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Long-Term Nutrient Enrichment of an Oligotroph-Dominated Wetland Increases Bacterial Diversity in Bulk Soils and Plant Rhizospheres

Regina B. Bledsoe,[a] Carol Goodwillie,[a] Ariane L. Peralta[a]

[a]Department of Biology, East Carolina University, Greenville, North Carolina, USA

ABSTRACT In nutrient-limited conditions, plants rely on rhizosphere microbial members to facilitate nutrient acquisition, and in return, plants provide carbon resources to these root-associated microorganisms. However, atmospheric nutrient deposition can affect plant-microbe relationships by changing soil bacterial composition and by reducing cooperation between microbial taxa and plants. To examine how long-term nutrient addition shapes rhizosphere community composition, we compared traits associated with bacterial (fast-growing copiotrophs, slow-growing oligotrophs) and plant (C_3 forb, C_4 grass) communities residing in a nutrient-poor wetland ecosystem. Results revealed that oligotrophic taxa dominated soil bacterial communities and that fertilization increased the presence of oligotrophs in bulk and rhizosphere communities. Additionally, bacterial species diversity was greatest in fertilized soils, particularly in bulk soils. Nutrient enrichment (fertilized versus unfertilized) and plant association (bulk versus rhizosphere) determined bacterial community composition; bacterial community structure associated with plant functional group (grass versus forb) was similar within treatments but differed between fertilization treatments. The core forb microbiome consisted of 602 unique taxa, and the core grass microbiome consisted of 372 unique taxa. Forb rhizospheres were enriched in potentially disease-suppressive bacterial taxa, and grass rhizospheres were enriched in bacterial taxa associated with complex carbon decomposition. Results from this study demonstrate that fertilization serves as a strong environmental filter on the soil microbiome, which leads to distinct rhizosphere communities and can shift plant effects on the rhizosphere microbiome. These taxonomic shifts within plant rhizospheres could have implications for plant health and ecosystem functions associated with carbon and nitrogen cycling.

IMPORTANCE Over the last century, humans have substantially altered nitrogen and phosphorus cycling. Use of synthetic fertilizer and burning of fossil fuels and biomass have increased nitrogen and phosphorus deposition, which results in unintended fertilization of historically low-nutrient ecosystems. With increased nutrient availability, plant biodiversity is expected to decline, and the abundance of copiotrophic taxa is anticipated to increase in bacterial communities. Here, we address how bacterial communities associated with different plant functional types (forb, grass) shift due to long-term nutrient enrichment. Unlike other studies, results revealed an increase in bacterial diversity, particularly of oligotrophic bacteria in fertilized plots. We observed that nutrient addition strongly determines forb and grass rhizosphere composition, which could indicate different metabolic preferences in the bacterial communities. This study highlights how long-term fertilization of oligotroph-dominated wetlands could alter diversity and metabolism of rhizosphere bacterial communities in unexpected ways.

KEYWORDS copiotroph, fertilization, oligotroph, plant-microbe, rhizosphere

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Address correspondence to Regina B. Bledsoe, ginabbledsoe@gmail.com.

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The soil microbiome is critical for plant health, fitness, and diversity, especially in nutrient-limited environments (1–4). In particular, within the rhizosphere, plants provide carbon (C) resources to soil microorganisms in exchange for nutrients such as nitrogen (N) and phosphorus (P). However, nutrient enrichment has been documented to disrupt plant-microbe mutualisms (2). Over the last century, agricultural fertilization and the burning of fossil fuels and biomass have indirectly led to nutrient deposition onto historically low-nutrient ecosystems (5–8). Nutrient enrichment generally causes reduced plant species diversity (9, 10) sometimes as a shift in plant functional types with an increase in grass biomass and loss of forb diversity (11–13). Fertilization has also been shown to decrease soil microbial diversity across cropland, grassland, forest, and tundra ecosystems (14–16). Despite patterns that have emerged from these bulk soil studies, it is less clear how changes in soil microbial diversity due to nutrient additions influence rhizosphere microbial community assembly and diversity. We address this knowledge gap by comparing changes in rhizosphere bacterial community composition of a grass and forb within a long-term fertilization experiment.

Both bulk soil matrix (i.e., not in contact with plant roots) properties and plant identity influence rhizosphere microbial communities. The bulk soil matrix is the reservoir of microbial diversity from which rhizosphere-associated microbial communities are selected; therefore, shifts in bulk soil microbial communities affect rhizosphere assemblages (17–19). In many cases, N, N and P, and N-P-K (nitrogen-phosphorus-potassium) fertilization decreases soil bacterial diversity (14–16). Additionally, nutrient enrichment selects for more copiotrophic (i.e., fast-growing, r-strategists) microbial heterotrophs that preferentially metabolize labile C sources versus oligotrophic (i.e., slow-growing, K-strategist) microbial species, which can metabolize complex C sources (20–23). A molecular marker to identify life history strategy (i.e., copiotroph or oligotroph) is rRNA (*rrn*) gene copy number (23–26). Bacterial taxa are estimated to contain 1 to 15 rRNA gene copies, with faster-growing taxa containing higher numbers of gene copies than slower-growing taxa (20, 23–27). Specifically, bacterial growth rate is limited by the transcription rate of rRNA, such that growth rate is estimated to double with doubling of rRNA gene copy number (23). Further, several studies indicate that fertilization increases the abundance of copiotrophic bacterial groups within *Actinobacteria*, *Alphaproteobacteria*, and *Gammaproteobacteria* and decreases abundance in oligotrophic bacterial groups within *Acidobacteria*, *Nitrospirae*, *Planctomycetes*, and *Deltaproteobacteria* of bulk soils (15, 21, 28, 29). Additionally, copiotrophic taxa within *Alpha-*, *Beta-*, and *Gammaproteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* are dominant members of some rhizosphere communities (17, 30, 31).

