EXAMINING CHRONIC NUTRIENT ENRICHMENT EFFECTS ON WETLAND PLANT-MICROBE INTERACTIONS USING A TRAIT-BASED APPROACH

by

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Mutualistic plant-microbe relationships that have evolved under nutrient starved environmental conditions are important for generating and maintaining plant and microbial biodiversity. However, human activities associated with land use change have altered nutrient cycles in ways that disrupt these long-standing mutualisms. Mutualistic plant-microbe relationships are especially necessary in low-nutrient ecosystems, and previous studies have shown that root-associated microbes enhance plant growth by inhibiting pathogenic fungi, improving nutrient uptake, and increasing plant species richness. Therefore, a deeper understanding of how nutrient enrichment alters the soil microbiome in the context of plant-microbe associations is needed. The goal of this study is to examine how long-term fertilization of a historically low nutrient coastal plain wetland influences plant-microbe associations. An experimental approach was used to test the hypothesis that long-term fertilization alters bacterial traits (e.g., growth rates) that disrupt beneficial plant-microbe associations. Four bacterial isolates from bulk soil were isolated from a long-term fertilization experiment (established in 2003) conducted in a coastal plain wetland located at East Carolina University's West Research Campus (Greenville, North Carolina, USA). For this study, we compared functional traits (e.g.,

growth rates) of phylogenetically identical soil bacterial isolates (>99% similar in the 16S rRNA gene) that were previously taxonomically classified into fast-growing copiotrophs and slow-growing oligotrophs. These simplified bacterial communities cultured from different soil sources (fertilized, unfertilized) were added to *Chasmanthium laxum* seedlings that were exposed to a nutrient gradient over five months. Results showed that bacterial isolates cultured from different nutrient enrichment histories displayed different growth rates depending on their life history strategy (i.e., slow growing oligotroph vs. fast growing copiotroph). After five months of growth, *Chasmanthium laxum* aboveground plant biomass was highest at low fertilization treatments (0x, 0.5x) when exposed to bacterial inocula sourced from unfertilized compared to fertilized soils. Addition of the simplified bacterial community also increased belowground root biomass compared to no bacterial additions. This study revealed that long-term nutrient enrichment does alter soil bacterial traits of cultured isolates and modifies plant-microbe relationships from mutualistic to competitive.

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INTRODUCTION

Mutualistic plant-microbe relationships have evolved under low nutrient environmental conditions and are important for maintaining plant and microbial biodiversity. However, industrial agriculture, deforestation, and fossil fuel combustion have caused an imbalance to global nutrient cycles (O'Sullivan et al. 2016, Guignard et al. 2017). For example, excess fertilizer use and land use conversion have resulted in nutrient enrichment of historically low nutrient ecosystems (O'Sullivan et al. 2016, Guignard et al. 2017). Despite the low nutrient status, coastal plain wetland environments have exceptional carbon storage potential due to symbiotic plant-microbe relationships, high plant productivity, and slow decomposition rates (Jansson and Hofmockel 2020). If nutrient enrichment inadvertently affects plant-microbe relationships resulting in increased carbon turnover, then enhanced carbon losses from wetlands are expected (Jansson and Hofmockel 2020). While characterizing soil microbial diversity has become routine, predicting how environmental change affects microbial community structure and function continues to be challenging (Martiny and Walters 2018).

There are several abiotic (e.g., soil pH, moisture) and biotic (e.g., plant species) factors that are strong environmental filters that influence bacterial community structure and function (Philippot and Hallin 2011). For example, carbon resources and soil nutrient availability influence microbial community composition, and resource and nutrient exchange underlie plant-microbe relationships (Berg 2009). Within the soil, there is a "microbial seed bank" where plants are able to recruit microbes that are best suited for their growth and nutrient uptake (Philippot et al. 2013). The mutualistic relationships between plants and microbes can also help a plant establish immunity by releasing the hormones salicylic and jasmonic acid that signal antimicrobial protection (Bakker et al. 2018). However, ongoing environmental changes could be

modifying established plant-microbe relationships in critical ways (**Figure 1**). For example, fertilization of nutrient poor ecosystems can modify plant-microbe relationships and lead to a more competitive environment (rather than a mutualistic one) (Ramoneda Massague et al. 2019). Therefore, examining how chronic nutrient enrichment alters the soil microbiome and plant-microbe relationships can improve our ability to predict climate change effects on nutrient-limited and biodiversity-rich coastal plain wetland ecosystems (Bakker et al. 2014, Xu et al. 2020, Hicks et al. 2020).

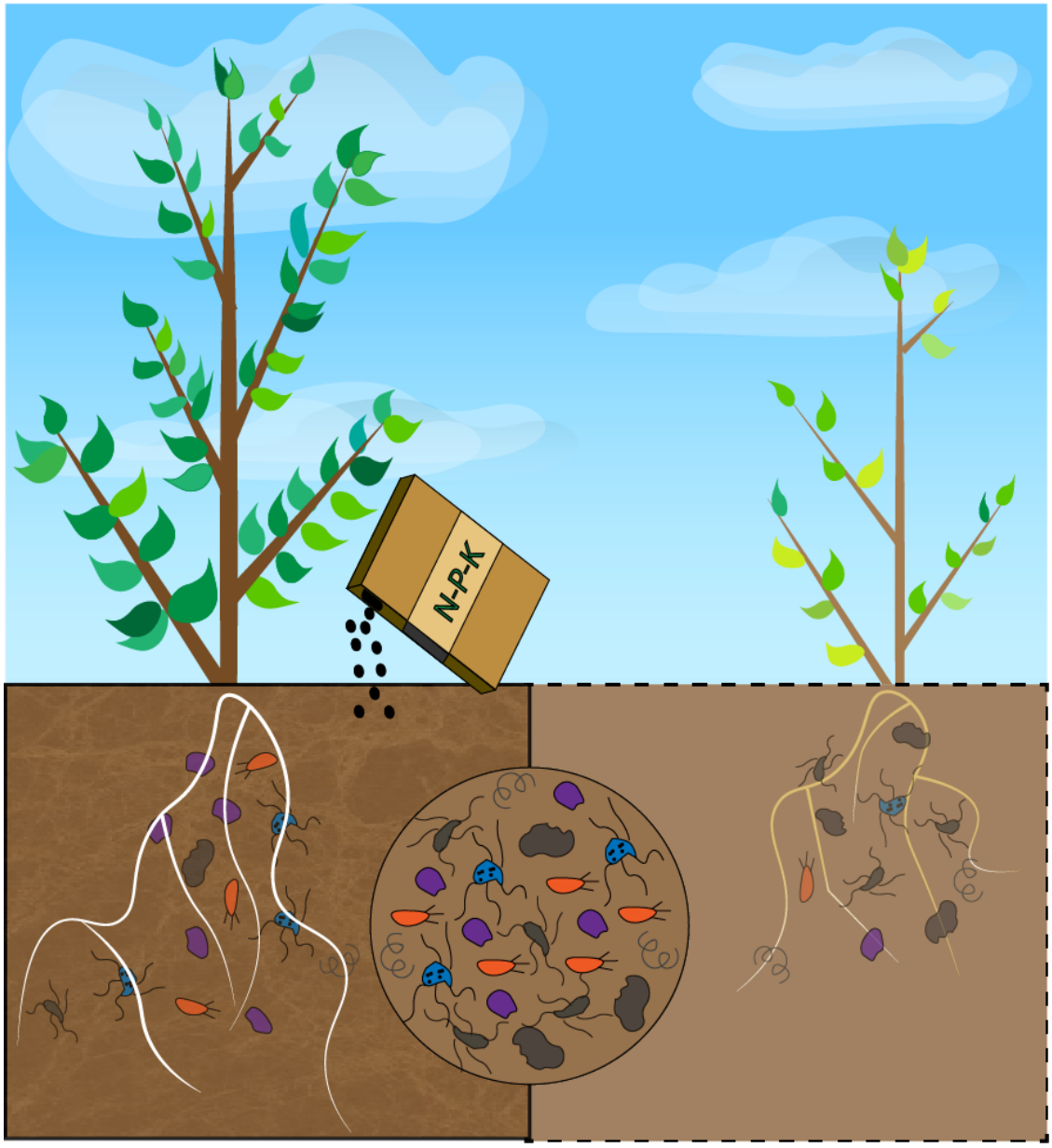

Figure 1: Schematic diagram depicting how addition of fertilizer could be altering plant-microbe interactions. The circle in the middle is an example of the native bacterial community composition of bulk soils. The panel on the left represents how the addition of fertilization would alter the bacterial community composition and what microbes would be available for recruitment by plants. On the right, the bacterial community composition is representative of native, undisrupted soil microbiome. Fertilization of historically nutrient-limited ecosystems can disrupt mutualistic plant-microbe relationship since plants and soil microbes can acquire nutrients directly from the soil.

In cases where soil microbes are starved for nutrients, forming mutualistic relationships with plants enable access to plant-derived nutrients (Berendsen et al. 2012). Benefits for plants would be protection from diseases and enhanced nutrient and mineral uptake, especially around the plant rhizosphere, which is the narrow zone of soil immediately surrounding a plant's root (Liu et al. 2019). Plants can release compounds (e.g., metabolites, flavonoids, hormones) that recruit microbes to the rhizosphere (Philippot et al. 2013, Bakker et al. 2018). However, long-term fertilization can result in major differences between bacterial genomic traits (e.g., chemotaxis, resource acquisition) due to the lack of signaling from plants in fertilized compared to unfertilized conditions (Malik et al. 2020). Therefore, increased fertilization can undermine beneficial plant-microbe relationships.

Studying microbial biodiversity using a trait-based approach informs how the soil microbiome relates to ecosystem functions (Philippot et al. 2013, Martiny et al. 2015). Functional traits can provide information on growth, survival, and reproductive rates which reveal how the microbes affect the ecosystem and how the ecosystem influences the soil microbiome (Krause et al. 2014, Martiny et al. 2015). Identifying changes in these traits could be helpful in connecting the effect of nutrient enrichment on microbial processes (Malik et al. 2020). These traits are also indicative of how organisms will interact with their surroundings; therefore, it is important to study their activity along a gradient of factors (Philippot et al. 2013). Additionally, it is important to recognize the differences between the bulk soil microbiome and rhizosphere so that key taxa for plant health are identified (Berg and Smalla 2009). The continual modifications to the soil and rhizosphere microbiome will challenge the maintenance of beneficial plant-microbe relationships (Fry et al. 2019).

A few examples of important soil taxa are *Pseudomonas spp., Bacillus spp., Collimonas spp.,* and *Arthrobacter spp.* Several strains of *Pseudomonas spp.* (e.g., *P. koreensis and P. fluorescens*) exhibit functional traits that aid plants in the uptake of selected carbohydrates, as well as, respond to root exudates by inducing a fructose-specific system (Mavrodi et al. 2021). *P. fluorescens* and *Collimonas pratensis* are able to produce volatile organic compounds (VOCs) that inhibit fungal pathogens and require root exudates for productions of VOCs (Garbeva et al. 2014, Das et al. 2020). *Pseudomonas spp.*, fast growing copiotrophs, and *Collimonas spp.*, slow growing oligotrophs, are also important phosphate-solubilizing microorganisms and long-term fertilization could affect their composition and abundance (Guo et al. 2022). *Bacillus spp.* and *Arthrobacter spp.*, slow growing oligotroph, are two examples of plant growth-promoting rhizobacteria (PGPR) that have potential to enhance crop productivity (Akinrinlola et al. 2018). Specifically, *Arthrobacter spp.* are important for plant health due to their ability to protect plants from abiotic stresses (Roy and Kumar 2020). Understanding how long-term fertilization influences the functional traits of these soil and rhizosphere bacteria will provide useful insight on how to maintain commensal and beneficial plant-microbe relationships.

