and *Rhodospirillales* and orders *Myxococcales* and

310 *Rhodospirillales*; and for the 'Added Inocula' treatment, top indicator species represented genera
311 *Geobacter*, *Prosthecobacter* and family *Gp1* (Table S2).
312
313 **DISCUSSION**
314 While understanding the underpinnings of microbial ecology is still in its relative infancy
315 compared to more contemporary scientific disciplines, the roles of belowground microbial
316 communities in coastal wetland restoration have been of great interest (Birnbaum & Trevathan-
317 Tackett, 2023). Many empirical studies have demonstrated the reliance of plants on soil
318 microbiomes in tolerating environmental stresses, including salinity, inundation, drought, and
319 limited nutrient availability (reviewed in Farrer et al., 2022; Rodriguez et al., 2008). Furthermore, the
320 adaptive and evolutionary responses of plants to changing environmental conditions have been
321 shown to rely heavily on the context of microbial community assemblage, with plants exhibiting
322 increased fitness when associated with stress-adapted microbiomes (Lau & Lennon, 2012).
323 Presently, gaps in knowledge hinder the success of complete ecosystem restoration projects
324 (Duarte et al., 2015); therefore, the foundational knowledge gained from biological conservation
325 and species restoration studies is of utmost relevance and importance. In our investigation, the
326 interactive effects of microbial inocula additions and salinity treatment on *S. alterniflorus* height,
327 biomass, and root-associated microbiomes produced various results. We hypothesized that
328 inoculating *S. alterniflorus* plugs with salt marsh microbial communities would buffer salt stress
329 and promote plant growth and survivorship; a subset of our results supports this hypothesis.
330 Upon analyzing plant height data, we concluded that the addition of marsh microbes
331 'Added Inocula' at early plant development stages may ameliorate salinity stress (as demonstrated
332 by the reversal trend in median plant heights). Conversely, autoclaved microbial media and salinity
333 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 plugs. 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 'Autoclaved Inocula' treatment tended to decrease the ranges of H' and J', while greater ranges existed in the 'Added Inocula' 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.

CONCLUSION

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. 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 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 and protect shorelines from erosion, provide tourism, and facilitate many other functions

381 (Arkema et al., 2013; Buchanan et al., 2022). Therefore, preserving and restoring such ecological
382 habitats are vital to humans, wildlife, and the environment, emphasizing the concept of One Health
383 (Peixoto et al., 2022). By evaluating the interactions between *Sporobolus alterniflorus*, marsh
384 sediment microbiomes, and environmental stressors, this study provides insight into ecological
385 dynamics and may aid in salt marsh restoration efforts considering climate and anthropogenic
386 change.
387

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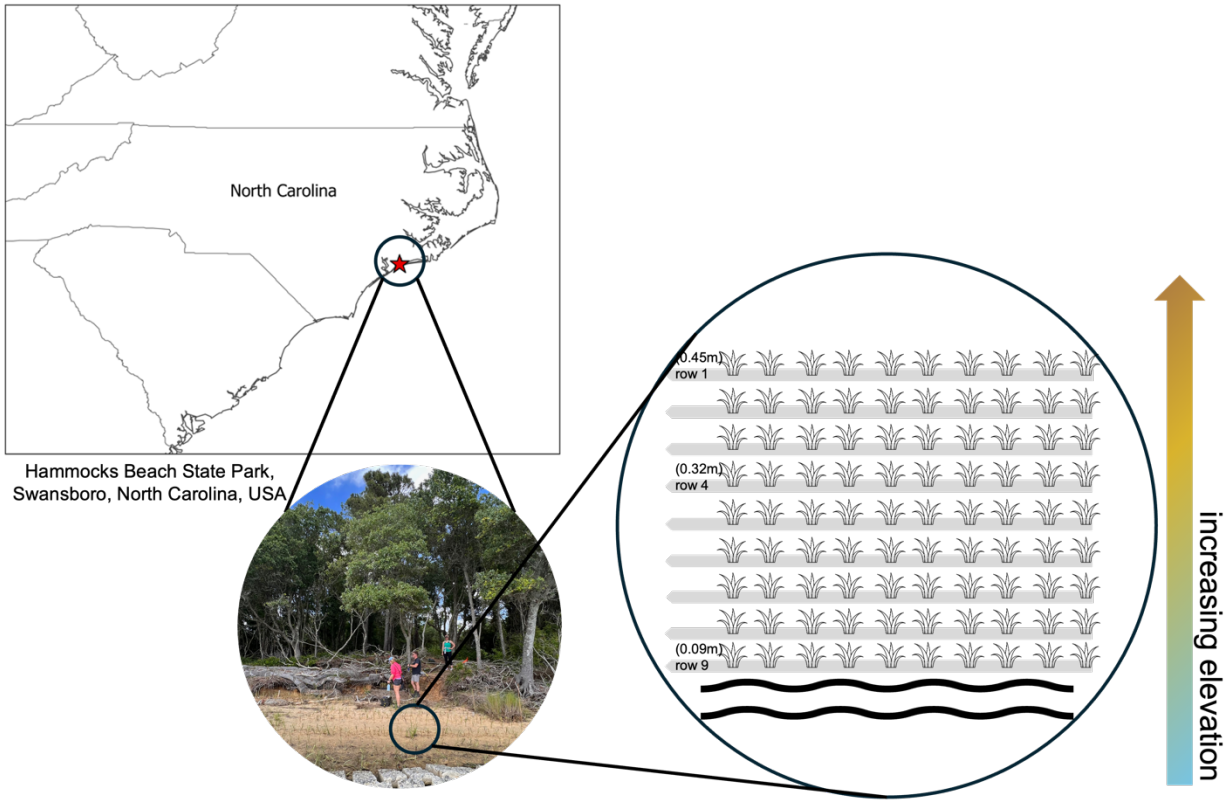
526 **FIGURES**

