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RESEARCH ARTICLE

Plant diversity and functional identity drive grassland rhizobacterial community responses after 15 years of CO_2 and nitrogen enrichment

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Abstract

1. Improved understanding of bacterial community responses to multiple environmental filters over long time periods is a fundamental step to develop mechanistic explanations of plant–bacterial interactions as environmental change progresses.
2. This is the first study to examine responses of grassland root-associated bacterial communities to 15 years of experimental manipulations of plant species richness, functional group and factorial enrichment of atmospheric CO_2 (eCO_2) and soil nitrogen (+N).
3. Across the experiment, plant species richness was the strongest predictor of rhizobacterial community composition, followed by +N, with no observed effect of eCO_2. Monocultures of C_3 and C_4 grasses and legumes all exhibited dissimilar rhizobacterial communities within and among those groups. Functional responses were also dependent on plant functional group, where N_2-fixation genes, NO_{3^-}-reducing genes and P-solubilizing predicted gene abundances increased under resource-enriched conditions for grasses, but generally declined for legumes. In diverse plots with 16 plant species, the interaction of eCO_2+N altered rhizobacterial composition, while +N increased the predicted abundance of nitrogenase-encoding genes, and eCO_2+N increased the predicted abundance of bacterial P-solubilizing genes.
4. *Synthesis*: Our findings suggest that rhizobacterial community structure and function will be affected by important global environmental change factors such as eCO_2, but these responses are primarily contingent on plant species richness and the selective influence of different plant functional groups.

KEYWORDS
elevated CO_2, environmental change, nitrogen deposition, optimal resource allocation, plant functional group, Rhizobacteria

1 | INTRODUCTION

Rhizobacteria, the bacterial communities associated with plant roots, are recognized as integral parts of the plant microbiome, and important contributors to plant productivity and health (Bahram et al., 2018; Lugtenberg & Kamilova, 2009; van der Heijden & Schlaeppi, 2015). Many rhizobacteria directly and indirectly affect plant resource availability through processes such as nitrogen (N) fixation or phosphorus (P) solubilization, while others contribute to plant pathogen defences or stimulate root growth. The broad group of beneficial soil bacteria known as plant-growth-promoting rhizobacteria (PGPR) have been identified and functionally characterized across many systems (Leff et al., 2015; Luo et al., 2014; Ramirez et al., 2017). For example, bacteria in the order Rhizobiales have long been known as symbiotic N-fixers with plants in the Fabaceae, but a vast diversity of free-living N-fixing bacteria such as *Pseudomonas*, *Azospirillum*, *Enterobacter* and many *Bacillus* in the rhizosphere are increasingly recognized for their importance to the N or P nutrition of plants and being adopted as amendments in agriculture and ecological restoration (Reed et al., 2011; Smercina et al., 2019). Importantly, rhizobacteria can be affected by multiple environmental drivers, including plant diversity, local soil properties and also altered resource availability resulting from global changes including elevated atmospheric CO_2 and nitrogen deposition.

Plant richness, composition and functional identity have notable impacts in terrestrial ecology (Tilman et al., 2014), and confer important effects on soil physical structure and processes such as carbon and nutrient cycling, but these plant properties can also alter soil microbial communities in various ways (Trivedi et al., 2020; Xiong et al., 2021). Microbes in the rhizosphere simultaneously compete for resources in soil and facilitate their acquisition and transfer to plants (Bonfante & Anca, 2009), but prior to microbiome filtering based on trade and functional rewards (i.e. Kiers et al., 2011), they are structured by the composition of root chemicals and physical deposits in the soil (Semchenko et al., 2021). Importantly, root exudates and rhizodeposits are species-specific and have the potential to drastically shape rhizosphere microbial community assembly (Sasse et al., 2018), and it has been shown that the abundance of certain bacterial taxa are selectively increased by host-specific rhizodeposits (Dennis et al., 2010; Paterson et al., 2007). Therefore, the diversity and richness of *plant roots in soil*, or presence of different plant functional groups, such as C_3 or C_4 grasses, forbs or legumes that maintain different physiological strategies and resource requirements, are likely to exert strong and distinctive controls on rhizobacterial communities (Berg et al., 2014).

Abiotic factors such as soil pH or precipitation rates are known to structure rhizobacterial communities across ecosystems (Bahram et al., 2018; Lozupone & Knight, 2007), and there is gaining evidence that soil bacteria respond to global change factors such as elevated atmospheric CO_2 (eCO_2) and anthropogenic N deposition (+N) (He et al., 2012; Jansson & Hofmockel, 2020; Ramirez et al., 2012). Recently, studies have shown that eCO_2 can alter bacterial taxonomic abundances as well as shift microbial functions associated with carbon and nutrient cycling. In an agricultural setting, eCO_2 was shown to shift bacterial community composition, mostly by promoting taxa specifically associated with increased N cycling functionality, including members of the Rhizobiales, Burkholderiales and Pseudomonadales (Usyskin-Tonne et al., 2021). In temperate grassland, Tu et al. (2017) showed that eCO_2 selectively affected N cycling functions, stimulating the abundance of N_2-fixation as well as nitrate reduction and nitrite reduction genes. In a large meta-analysis investigating the global effects of N deposition on soil microbes, Zhang et al. (2018) revealed that microbial biomass and microbial respiration decreased with N deposition and that increasing N deposition increased the relative abundance of gram-positive bacteria. Globally, +N has also been shown to shift microbial diversity in a way that breaks the prior positive association between soil C and microbial functions, with implications for the long-term effects of N enrichment (Yang et al., 2022). In most cases, the enrichment of CO_2 or N increases plant productivity through shifts in allocation of photosynthetically-derived carbon (i.e. greater shoot or root biomass, or increased rhizodeposition and microbial recruitment; Wang et al., 2021), but importantly, while resource enrichment can alleviate growth-limitations, it can also shift resource requirements to other nutrients (i.e. eCO_2-induced N-limitation, or +N-induced P-limitation; Terrer et al., 2019; Vitousek et al., 2010).

Given the foundational importance of soil microbial services including decomposition, carbon sequestration, nutrient cycling and pathogen protection, it will be critical to understand the factorial impacts of plant diversity, eCO_2 and +N on soil prokaryotic diversity and functions (Delgado-Baquerizo et al., 2020). Guerra et al. (2020) recently identified large research 'blind spots' that miss the links among multiple aboveground biotic and abiotic factors to microbial diversity or function, suggesting that exploring multi-factor effects in soil ecology, particularly in the context of environmental change, is of high importance. Despite this, there are surprisingly few studies that have examined the interactive influence of plant diversity metrics on rhizobacterial communities experiencing resource enrichments such as eCO_2 or +N (Fitzpatrick et al., 2018). Further, long-term studies of global change can provide an important glimpse into the Anthropocene and help us better predict the ways biological communities will develop and interact in our changing world (Reich et al., 2018).

In this study, we utilize the BioCON (biodiversity, CO_2, N) environmental change experiment (Reich, Knops, et al., 2001) and next-generation sequencing to examine how plant richness and changing resource availability influences communities of root-associated bacteria. We focus on plant richness levels of 16, 9 and 1 (hereafter referred to as R16, R9, and R1), and factorial combinations of ambient and enriched CO_2 (eCO_2) and ambient and enriched N (+N) treatments (Figure S1). We ground our predictions in the concept of optimal resource allocation, which posits that plant–microbial relationships will be selected through preferential plant C allocation that optimizes the acquisition of resources most limiting growth, either directly through roots or indirectly through the microbiome (Bloom et al., 1985; Friel & Friesen, 2019; Johnson et al., 2013). By

alleviating C or N limitations through the enrichment of CO_2 and +N, plants may preferentially select functionally important bacterial taxa or passively alter the rhizobacterial community via shifting plant C allocation to either above- or below-ground biomass (Figure 1).