Recent work from our lab showed that microbial activity is higher in fertilized compared to unfertilized rhizospheres in a coastal plain wetland, where bacterial communities in bulk soils are oligotroph-dominated (Bledsoe 2020, Bledsoe et al. 2020). These past results revealed that bacterial taxonomic classification of an oligotroph (slow grower) does not match with measured phenotype (high activity). The current study examined how long-term fertilization influenced the relationship between bacterial life history strategy (based on taxonomic classification) and bacterial phenotype (based on growth rates) on plant-bacterial associations. **The overarching goal of this study was to examine how long-term fertilization of a historically low nutrient**

wetland influences plant-microbe relationships. We hypothesized that plant-microbe symbiosis will weaken with increasing soil fertilization, and the relationships between plants and microbes will go from mutualistic to competitive. To test this hypothesis, we 1) isolated and identified bulk soil microbes via culture-dependent analyses; 2) selected bacterial isolates that are taxonomically identical and created two simplified bacterial communities for inoculation of a wetland grass (*Chasmanthum laxum)*; and 3) inoculated *C. laxum* with the simplified communities along a nutrient gradient to measure consequences of plant-bacterial associations. We used a combination of computational and lab-based approaches to examine how long-term fertilization has affected the functional traits of bulk soil microbes in a historically low nutrient coastal plain wetland.

METHODS

FIELD SITE AND LONG-TERM ECOLOGY EXPERIMENT

Study Site and Experimental Design

We collected soil samples from a long-term ecology experiment at East Carolina University's West Research Campus that was established in 2003. The campus is located in the North Carolina coastal plain and more than 60% of the land has been classified as jurisdictional wetlands (Goodwillie et al. 2020). The tract of land is mowed and raked once a year to simulate removal of litter by wildfires (Goodwillie et al. 2020). A 10-10-10 NPK pellet fertilizer is added three times a year, resulting in a 45.4 kg/ha addition for each nutrient (Goodwillie et al. 2020). The treatments are set up as 2 x 2 factorial randomized block design: (1) unmowed/unfertilized, (2) unmowed/fertilized, (3) mowed/unfertilized, (4) mowed/fertilized (Goodwillie et al. 2020). These treatments are replicated on eight 20 x 30 m blocks (**Figure 2**).

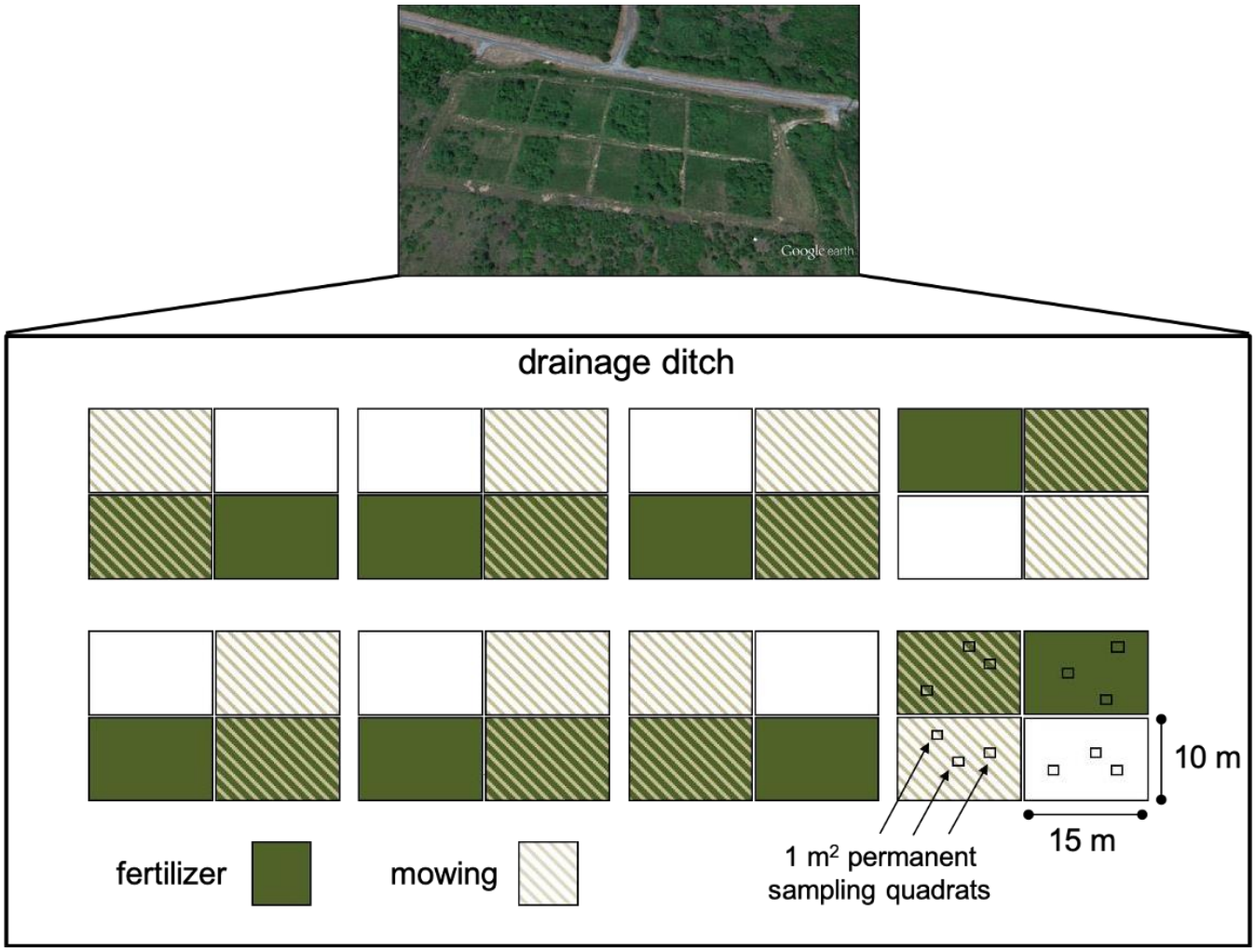

Figure 2: East Carolina University's West Research Campus long term experiment design. There are four treatment plots replicated on eight blocks: control (white), unmowed/fertilized (white with green stripes), mowed/unfertilized (green), mowed/fertilized (green with stripes). This study focuses on mowed/fertilized and mowed/unfertilized treatment plots.

Within these plots are randomized permanent sampling quadrats (1-m^2) where vegetation surveys (since 2004) and soil sampling (since 2014) take place once per year (Goodwillie et al. 2020). There is a ditch present on one side of the study site which creates a hydrology gradient (Goodwillie et al. 2020). The four blocks that are closer to the ditch have drier soil than the four blocks further away from the ditch (Bledsoe 2020). The plant community within the treatment blocks is a mosaic of mixed hardwood, wet pine flatwood, and pine savanna (Goodwillie et al. 2020). The soils are historically acidic and low in nutrients (Chester 2004). This study was focused on soil bacterial isolates and native wetland grass (*Chasmanthum laxum*) collected from the mowed/fertilized (MF) plots in December 2021.

From each of the treatment plots, we collected two soil cores (12 cm depth, 3.1 cm diameter) from each of the permanent sampling quadrats and combined the six cores into a single composite bulk soil sample (Bledsoe 2020). We passed each bulk soil sample through a 4 mm sieve, homogenize the sample, and remove any plant debris (Bledsoe 2020). Then, for each treatment, we transferred 10 g of each subsample to combine all replicates into a single combined sample and into stored at 4 °C for bacterial culturing.

BACTERIAL CULTURING FROM SOIL

Isolation and Identification

We cultured bacterial isolates from bulk soils collected in 2018 using a serial dilution approach. We weighed 1 g of soil from the mowed/fertilized plots or the mowed/unfertilized plots into a 15 mL falcon tube and added 9 mL of 1X PBS to the stock tubes, which represented a 1:10 dilution (10^{-1}). We prepared a series of dilution tubes by adding 1 mL of inocula to 9 mL of 1X PBS to achieve 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} dilutions. Based on previous work, there was little

to no bacterial growth was observed after the 10^{-5} dilution. We chose the dilution tubes 10^{-3}, 10^{-4}, and 10^{-5} for spread plating due to low fungal growth and optimal growth for slow and fast-growing bacterial colonies. From each dilution tube, we added 100 mL of culture and plated onto R2A media. R2A agar was used due to it being a low nutrient media that promotes the growth of oligotrophic microbes (Horgan et al. 1999). The R2A spread plates were incubated in the dark at room temperature for 5 days. Then, we randomly selected single bacterial colonies, streaked single colonies onto new R2A plates, and repeated the transfer of a single bacterial colony 3 times to ensure a pure bacterial isolate.

We selected a single colony from the streak plates and inoculated 9 mL culture tubes with R2 broth. The inoculated R2 broth tubes were incubated at room temperature for three days in environ shaker. We centrifuged 1 mL of inoculated R2 broth in a 2 mL microcentrifuge tube, discarded the R2 broth, and repeated the process four times to create a pellet in preparation for DNA extraction.

We used the Qiagen UltraClean Microbial DNA Isolation Kit to extract DNA from the isolated bacterial colonies and diluted the samples to 10 ng/μL. The diluted DNA samples were used as template DNA for PCR reactions to amplify the 16S rRNA gene. The PCR master mix included 29.5 μL molecular grade water, 10 μL 5x Colorless GoTaq Flexi buffer, 5 μL $MgCl_2$ (25mM), 1.25 μL dNTPs (10mM), 1 μL 8 forward primer (10 uM), 1 μL 1492 reverse primer (10 mM), 0.25 μL GoTaq DNA polymerase, and 2 μL DNA template, and 1 μL of 10 ng/μL DNA template. Thermocycler conditions for PCR reactions were as follows: initial denaturation (95 °C 2 minutes); 30 cycles of 94 °C for 35 seconds, 52 °C for 45 seconds, 72 °C for 2 minutes; final elongation (72 °C, 2 minutes). We cleaned the PCR samples using the Axygen AxyPrep Magnetic Bead Purification kit. Following cleaning, the concentration and purity of PCR

samples were quantified using Quant-iT dsDNA BR (broad-range) assay (Thermo Scientific, Waltham, MA, USA) in preparation for Sanger Sequencing standard BigDye reactions. Samples were sequenced on a SeqStudio Genetic Analyzer (Applied Biosystems) at the East Carolina University Genomics Core. For each bacterial isolate, we created a concatenated sequence using the forward and reverse reads to ensure quality and length of the target product using Geneious Prime (Geneious Prime 2022.0.2). Then, we identified each isolate to genus and species using tblastx in Geneious Prime and the SILVA database. We used grade, sequence length and % pairwise provided by Geneious Prime to select the most probable genus and species and checked with SILVA ACT database (Pruesse et al. 2012) (**Table 1; Table S1**).

Table 1: The bacterial isolates selected for the simplified community were identified using the databases tblastx in Geneious Prime and SILVA. The treatments are mowed/fertilized (MF) and mowed/unfertilized (M).

