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Elevated CO₂ and nitrogen addition have minimal influence on the rhizospheric effects of *Bothriochloa ischaemum*

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The influence of elevated CO₂ and nitrogen (N) addition on soil microbial communities and the rhizospheric effects of *Bothriochloa ischaemum* were investigated. A pot-cultivation experiment was conducted in climate-controlled chambers under two levels of CO₂ (400 and 800 μmol mol⁻¹) and three levels of N addition (0, 2.5, and 5 g N m⁻² y⁻¹). Soil samples (rhizospheric and bulk soil) were collected for the assessment of soil organic carbon (SOC), total N (TN), total phosphorus (TP), basal respiration (BR), and phospholipid fatty acids (PLFAs) 106 days after treatments were conducted. Elevated CO₂ significantly increased total and fungal PLFAs in the rhizosphere when combined with N addition, and N addition significantly increased BR in the rhizosphere and total, bacterial, fungal, Gram-positive (G⁺), and Gram-negative (G⁻) PLFAs in both rhizospheric and bulk soil. BR and total, bacterial, G⁺, and G⁺/G⁻ PLFAs were significantly higher in rhizospheric than bulk soil, but neither elevated CO₂ nor N addition affected the positive rhizospheric effects on bacterial, G⁺, or G⁺/G⁻ PLFAs. N addition had a greater effect on soil microbial communities than elevated CO₂, and elevated CO₂ and N addition had minor contributions to the changes in the magnitude of the rhizospheric effects in *B. ischaemum*.

Changes in global climate such as elevated CO₂ concentrations, nitrogen (N) deposition, drought, and warming will have dramatic impacts on biological nutrient cycling in terrestrial ecosystems¹. Soil microbial communities, especially rhizospheric microorganisms, serve as bridges between plants and soil, are key drivers of the global bio-geochemical cycles of mineral elements². Much attention, however, has been given to characterize the responses of these communities to various scenarios of climate change^{3–5}, but the responses of the communities to elevated CO₂ and N deposition are poorly understood.

The individual effects of either elevated CO₂ or N addition on soil microbial communities have been widely studied^{6,7}. CO₂ concentrations are generally much higher in the pore spaces of soil (2000–3800 μmol mol⁻¹) than in the atmosphere, so elevated CO₂ usually influences the richness, composition, and structure of the communities indirectly, such as by increasing plant carbon (C) inputs to the soil and altering soil properties⁷. Phospholipid fatty acid (PLFA) analysis and denaturing gradient gel electrophoresis indicated that CO₂ enrichment had no detectable effects on the composition and structure of the community^{8–12}, but usually stimulated soil respiration^{11,13}. Analyses of functional genes indicated that elevated CO₂ decreased the expression of genes involved in the C and N cycles^{14,15}, but Liu *et al.*¹⁶ reported that elevated CO₂ increased the abundance of ammonia-oxidizing archaea and bacteria in a Chinese paddy field. These contradicting results might be due to the duration of CO₂ enrichment⁷, the soil¹⁵, or the vegetation type^{17,18}.

N deposition can increase the concentrations of NH₄⁺-N and NO₃⁻-N in soil and improve the amount and quality of substrates available to soil microorganisms and thereby increase soil microbial biomass and activity¹⁹. Zhou *et al.*²⁰ and Ma *et al.*²¹ reported that N additions increased the abundances of the microbial functional groups involved in the soil N cycle. N deposition, however, can also significantly decrease soil pH and thus dramatically decrease microbial biomass and the diversity of soil microbial communities^{22–24}. The effects of N

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Indicator	Rhizosphere			Bulk soil		
	C	N	C × N	C	N	C × N
SOC content	0.270	0.820	0.879	0.235	0.221	0.700
TN content	0.267	0.970	0.865	0.453	0.906	0.712
TP content	0.023	0.085	0.565	0.035	0.200	0.659
BR	0.740	< 0.001	0.204	0.441	0.340	0.769
SIR	0.561	0.785	0.504	0.131	0.533	0.920
AWCD	0.132	0.841	0.846	0.844	0.260	0.574
<i>H</i>	0.674	0.826	0.544	0.717	0.499	0.436
<i>D</i>	0.846	0.954	0.546	0.658	0.580	0.322
Total PLFA	0.004	< 0.001	0.154	0.622	< 0.001	0.932
Bacterial PLFA	0.087	< 0.001	0.900	0.889	< 0.001	0.878
Fungal PLFA	0.007	0.004	0.442	0.944	0.020	0.208
G ⁺ PLFA	0.401	0.010	0.103	0.362	0.002	0.172
G ⁻ PLFA	0.057	0.001	0.152	0.300	< 0.001	0.097
Fungal/bacterial PLFA	0.074	0.364	0.522	0.925	0.424	0.140
G ⁺ /G ⁻ PLFA	0.240	0.404	0.837	0.170	0.139	0.439

Table 1. *P* values for the effects of elevated CO₂ (C), N addition (N), and their interaction on chemical and microbial properties in the rhizosphere and bulk soil. Significant *P* values are highlighted in bold.

addition on these communities depended significantly on the amount added. Low or moderate N addition usually increases microbial biomass and diversity, but excessive N addition decreases them^{25,26}.