527 **Figure 1.**

528



529

530 **Figure 1.** Location of field marsh sediment and plant sampling at Hammocks Beach State Park,

531 Swansboro, North Carolina, USA. *Sporobolus alterniflorus* transplants were planted along an

532 elevation gradient. Row 1 represented the highest elevation and row 9 represented the lowest

533 elevation. At three time points (12 June 2023, 13 July 2023, 5 October 2023), one plant sample and

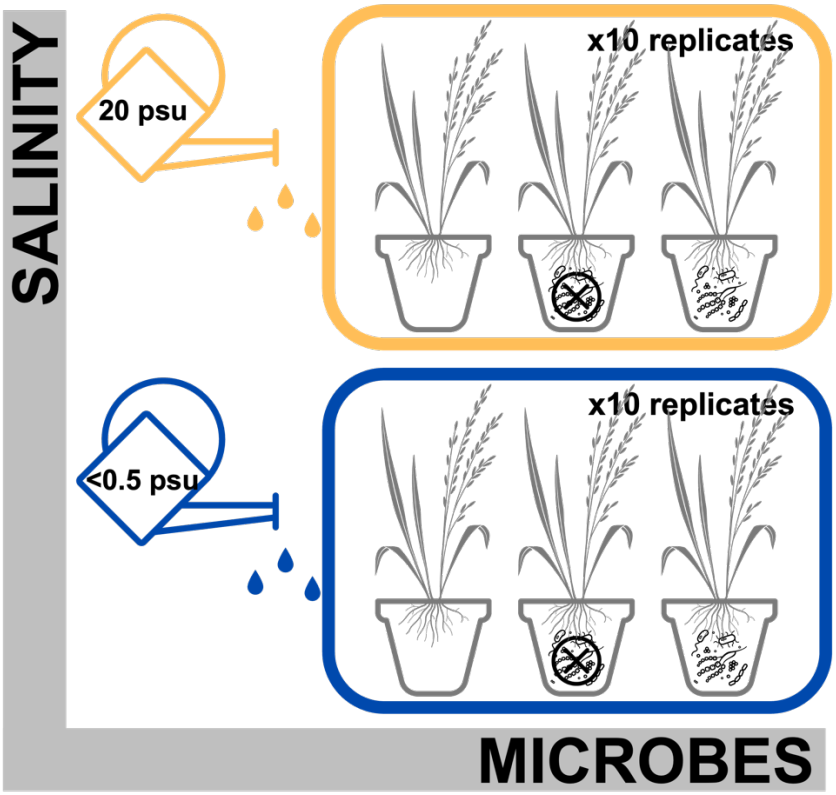
534 associated bulk soil were collected and processed to examine bulk and rhizosphere microbiomes.

535 Marsh sediment from this field site was used as the microbial inocula treatment in the salinity x

536 microbe experiment.