Given the notable effects of plant diversity metrics and plant functional groups on the selection of specific rhizobacterial consortia, as well as the observed impacts of elevated atmospheric CO_2 and N deposition on plant–microbial interactions, we hypothesize that H_1: plant species richness will exert a strong influence in shaping rhizobacterial community composition and shift the predicted abundance of bacterial functional genes at BioCON to alleviate plant-specific nutrient limitation. R16 plots are composed of many plant taxa, but also maintain more consistent compositionality across plots, and are therefore predicted to host more homogenous rhizobacterial communities than R1 plots, which are predicted to maintain the most dissimilar and phylogenetically distant consortia of bacterial taxa. We also predict that H_2: distinct rhizobacterial communities can be expected to develop on the roots of C_3 and C_4 grasses, forbs and legumes. Finally, as plant functional groups can exhibit distinct growth responses to eCO_2 and +N (Reich et al., 2004, 2018; Wei et al., 2017), we propose that H_3: the composition of the rhizobacterial community will also respond to eCO_2 and +N in such a way that acquisition of the most limiting resources can be optimized by the plant functional groups via selection of beneficial rhizobacterial taxa and associated functions such as phosphate solubilization, N_2-fixation, or NO_3-reduction (Figure 1). With this research, we aim to highlight the mediating role of plant properties on rhizobacterial community response to long-term, multi-factor environmental change, and promote a more mechanistic framing of plant–microbial interactions for future global change impact studies.

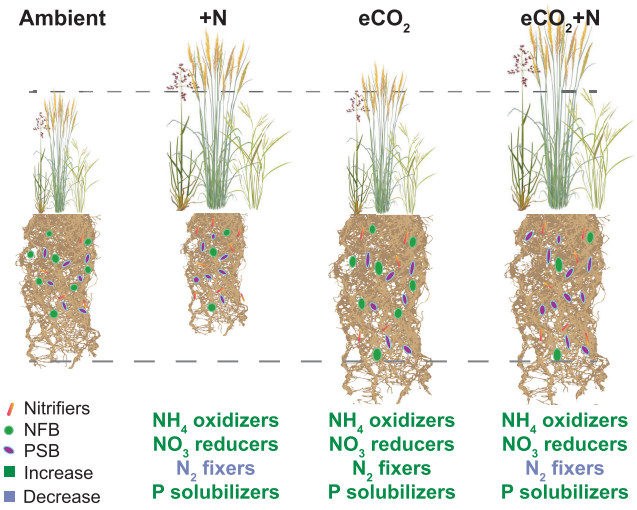

FIGURE 1 Effects of nitrogen fertilization (+N), elevated CO_2 (eCO_2), and the combination of both treatments on plant biomass allocation, microbial functional gene abundances, as well as relative composition of putative functional bacteria on roots predicted by the optimal resource allocation model of the extended plant phenotype. Dashed lines represent baseline above- and below-ground biomass allocation predictions under ambient conditions. NFB, nitrogen-fixing bacteria; PSB, phosphate-solubilizing bacteria.

2 | MATERIALS AND METHODS

2.1 | Study site

We studied a long-term (15 year) environmental change experiment in a temperate grassland to begin to elucidate how changes in resource availability and plant community structure have affected the composition of rhizobacterial communities. BioCON is a field experiment that began in 1997 at the Cedar Creek Ecosystem Science Reserve (East Bethel, MN, USA) to examine the effects of three globally occurring environmental changes; decreasing plant species diversity, elevated atmospheric CO_2 and increasing N deposition rates (Reich, Knops, et al., 2001). BioCON was designed to determine the environmental change effects on individual plant species and across plant functional groups in monoculture (R1) plots, while the more species-rich, R9 and R16 plots simulate the community and functional responses from diverse native prairie grass systems of the Midwestern United States. We acknowledge there are differences between planted richness and observed richness, and perform supplementary analyses to show that both metrics induce similar effects on rhizobacterial communities. The Free-Air Carbon dioxide Enrichment (FACE) treatments increase atmospheric CO_2 by 180 $\mu mol\,mol^{-1}$ daily, during daylight hours, for the full growing season (May–October). We sampled from two ambient CO_2 rings and two eCO_2 rings. The +N treatment applies 4 $g\,m^{-2}\,year^{-1}$ via 34% NH_4NO_3 pellets three times per growing season to half of all 1 m × 1 m planted plots within ambient and eCO_2 FACE rings (see Figure S1 for study schematic). Notably, the soil at BioCON is N-limited, where available soil N equals ~10 μg NH_4-N + NO_3-N g^{-1}. In this study, we use root samples from 132 plots consisting of three factorial treatments; plant species richness levels of R16, R9 or R1, ambient and eCO_2, and unfertilized or +N plots.

2.2 | Sampling and rhizobacterial processing methods

Next-generation sequencing was performed on DNA extracted from dry roots that were not surface-sterilized, and therefore retained an intact rhizoplane community along with endophytic bacteria. Samples were collected from two eCO_2 FACE rings (#3, #5) and two ambient control rings (#2, #4) at BioCON, and included all R16, R9 and R1 plots that were either unfertilized or +N ($n = 132$). Roots were collected in summer 2013 by root-coring (5 cm) to a depth of 20 cm. No licensing or permits were required for field work. The roots of three replicate cores from each plot were homogenized, rinsed with DI water at Cedar Creek Ecosystem Science Reserve and dried in solar ovens at ~35°C in plot-specific wax-paper bags. Dried root samples were

bagged individually by plot number and shipped to Northern Arizona University and were then processed for DNA extraction using sterile technique. DNA was extracted from roots using the MO BIO PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) with a slightly modified protocol. Briefly, five sterile stainless steel beads (2 mm) were added to each well during cellular lysis, and 96-well plates were heated to 60°C for 15 min after mechanical lysis. Genomic DNA was measured by NanoDrop and purified using magnetic beads. PCR was carried out utilizing the 515F-806R primers to amplify the V4 region of the 16S SSU rRNA (Gilbert et al., 2014). Final DNA quantitation was performed using PicoGreen (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and all samples were normalized to 2 ng DNA/μL prior to sequencing. Samples were 150 bp paired-end sequenced using MiSeq (Illumina, Inc., San Diego, CA, USA).

2.3 | Data processing

Read pairs were merged in akutils using the *join_paired_reads* command. Demultiplexing and quality filtering was carried out with the split_libraries_fastq.py command in QIIME 1.9.1 (Caporaso, 2010) using a minimum quality threshold of q20, 0 bad characters allowed, and retaining only reads which satisfied these requirements for at least 95% of their length. OTU picking was performed de novo with Swarm (Mahé et al., 2014) at d4 resolution (~98.4% similarity for bacteria/archaea), providing similar resolution to the QIIME2 DADA2-ASV approach for 16S rRNA (Bolyen et al., 2019). Taxonomic identities were assigned with BLAST against the 97% Greengenes database (McDonald et al., 2012). 16S OTU sequences were aligned using PyNAST (Caporaso et al., 2010), and phylogenetic tree was constructed with FastTree (Price et al., 2009). Taxa tables were rarefied to the lowest sample depth (5442) for alpha diversity analyses. Relative abundance of rhizobacterial taxa by treatments was analysed within the different plant species richness levels (R16, R9, R1), environmental change treatments (for R16 plots) and environmental change treatments under four plant functional groups (R1 plots) using the *group_significance.py* command in QIIME. Tests of β-diversity and differential abundance were performed on OTU tables after cumulative sum scaling (CSS) normalization (Paulson et al., 2013). Diversity analyses were conducted with the *core_diversity* command in akutils. Analyses were performed using NAU Advanced Research Computing High-Performance Computing cluster (https://in.nau.edu/arc/). R statistical software was used for all analyses unless described (R Core Team, 2020).

2.4 | Phylogenetic dispersion

Phylogenetic clustering of rhizobacterial taxa relative to plant species richness, eCO_2, +N or eCO_2 +N was analysed to determine whether rhizobacterial taxa (OTUs) become more related to each other as richness decreases or resource availability shifts than would be predicted by random models. We utilized net relatedness index (NRI) to measure rhizobacterial phylogenetic dispersion under the treatments at BioCON. NRI values were calculated (−mpd.obs.z) to provide an index of basal clustering of taxa on the phylogenetic tree. Phylogenies were created with FastTree as above and 'pruned' to match representative taxa in this study in R (R Core Team, 2020) using the ape and picante packages (Kembel et al., 2010; Paradis et al., 2004). The function *ses.mpd* was used in R, and negative values of the standardized effects size of mean phylogenetic distance versus null communities (−(mpd.obs−mpd.rand.mean)/mpd.rand.sd) were calculated for NRI. NRI > 0 indicates phylogenetic clustering, and NRI < 0 indicates phylogenetic over-dispersion, while NRI no different than zero indicates a random phylogenetic dispersion.