While the bulk soil environment is the primary source of rhizosphere diversity, plant species also influence rhizosphere bacterial community assembly due to variation in rhizodeposition (30–33). Rhizodeposits include nutrients, exudates, root cells, and mucilage released by plant roots (34). Plants allocate 5 to 20% of photosynthetically fixed C belowground (35–37). Some estimates suggest that up to 40% of fixed C is translocated belowground (38), and grasses are suggested to be near that upper limit with ~30% of fixed C allocated belowground (39). These rhizodeposits also include root exudates which are composed of sugars, organic acids, phenolic compounds, and amino acids (1, 17, 40, 41). Differences in plant physiology influencing the quantity and composition of root exudates can affect rhizosphere bacterial community composition. For example, C_4 grasses have higher photosynthetic rates (i.e., fix more C) and greater root biomass allocation compared to C_3 plants, resulting in a greater quantity of root exudates (42, 43). C_3 plant root exudates can contain a greater variety of organic acids and amino acids along with the sugars mannose, maltose, and ribose compared to C_4 plant root exudates, which can contain several sugar alcohols (i.e., inositol, erythritol, and ribitol) (44). However, N fertilization has been shown to increase C assimilation in plants but decrease belowground allocation of assimilated C while increasing total C into soils as rhizodeposits (39, 45). Prior studies revealed that root exudation of organic C can be higher in both low-nutrient scenarios (46, 47) and high-nutrient scenarios (48,

TABLE 1 Soil physiochemical properties after 12 years of fertilization and mowing disturbance[a]

Soil physiochemical property	Value for:					
	Unfertilized plot			Fertilized plot		
	Bulk	Grass	Forb	Bulk	Grass	Forb
Temp (°C)	23.3 ± 0.4			22.8 ± 0.6		
Moisture (%)	19.53 ± 0.39	19.18 ± 0.10	19.18 ± 0.13	19.45 ± 0.26	19.15 ± 0.10 **B**	19.18 ± 0.13
pH	**5.17 ± 0.15 A**	**4.50 ± 0.31 B**	**4.62 ± 0.39 B**	**5.38 ± 0.08 A**	**4.81 ± 0.25 B**	**4.62 ± 0.39 B**
NO_3^--N (μg/g soil [dry wt])	0.31 ± 0.26 **B**	1.83 ± 0.55 **A**	0.97 ± 0.28 **AB**	0.41 ± 0.28 **B**	0.97 ± 0.25 **A**	0.97 ± 0.28 **AB**
NH_4^+-N (μg/g soil [dry wt])	2.51 ± 0.71	2.45 ± 0.90	2.37 ± 0.14	2.64 ± 0.95	2.53 ± 0.82	2.37 ± 0.14
Total C (%)	3.52 ± 0.86 **A**	5.24 ± 1.03 **B**	5.00 ± 1.02 **AB**	3.81 ± 0.59 **A**	5.82 ± 2.71 **B**	5.00 ± 1.02 **AB**
Total N (%)	0.20 ± 0.05 **A**	0.29 ± 0.06 **B**	0.27 ± 0.06 **AB**	0.22 ± 0.03 **A**	0.33 ± 0.15 **B**	0.27 ± 0.06 **AB**
Soil C/N ratio (wt/wt)	17.84 ± 1.21	18.13 ± 1.02	18.91 ± 0.35	17.31 ± 1.47	17.62 ± 0.49	18.91 ± 0.35

[a]Average (mean ± standard deviation) soil properties (temperature, gravimetric moisture, pH, extractable nitrate and ammonium concentrations, total soil C and N, and C/N ratio) across unfertilized and fertilized plots and among soil sources (bulk soil, grass rhizosphere, and forb rhizosphere) in mowed plots. The fertilization main effect that is significantly different (ANOVA $P < 0.05$) is shown in bold type. Letters represent significant differences between soil sources (Tukey's HSD test, $P < 0.05$).

49). Further, differences in soil nutrient status can change the composition (i.e., carbohydrates, organic acids, and amino acid concentrations) of root exudates (46, 50). Thus, fertilization and plant-specific rhizodeposition patterns of C_3 forbs and C_4 grasses are predicted to differentially affect rhizosphere bacterial community structure.