537

538 **Figure 2.**

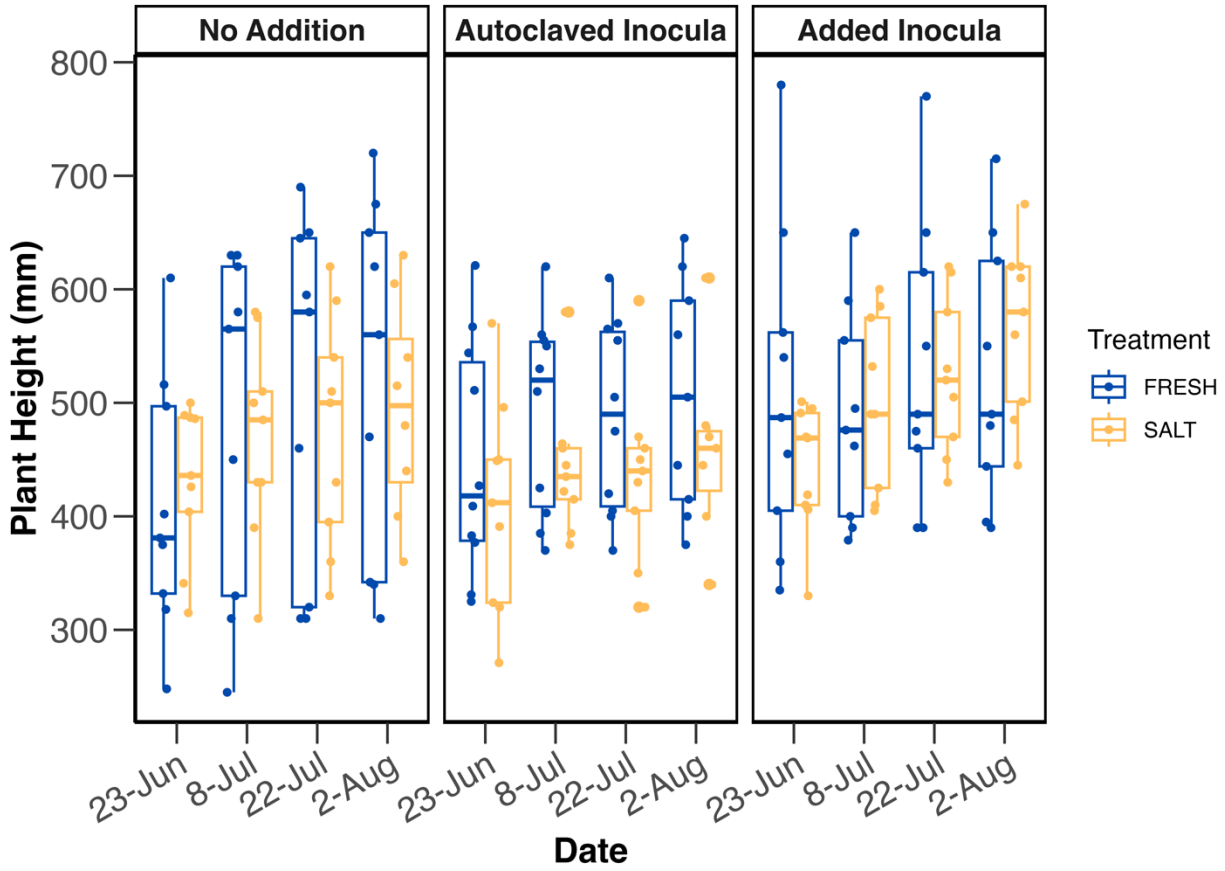


539

540 **Figure 2.** Experimental design of salinity/microbe treatments. Factorial experiment with three levels
541 of microbial addition (No Addition, Autoclaved Inocula, Added Inocula) and two levels of salinity
542 (<0.5 psu, 20 psu). Treatments were replicated ten times. Microbial inocula were combined with
543 potting mix and established for four weeks, followed by weekly salt stress treatment, and harvested
544 after eight weeks.