2.5 | Predicted bacterial metagenomic expression

The PICRUSt2 method provides a predicted metagenomic profile of the rhizobacterial community, which can be compared with previous functional gene profiling studies from BioCON or generate hypotheses for future work utilizing qPCR and measured N-cycling rates (e.g. N mineralization, nitrification and denitrification). Protocol for PICRUSt2 was followed as in Douglas et al. (2020) and tutorials on https://github.com/picrust/picrust2 for 97% similarity OTUs. The OTU table was normalized by copy, and a 'virtual' metagenome of KEGG Ortholog (KO) relative abundances for each sample in the provided OTU table was predicted. Below, we will refer to gene 'abundances', but are aware that predicted KOs are proxies calculated from relative abundances and are not absolute. Metagenome contributions by rhizobacterial taxa were calculated for N-cycling genes: *nifH*, *napA*, *narG*, *nirK*, and *nosZ*, and P solubilizing genes: acid phosphatase, glucose dehydronase, *phoD* and phytase. Bacterial taxonomic contributions to predicted functional genes are presented using only bacterial families that contributed >20% abundance per sample to respective predicted functional genes. Weighted nearest sequence taxon index (NSTI) values were calculated as a means of determining confidence in the metagenome prediction in this study. Figures are presented with treatments (+N, eCO_2 or eCO_2+N) as per cent change from the control (ambient CO_2, unfertilized), and calculated as $(treatment_{mean} - control_{mean}/control_{mean})*100$, though statistical analyses were performed on raw data. See Douglas et al. (2020) for a detailed explanation of PICRUSt2.

2.6 | Plant biomass allocation

Above-ground and below-ground plant biomass, and per cent tissue N and C data are sampled annually in all BioCON plots (e.g. Reich et al., 2018; Reich & Hobbie, 2013). To minimize biomass anomalies across years and to match our sampling in 2013, we calculated the

mean values of each plot used in this study ($n = 132$) for the years 2012–2014. Per cent change from control plots (ambient CO_2, unfertilized) was calculated for plant responses to treatments as ($treatment_{mean} - control_{mean}/control_{mean}$)*100 and presented in figures for ease of interpretation, though statistical analyses were performed on raw data.

2.7 | Statistical analyses

Analyses were performed across plant species richness levels (R16, R9, R1), and within R16 and R1 plots to determine the role of diverse plant communities and the effects of plant functional groups; respectively. Plant biomass, nutrient concentration, soil net N mineralization, NRI values and predicted gene abundances were all tested using ANOVA and Tukey's HSD post hoc (*aov* and *TukeyHSD* in R) to determine main treatment effects of eCO_2, +N or eCO_2+N relative to control. Linear models were used to determine relationships between predicted relative abundances of NO_3-reducing genes and P-solubilizing genes. Estimated marginal means were calculated to determine pairwise effects of +N, eCO_2 or eCO_2+N on slopes of linear regressions with *emmeans* in R (Lenth et al., 2017). A *t*-test was performed on NRI values to determine statistical difference from zero. Data were log-transformed to meet normality assumptions. Rhizobacterial community alpha diversities were compared across treatments with Wilcoxon rank-sum test, and *t*-test for between treatment comparisons. Differences in rhizobacterial β-diversity were assessed by PERMANOVA (Anderson, 2001) using weighted and unweighted UniFrac (Lozupone & Knight, 2005), a β-diversity metric that accounts for phylogenetic distance between communities, and visualized using principal coordinate analysis. PERMANOVA was initially performed across all species richness levels ($n = 132$), to determine species richness and resource enrichment treatment (+N and eCO_2) effects despite variation in plant functional group. Permuted ($n = 999$) pairwise group dispersion centroids were calculated in Jupyter Notebook on weighted and unweighted UniFrac dissimilarities from R16 plots to obtain confidence intervals; ANOVA was then performed on these values to confirm PERMANOVA results. Within R1 plots, a pairwise PERMANOVA function was used to determine rhizobacterial dissimilarities between and within functional groups under enrichment treatments (https://github.com/pmartinezarbizu/pairwiseAdonis). Additionally, we performed PERMANOVA on unweighted UniFrac dissimilarity against observed plant species richness (mean from 2012 to 2014) to determine whether planted and observed richness affected rhizobacterial community composition in a similar manner. Measures of rhizobacterial diversity under R9 plots did not significantly differ from those in the R16 plots, and therefore, broader comparisons regarding plant species richness were drawn between the diverse R16 and R1 monoculture plots. All taxa annotated from sequencing were used for analyses of rhizobacterial communities, phylogenetic dispersion or predicted functions.

3 | RESULTS

3.1 | Experiment-wide rhizobacterial community responses

Across the study, the strongest effects on rhizobacterial communities came from plant species richness and the addition of N. The main effects of plant species richness and +N significantly influenced unweighted UniFrac rhizobacterial β-diversity (pseudo-$F_{2,130} = 5.45$, $p < 0.001$; pseudo-$F_{1,131} = 2.8$, $p < 0.005$, respectively), while we observed no main effect from 15 years of eCO_2 on rhizobacterial diversity (Figure S2). As predicted in H_1, rhizobacterial β-diversity under R16 plots was significantly different than R1 plots (Figure 2; pseudo-$F_{2,94} = 2.62$, $p < 0.005$). Despite differences in planted richness and observed richness at the time of sampling (see Table S1), observed plant richness revealed the same significant pattern found for planted species richness, where community dissimilarity decreased as species diversity increases (Figure S3; pseudo-$F_{1,131} = 3.4$, $p < 0.001$). The main effect of planted species richness in shaping rhizobacterial betadiversity after 15 years (Figure 2) suggests a strong selective force in temperate grassland soils, and by focusing on original planted richness we highlight the long-term influence of diverse or monoculture root systems on belowground rhizobacteria. Across the study, both R16 and R9 plots had more phylogenetically clustered rhizobacterial communities than R1 plots (Figure S4; $F_{2,130} = 14.01$, $p < 0.001$), while rhizobacterial NRI under R1 plots was not significantly different than zero, suggesting a random phylogenetic dispersion of rhizobacterial taxa.

3.2 | Plant richness-dependent responses of rhizobacterial communities

Under the diverse R16 plots, +N and the interaction eCO_2+N had the strongest effects on rhizobacteria. +N significantly altered rhizobacterial community composition (pseudo-$F_{3,31} = 2.3$, $p = 0.05$), while eCO_2 had no significant effect on β-diversity (Figure S5). Rhizobacterial community dissimilarity (unweighted UniFrac) was significantly different between the control and +N treatments (Figure S5; pseudo-$F_{1,108} = 7.03$, $p < 0.01$) and also between +N and eCO_2+N treatments (Figure S5B; pseudo-$F_{1,108} = 5.08$, $p < 0.05$). Abundance-weighted UniFrac dissimilarity was significantly affected by the eCO_2+N treatment (Figure S6; pseudo-$F_{1,108} = 11.655$, $p < 0.001$) suggesting strong interaction effects for resource enrichments on rhizobacterial community organization under more diverse plant communities. We observed a strong effect of N on rhizobacterial phylogenetic dispersion in R16 plots, where +N and the interaction of eCO_2+N both increased phylogenetic clustering of rhizobacterial taxa (Figure 3b; $F_{3,28} = 2.41$, $p = 0.02$; $F_{3,28} = 2.74$, $p = 0.01$; respectively).