In this study, we address the following question: to what extent does long-term fertilization (N-P-K) of bulk soil shift rhizosphere bacterial communities of two plant species representing distinct functional types (i.e., a C_3 forb and a C_4 grass)? First, we hypothesize that nutrient addition will decrease bacterial species diversity and increase the abundance of copiotrophic taxa in all soils, especially rhizosphere soils due to increased availability of labile C from root exudates. We expect that fertilization will stimulate microbial activity of faster-growing copiotrophic species, which would outcompete slower-growing oligotrophic species and result in decreased bacterial diversity. This effect is predicted to be amplified within plant rhizospheres due to the availability of labile C substrates in root exudates, which should preferentially select for copiotrophic bacteria. Second, we hypothesize that fertilization will be the primary factor determining differences in rhizosphere communities and plant identity will secondarily influence the rhizosphere community. If bulk soil is the reservoir for the rhizosphere community, then fertilization will more strongly determine rhizosphere bacterial diversity and community composition. In addition, plant type can also affect rhizosphere communities due to differences in root exudate composition; however, fertilization effects will constrain rhizosphere effects. As a result, plant species are expected to associate with unique core microbiomes that differ between fertilization treatments.

To test these hypotheses, bulk and rhizosphere soils were sampled from two plant species (a grass and a forb) from fertilized and unfertilized plots at a long-term disturbance and fertilization experiment (established in 2003). Bacterial communities were identified using 16S rRNA amplicon sequencing which allowed binning of bacterial taxa as copiotrophic or oligotrophic by estimating the average rRNA (*rrn*) gene copy number. By evaluating differences in taxonomic information and 16S rRNA gene copy numbers of bulk and rhizosphere soils of two plant species with associated soil properties (i.e., ammonium, nitrate, soil pH, carbon, and moisture), we provide insight into biotic and abiotic processes that are contributing to rhizosphere bacterial community assembly.

RESULTS

Soil source and fertilization distinguish soil properties. The main effect of fertilization was significantly different in the soil physiochemical property of pH ($P = 0.02$), and the main effect of soil source (bulk versus rhizosphere) was significantly different in the soil physiochemical properties of pH ($P < 0.001$), nitrate ($P < 0.0001$), C percent ($P = 0.03$), and N percent ($P = 0.04$; Table 1; see also Table S1 in the

supplemental material). Rhizosphere soils were more similar to each other in soil properties than to bulk soils (Table 1, Table S1, Tukey's honestly significant difference [HSD] test, $P < 0.05$). Specifically, bulk soil had lower total C and N and nitrate concentrations than forb rhizospheres with grass rhizospheres having the highest values (Table 1, Table S1, Tukey's HSD test, $P < 0.05$). Soil pH was lowest in rhizosphere soils compared to bulk soils but higher in fertilized soils compared to unfertilized soils within soil sources (Table 1, Table S1, Tukey HSD, $P < 0.05$).

Fertilization increased soil bacterial diversity in bulk and rhizosphere soils. Chao1 bacterial richness ($P < 0.0001$) and Shannon H′ diversity ($P < 0.0001$) were higher in fertilized soils than in unfertilized soils (Fig. 1A, Table S2). In addition, the main effect of soil source influenced bacterial diversity; bulk soil bacterial diversity was significantly higher than rhizosphere soil diversity (Tukey's HSD test, $P < 0.05$, Fig. 1B, Table S2). Finally, results revealed a positive relationship between Shannon H′ diversity and pH, where pH explained 71% ($P = 0.0003$) and 32% ($P = 0.03$) of the variation in bacterial diversity in unfertilized and fertilized treatments, respectively, across all soil sources (Fig. 1C).

Copiotroph-to-oligotroph ratios indicated oligotroph-dominated bacterial communities. Across all samples, we detected 9 to 30 copiotrophic taxa and 82 to 190 oligotrophic taxa at the class level. This resulted in copiotroph-to-oligotroph ratios of <0.2 within all treatment combinations. Nutrient additions significantly decreased the ratio of copiotrophs to oligotrophs in bulk soils compared to rhizosphere soils (Tukey's HSD test, $P < 0.05$; Table S4; Fig. 2). Finally, there was no relationship between bacterial Shannon H′ diversity and copiotroph-to-oligotroph ratio (fertilized, $R^2 = -0.01$, $P = 0.38$; unfertilized, $R^2 = 0.14$, $P = 0.13$) (see Fig. S1 in the supplemental material).

Fertilization treatment and soil source influenced bacterial community composition. Specifically, fertilization treatment (along principal-coordinate analysis [PCoA] axis 1) explained 31.6% of variation in bacterial community composition, while soil source (primarily bulk versus rhizosphere) separated bacterial composition (along PCoA axis 2) and explained 22.5% of bacterial community variation (Fig. 3). The main effects of soil source (permutational multivariate analysis of variance [PERMANOVA], $R^2 = 0.23$, $P = 0.001$) and fertilization treatment (PERMANOVA, $R^2 = 0.281$, $P = 0.001$) influenced bacterial community composition (Table S3A). According to pairwise comparisons, rhizosphere bacterial community composition was similar between grass and forb rhizosphere samples within fertilization treatments (Table S3B). When examining relationships between community composition and soil characteristics, higher soil pH and moisture were correlated with fertilized bulk soils (Fig. 3). Further, higher concentrations of soil C and N were correlated with rhizosphere community composition (Fig. 3).