Genus	Species	Sample ID	Treatment
Collimonas	*pratensis*	WRC_281	fertilized/mowed
Pseudomonas	*fluorescens*	WRC_246	fertilized/mowed
Pseudomonas	*koreensis*	WRC_315	fertilized/mowed
Arthrobacter	*ramosus*	WRC_267	fertilized/mowed
Collimonas	*pratensis*	WRC_283	unfertilized/mowed
Pseudomonas	*fluorescens*	WRC_288	unfertilized/mowed
Pseudomonas	*koreensis*	WRC_271	unfertilized/mowed
Arthrobacter	*ramosus*	WRC_263	unfertilized/mowed

PHYLOGENETIC ANALYSIS

All statistical calculations and graphing were conducted in the R environment (R version 4.0.2, 2020 and RStudio version 1.3.1073; R version 4.2.2, 2022 and RStudio version 2022.07.2-576). Bacterial isolates were selected by creating a data frame of Sanger sequences using the mafft() function in R and then converted to a file of multiple sequence alignment. We used the .phy file to create a cladogram using IQ Tree version 2.1.2 (Minh et al. 2020). IQ Tree is a stochastic algorithm that uses maximum likelihood to analyze trees, and we used ggtree R package to visualize the cladogram and phylogram (Yu et al. 2017). We used R packages ape and geiger to analyze the difference in branch lengths (Paradis et al. 2004, Harmon et al. 2008). The tree is unrooted, and we identified bacterial isolates from the different treatments with minimal genetic difference according to the branch length **(Figure 3**). In **Figure 4**, isolates 283 (M) and 281 (MF) displayed no difference in branch lengths and were identified as being the same bacterial isolate. In **Figure 5**, isolates 315 (MF) and 271 (M) have minor differences between branch lengths and were identified as being the same bacterial species (>99% similarity in the 16S rRNA gene). We used this phylogenetic approach to select the bacterial isolates that were used for a simplified community.

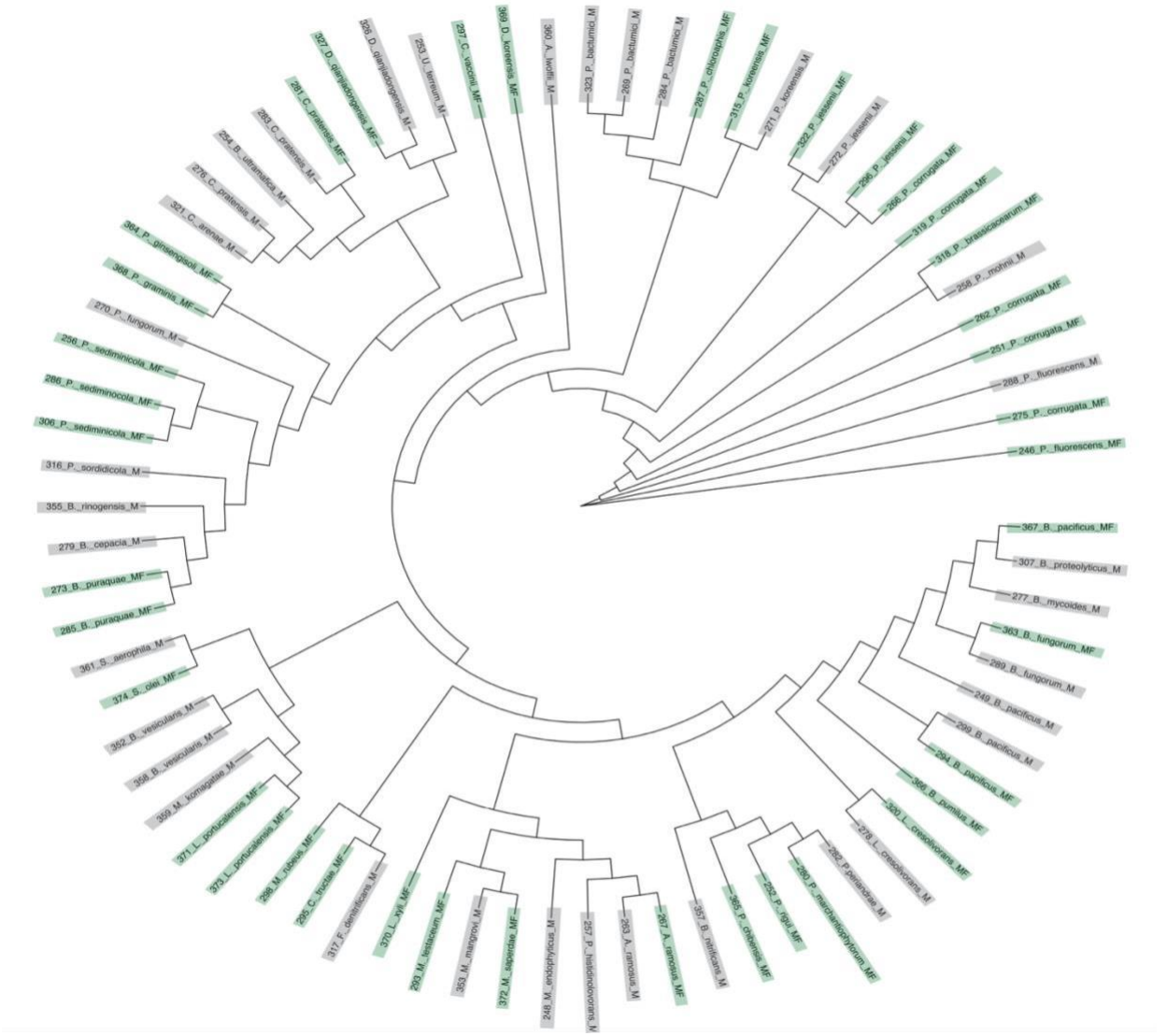

Figure 3: Unrooted cladogram used to identify bacterial isolates for the simplified community. Green highlights indicate soil bacteria isolated from mowed/fertilized (MF) treatment plots and gray highlights indicate soil bacteria isolates from mowed/unfertilized (M) treatment plots.

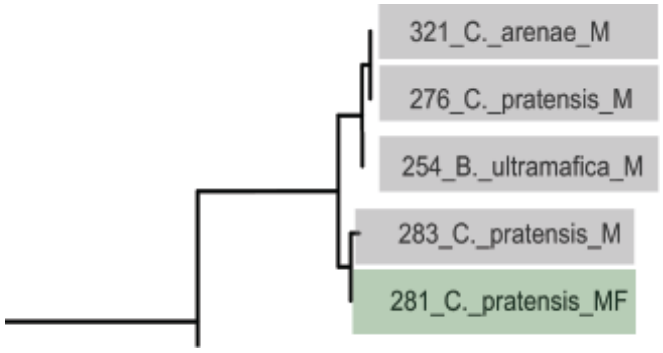

Figure 4: *Collimonas pratensis* was selected based on the minimal difference (>99% similarity) in tree branch length and having been isolated from different treatment plot.

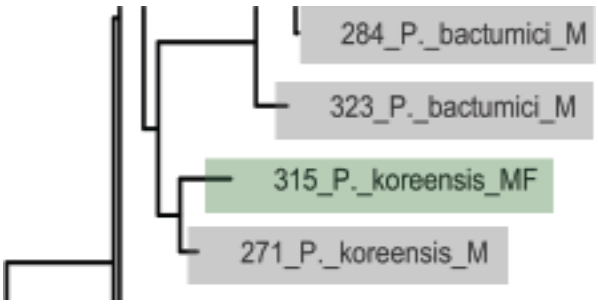

Figure 5: *Pseudomonas koreensis* was selected due to the minimal difference (>99% similarity) branch length and having been isolated from different treatment plots.

BACTERIAL TRAITS

Colony Morphology

For each bacterial isolate, we recorded the color, elevation, form, margin, and colony size. We compared the morphology of the isolates selected from the cladogram to ensure the bacterial isolates had similar morphological traits.

Bacterial Growth Rates

We analyzed the growth rates of the bacterial isolates using a microplate-based spectrophotometric method using a BioTek microplate reader and Gen5 software. We prepared culture tubes with 9 mL of R2 broth media. We inoculated an R2 broth culture tube with the similar bacterial isolates of interest 24 hours in advance from each treatment. A 48-well plate (Bio-One Cellstar Culture Plate) was used for the plate read, and we filled the outer wells with 700 μL of nanopure water to prevent evaporation of bacterial sample wells. The middle wells were filled with 500 μL of sterilized R2 broth media, then we added 50 μL of inoculated R2 broth media into two separate rows to avoid contamination. Within one plate, we prepared three replicates for the mowed/unfertilized bacterial isolate and three replicates for the mowed/fertilized bacterial isolate. The plate reader ran for 24 hours and measured absorbance readings every 30 minutes. Prior to each reading, the instrument was set to shake the plate for 3 seconds and then record a reading at 600 nm absorbance. Data were exported and processed in the R statistical environment. We used the function summarizegrowth() which uses a logistic curve equation and fits the curve to our measurements (Sprouffske and Wagner 2016).

PLANT-BACTERIAL BIOASSAY

We tested how long-term fertilization of soil bacteria has influenced the biomass of a wetland grass *Chasmanthium laxum* (*C. laxum). Chasmanthium laxum* is a native grass found in coastal plain wetlands that is categorized as a facultative wetland plant (Lichvar 2013). We chose this native grass because it is adapted to a low nutrient environment and is a representative of the plant community present at the study site. For the simplified bacterial community, we chose the bacterial isolates based on the phylogenetic approach previously detailed and based on current literature describing their role in plant growth promotion. To study the effects of fertilization, we created a nutrient gradient ranging from no fertilizer to 2 times the concentration applied in the field experiment (see *Plant-Microbe Nutrient Gradient Treatment* for more details).

Seed Sterilization

We collected *C. laxum* from the mowed/fertilized plots in December 2021. Then, we sterilized seeds using a diluted bleach solution for ten minutes while manually shaking to ensure all seeds were covered in the wash solution. We rinsed the seeds five times with autoclaved nanopure water to remove any bleach. To ensure the seeds were sterile, we placed a subset of seeds onto R2A agar petri dishes and checked for microbial growth. After confirmation of seed sterilization, we planted the seeds in sterilized (1.5 in. diameter, 8.25 in. length) conical planters (bleach washed, rinsed in nanopure water, 70% ethanol sprayed and air-dried) combined with autoclaved (120 °C gravity, 30 min.) 3B Fafard soilless media and watered seeds as needed with autoclaved tap water (120 °C liquid, 15 min.). We planted about 40 seeds in each “Super Cell” UV cone-tainer, and then thinned out to a single individual after 10 weeks of growth. Based on a previous experiment, *C. laxum* germination rates were about 10%.

Plant-Microbe Nutrient Gradient Treatment

We applied a nutrient gradient to the cone-tainers which increased from 0X, 0.5X, 1X, 1.5X and 2X the concentration added in the ecological experiment. We crossed the nutrient gradient treatment with bacterial inoculation treatment representing bacteria cultured from mowed/unfertilized, mowed/fertilized, and no bacterial control **(Figure 6**). We replicated the nutrient gradient by bacterial inocula treatments five times. For the nutrient gradient, we used Greenlight SuperBlood plant food, which is a NPK (12-55-6) powder fertilizer. To create a nutrient enrichment treatment, we prepare one liter of 2X concentration solution using 2.22 g of the plant food and one liter of autoclaved tap water (120 °C liquid, 15 min.). We prepared the nutrient gradient for each concentration and added 5 mL in the appropriate cone-tainer biweekly for three months (**Table 2**).

Table 2: We prepared 80 mL of fertilizer for each treatment (Control, MF, M) and 5 mL was added to the appropriate replicate for the nutrient gradient. Fertilization gradient represents concentrations relative to field conditions (1X = fertilization concentration equivalent to field conditions).