545

546 **Figure 3.**



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548

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

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

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

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

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

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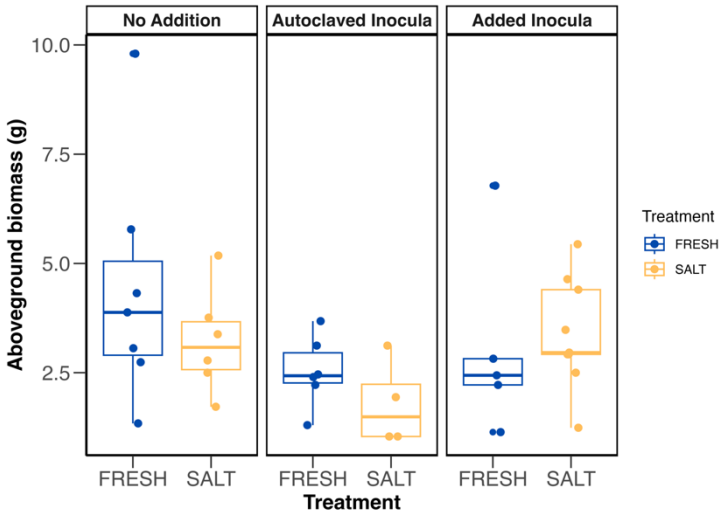
Figure 4.

(a)



(b)

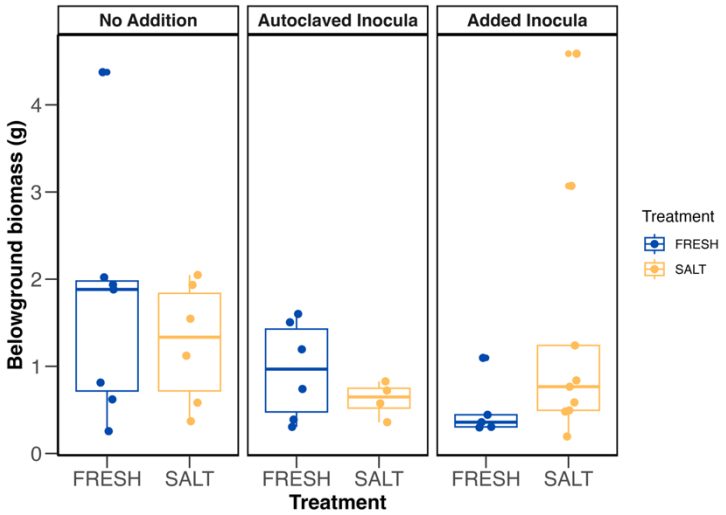


Figure 4. Boxplots depicting aboveground (a) and belowground root (b) biomass (in g) on the y-axis in response to microbial (No Addition, Autoclaved Inocula, Added Inocula) and salinity (<0.5 psu, 20 psu) treatments. Color represents salinity stress (orange) and freshwater control (blue). The boxplot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

582 **Figure 5.**

583 **(a)**

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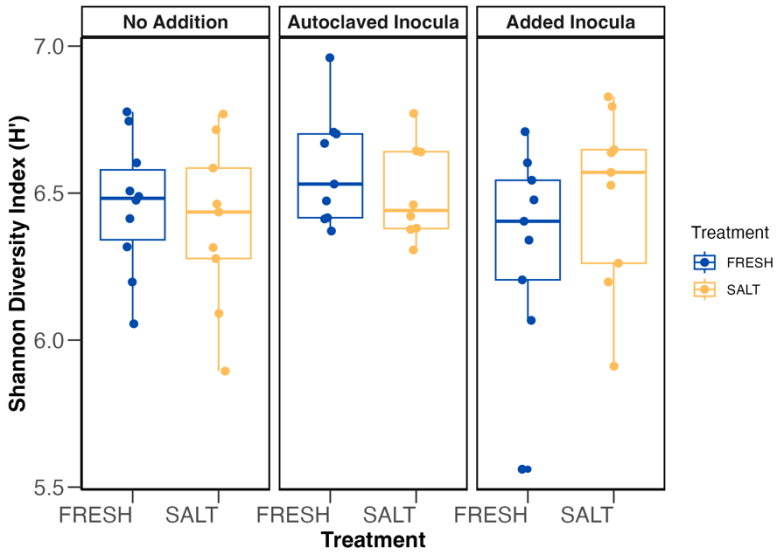