Different bacterial taxa (OTUs) represented fertilization treatments and plant species. We compared bacterial community taxonomic shifts in unfertilized and fertilized bulk soils and then grass and forb rhizospheres, concluding with differences in microbiome structure between the two plant species. Within bulk soil samples, important indicator species for bacterial communities within unfertilized plots were from the class *Alphaproteobacteria* with 1 operational taxonomic unit (OTU) from the order *Rhizobiales* and 2 OTUs from *Rhodospirillales* and 3 OTUs from the class *Spartobacteria* (Table S5). In contrast, fertilized bulk soils were best represented by members of the class *Actinobacteria* with 1 OTU from the order *Actinomycetales* and 2 OTUs from the order *Solirubrobacterales*. While OTUs within *Rhizobiales* were identified as indicator species for bacterial communities in unfertilized bulk soils, this order was in greatest relative abundance compared to other orders within both fertilization treatments (Fig. S2).

Comparisons of rhizosphere bacterial OTU presence/absence data revealed that forb (1,249 OTUs) and grass (1,019 OTUs) rhizospheres have distinct but overlapping microbiomes. Of the 1,621 total OTUs found in rhizosphere soils, 647 are broadly distributed and are observed in all plant rhizospheres and bulk soils regardless of treatment. Therefore, less than half of the forb (48%) and grass (37%) rhizosphere members were

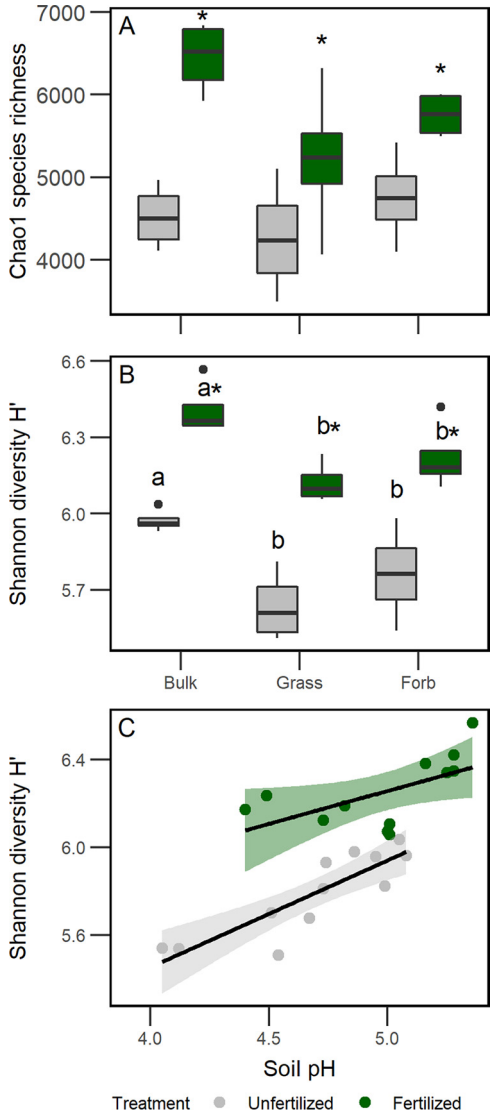

FIG 1 Bacterial diversity patterns according to soil source, fertilization, and soil pH. Boxplots of bacterial diversity for Chao1 richness (A) and Shannon diversity H′ (B) associated with soil source (bulk, grass rhizosphere, forb rhizosphere) and fertilization treatment. (C) Linear regression of soil pH and bacterial community Shannon diversity H′ by fertilization treatment with 95% confidence intervals (fertilized, $R^2 = 0.32$, $P = 0.03$; unfertilized: $R^2 = 0.71$, $P = 0.0003$). Colors indicate fertilization treatment. Asterisks indicate significant differences between fertilization treatments, and lowercase letters represent significant differences between soil sources (Tukey's HSD test, $P < 0.05$).

unique to that plant functional type, and broadly distributed OTUs dominate plant microbiomes especially in grasses.

Of OTUs that were represented only in the grass microbiome ($n = 372$), only 22 bacterial families are represented at $>0.075\%$ relative abundance. Within those top OTUs, unfertilized grass rhizospheres were enriched in 9 families, while fertilized plots were enriched in 19 families (Fig. 4). Indicator species for unfertilized grass rhizospheres included 2 OTUs, one in the genus *Singulisphaera* and family *Planctomycetaceae* (indicator value [IndVal] = 0.38, $P = 0.026$) and an unclassified *Spartobacteria* OTU (IndVal = 0.44, $P = 0.008$; Table S5). Indicator species for fertilized grass rhizospheres included two OTUs, one in the genus *Planctomyces* and family *Planctomycetaceae* (IndVal = 0.42, $P = 0.011$) and one in the genus *Actinoallomurus* and family *Thermomonosporaceae* (IndVal = 0.36, $P = 0.045$; Table S5).