Similar to the more diverse R16 plots, +N significantly altered rhizobacterial composition in R9 plots (pseudo-$F_{2,38} = 4.25$, $p = 0.001$). Relative to controls, eCO_2 and eCO_2+N had no significant effects on rhizobacterial β-diversity at the R9 level, and no treatments affected

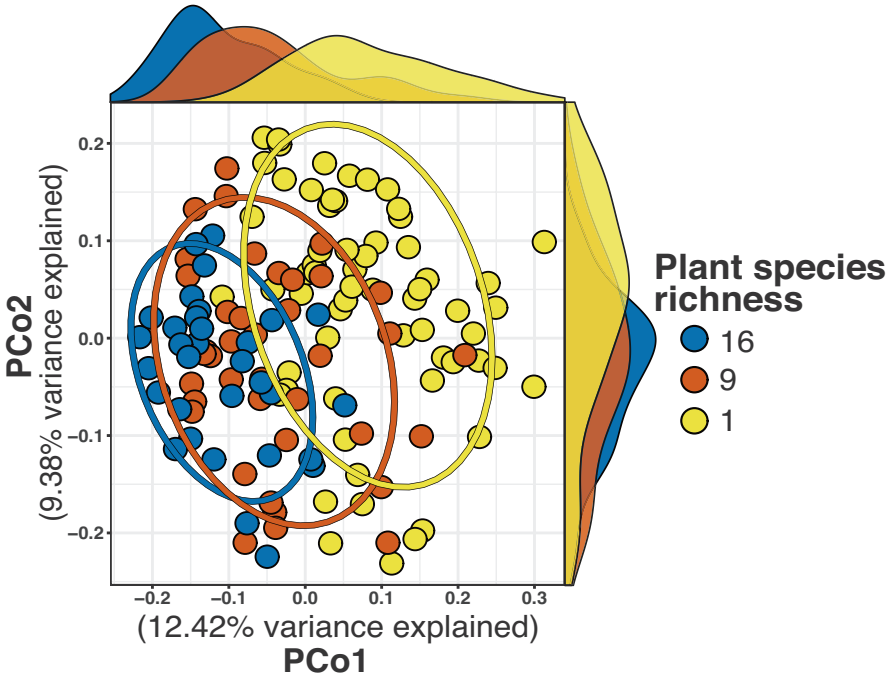

FIGURE 2 Principal coordinate analysis of rhizobacterial unweighted UniFrac community dissimilarity under three plant species richness levels, R16, R9 and R1 ($n = 132$). Ellipses represent 95% confidence areas of plant species richness level, with value densities on respective axes.

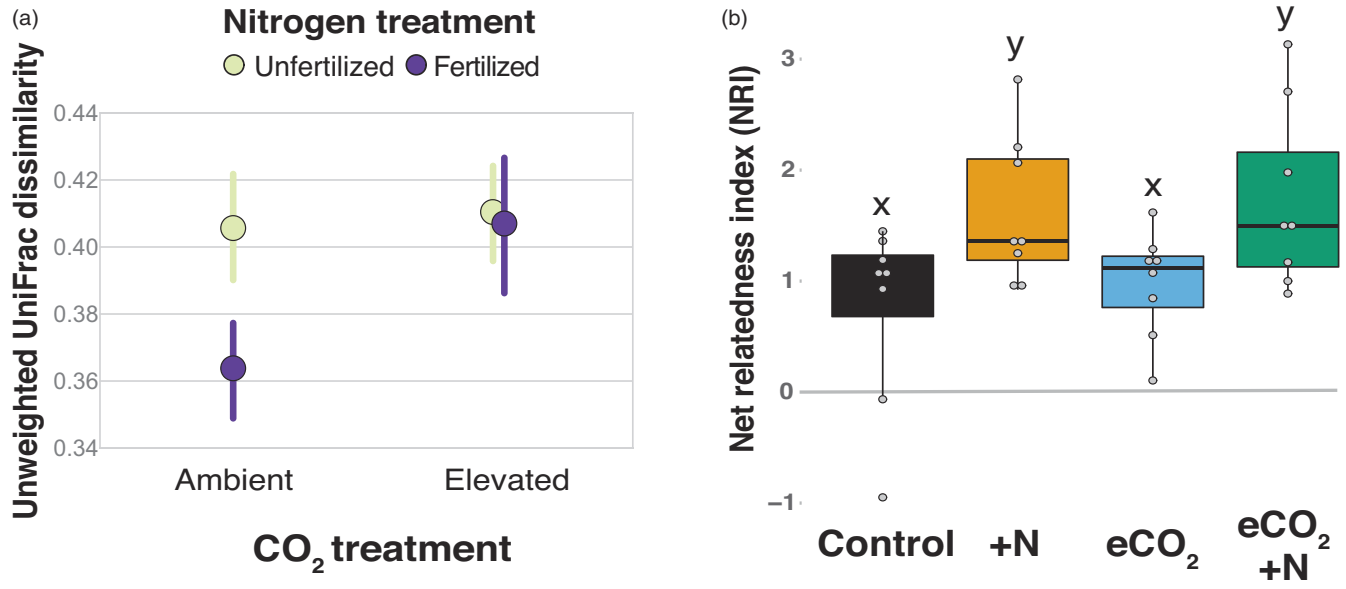

FIGURE 3 Rhizobacterial community responses from R16 plots ($n = 32$). (a) Mean pairwise boot-strapped ($n = 999$) unweighted UniFrac distances (points) with 95% confidence intervals (lines) under ambient or elevated CO_2 and coloured by nitrogen fertilization treatment. (b) Phylogenetic dispersion of rhizobacterial communities (Net Relatedness Index) under control (ambient CO_2 and unfertilized), nitrogen fertilized (ambient CO_2+N), elevated atmospheric CO_2 (unfertilized+eCO_2), or eCO_2+N. Grey points represent raw data. Letters indicate significant differences between treatments ($p \leq 0.05$).

rhizobacterial alpha diversity, using either nonparametric phylogenetic distance (PD; Faith & Baker, 2006) or observed 'species' metrics.

3.3 | Plant functional group strongly influenced rhizobacterial community responses

Across the R1 monoculture plots, the strongest effects on rhizobacterial community composition were observed for plant functional group (Figure 4) and the eCO_2+N treatment (pseudo-$F_{3,61} = 3.27$, $p < 0.001$; pseudo-$F_{3,61} = 1.99$, $p < 0.001$, respectively). We also observed a marginally significant interaction between plant functional group and eCO_2+N (pseudo-$F_{3,61} = 1.14$, $p = 0.061$), suggesting some important differences the responses to resource enrichments for plant functional groups. Between functional group comparisons are presented in Table 1, but briefly, C_3 and C_4 grass rhizobacterial communities were significantly, albeit weakly, different from each other, while rhizobacteria associated with C_3 and C_4 grass were significantly different from

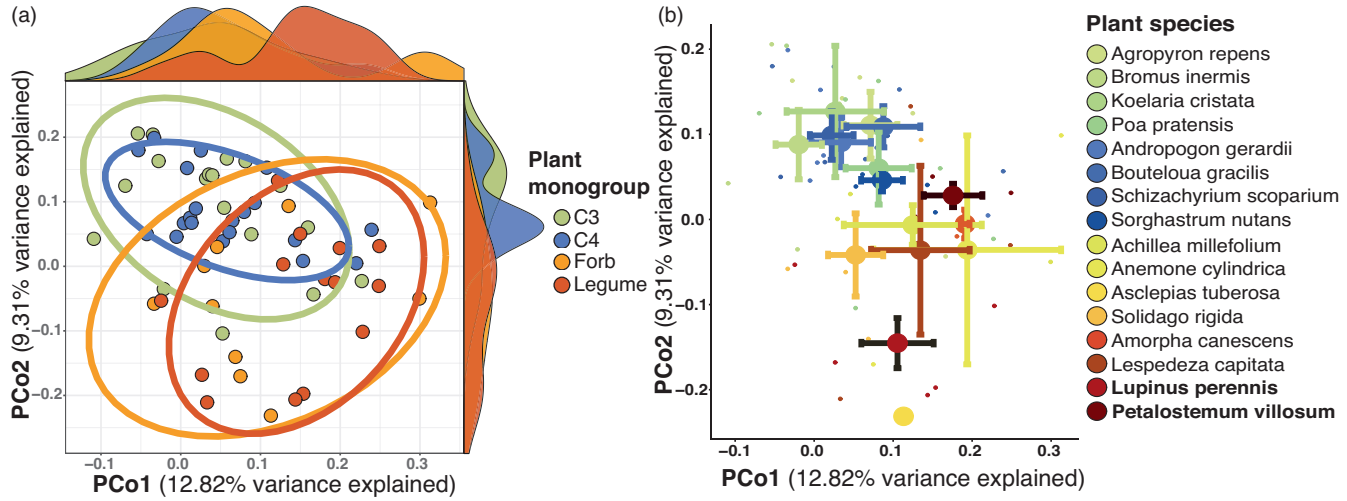

FIGURE 4 Principal coordinate analysis of rhizobacterial betadiversity under different plant functional groups and plant species from monoculture plots ($n=64$). (a) Unweighted UniFrac community dissimilarity coloured by the four plant functional groups: C_3 grasses are green, C_4 grasses are blue, forbs are yellow, and legumes are red. Ellipses represent 95% confidence areas around respective plant functional group. Densities of principal coordinates are presented along respective *x*- and *y*-axes. (b) Unweighted UniFrac community dissimilarity for all plant species. Large points are mean dispersion centroids with standard error bars for each plant species, while small points represent individual plants. Plant species are coloured along a gradient within plant functional group, similar to those in 4A. Plant species in bold correspond to centroids with black error bars and indicate significantly dissimilar rhizobacterial communities.