Concentration	Autoclaved Water (mL)	2X Stock Fertilizer (mL)
0X	80	0
0.5X	60	20
1X	40	40
1.5X	20	60
2X	0	80

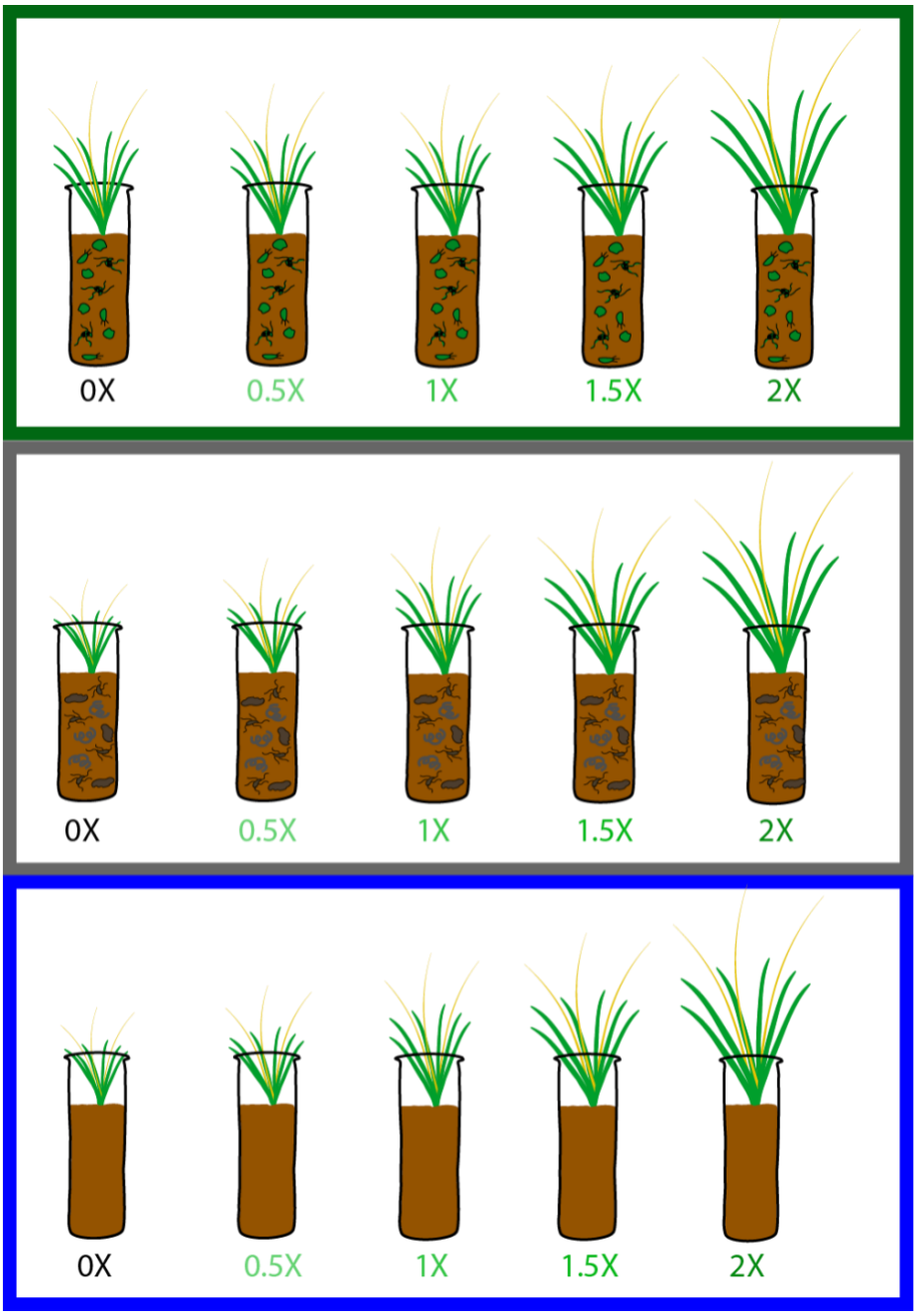

Figure 6: *Chasmanthium laxum* was grown and exposed to nutrient gradient and inoculated with simplified bacterial communities comprised of isolates from fertilized plots (green), unfertilized plots (gray), or no bacterial inoculation (blue). The fertilization gradient represents concentrations relative to field conditions (1 = fertilization concentration equivalent to field conditions).

Simplified Bacterial Community Inoculation

We inoculated the cone-tainers biweekly for 3 months. We added all bacterial isolates in equal cell number. We calculated the colony forming units using a spot plate method. First, 9 mL R2 broth culture tubes were inoculated with a pure bacterial colony 24 hours in advance and incubated at room temperature in an orbital shaker. Next, we did serial dilutions for each bacterial isolate and mixed with the pipette after each dilution. The serial dilutions ranged from the stock dilution to 10^{-11}. The R2A plates were divided into four sections, and we used 10 μL of inoculated broth to create five spot replicates for each dilution (**Figure 7**). The spot plates were incubated for two days at room temperature. Then, we selected the dilutions that contained 3-30 colonies per replicate. We identified the dilutions that shared a similar average of colonies for each pair of bacterial isolates and repeated the process three more times to check variability. The dilutions with the desired cell number ranged from 10^{-3} to 10^{-11} (**Table S2**). We prepared ten 250 mL flasks with 100 mL of R2 broth and inoculated the flasks with the selected bacterial isolates. The flasks were incubated for 24 hours at room temperature in an orbital shaker and were used as the stock solutions, where 1 mL of bacterial was added to 99 mL of 1X PBS (**Figure 8**). We added four mL of the inoculated PBS + simplified bacterial community (MF, F, no bacteria) to its respective cone-tainer.

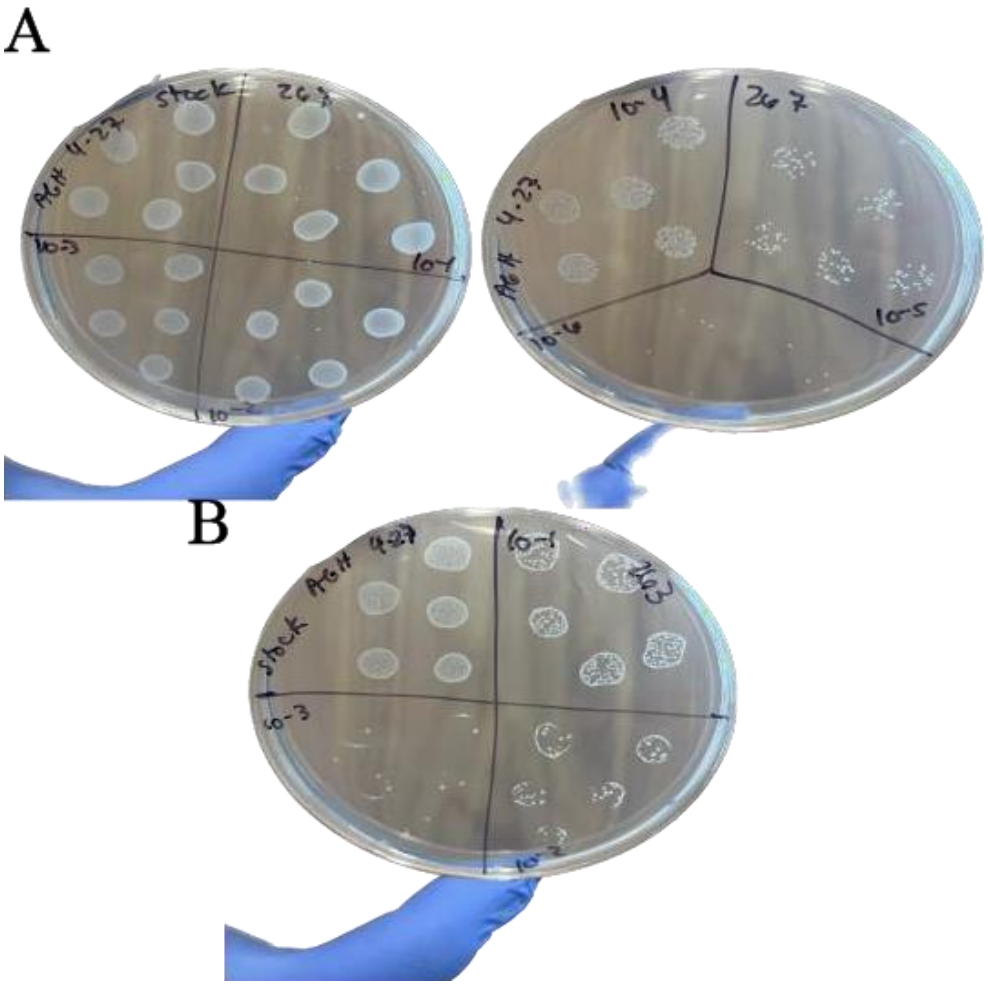

Figure 7: Example images of bacterial spot plates used to quantify calculate colony forming units. In panel A are the spot plates for bacterial isolate *Arthrobacter ramosus* (WRC_267_MF) and in panel B are the spot plates for bacterial isolate *Arthrobacter ramosus* (WRC_263_M). We divided R2A plates into three or four sections to accommodate several dilutions. In each section, we added 5 spots of 100uL and checked for spots that had between 3-30 colonies.

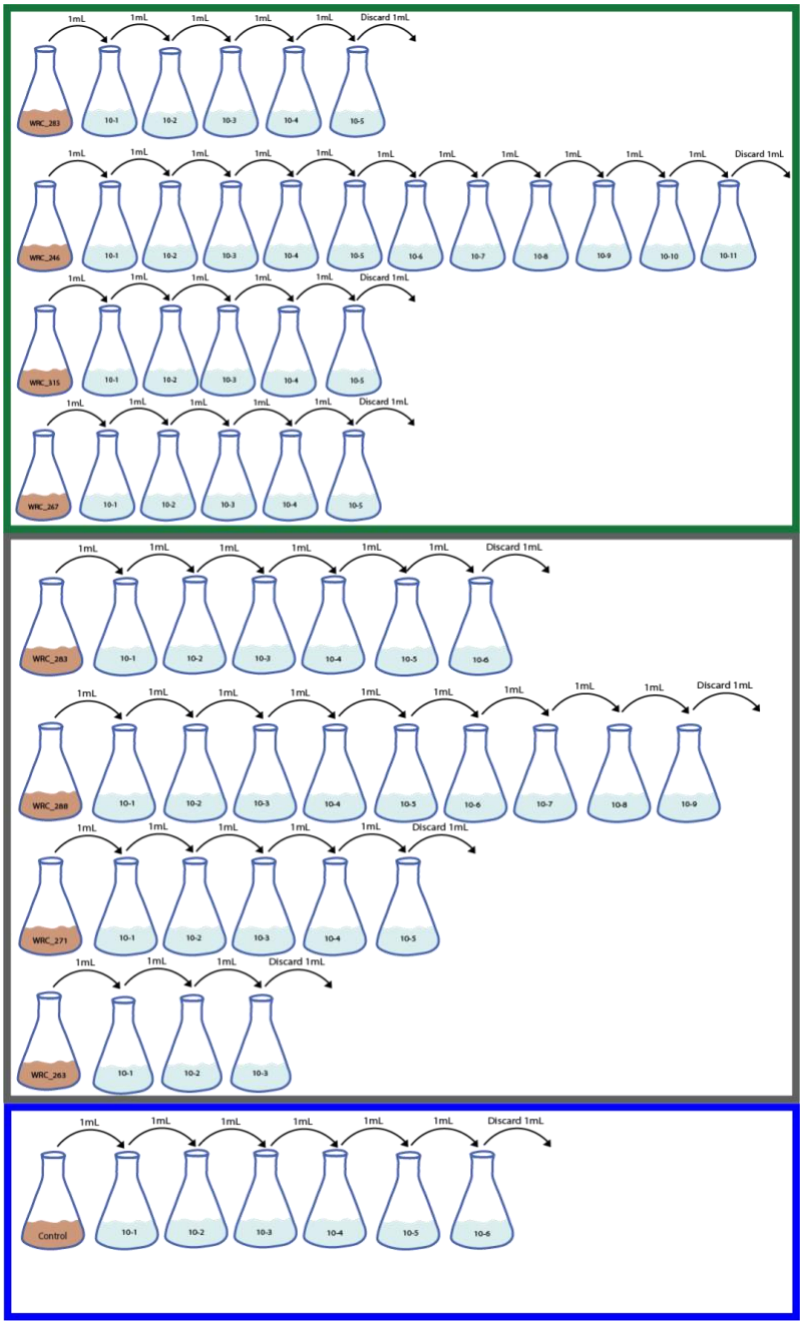

Figure 8: Serial dilution of bacterial isolates for inoculation of cone-tainers. Green box contains bacterial isolates from mowed/fertilized (MF) plots, gray box contains bacterial isolates from mowed/unfertilized (M) plots, and the blue box is the control.