Of the OTUs that were represented only in the forb microbiome ($n = 602$), only 21 bacterial families are represented at >0.1% relative abundance. Within those top OTUs,

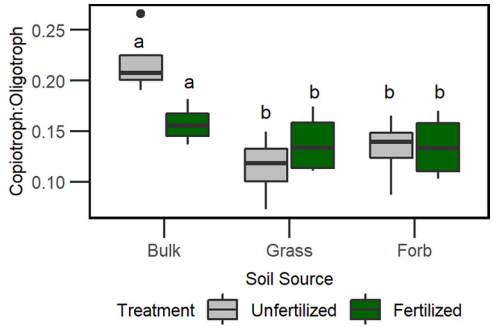

FIG 2 Comparison of bacterial life history traits. Boxplots of copiotroph-to-oligotroph ratios (based on 16S rRNA sequences) according to soil source (bulk, grass rhizosphere, and forb rhizosphere) and fertilization treatment. Boxplots are colored according to fertilization treatment. Lowercase letters indicate significant differences among soil sources (Tukey's HSD test, $P < 0.05$).

unfertilized forb rhizospheres were enriched in 10 families, while fertilized plots were enriched in 16 families (Fig. 5). Indicator species for unfertilized forb rhizospheres included two OTUs, *Acidobacteria* Gp1 (IndVal = 0.42, $P = 0.02$), and an unclassified *Proteobacteria* (IndVal = 0.46, $P = 0.033$; Table S5). Indicator species for fertilized forb rhizospheres included an OTU in *Acidobacteria* Gp1 (IndVal = 0.34, $P = 0.041$) class and an unclassified bacterial OTU (IndVal = 0.60, $P = 0.017$; Table S5).

DISCUSSION

In this study, nutrient addition increased bacterial species diversity (H′) and richness in bulk and rhizosphere soils. These results were similar to the results of O'Brien et al. (51) but contrary to our prediction and the results of other studies (14–16). Overall, bulk soils had the greatest bacterial diversity and highest pH values compared to rhizosphere soils. Since pH is known to be a strong driver of bacterial diversity, which can have a positive relationship with pH (52, 53), this increase in diversity may be due, in part, to the greater bulk soil pH compared to rhizosphere soil pH. The difference in pH between soil types is possibly due to organic acids in plant root exudates released into the rhizosphere (41); however, we did not analyze the composition of root exudates. Additionally, pH tended to be lower in unfertilized treatments, and diversity was more strongly related to pH in unfertilized soils than in fertilized soils. This may be due to the sensitivity of bacteria to acidic soils (53). The increase in bacterial diversity is likely the result of soil pH and niche differentiation due to fertilization increasing nutrient

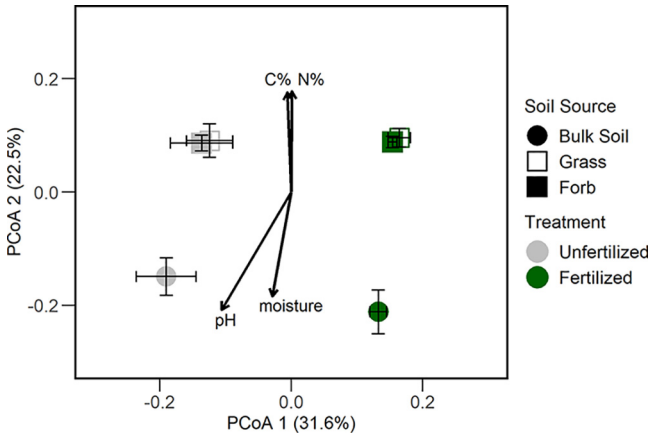

FIG 3 Ordination based on principal-coordinate analysis depicting bacterial community composition. Colors represent fertilization treatment, and symbols represent soil source (bulk soil, grass rhizosphere, and forb rhizosphere). Vectors represent soil factors that are correlated with patterns in bacterial community composition ($P < 0.05$) (soil pH [pH], soil gravimetric moisture percentage [moisture], total soil carbon [C%], total soil nitrogen [N%]).

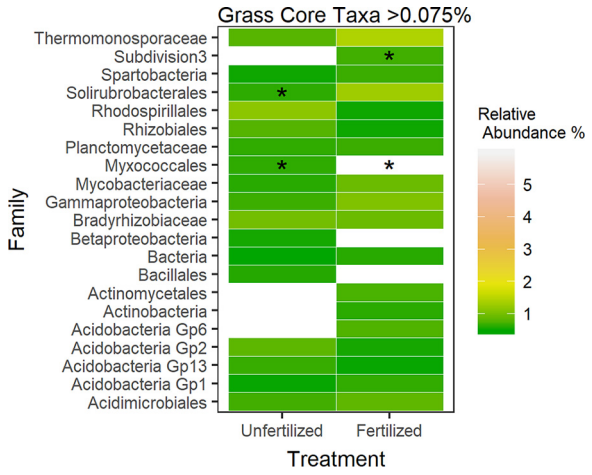

FIG 4 Comparisons of top OTU relative abundances (>0.075%) at the family level between fertilization treatments for grass rhizosphere bacterial communities. Asterisks represent indicator species present within the family (see Table S5 in the supplemental material). Colors indicate relative abundance increases from cool to warm (green yellow, orange, and red). White boxes indicate taxa present at <0.075% relative abundance.

availability and rhizodeposition by plants, which introduces organic C resources for heterotrophs (17, 32). In dilution to extinction experiments, decreases in microbial diversity can result in loss of microbial functional diversity (54, 55). Therefore, increases in microbial diversity could result in increased microbial functional diversity, which could increase C cycling and promote N mining particularly in plant rhizospheres (56).