TABLE 1 Significant results from between and within plant functional group effects on rhizobacterial community dissimilarity (unweighted UniFrac) from monoculture plots calculated using pairwise PERMANOVA.

	Pseudo-*F*	r^2	*p*-value
Between functional group			
C_3 versus C_4	2.07	0.05	0.018
C_3 versus Forb	3.43	0.13	0.001
C_4 versus Forb	4.17	0.14	0.001
C_3 versus Legume	3.30	0.10	0.001
C_4 versus Legume	3.89	0.11	0.001
Within functional group			
C3			
Control versus +N	1.77	0.16	0.032
Control versus eCO_2+N	1.53	0.14	0.051
C4			
Control versus +N	1.61	0.17	0.032
Control versus eCO_2+N	1.52	0.23	0.025
eCO_2 versus +N	1.65	0.14	0.008
eCO_2 versus eCO_2+N	1.52	0.17	0.022
Legume			
Control versus eCO_2	2.27	0.311	0.039

both forb or legume communities. Forb and legume rhizobacterial communities were not different from each other (Table 1, Figure 4a). Analysis of phylogenetic dispersion shows that clustering of rhizobacterial taxa under C_4 grasses with +N was greater than in control and eCO_2 plots (Figure S7; $F_{3,15}=5.1$, $p<0.05$; $F_{3,15}=5.1$, $p=0.04$; respectively). For legumes, phylogenetic clustering was greater in the eCO_2+N treatment than in +N (Figure S7; $F_{3,15}=3.6$, $p=0.046$).

Environmental change treatments differentially affected the rhizobacterial communities associated with particular plant functional groups, with grass rhizobacteria tending to respond most strongly. We observed significant effects of +N and eCO_2+N on rhizobacterial communities associated with C_3 and C_4 grasses, and a significant effect of eCO_2 on rhizobacterial communities for legumes (Table 1; Figure 5). Plant functional group also significantly altered rhizobacterial alpha diversity, where we observed both lower PD and fewer observed bacterial taxa under forbs and legumes than C_3 and C_4 grasses (Forb-C_3: $t=-3.74$, $p=0.006$; Forb-C_4: $t=-5.03$, $p<0.005$; Legume-C_3: $t=-3.38$, $p<0.05$; Legume-C_4: $t=-4.48$, $p=0.006$). Across all plant species in monoculture, we observed distinct rhizobacterial communities for two plant species, *Lupinus perennis* and *Petalostemum villosum* (Figure 4b; pseudo-$F_{1,63}=2.11$, $p<0.05$; pseudo-$F_{1,63}=1.7$, $p=0.05$, respectively).

3.4 | Plant richness-dependent responses of rhizobacterial functional genes to environmental change factors

Across the study, few predicted gene abundances were significantly affected by resource enrichment treatments in this study, but as predicted, +N and eCO_2+N had particularly strong effects. Given the importance of plant species richness to rhizobacterial diversity, analyses of predicted gene abundances were performed in the context of the diverse R16 plots and the within the R1 monoculture plots. In the R16 plots, +N significantly increased predicted gene abundance of nitrogenase encoding *nifH* gene (N_2-fixation) by 40% (Figure 6a; $F_{3,26}=3.095$,

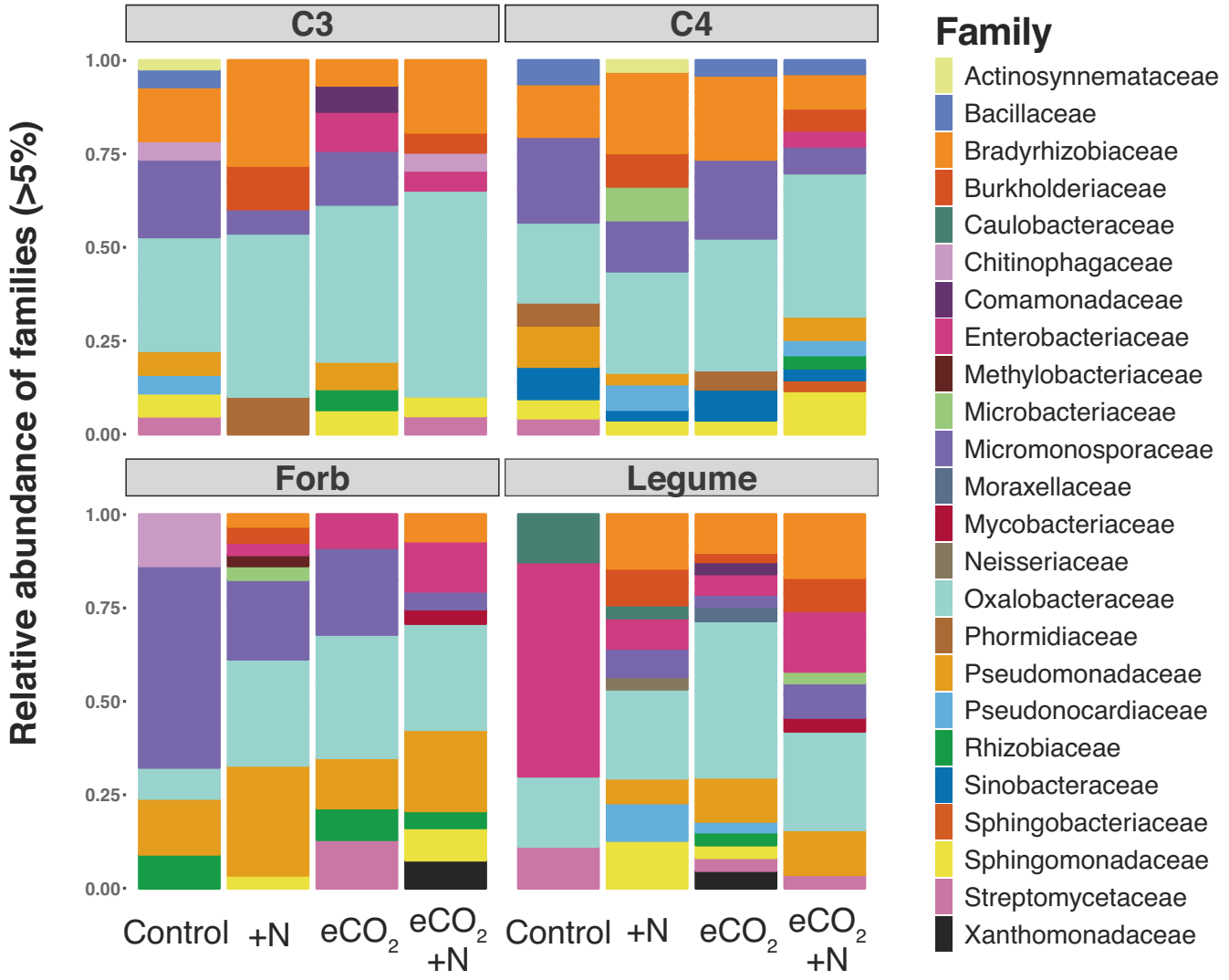

FIGURE 5 Rhizobacterial community composition of plant functional groups from monoculture plots ($n=64$) under control or resource-enriched conditions. Relative abundance of bacterial families, facetted by four plant functional groups (C_3, C_4, forb, legume). Here, we present only those taxa that contributed greater than 5% relative abundance to each sample. Bars represent rhizobacterial communities under control, +N, eCO_2, and eCO_2+N treatments.