Biomass Collection

After a total of five months of growth, we collected the above and below ground biomass of *C. laxum*. The soil and grass were carefully removed from the cone-tainer. We separated the above and below ground biomass with scissors and carefully added roots to a 50 mL tube with nanopure water to remove excess soilless media (**Figure S1**). We transferred belowground and aboveground biomass into individual paper envelopes (**Figure S2**). We dried the biomass for at least 48 hours at 60 °C in a convection oven. Using an analytical balance, we weighed and recorded the biomass of each replicate (n=60).

STATISTICAL ANALYSIS

All statistical calculations were conducted and graphs made in the R environment (R version 4.0.2, 2020 and RStudio version 1.3.1073, R version 4.2.2, 2022 and RStudio 2022.07.2-576). The growth rate curves were plotted using R packages ggplot and growthcurver after finding the means of the replicates (Wickham 2016, Sprouffske and Wagner 2016). We ran a nonlinear logistic regression model and obtained the parameters for carrying capacity (K), initial population size (N_0), growth rate (r), residual standard error (n_se) from the nonlinear regression model, and the inflection point (t_mid) of the growth curve. We performed an analysis of variance (ANOVA) (using the aov() function) on the *C. laxum* biomass to examine how bacterial inocula source (no bacterial inoculation, mowed/fertilized bacterial inoculation, mowed/unfertilized inoculation), and fertilization influenced above- and belowground *C. laxum* biomass. Boxplots were created using the ggplot2 package to visualize the differences between the treatments. To test for interaction between treatments using estimated marginal means (EMMs), we used the R package emmeans (Searle et al. 1980).

RESULTS

Colony Morphology and Traits

We selected four bacterial isolates that had similar colony morphology traits. Based on the available literature, these genera have shown to be plant growth-promoting rhizobacteria (Garbeva et al. 2014, Roy and Kumar 2020, Das et al. 2020). The color, margin, elevation, and form of the bacterial isolates were relatively similar (**Table 3**). In contrast, the colony size of bacterial isolates from mowed/fertilized (MF) plots was smaller (□1 mm) than those from mowed/unfertilized (2-4 mm) plots. The biochemical results showed minimal differences among the bacterial isolates for respiration, nitrate reduction, and salt tolerance. (**Table S3**).

Table 3: Bacterial isolates for the simplified communities were selected due to the similar colony morphologies. The treatment plots that the bacterial isolates were collected from was either mowed/fertilized (MF) plots or mowed/unfertilized (M) plots.

Genus species	Sample ID	Treatment	Color	Margin	Elevation	Form	Colony Size (mm)
C. pratensis	WRC_281	MF	clear	smooth	raised	round	<1
P. fluorescens	WRC_246	MF	yellow	smooth	flat	round	1
P. koreensis	WRC_315	MF	beige	smooth	raised	round	0.8
A. ramosus	WRC_267	MF	white	smooth	raised	round	1
C. pratensis	WRC_283	M	clear	undulated	flat	round	2
P. fluorescens	WRC_288	M	yellow	smooth	flat	round	3
P. koreensis	WRC_271	M	yellow	smooth	flat	round	2
A. ramosus	WRC_263	M	white	smooth	convex	round	4

Phylogenetic Analysis

The four pairs of bacterial isolates showed minimal differences in grade, % pairwise, and sequence length (**Table S1**). The IQTree (Minh et al. 2020) created a maximum likelihood tree in Newick format. The best fitting parsimony tree model was TIM2e+R4 which had a BIC of 46146.040 and an optimal log-likelihood of -22486.799 which was found after 154 iterations (**Figure S3**). The patristic distances between the pairs of bacterial isolates are as follows: *P. fluorescens* 8.7288E-06; *P. koreensis* 9.0742E-3; *C. pratensis* 1.1851E-3; *A. ramosus* 6.5834E-4. The branch length difference between 1) *P. fluorescens* M and *P. fluorescens* MF was 0; 2) *A. ramosus* M and *A. ramosus* MF was 5.8104E-4; 3) *C. pratensis* M and *C. pratensis* MF was 1.1573E-3; and 4) *P. koreensis* M and *P. koreensis* MF was 4.9400E-3 (**Figure 9**). The minor differences in branch lengths were a determining factor in selecting these four soil bacterial isolates. These results revealed that the paired mowed/unfertilized and mowed/fertilized (M and MF) bacterial isolates were genetically similar.

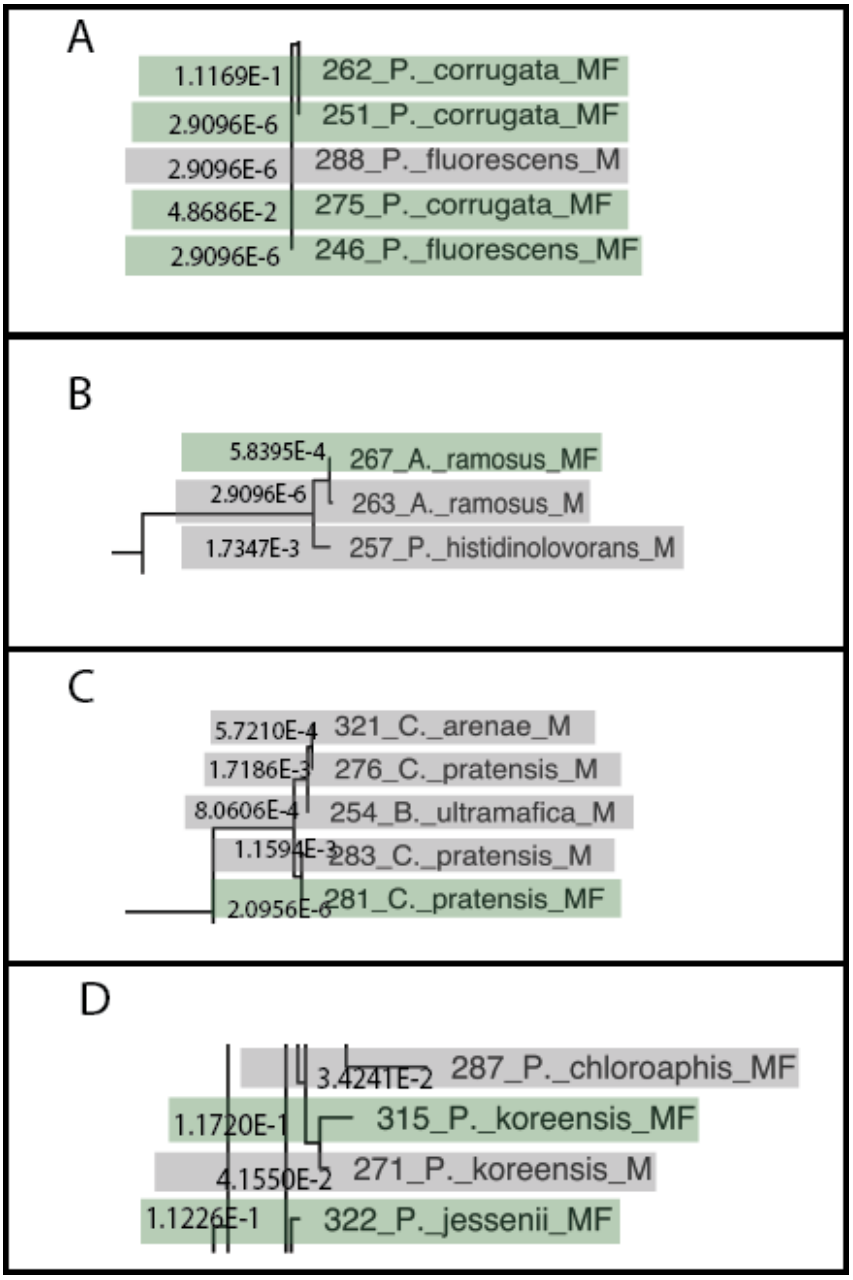

Figure 9: Bacterial isolates for the simplified communities were selected due to the similar colony morphologies. The treatment plots that the bacterial isolates were collected from mowed/fertilized (MF) plots highlighted in green or mowed/unfertilized (M) plots highlighted in gray.

Bacterial Growth Rates

We observed variation in growth rate parameters of soil bacterial isolates cultured from mowed/unfertilized and mowed/fertilized according to life history strategy (**Table 4; Figure 10**; **Table S4**). For the putative fast growing copiotroph *P. fluorescens* **(Figure 10A**), the growth rates and points of inflection were similar between mowed/unfertilized ($r=0.247\ hr^{-1}$) and mowed/fertilized ($r=0.242\ hr^{-1}$) (**Table 4**). The inflection points for the mowed/unfertilized *P. fluorescens* isolate were x=13.141 and y=0.178; for the mowed/fertilized isolate, they were x=13.138 and y=0.170 (**Table 4**). *P. fluorescens* from the mowed/unfertilized treatment had an initial population size of 0.130 and carrying capacity of 0.356, while *P. fluorescens* from the mowed/fertilized treatment had an initial population size of 0.014 and carrying capacity of 0.339 (**Table 4**). For the putative copiotroph *P. koreensis*, growth rates were higher for the mowed/unfertilized isolate ($r=0.641\ hr^{-1}$) compared to the mowed/fertilized isolate ($r=0.274\ hr^{-1}$) (**Table 4**). The inflection points for *P. koreensis* mowed/unfertilized isolate were x=7.141 and y=0.0785 and for the mowed/fertilized isolate they were x=10.631 and y=0.095 (**Table 4**). The *P. koreensis* isolate cultured from the mowed/unfertilized treatment had an initial population size of 0.002 and carrying capacity of 0.157, while the *P. koreensis* isolate cultured from the mowed/fertilized treatment had an initial population size of 0.010 and carrying capacity of 0.190 (**Table 4**). The *P. koreensis* isolated from mowed/fertilized plots had a higher carrying capacity, slower growth rate, and reached its point of inflection sooner than *P. koreensis* isolated from mowed/unfertilized treatment plots (**Figure 10B; Table 4**). For putative slow growing oligotrophs, we observed lower growth rates but higher inflection points for *A. ramosus* cultivated from the mowed/unfertilized ($r=0.258\ hr^{-1}$) compared to *A. ramosus* cultivated from the mowed/fertilized ($r=0.459\ hr^{-1}$) treatment (**Figure 10C; Table 4**). The inflection points for *A.*

ramosus mowed/unfertilized isolates were x=29.751 and y=0.0965 and for the mowed/fertilized isolates were x=9.655 and y=0.167 (**Table 4**). The *A. ramosus* isolate cultured from the mowed/unfertilized treatment had an initial population size of 0 and a carrying capacity of 0.193, while the *A. ramosus* isolate cultured from the mowed/fertilized treatment had an initial population size of 0.004 and a carrying capacity of 0.334 (**Table 4**). For putative slow growing oligotroph *C. pratensis*, we observed higher growth rate from the mowed/unfertilized (r=0.667 hr^{-1}) compared to isolates cultivated from the mowed/fertilized (r=0.380 hr^{-1}) treatment (**Figure 10D; Table 4**). The inflection points for the mowed/unfertilized *C. pratensis* isolate were x=8.776 and y=0.112 and for the mowed/fertilized isolate were x=10.003 and y=0.130 (**Table 4**). The *C. pratensis* isolate cultured from the mowed/unfertilized treatment had an initial population size of 0.001 and a carrying capacity of 0.223, while the *C. pratensis* isolate cultured from the mowed/fertilized treatment had an initial population size of 0.006 and a carrying capacity of 0.259 (**Table 4**).