Bacterial taxa identified in rhizosphere samples are putatively involved in nutrient cycling and disease-suppressive functions. For example, fertilized forb rhizospheres were enriched in taxa from the family *Streptomycetaceae*, of which many produce antibiotics (57) and *Sphingomonadaceae*, which include taxa with disease suppression potential against fungal pathogens (58) (Fig. 5). This increase in disease-suppressive bacterial taxa suggests a potential increase in plant-pathogenic taxa within fertilized rhizospheres; however, this study did not specifically address disease suppression in soils. In contrast, fertilized grass rhizospheres were enriched with taxa putatively involved in N_2 fixation (*Acetobacteraceae*) (59) and also *Chitiniphagaceae* and *Conexibacteraceae*, which have been implicated in decomposition of recalcitrant C sources (60,

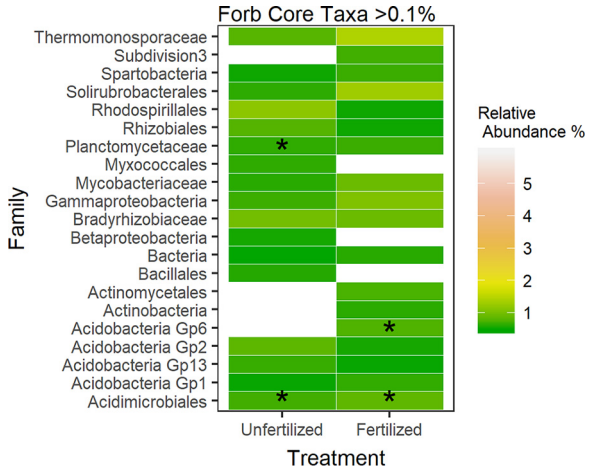

FIG 5 Comparisons of top OTU relative abundances (>0.1%) at the family level between fertilization treatments for forb rhizosphere bacterial communities. Asterisks represent indicator species present within the family (Table S5). Colors indicate relative abundance increases from cool to warm (green yellow, orange, and red). White boxes indicate taxa present at <0.1% relative abundance.

61) (Fig. 4). Bacterial taxa in the *Xanthomonadaceae* family, which have previously been found in environments containing glyphosate (62), and *Caulobacteraceae*, which grows optimally on pesticides (63), are also more abundant in fertilized grass rhizospheres (Fig. 4). Since fertilization increased bacterial diversity and shifted composition, it is possible that fertilization has stimulated root exudation. The relative increase in complex C-degrading bacterial taxa in the grass rhizosphere could also be due to greater inputs of phenolics and terpenoids used as allelochemicals by the plant as revealed in past studies (64, 65). These differences in bacterial composition between the two plant species could be due to differences in composition of root exudates released into the rhizosphere (37); however, we did not analyze the composition of root exudates in the present study. Together, results suggest that nutrient addition enriches forb rhizospheres with putatively disease-suppressive bacteria and grass rhizospheres with taxa capable of decomposing complex C sources.

Within bulk soil bacterial members, putative nitrogen cycling taxa in the order *Rhizobiales* were enriched across all fertilization treatments (66, 67). This is not surprising, considering the limited amount of nitrogen in both unfertilized and fertilized soils at the study site. Despite the increase in taxa capable of N_2 fixation in fertilized rhizospheres, these bacteria will acquire soil N if it is available (68). Therefore, these taxa may be less cooperative with plant associates than the same taxa from unfertilized soils, thereby reducing plant benefit (2, 69). This was not specifically tested in this study but could be an important future research topic.

Contrary to our prediction, bulk soils had a higher copiotroph-to-oligotroph ratio (based on *rrn* gene copy number) than rhizospheres. Characteristic of the copiotrophic life history strategy is the ability to rapidly decompose labile C sources; therefore, we expected that C-rich root exudates in the rhizosphere would support higher proportions of copiotrophic species (17). Additionally, fertilization did not increase the relative abundance of copiotrophic taxa. Rather, the observed copiotroph-to-oligotroph ratios were low in all samples with unfertilized bulk soils having the greatest proportion (0.22) and unfertilized grass rhizospheres having the lowest (0.13) copiotroph-to-oligotroph ratios. We suggest that the dominance of oligotrophs reflects the low-nutrient history of this wetland (29, 70), which is in contrast to agricultural systems that undergo regular fertilization at target rates intended to support high nutrient requirements for enhanced crop production (e.g., corn).

These results are in contrast to our first hypothesis and in agreement with our second hypothesis. Analyses of bacterial diversity and copiotroph-to-oligotroph ratios revealed an increase in bacterial diversity in response to fertilization and dominance of oligotrophs across all treatments within the study wetland. The low-nutrient history of the study site is likely the primary factor shaping bacterial community composition within the wetland. In agreement with our second hypothesis, comparisons of bulk and rhizosphere bacterial communities revealed that rhizospheres were more similar to each other than to bulk soil bacterial communities within fertilization treatments. Core plant microbiomes were predominantly composed of broadly distributed taxa; therefore, changes in bulk soil bacterial composition due to nutrient enrichment can directly alter plant microbiome composition and indirectly diminish benefits to plants if nutrient enrichment selects for more competitive bacterial taxa. These results highlight the importance of bulk soils as reservoirs of diversity for plant rhizospheres, which could have further implications for agricultural plant species in maintaining beneficial microbial communities.