$p<0.05$), and although eCO_2+N increased *nifH* abundance by 29%, this effect was not significant. The predicted gene abundance of the nitrite-reductase encoding *nirK* gene increased by 32% under +N (Figure 6b; $F_{3,26}=2.41$, $p=0.05$). eCO_2+N significantly increased total P-solubilizing gene abundance by 10% (Figure 6c; $F_{3,26}=3.06$, $p<0.05$). Across the R16 plots, we also identified a significant, positive correlation between the predicted abundance of total P-solubilizing genes and total NO_{3-} reducing genes (Figure 6f; $F_{1,28}=13.84$, adj-$r^2=0.31$, $p<0.001$). Within this relationship, we observed a significant interaction effect of eCO_2 (Figure 6f; $F_{2,27}=16.77$, adj-$r^2=0.52$, $p=0.002$), and determined that the slope of the relationship between P-solubilizing genes and total NO_{3-} reducing genes under eCO_2 decreased significantly when compared to ambient CO_2 conditions (Figure 6f; $t=-3.42$, $p<0.005$).

Predicted abundance of genes associated with N and P cycling responded variably to resource enrichments within plant functional groups, and we observed the strongest rhizobacterial functional responses under the C_3 and C_4 grasses (Figure 7). The nitrogenase encoding gene, *nifH*, increased by approximately 80% under eCO_2 for C_3 grasses (Figure 7a; $F_{3,14}=3.38$, $p=0.049$), while NO_{3-}-reducing genes increased by ~140% for C_3 grasses and ~70% for C_4 grasses under eCO_2+N (Figure 7b; $F_{3,14}=4.57$, $p=0.01$; $F_{3,17}=3.95$, $p=0.04$, respectively). For C_4 grasses, P-solubilizing genes increased by approximately 100% under eCO_2+N (Figure 7c; $F_{3,17}=3.0$, $p=0.049$). Interestingly for legumes, eCO_2+N decreased both NO_{3-}-reducing and P-solubilizing genes by ~50% (Figure 7b,c; $F_{3,10}=3.57$, $p=0.05$, $F_{3,10}=3.1$, $p=0.05$; respectively), suggesting a potentially decreased reliance on bacterial nutrient cycling functions when both C and N limitations are alleviated.

3.5 | Plant optimal resource allocation responses were dependent on plant richness, but inconsistent

Patterns emerged that occasionally aligned with optimal allocation predictions for plant productivity and biomass allocation

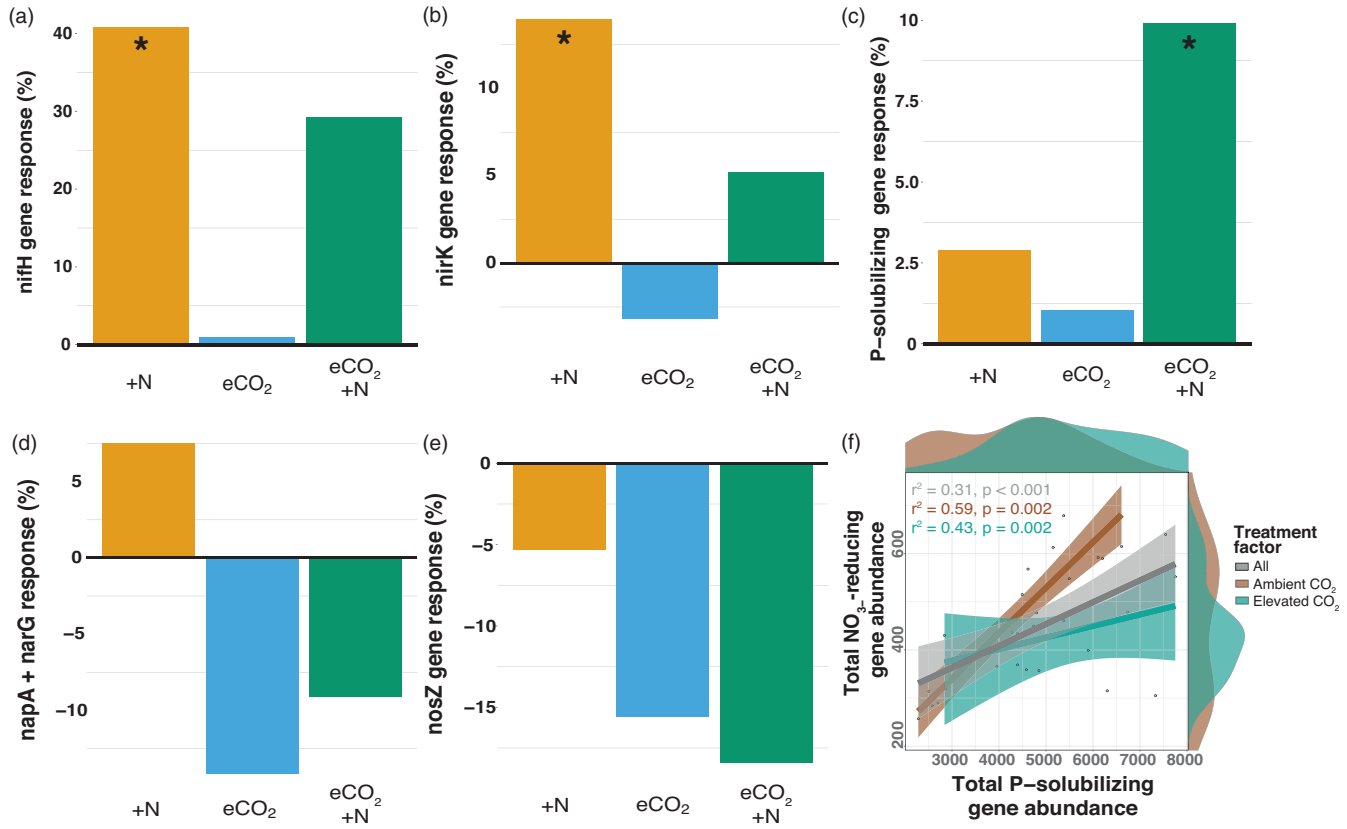

FIGURE 6 (a–e) Percentage change of mean predicted rhizobacterial gene abundance from control (zero bar) under nitrogen fertilization (+N), elevated atmospheric CO_2 (eCO_2), or eCO_2+N from diverse R16 plots ($n=32$). (a) Responses of predicted *nifH* gene abundance, (b) nitrite reductase-coding gene *nirK*, (c) total P-solubilizing genes (*pho*+acid phosphatase+phytase+glucose dehydronase), (d) the nitrate reductase-coding genes (*napA*+*narG*), and (e) nitric oxide reductase-coding gene *nosZ*. (f) Linear regression of total P-solubilizing genes on total nitrate (NO_{3-}) reducing genes, with value densities on respective axes and coloured as aggregated ambient CO_2 and aggregated elevated CO_2, with no treatment factor considered in grey ('All'). 95% confidence intervals are shaded areas around regression line, and correlation statistics for each treatment are in the top left of the panel. In (a–e), *significant differences from control with ANOVA ($p \leq 0.05$).

shifts in the diverse R16 plots. For example, as predicted, eCO_2 marginally increased total plant biomass by 21%, and the combination of eCO_2+N increased total plant biomass by 31% (Figure S9A; $F_{3,32}=2.67$, $p<0.05$). Contrary to optimal allocation predictions, eCO_2+N only marginally increased plant root: shoot biomass ratio by 5% (Figure S9B; $p=0.07$). Aboveground percent N increased by ~22% under +N, but this was not significant ($p=0.08$). Plant C:N ratio decreased under all resource enrichment treatments, but these changes were also not significant (Figure S9D).

In the R1 monoculture plots, the expected optimal allocation predictions of plant productivity, biomass allocation, %N, or plant C:N ratio were not consistently observed within plant functional groups. For example, pairwise comparisons within C_3 grasses show, as expected, that total plant biomass under eCO_2+N was marginally higher than controls (Figure S10A; $F_{3,14}=2.65$, $p=0.069$, Tukey adj-$p=0.061$); but, contrary to predictions, C_3 grass root: shoot ratio was lower under eCO_2 than all other treatments (Figure S10B; see Table S2), and aboveground plant %N was 45% higher under eCO_2 than in control plots (Figure S10; $F_{3,14}=4.9$, $p=0.015$, Tukey adj-$p=0.049$). Based on optimal allocation predictions, we would have expected consistent eCO_2 and eCO_2+N increases in biomass in C_3 grasses where any alleviation of C limitation would have drastically increased productivity, above- and below-ground, but instead we only observed increased C_3 plant biomass after eCO_2+N (Figure S10A). For C_4 grasses, total plant biomass was marginally higher under eCO_2+N than other treatments (Figure S8); with no observed changes in root: shoot ratios, aboveground %N, or tissue C:N ratio. Forb total biomass increased by 33% under eCO_2+N, but this was not significant. Legume total biomass increased by approximately 60% under all enrichment treatments, but not significantly, and legume root: shoot ratio was marginally higher than controls under +N and eCO_2+N treatments (Table S2; Figure S10B; $F_{3,10}=3.8$, model $p=0.047$, Tukey adj-$p=0.63$; $F_{3,10}=3.8$, model $p=0.047$, Tukey adj-$p=0.069$; respectively).