Table 4: Summary of statistical values showing growth rate parameters of the paired bacterial isolates (from mowed/fertilized (MF) and mowed/unfertilized (M) treatments (trt), where K = carrying capacity, K_p = p-value of carrying capacity parameter, N0 = initial population size, N0_p = p-value of initial population size, r = growth rate, r_p = p-value of growth rate, sigma = residual standard error, t_mid = time at the inflection point.

Genus Species (trt)	**K**	**K_p**	**N0**	**N0_p**	**r (hr^{-1})**	**r_p**	**sigma**	**t_mid**
P. fluorescens (MF)	0.339	8e-35	0.014	1e-09	0.242	5e-23	0.013	13.138
P. fluorescens (M)	0.356	2e-38	0.013	4e-11	0.247	5e-26	0.012	13.141
A. ramosus (MF)	0.334	2e-79	0.004	4e-22	0.459	7e-50	0.004	9.655
A. ramosus (M)	0.193	7e-03	0	2e-07	0.258	9e-30	0	29.751
P. koreensis (MF)	0.157	1e-66	0.002	2e-07	0.641	2e-30	0.004	7.141
P. koreensis (M)	0.190	1e-27	0.010	7e-04	0.274	1e-11	0.017	10.631
C. pratensis (MF)	0.259	8e-60	0.006	3e-12	0.380	8e-34	0.007	10.003
C. pratensis (M)	0.223	8e-62	0.001	3e-04	0.667	1e-26	0.007	8.776

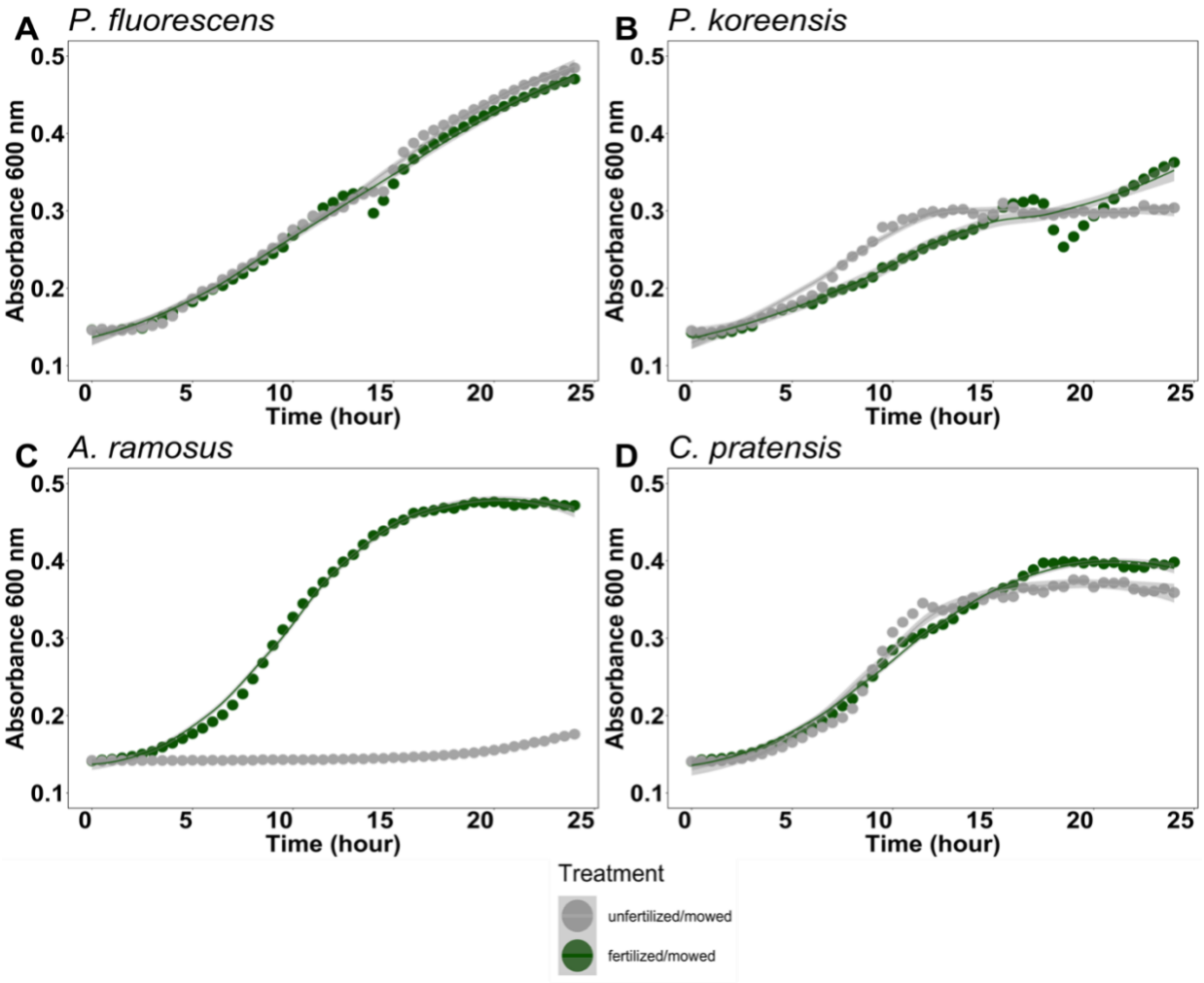

Figure 10: Bacterial growth rates comparing bacterial isolates from mowed/unfertilized and mowed/fertilized plots measured every 30 minutes for 24 hours. The growth curves represent an average of three replicates of the bacterial isolates collected from mowed/unfertilized (gray) and from mowed/fertilized (green) treatment plots.

Plant Response to Simplified Bacterial Communities

Aboveground ($F_{2,60}$ =12.174, P<0.0001) and belowground ($F_{2,60}$ =3.566, P=0.0344) plant biomass were higher in the presence of soil bacteria compared to no bacterial addition across the fertilization gradient (**Figures 11, 12; Table S5**). Specifically, aboveground biomass tended to be higher at low fertilization treatments (0x, 0.5x) when bacterial inocula were added from mowed/unfertilized compared to mowed/fertilized soil sources (**Figure 11**). The aboveground biomass was higher in fertilized compared to control treatments, and the estimated contrast between the fertilized simplified community inoculation and the control (no bacteria) treatment was -0.0790 (P=0.0348) (**Table S5**). The aboveground biomass was higher in unfertilized simplified bacterial community inoculation compared to control (no bacterial) treatment, and the estimated contrast was -0.1527 (P<0.0001) (**Table S5**). In addition, aboveground biomass was higher in unfertilized compared to fertilized simplified bacterial community inoculation, and the contrast between the fertilized and unfertilized simplified community inoculation was -0.0737 (P=0.0528) (**Table S5**). In contrast, we observed overlap in belowground biomass between fertilized and unfertilized simplified bacterial community additions along the fertilization gradient, and the contrast between the fertilized and unfertilized bacterial community inoculation was 0.0082 (P=0.6855) (**Figure 12; Table S5**). For the belowground, the biomass was higher in the unfertilized simplified bacterial community compared to the control (no bacteria), and the estimated contrast was -0.0259 (P=0.0300), while belowground biomass was similar between the fertilized and control treatments and the estimated contrast between the control and the fertilized community inoculation was -0.0177 (P=0.1841) (**Table S5**). Results also showed that fertilizer addition had a positive influence on belowground biomass ($F_{4,60}$ =2.513, P=0.0509) (**Table S5**).

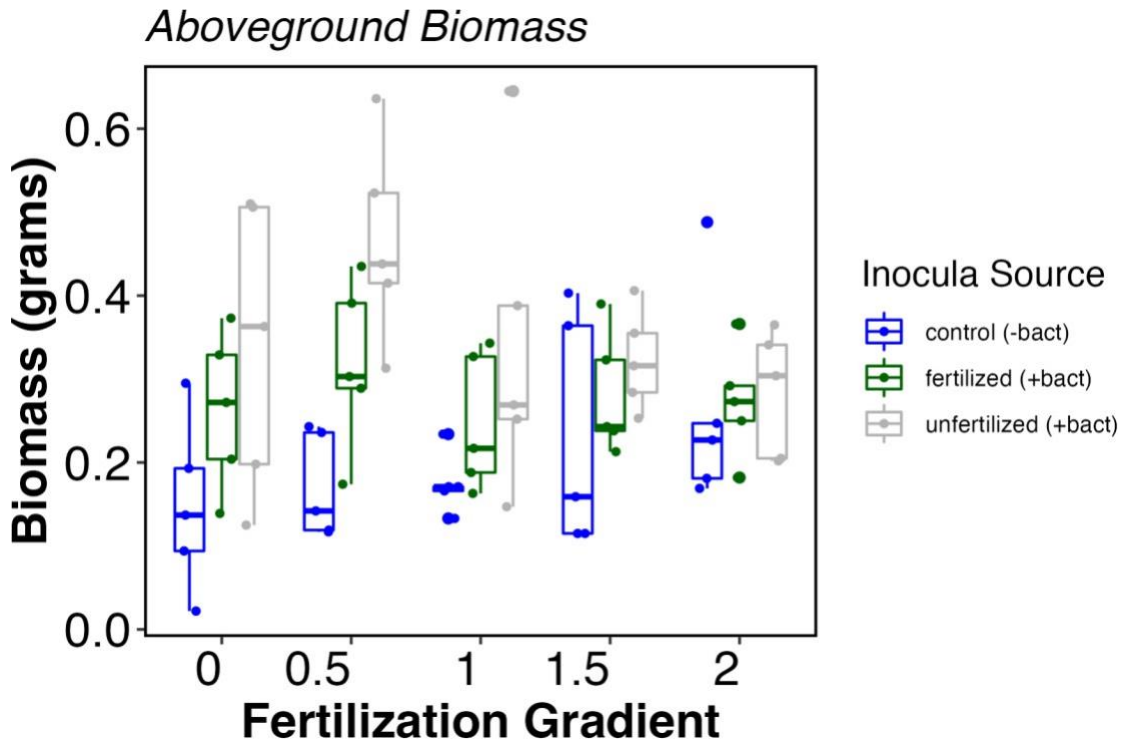

Figure 11: Boxplots depicting aboveground biomass measured after 4 months of growth. Plants exposed to nutrient gradient and inoculated with simplified bacterial communities comprised of isolates from unfertilized plots (gray), fertilized plots (green), or no bacterial inoculation (blue). Fertilization gradient represents concentrations relative to field conditions (1 = fertilization concentration equivalent to field conditions). 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 raw data points from five replicate samples. Summary of statistical output in Appendix S1: Table S5.