Overall, this study revealed that long-term fertilization of oligotroph-dominated soils in low-nutrient wetlands increases bacterial species diversity. This increase in bacterial diversity has the potential to result in increased C and nutrient cycling that could lead to declines of wetland C storage potential. Nutrient enrichment also differentially alters plant rhizosphere composition in a way that suggests metabolic changes within soil bacterial communities. These metabolic changes could indirectly impact plant species diversity by providing an advantage to one species versus another through disease suppression or by increasing plant-available N through promotion of soil organic matter decomposition. If indirect fertilization supports rhizosphere bacterial commu-

nities that can enhance recalcitrant or labile C decomposition, wetland C storage potential could decline. Based on this study, bacterial taxonomic characterization sheds light on fertilization effects on plant-bacterial relationships. As such, nutrient enrichment effects on the metabolic diversity of bacterial communities could be even more pronounced in naturally low-nutrient ecosystems and warrants further investigation.

MATERIALS AND METHODS

Study site and experimental design. A long-term experimental site was established in 2003 to test the effects of fertilization, mowing, and the interaction on wetland plant communities. The site is located at East Carolina University's West Research Campus in Greenville, NC, USA (35.6298N, 77.4836W). A description of the study site and experimental design can be found in Goodwillie and Franch (71) and is summarized here. This site is classified as a jurisdictional wetland but historically described as a mosaic of wet pine flatwood habitat, pine savanna, and hardwood communities. Soils were characterized as fine, kaolinitic, thermic Typic Paleaquults (Coxville series) with a fine sandy loam texture which are ultisols that are acidic and moderate to poorly drained soil types (https://soilseries.sc.egov.usda.gov/osdname.aspx). The annual mean temperature is 17.2°C, and annual precipitation is 176 cm (https://www.climate.gov/maps-data/dataset/). Treatments are replicated on eight 20×30 m blocks, and the nitrogen-phosphorus-potassium (N-P-K) 10-10-10 pellet fertilizer is applied three times per year (February, June, and October) for a total annual supplementation of 45.4 kg ha^{-1} for each nutrient. Plots are mowed by bush-hog and raked annually to simulate a fire disturbance (71).

We compared rhizosphere and bulk soil microbiomes in mowed unfertilized and fertilized plots, where herbaceous species dominated. Soil samples were collected at mowed/unfertilized and mowed/fertilized plots in four out of eight replicate blocks to reduce variability due to hydrology. Half the site is located adjacent to a ditch (drier soils) compared to away from the ditch, where soil conditions are wetter. Since this hydrologic gradient has resulted in distinct plant communities (C. Goodwillie, M.W. McCoy, and A. L. Peralta, submitted for publication), we collected samples from the wetter plots (away from the drainage ditch).

Bulk and rhizosphere soil sampling. We collected soil samples on 29 September 2015, approximately 3 months after the last fertilization treatment. Due to annual mowing and raking in sample plots, there was limited biomass accumulated in the organic horizon. We focused soil sampling and analysis on the mineral horizon. For a single composite bulk soil sample, we collected two soil cores (12-cm depth, 3.1-cm diameter) near each of the three permanently installed 1-m^2 quadrats used for annual plant surveys. Each composite bulk soil sample was homogenized and passed through a 4-mm sieve, and any plant material was removed before further analysis. At each plot, rhizosphere soils were collected from the C_3 forb *Euthamia caroliniana* (L.) Greene ex Porter & Britton and C_4 grass *Andropogon virginicus* L. Rhizosphere soils were a composite of three root systems of the same species. Roots were gently dislodged from soil and neighboring roots and placed in a paper bag. After vigorous shaking, soil in the bag was processed for abiotic analysis. The roots were placed into 50-ml centrifuge tubes with 30 ml sterilized Nanopure water and shaken at 100 rpm for 1 h. Washed roots were removed, and the soil and water mixture was freeze dried to remove water. Freeze-dried rhizosphere samples were stored at −80°C until DNA extraction.

Soil chemical and physical characteristics. We measured gravimetric soil moisture by drying 20 to 30 g of field soil (moist soil) at 105°C for 24 h. We calculated percent moisture as the difference in weight of moist and dried soils divided by the oven-dried soil weight. Oven-dried samples were ground and measured for pH by mixing a 1:1 (soil-water) solution. A subsample of oven-dried soil was sieved with a 500-μm mesh and analyzed for total carbon and total nitrogen using an elemental analyzer (2400 CHNS Analyzer; Perkin-Elmer, Waltham, MA, USA) at the Environmental and Agricultural Testing Service laboratory (Department of Crop and Soil Sciences at NC State). Approximately 5 g of field soil (moist soil) was extracted with 45 ml of 2 M KCl, and available ammonium (NH_4^+) and nitrate (NO_3^-) ions were colorimetrically measured using a SmartChem 200 auto analyzer (Unity Scientific, Milford, MA, USA) at the East Carolina University Environmental Resources Laboratory.