4 | DISCUSSION

Overall, our findings from this long-term environmental change study highlight the importance of interpreting rhizobacterial responses to resource enrichment in the context of plant diversity.

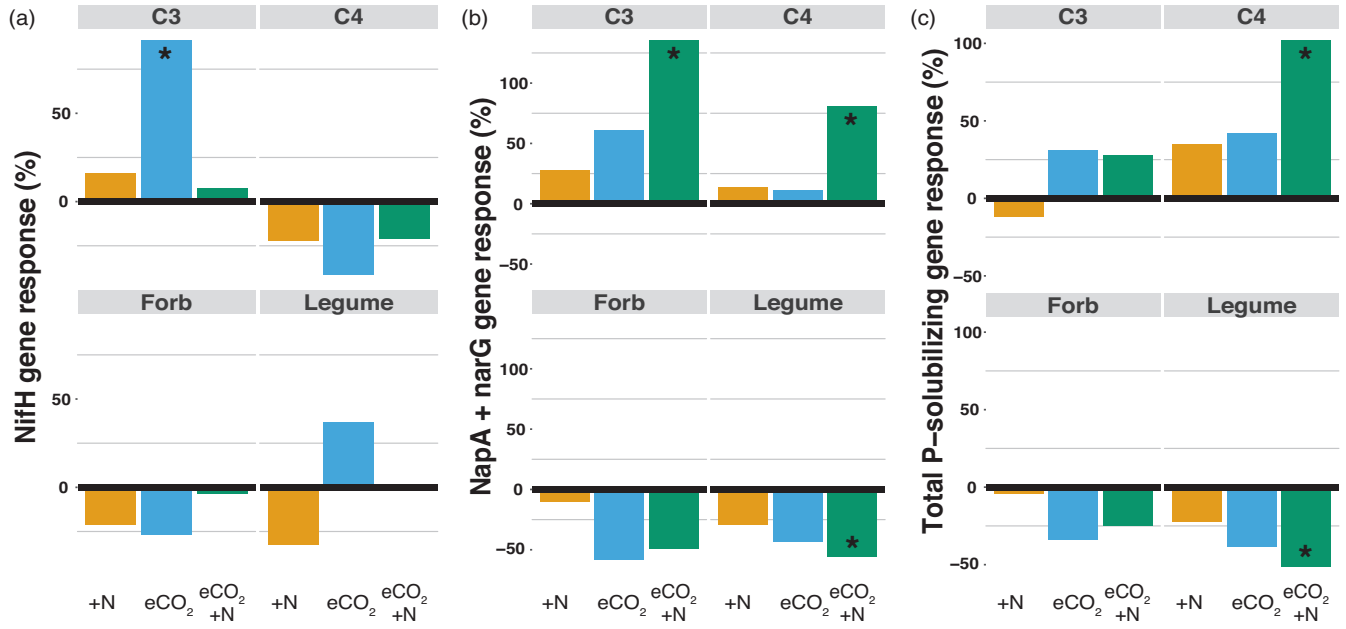

FIGURE 7 Rhizobacterial gene abundance response (percent change) of nitrogen and phosphorus cycling genes under nitrogen fertilization (+N), elevated atmospheric CO_2 (eCO_2), or eCO_2+N from R1 monoculture plots ($n = 64$), and facetted by plant functional group (C_3, C_4, forb, legume). Responses of (a) predicted *nifH* gene abundance, (b) nitrate reducing genes (*napA* + *narG*), and (c) total P-solubilizing genes (*pho* + acid phosphatase + phytase + glucose dehydronase). These data are presented as percent change relative to control treatment plots. *significant differences from control (zero line) with ANOVA ($p \leq 0.05$).

While we were unable to define the exact mechanisms influencing functional group-specific rhizobacterial responses to environmental change, our study is to our knowledge, the first to comprehensively describe the long-term interactive effects of plant diversity, elevated CO_2, and nitrogen deposition on rhizosphere bacteria. We show that plant species richness and functional group composition had a stronger influence on rhizobacterial community structure than 15 years of enriched N or atmospheric CO_2. The enrichment of N or the interaction of eCO_2+N consistently affected rhizobacterial communities and functional genes in our study, and we suspect this was largely due to the relative N-limitation at our study site, but also from changes in plant resource requirements leading to the selection of functionally unique microbial consortia. Together, our results lead us to prescribe future work that more finitely identifies the interactions between resource enrichment, plant C allocation and microbial selectivity by diverse plant assemblages or functional groups.

We were surprised by the general lack of eCO_2 effects across the study, particularly given the known effects of eCO_2 on plant productivity from studies across the world, including this experiment (Eisenhauer et al., 2012; Reich et al., 2004; Terrer et al., 2021). It has been shown that plant productivity responses to eCO_2 are constrained by the vegetation type, the N or P status in soils, and the mycorrhizal type of the dominant plants (Reich & Hobbie, 2013; Terrer et al., 2019), which in many ways aligns well with our findings. Mycorrhizal status was a major factor modulating plant responses to eCO_2 in a global analysis by Terrer et al. (2021), and this was particularly true in grasslands and for arbuscular mycorrhizal plants, which suggests that when grassland plants are N-limited, they must adopt strategies to alleviate growth constraints that involve their microbial partners in soil. Importantly though, soil microbes also deal with their own set of nutrient limitations, for example, microbial decomposition in a grassland under eCO_2 was significantly lower when constrained by N availability (Chiariello et al., 2002). Therefore, it appears that not all responses of the complex plant–soil system (holobiont) to environmental change related resource enrichments can be easily predicted. Our study and others indicate that it will be critical for researchers to consider plant composition (both above- and belowground), plant resource requirements in the local environment, and a finer-scale accounting of belowground C allocation by plants in order to identify the degree of control that plants and microbes exert over resource exchange in future environmental change research (Kivlin et al., 2022).

We showed that in diverse grasslands it may prove most useful to characterize plant-microbial interactions in the context of plant diversity and functional identity, particularly when experiencing long-term shifts in resource availability under environmental change. As predicted by H_1, the rhizobacterial communities from R16 plots were more homogenous than those in the R1 plots (Figure 2, Figure S3). This suggests a strong influence of the functional groups comprising R1 plots to filter for particular rhizobacterial communities, and indeed, across all plant richness levels, plant functional group was more important in structuring rhizobacterial communities than the environmental change treatments of +N and eCO_2. Plant functional groups are notably important factors in temperate grasslands (Adair et al., 2009; Isbell et al., 2013; Reich et al., 2004, 2018), and their productivity has

been previously shown to respond differentially when atmospheric CO_2 and soil N are enriched (Reich, Tilman, et al., 2001). As predicted by H_2, distinctly different bacterial communities were observed on grasses and forbs (Figure 4), with C_3 and C_4 grasses having similar rhizobacterial communities, while legume rhizobacterial communities were significantly different than those on grasses (Figure 4a; Table 1). Across all plant species, *Lupinis perennis* and *Petalostemum villosum*, exhibited statistically distinct rhizobacterial communities (Figure 4b), indicating a fine degree of rhizobacterial selectivity for these legumes. These results suggest the need for analyses of plant-microbial interaction that span functional and taxonomic levels to identify 'core' grassland microbial communities, and to specifically define microbial functions to best contextualize responses to changing environments.