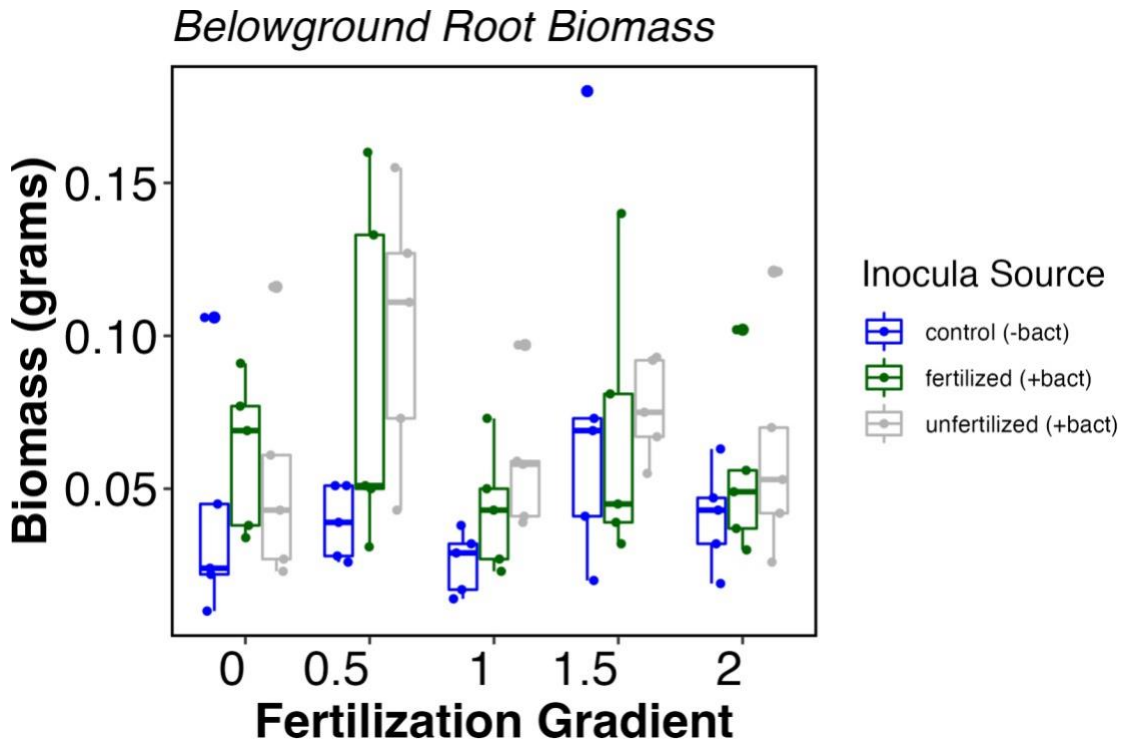

Figure 12: Boxplots depicting belowground root biomass measured after 4 months of growth. Plants exposed to nutrient gradient and inoculated with simplified bacterial communities comprised of isolates from unfertilized plots (gray), fertilized plots (green), or no bacterial inoculation (blue). Fertilization gradient represents concentrations relative to field conditions (1 = fertilization concentration equivalent to field conditions). 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 raw data points from five replicate samples. Summary of statistical output in Appendix S1: Table S5.

DISCUSSION

This study focused on how long-term fertilization of a historically low nutrient wetland is changing plant-microbe interactions. Results showed that long-term fertilization of a wetland ecosystem influenced bacterial traits in ways that modified plant-bacterial associations. In this study, the phylogenetic analysis enabled the identification of bacterial isolates from mowed/unfertilized treatment plots that had a taxonomically identical 16S rRNA region to bacterial isolates from mowed/fertilized treatment plots. This gave us the opportunity to characterize how long-term nutrient enrichment influenced bacterial activity and plant-microbe associations. By using a trait-based approach to understand changes in the functional role of soil bacterial cultivated from bulk wetland soils, we gained insight into the consequences of nutrient enrichment effects on soil bacteria and plant growth (Philippot et al. 2013, Geisen et al. 2019). The current study challenges the approach of using taxonomic classification to infer bacterial life history strategy, and instead takes a bacterial trait-based functional approach to examine plant-microbe interactions.

Bacterial isolates cultured from different nutrient enrichment histories exhibited different growth rates depending on their life history strategy (i.e., slow growing oligotroph vs. fast growing copiotroph) (Fierer et al. 2007). The difference in growth rates when using common media could be explained with changes to genes outside the 16S rRNA region; however, that was not tested during this study. Long-term fertilization of a coastal wetland ecosystem influenced soil bacterial growth rates to different degrees: copiotrophic *P. koreensis* exhibited increased growth rates and *P. fluorescens* showed similar growth rates in response to fertilizer, while oligotrophic *A. ramosu*s increased but *C. pratensis* decreased growth rates and carrying capacity increased in response to fertilizer. Other long-term fertilization experiments around the world

(e.g. arctic soils and agriculture fields in China) that have been ongoing for over 25 years show that chronic fertilization has led to a decrease in soil bacterial diversity and increased bacterial growth rates (Xu et al. 2020, Hicks et al. 2020). Using a life-history approach, groups microbes based on functional traits and previous studies have revealed that nutrient additions promotes copiotrophs over oligotrophs (Habteselassie et al. 2022). Short incubation periods are a limitation of the approach to measure bacterial growth rates. For bacterial isolates *P. fluorescens* and *A. ramosus*, a longer incubation period would allow for a more detailed growth curve that allow carry capacity to be reached. Differences in the initial population size were largest in bacterial isolates *P. koreensis* and *C. pratensis.* A larger initial population size could skew the growth rate curve by reaching carrying capacity sooner than its experimental counterpart. However, since growth rate is the parameter of interest, the difference in initial population is not expected to affect the measured bacterial growth rate.

In this study, the soil bacterial isolates from distinct fertilization histories modified plant biomass in different ways, especially when plants were exposed to no or low nutrient conditions. For the plant-microbe biomass experiment, the aboveground plant biomass was highest at the lower additions of fertilization when inoculated with unfertilized bacterial isolates. As expected, bacterial isolates collected from unfertilized treatment plots interacted with the native grass in a beneficial manner that resulted in increased plant biomass. The belowground plant biomass was highest when inoculated with microbes versus no bacterial inoculation. This experiment has shown that plant-microbe relationships are necessary for healthy plant growth especially when plants and microbial partners are in a nutrient-starved soil environment. These observations are supported by past studies where plant-microbe interactions have been altered due to long-term fertilization (Wei et al. 2013, Huang et al. 2019b, Paul Chowdhury et al. 2019). Access to plant-

derived C compounds could be limited by N enrichment induced soil acidification (Wei et al. 2013). Long-term fertilization has also been shown to decrease the interactions between plants and microbes in relation to C cycling and degradation (Huang et al. 2019b). Rhizosphere microbiomes have shown a shift in composition due to long-term fertilization, but not much is known on how it could be affecting aboveground plant performance (Paul Chowdhury et al. 2019, Bledsoe et al. 2020). This study revealed that fertilization increased belowground root biomass, but without bacterial inoculation root biomass decreased relative to plants exposed to soil bacteria. We expected plant biomass to increase along the nutrient gradient and show differences between bacterial inoculation. However, aboveground biomass was higher in fertilization levels 0x and 0.5x. Fertilization levels 1x and up, show a lower biomass. This occurs in the root biomass as well. Interactions between fertilization and bacterial inoculation could affect how the results from the plant-microbe experiment are interpretated. Therefore, estimated marginal means and post-hoc contrasts were tested (Supplementary Table S6; Supplementary Table S7). This study demonstrated that bacterial inoculation is important for healthy belowground root biomass. These results and past studies support that root-microbe relationships are important for healthy plant growth and nutrient uptake (Philippot et al. 2013, Yan et al. 2017).

This study also revealed that taxonomic classification might not accurately predict how bulk soil bacteria are functioning with plant partners that have co-evolved with microbes under nutrient-limited conditions. Past studies showed that long-term fertilization weakened plant-microbe relationships and decreased relative abundance of functional genes (e.g. N fixation and P utilization) in soil microbes (Huang et al. 2019b, 2019a, Ji et al. 2020). Based on the results of this study, the next step could be to study genes outside the 16S rRNA region to identify

differences in functional genes among the selected bacterial isolates. This experiment demonstrated that plant-microbe interactions are being affected by nutrient additions.

Using a simplified bacterial community cultured from distinct soil nutrient histories does limit the generalization of these findings to the wetland ecosystem level. However, this approach was effective in identifying how bulk soil bacteria that differ in functional traits could change plant growth potential due to initial environmental changes. This study was limited to evaluating the simplified bacterial community effects during the early plant growth stage. Since soil biodiversity is immense, the role that soil bacteria play individually and together provide more insight into how long-term fertilization affects bacterial taxonomy, phylogeny, and function. While we used Sanger sequencing of the 16S rRNA gene for classification, we recognized that this sequencing technique is helpful in identifying the genus, but classification of species can be biased (Gupta et al. 2019).

Future Work

There is an opportunity to apply high yield, resource acquisition, and stress tolerance (Y-A-S) strategies to better represent soil bacterial functional potential to better predict environmental stressor effects on plant-microbe relationships (Ramin and Allison 2019, Malik et al. 2020, Bittleston et al. 2021). It is challenging to assign bacterial isolates to the appropriate life history strategies; therefore, it is important to use several genomic approaches to accurately classify microbes (Leff et al. 2015). For example, we can perform whole genome sequencing on the bacterial isolate pairs and analyze which genes are present. Additionally, we could analyze the bacterial community composition of the soilless media and rhizosphere using Illumina sequencing and qPCR in order to understand which bacterial isolates are being recruited. This

would provide a deeper understanding on how these microbial pairs differ in functional traits. Additional biochemical tests could reveal differences in phylogenetically identical bacterial pairs functions. In addition, replicating the plant-microbe biomass experiment with a different set of bacterial isolates will increase our understanding of how the bulk soil bacterial composition has changed in phylogeny and function. Atmospheric deposition of nutrients and other contaminants continue to deposit onto ecosystems far from the source. Taken together, additional genomic and biochemical approaches can be applied to bridge the knowledge gap of how long-term fertilization affects microbial phylogeny and function.

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APPENDIX

Table S1: Geneious Prime statistics used to identify genus and species of bulk soil bacterial isolates.

Genus Species	Sample_ID	Treatment	Sequence Length	% Pairwise	% Grade
C. pratensis	WRC_281	MF	1455	99.2	99.6
P. fluorescens	WRC_246	MF	1457	99.4	99.6
P. koreensis	WRC_315	MF	1463	99.5	99.8
A. ramosus	WRC_267	MF	1453	99.4	99.7
C. pratensis	WRC_283	M	1459	99.3	99.7
P. fluorescens	WRC_288	M	1458	99.6	99.7
P. koreensis	WRC_271	M	1455	99.8	99.8
A. ramosus	WRC_263	M	1429	99.5	99.8

Table S2: Serial dilution colony forming units (CFUs) of bacterial isolates for preparation of simplified community inoculation.

Genus	Species	Sample ID	Treatment	Dilution	CFU/mL
Collimonas	*pratensis*	WRC_281	MF	10-6	$6.2e10^{8}$
Pseudomonas	*fluorescens*	WRC_246	MF	10-11	$9.6e10^{13}$
Pseudomonas	*koreensis*	WRC_315	MF	10-5	$1.06e10^{8}$
Arthrobacter	*ramosus*	WRC_267	MF	10-5	$2.30e10^{8}$
Collimonas	*pratensis*	WRC_283	M	10-6	$1.08e10^{9}$
Pseudomonas	*fluorescens*	WRC_288	M	10-9	$1.00e10^{12}$
Pseudomonas	*koreensis*	WRC_271	M	10-5	$1.26e10^{8}$
Arthrobacter	*ramosus*	WRC_263	M	10-3	$1.32e10^{6}$

Table S3: Additional biochemical traits for comparison of selected bacterial isolates.

Genus Species	Sample_ID	Treatment	Respiration	Nitrate Reduction	Salt Tolerant
C. pratensis	WRC_281	MF	Facultative anaerobe	Nitrite	Yes
P. fluorescens	WRC_246	MF	Facultative anaerobe	Nitrite	No
P. koreensis	WRC_315	MF	NA	Nitrate	Yes
A. ramosus	WRC_267	MF	Facultative anaerobe	Nitrate	Yes
C. pratensis	WRC_283	M	Facultative anaerobe	Nitrate	Yes
P. fluorescens	WRC_288	M	NA	Nitrite	Yes
P. koreensis	WRC_271	M	Facultative aerobe	Nitrate	Yes
A. ramosus	WRC_263	M	Facultative anaerobe	Nitrate	Yes

Table S4: Summary of additional growth rate statistics, where K_se= standard error of carrying capacity, N0_se= standard error of initial population, r_se= standard error of growth rate, t_gen= doubling time, auc_l= area under the curve of the fitted logistic equation from time 0 to time t, and auc_e= area under the curve of the measurements. Growth rate units in hr^{-1} and time is hr.