591 **(b)**

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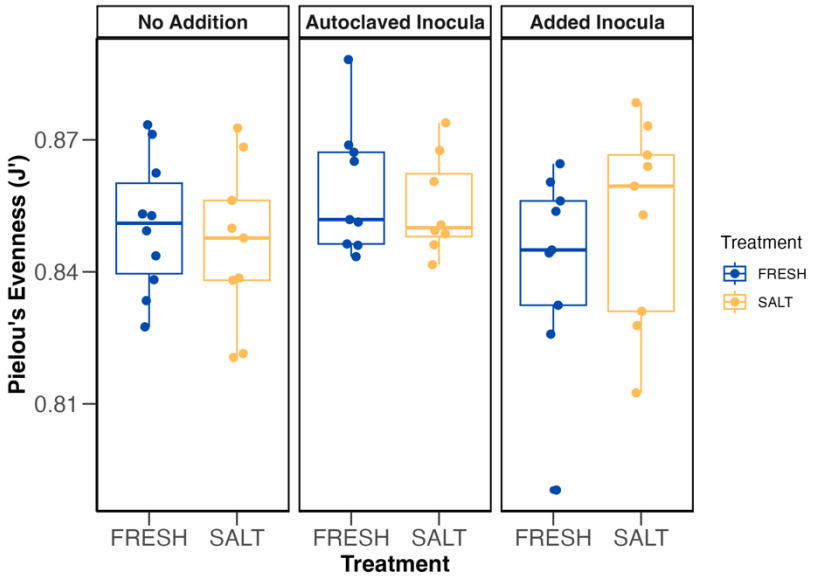


600 **Figure 5.** Boxplots for bacterial diversity representing Shannon Diversity Index (H') (a) and Pielou's

601 Evenness (J') (b) associated with microbial (No Addition, Autoclaved Inocula, Added Inocula) and

602 salinity (<0.5 psu, 20 psu) treatments. Colors indicate salinity treatment (fresh in blue, salt in

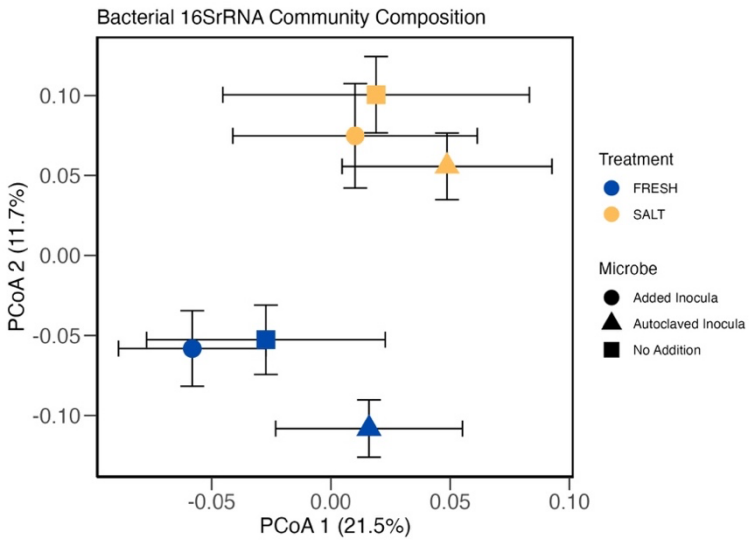
603 orange). Symbols represent individual data points.

604

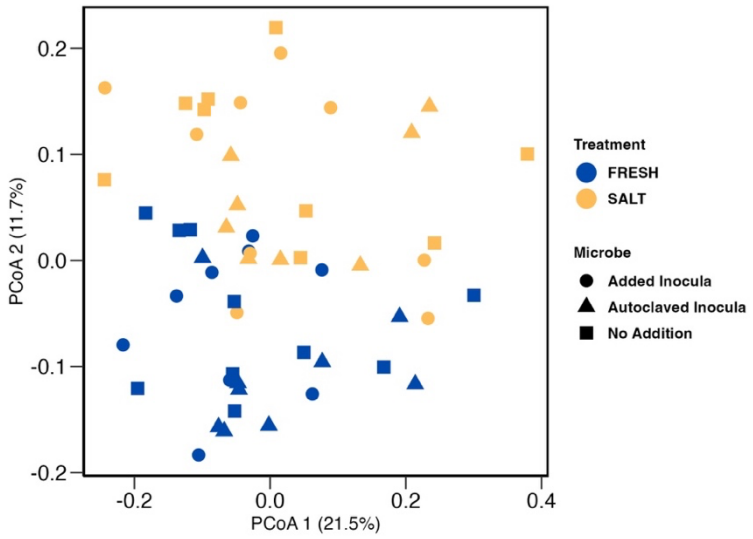
605

606 **Figure 6.**

607 **(a)**



615 **(b)**



623

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

Table 1. 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	0.1290
Residual	48	3.8147	0.7718		
Total	53	4.9429	1.0000		