Bacterial community analyses. We extracted DNA from soils using the Qiagen DNeasy PowerSoil kit. We used this DNA as the template in PCRs using barcoded primers (bacterial 515FB/806R) originally developed by the Earth Microbiome Project to target the V4 region of the bacterial 16S subunit of the rRNA gene (72). For each sample, three 50-μl PCR libraries were prepared by combining 30.75 μl molecular grade water, 5 μl Perfect *Taq* 10× buffer, 10 μl Perfect *Taq* 5× buffer, 1 μl deoxynucleoside triphosphates (dNTPs) (40 mM total, 10 mM each), 0.25 μl Perfect *Taq* polymerase, 1 μl forward barcoded primer (10 μM), 1 μl reverse primer (10 μM), and 1 μl DNA template (10 ng μl^{-1}). Thermocycler conditions for PCRs were as follows: (i) initial denaturation (94°C for 3 min); (ii) 30 cycles with 1 cycle consisting of 94°C for 45 s, 50°C for 30 s, and 72°C for 90 s; (iii) final elongation (72°C, 10 min). Triplicate PCRs were combined and cleaned using the AMPure XP magnetic bead protocol (Axygen, Union City, CA, USA). Cleaned PCR product were quantified using QuantIT dsDNA BR assay (Thermo Scientific, Waltham, MA, USA) and diluted to a concentration of 10 ng μl^{-1} before pooling libraries in equimolar concentration of 5 ng μl^{-1}. We sequenced pooled libraries using the Illumina MiSeq platform using paired-end reads (Illumina reagent kit v2, 500 reaction kit) at the Indiana University Center for Genomics and Bioinformatics Sequencing Facility. Sequences were processed using mothur (v1.40.1) (73) MiSeq pipeline (74). We assembled contigs from the paired-end reads, quality trimmed using a moving average quality score (minimum quality score, 35), aligned sequences to the SILVA rRNA database (v128) (75), and removed chimeric sequences using the VSEARCH algorithm (76). We created operational taxonomic units

(OTUs) by first splitting sequences based on taxonomic class and then binning into OTUs based on 97% sequence similarity. The SILVA rRNA database was then used to assign taxonomic designations to OTUs.

Samples were rarefied to 43,811 reads (lowest read count among all samples) and resampled. We used *vegan::diversity* (77) to calculate bacterial species diversity by calculating Shannon diversity index (H′) because it accounts for species abundance and evenness and rare species (78, 79). We estimated bacterial richness using Chao1 species richness because it is nonparametric and also considers rare species (79, 80). Shannon diversity was calculated using the *vegan::diversity* function and Chao1 OTU richness using *vegan::estimate* (77). We assigned gene copy number to each OTU using RDP classifier (v2.12) (81) integrated with the *rrn* operon database developed by the Schimdt Laboratory at the Michigan Microbiome Project, University of Michigan (23, 27). Higher gene copy numbers ($\geq$5) represent the copiotrophic lifestyle, and lower gene copy numbers ($<$5) represent the oligotrophic lifestyle (20, 24, 82). The numbers of copiotrophs and oligotrophs were summed for each soil sample to calculate the copiotroph-to-oligotroph ratio within a soil bacterial community.

Statistical analyses. All statistical analyses were performed in the R statistical environment (RStudio v1.1.383, Rv3.4.0) (83). We used a two-way model of analysis of variance (ANOVA) to compare the main effects of soil source, fertilization treatment, and the interaction to test for differences in OTU diversity and richness, copiotroph-to-oligotroph ratios, and soil parameters (soil pH, total carbon, total nitrogen, extractable ammonium and nitrate, and soil moisture). Significant interactions were compared with Tukey's *post hoc* analysis using the *agricolae::HSD.test* R function (84). We examined diversity by visualizing bacterial community responses to fertilization and rhizosphere association using principal-coordinate analysis (PCoA) based on Bray-Curtis dissimilarity. We used permutational multivariate analysis of variance (PERMANOVA) to test for differences in bacterial community composition among treatments and within treatment using pairwise comparisons. Hypothesis testing using PERMANOVA was performed using the *vegan::adonis* function (77). We examined the relationship between soil parameters and bacterial Bray-Curtis dissimilarity patterns using the *vegan::envfit* function (77). Soil parameters with $P < 0.05$ were represented on the PCoA plot as vectors scaled by strength of correlation. We performed Dufrene-Legendre indicator species analysis using the *labdsv::indval* function (85) to identify specific community members that represented each soil source and fertilization treatment combination.

Data availability. An .Rmd file that includes annotated code and R scripts, data files, and the sequence processing batch file used in this study can be found in a public GitHub repository at https://github.com/PeraltaLab/WRC15_Rhizo. Raw sequence files for each sample can be accessed through the NCBI SRA BioProject accession number PRJNA599142.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, PDF file, 0.01 MB.

FIG S2, PDF file, 0.01 MB.

TABLE S1, DOCX file, 0.01 MB.

TABLE S2, DOCX file, 0.01 MB.

TABLE S3, DOCX file, 0.01 MB.

TABLE S4, DOCX file, 0.01 MB.

TABLE S5, DOCX file, 0.01 MB.

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R.B.B., C.G., and A.L.P. conceived and designed the research. R.B.B. collected and analyzed the data. R.B.B. wrote the manuscript. All authors performed field work and edited the manuscript.

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