Genus Species	Trt	K_se	N0_se	r_se	t_gen	auc_l	auc_e
P. fluorescens	MF	0.01	0.002	0.013	2.864	3.723	3.679
P. fluorescens	M	0.008	0.002	0.011	2.804	3.902	3.860
A. ramosus	MF	0.001	0	0.006	1.512	4.782	4.770
A. ramosus	M	0.068	0	0.01	2.682	0.152	0.156
P. koreensis	MF	0.008	0.003	0.031	2.532	2.650	2.490
P. koreensis	M	0.001	0	0.023	1.081	2.650	2.653
C. pratensis	MF	0.002	0.001	0.011	1.823	3.610	3.591
C. pratensis	M	0.002	0.001	0.029	1.040	3.395	3.405

Table S5: Summary of analysis of variance table comparing bacterial inoculation and fertilization treatments for plant biomass.

Aboveground ANOVA					
Fixed Effect	**Df**	**SumSq**	**MeanSq**	**F-value**	**Pr(>F)**
Fertilization	4	0.0437	0.01092	0.911	0.463
Bacterial Inoculation	2	0.2917	0.14583	12.174	**3.65e-05*** **
Fertilization:Bacterial Inoculation	8	0.1081	0.01351	1.128	0.358
Residuals	60	0.7188	0.01198		
Belowground ANOVA					
Fixed Effect	**Df**	**SumSq**	**MeanSq**	**F-value**	**Pr(>F)**
Fertilization	4	0.01236	0.003090	2.513	0.0509
Bacterial Inoculation	2	0.00877	0.004385	3.566	**0.0344***
Fertilization:Bacterial Inoculation	8	0.00703	0.000879	0.714	0.6778
Residuals	60	0.07378	0.001230		

Table S6: Estimated Marginal Means (EMMs) for aboveground biomass, belowground biomass, and fertilization levels. For aboveground and belowground EMMs, control represents no bacterial inoculation, fertilized represents bacterial inoculation sourced from the fertilized source, and unfertilized represents bacterial inoculation from the unfertilized source.

Aboveground EMMs					
Treatment	**emmean**	**SE**	**Df**	**lower.CL**	**upper.CL**
Control	0.198	0.0219	60	0.154	0.241
Fertilized	0.277	0.0219	60	0.233	0.320
Unfertilized	0.350	0.0219	60	0.307	0.394
Belowground EMMs					
Treatment	**emmean**	**SE**	**Df**	**lower.CL**	**upper.CL**
Control	0.0448	0.00701	60	0.0307	0.0588
Fertilized	0.0624	0.00701	60	0.0484	0.0765
Unfertilized	0.070	0.00701	60	0.0567	0.0847
Nutrient Gradient EMMs					
Treatment	**emmean**	**SE**	**Df**	**lower.CL**	**upper.CL**
0x	0.0524	0.00905	60	0.0343	0.0705
0.5x	0.0753	0.00905	60	0.0572	0.0934
1x	0.0427	0.00905	60	0.0246	0.0608
1.5x	0.0735	0.00905	60	0.0554	0.0916
2x	0.0527	0.00905	60	0.0546	0.0708

Table S7: Contrasts for two-way interactions between treatments. For aboveground and belowground contrasts, control represents no bacterial inoculation, fertilized represents bacterial inoculation sourced from the fertilized source, and unfertilized represents bacterial inoculation from the unfertilized source.

Aboveground Contrasts					
Contrast	**Estimate**	**SE**	**Df**	**t.ratio**	**p.value**
Control:Fertilized	-0.0790	0.031	60	-2.553	0.0348
Control:Unfertilized	-0.1527	0.031	60	-4.933	<0.0001
Fertilized:Unfertilized	-0.0737	0.031	60	-2.380	0.0528
Belowground Contrasts					
Contrast	**Estimate**	**SE**	**Df**	**t.ratio**	**p.value**
Control:Fertilized	-0.01768	0.00992	60	-1.783	0.1841
Control:Unfertilized	-0.02592	0.00992	60	-2.613	0.0300
Fertilized:Unfertilized	-0.00824	0.00992	60	-0.831	0.6855
Nutrient Gradient Contrasts					
Contrast	**Estimate**	**SE**	**Df**	**t.ratio**	**p.value**
0x:0.05x	-0.022867	0.0128	60	-1.786	0.3913
0x:1x	0.009733	0.0128	60	0.760	0.9409
0x:1.5x	-0.021067	0.0128	60	-1.645	0.4751
0x:2x	-0.000267	0.0128	60	-0.021	1.0000
0.5x:1x	0.032600	0.0128	60	2.546	0.0941
0.5x:1.5x	0.001800	0.0128	60	0.141	0.9999
0.5x:2x	0.022600	0.0128	60	1.765	0.4033
1x:1.5x	-0.030800	0.0128	60	-2.405	0.1279
1x:2x	-0.010000	0.0128	60	-0.781	0.9351
1.5x:2x	0.020800	0.0128	60	1.624	0.4880

Figure S1: Process for separating above and below-ground biomass for weighing.. Image A shows *C. laxum* once it has been removed from the cone-tainer. Image B provides an example of how the aboveground and belowground are separated. Image C is a close-up of the plant shoots (white arrows pointing at shoots) which should be kept with the aboveground biomass. Image D shows how the plant should be washed and reveals remaining belowground biomass that should be removed and added to its respective belowground biomass envelope.

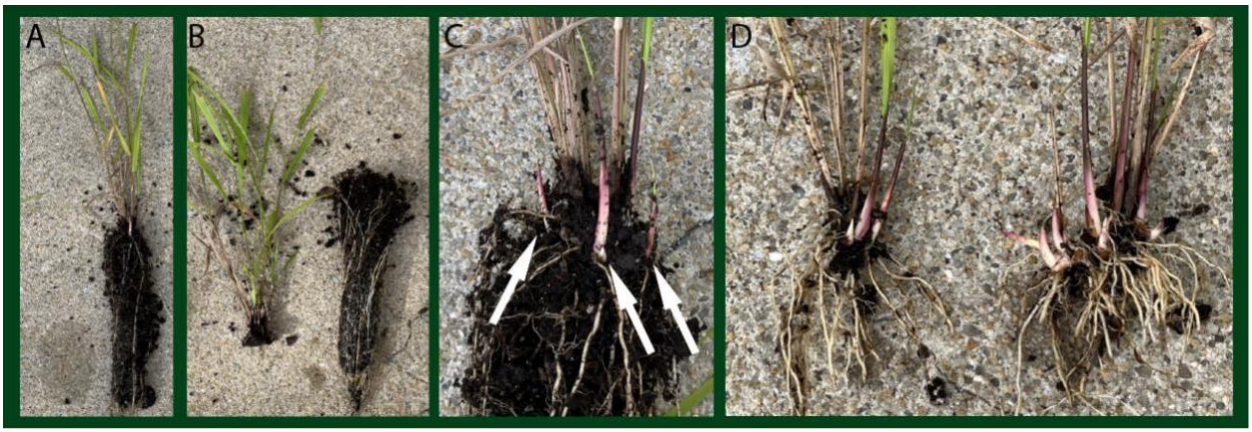

Figure S2: Above and belowground biomass preparation for drying and storage.

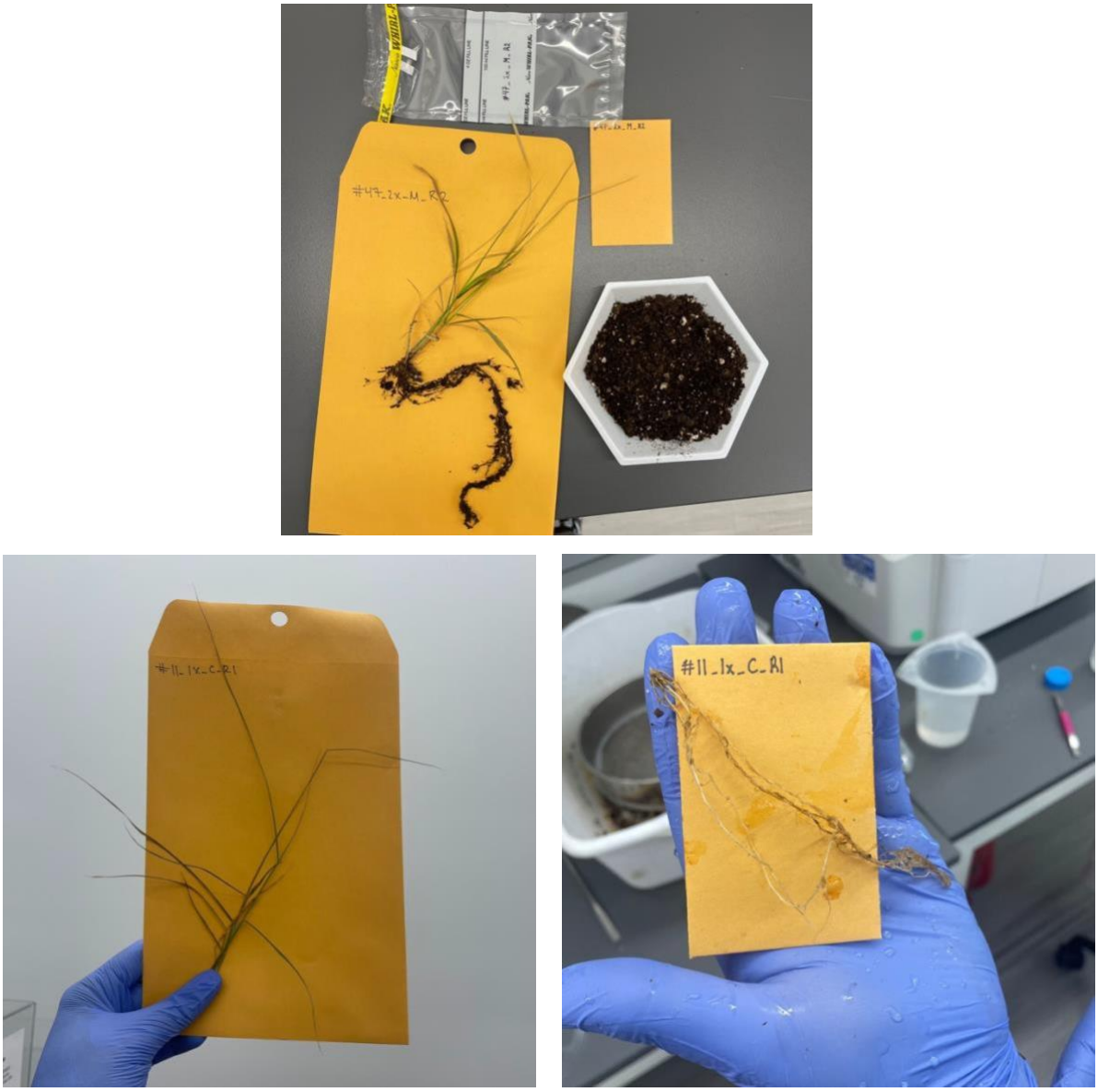

Figure S3: IQTree of bulk soil bacterial isolates and branch lengths. Green highlights represent bacterial isolates from mowed/fertilized treatment plots. Gray highlights represent bacterial isolates from mowed/unfertilized treatment plots.