Significant shifts in rhizobacterial taxa under different plant functional groups and environmental change treatments may indicate shifts in plant resource requirements and subsequent alterations in plant C allocation. In the legume group, there was a distinct and significant decline in the relative abundance of the Enterobacteriaceae under resource enrichments (Figure 4b). Enterobacter is the most abundant PGPR found in legumes, and many species from the Enterobacteriaceae are commonly associated with N_2-fixing nodule formation (Mishra et al., 2009). Further, in legumes under +N, eCO_2, and eCO_2+N, the relative abundance of Bradyrhizobiaceae, Burkholderiaceae, and Psuedomonadaceae increased significantly (Figure 4b). Each of these families have many putative free-living PGPR taxa that perform important N-cycling functions and are commonly found in PGPR inoculation studies (Lugtenberg & Kamilova, 2009; Rubin et al., 2017). While this observation is solely correlative, the distinct decline in the most abundant nodule-related bacterial taxa (Enterobaceriaceae) and increase in relative abundance of multiple bacterial families considered PGPR, including Rhizobiaceae in eCO_2 plots, could suggest a shift in N acquisition strategy for legumes under +N or eCO_2; from symbiotic nodule formation to increased reliance on free-living PGPR as shown in previous work highlighting legume control over nodule development and N nutrition (Ferguson et al., 2019; Liese et al., 2017). As suggested in Bulgarelli et al. (2013), it will be important to identify core sets of physiological traits of PGPR with whole genome information to best understand the direct and indirect effects of their recruitment, colonization, and growth-promotion.

Our analysis of root-associated soil bacteria is the first to truly utilize the complex and long-term design testing the relative importance of plant biodiversity, eCO_2, and +N at BioCON, as previous studies analysing microbial communities and/or functions here have solely focused on the main effect of eCO_2. A previous study found that eCO_2 significantly increased Rhizobiaceae abundance in diverse R16 plots (He et al., 2012), which is consistent with our results (Figure S5B). Tu et al. (2017) found that under diverse R16 plots, eCO_2 increased *nifH* gene abundance in bulk soil, but this does not agree with our results for rhizobacterial functions. We did find an ~80% increase in *nifH* abundance under C_3 grass plots with eCO_2 (Figure 7a), which could explain a significant increase of *nifH* from bulk soil (i.e. Tu et al., 2017), as C_3 grass roots account for ~40% of total root biomass in R16 plots. Along these lines, we feel that grass dominance in the root system, differentiation between C_3 and C_4 type, and a focus on their specific resource-driven selection for microbial taxa and functions could have important implications for interpretation of microbiome data in natural systems with high species diversity (Reich et al., 2018). Incongruities between our study and others may be attributed to different methodological approaches, sampling approaches (roots vs. soil), or changes over time in response to treatments (the studies above were conducted ~5 years earlier). Despite this, we expect our findings will be important in driving hypotheses and future testing regarding grassland soil microbial functional responses to environmental change.

Despite previous research showing strong agreement with predictions of the optimal allocation model across multiple ecosystem types and functional groups (Allen et al., 2020; Friel & Friesen, 2019; Johnson et al., 2015), the allocation of plant biomass, and composition of bacterial communities and their associated predicted functional genes did not consistently respond to eCO_2 and +N in such a way to suggest that acquisition of the most limiting resources per plant functional group were optimized. We found that under eCO_2+N, C_4 grasses tended to decrease root biomass allocation and had significantly higher P-solubilizing and nitrate reducing gene abundance (Figure 6), supporting the optimal allocation expectation that increases in rhizobacterial functions could be adopted to alleviate P requirements when N is no longer limiting and productivity is constrained by P. But, as the majority of our results were neutral or counter to predictions of optimal resource allocation for the extended plant phenotype, we believe that root biomass allocation, our proxy for C allocation belowground, was largely an insufficient measure. Venturi and Keel (2016) suggest that different forms and types of rhizodeposition, including root exudates, can have a significant influence on the formation of rhizobacterial community composition and function. And recently, Kong and Fridley (2019) found that carbon allocation to belowground exudate pools and fluxes that are not accounted for by root biomass can be substantial. Microbial-mediated plant resource acquisition strategies are clearly complex and we know that opportunistic, free-living taxa make up the majority of rhizobacteria (Noë & Kiers, 2018; Tedersoo et al., 2020), and so it will likely be best to study this system in the context of other more obligate microbial symbionts, such as mycorrhizal fungi (Terrer et al., 2021).

5 | CONCLUSIONS

We propose that rooting the interpretation of community or functional responses to environmental change in the context of plant diversity could be critical for future studies (Fitzpatrick et al., 2018; Revillini et al., 2019), and hope that our results can serve to generate hypotheses beyond descriptive analysis of bacterial structure and function to more ecologically relevant fields and timescales. For

example, what is the influence of dominant plant taxa on microbial community composition and function across multiple systems or global change factors? Fitzpatrick et al. (2017) showed that distinct assemblages of rhizobacteria can influence plant–soil feedback under drought conditions, again suggesting the importance of specificity. Might plant taxa have such a great influence on their rhizobacterial communities that recruitment of beneficial PGPR by one species can lead to the recruitment of more diverse plant communities, similar to the findings of Wubs et al. (2016)? A better understanding of plant functional group–specific responses of rhizobacterial communities can also help inform future predictive models that scale-up and address multifactor environmental changes across grasslands globally (e.g. Guerra et al., 2021). As global changes will continue to alter the soil environment in the Anthropocene, this study provides a framework to understand how responses to elevated atmospheric CO_2 and N deposition are locally constrained by soil nutrient status and plant community members, and may be best represented by an extended plant phenotype approach, which includes the soil microbiome (Kristin & Miranda, 2013; Vandenkoornhuyse et al., 2015).

AUTHOR CONTRIBUTIONS

Peter B. Reich conceived, designed and implemented the BioCON experiment and was responsible for acquisition of plant and soil chemical data. Daniel Revillini and Nancy Collins Johnson conceived of the rhizobacterial research focussed on in this study. Daniel Revillini carried out fieldwork, laboratory analysis, and statistical analyses. Daniel Revillini, Peter B. Reich and Nancy Collins Johnson contributed equally to manuscript preparation.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/1365-2745.14271.

DATA AVAILABILITY STATEMENT

The raw sequence data for this study are available under BioProject accession PRJNA549245 and SRA accession SRR9312786 in the NCBI database. All data used for statistical analyses and figures were uploaded to the FigShare database: https://doi.org/10.6084/m9.figshare.23921556 (Revillini, 2023).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. Schematic diagram of BioCON experimental FACE rings used in this study (eCO_2 = #3, #5; ambient CO_2 = #2, #4), indicating nitrogen fertilization, and plant richness treatments.

Figure S2. Principal coordinate analyses of unweighted UniFrac betadiversity of rhizobacterial communities from all soil samples in this study (n = 132).

Figure S3. Principal coordinate analyses of unweighted UniFrac betadiversity of rhizobacterial communities from all soil samples in this study (n = 132), colored by mean observed plant species richness from all plots in this study sampled from 2012 to 2014.

Figure S4. Net relatedness index of rhizobacterial communities under plant species richness levels of R16, R9, or R1.

Figure S5. Relative abundance of bacterial phyla (A) and families (B) from R16 plots. Presented are only those taxa that contributed more than (A) 2% or (B) 5% relative abundance to each sample.

Figure S6. (A) Principal coordinate analysis of rhizobacterial weighted UniFrac community dissimilarity under ambient CO_2 (left panel) and elevated CO_2 (right panel), and colored by nitrogen fertilization treatment.

Figure S7. Net relatedness index of rhizobacterial communities under control, nitrogen fertilized (+N), elevated atmospheric CO_2 (eCO_2), or eCO_2+N from monoculture plots, facetted by plant functional group.

Figure S8. Relative contribution of rhizobacterial orders (A) and families (B) to predicted N-cycling functional genes from 16 plant species plots: nifH, napA, narG, nirK, and nosZ.

Figure S9. Plant responses as percent change from control (zero line) under nitrogen fertilization (+N), elevated atmospheric CO_2 (eCO_2), or the combination of +N and eCO_2 from 16 plant species plots averaged from years 2012–2014.

Figure S10. Plant growth and %N responses (percent change) under +N, eCO_2, or +N + eCO_2 from monoculture plots (n = 64), facetted by plant functional groups (C_3, C_4, forb, legume).

Table S1. Planted and mean observed richness values for all plots used in this study across the years 2012–2014.

Table S2. Results of pairwise ANOVA of plant growth and nutrient responses under plant functional groups (C_3, C_4, forb and legume).

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