CO₂ concentrations and N deposition have both increased under global climate change²⁷. Elevated CO₂ combined with N deposition increases litter decomposition, improves the supply of C to the soil ecosystem, and lessens soil N limitation, thereby substantially increasing C and N cycling in soil ecosystems^{28,29}. Soil respiration can also increase significantly under combined elevated CO₂ and N addition³⁰, more than for either alone³¹. Haase *et al.*³² found that neither elevated CO₂ nor N supply affected the abundance of total and denitrifying bacteria in rhizospheric soil. Lee *et al.*²⁸ recently reported that elevated CO₂ and N addition affected bacterial and archaeal communities but not the fungal community. The effects of elevated CO₂ and N addition on microbial communities vary with plant type²⁸.

Processes in the rhizosphere mediate many important aspects of plant-soil interactions³³. The activity and diversity of soil microbial organisms are generally higher in rhizosphere than bulk soil³⁴. The release of low-molecular-weight organic compounds can change the biomass and composition of microbial communities, thus leading to further changes in rhizospheric microbial communities. Rhizodeposition is the main driver of rhizospheric effects³⁵, defined as the ratios of the values of a variable in the rhizosphere and bulk soil; a positive (negative) rhizospheric effect indicates that a variable was higher (lower) in the rhizosphere than the bulk soil. Factors that regulate rhizospheric nutrient fluxes may control the magnitude of rhizospheric effects^{4,36}. A comprehensive understanding of the responses of soil microbial communities to elevated CO₂ combined with N addition and exploring the changes of rhizospheric effects are important for understanding biogeochemical processes in terrestrial ecosystems in scenarios of global climate change.

Buthriochloa ischaemum (L.) Keng is a perennial herbaceous grass that is widespread in the hilly-gully regions of the Loess Plateau of China. It is characterized by quick reproduction and drought and trampling resistance and is a high-quality natural forage for livestock in this semiarid region. Its root system is well developed and forms a network that has a notable effect in preventing soil and water erosion. The soil of the plateau has low levels of total N³⁷. The current rate of N deposition in this area is 2.2 g N m⁻² y⁻¹^{38,39} and is expected to increase in the future⁴⁰. Elevated global levels of CO₂ and N deposition would synergistically lead to changes in the nutrient cycles in this temperate area. Elevated CO₂ and N addition can significantly increase the photosynthesis of *B. ischaemum* and mitigate the N deficiency on the plateau^{41,42}. The response of soil microbial communities, especially rhizospheric microorganisms, to combined elevated CO₂ and N addition, however, has rarely been studied. The purpose of the present study was to determine the response of soil respiration and microbial communities in the rhizosphere and bulk soil to an elevated CO₂ level in combination with N addition. We hypothesized that (1) N addition would have a larger impact on community composition than elevated CO₂ in the soil of this N-poor area, and (2) The effects of *B. ischaemum* rhizospheres on microbial-community variables should increase in the elevated-CO₂ and N-addition treatments due to increased rhizodeposition.

Results

Soil TOC, TN, and TP contents. A two-way ANOVA showed that elevated CO₂ did not have significant effect on SOC or TN content in either the rhizosphere or bulk soil, and N addition did not have significant effect on SOC, TN, or TP content (Table 1). Elevated CO₂ and N addition did not have interactive effect on SOC, TN, or TP content. Elevated CO₂ significantly affected TP content in both the rhizosphere and bulk soil (*P* < 0.05). TP content tended to decrease in response to elevated CO₂ (Table 2).

Microbial respiration. Elevated CO₂ did not have significant effect on basal respiration (BR) or substrate-induced respiration (SIR) in the rhizosphere or bulk soil (Table 1). N addition significantly increased

Treatment	SOC content		TN content		TP content	
	Rhizosphere	Bulk soil	Rhizosphere	Bulk soil	Rhizosphere	Bulk soil
AN0	1.51 ± 0.13	1.48 ± 0.07	0.18 ± 0.02	0.19 ± 0.03	0.56 ± 0.01b	0.57 ± 0.01b
AN1	1.47 ± 0.14	1.53 ± 0.07	0.18 ± 0.02	0.19 ± 0.03	0.56 ± 0.02ab	0.57 ± 0.00b
AN2	1.44 ± 0.17	1.55 ± 0.12	0.19 ± 0.01	0.20 ± 0.02	0.54 ± 0.02ab	0.57 ± 0.01ab
EN0	1.54 ± 0.11	1.54 ± 0.02	0.20 ± 0.03	0.20 ± 0.03	0.55 ± 0.01ab	0.57 ± 0.01ab
EN1	1.53 ± 0.06	1.54 ± 0.06	0.19 ± 0.02	0.20 ± 0.02	0.53 ± 0.03a	0.56 ± 0.01ab
EN2	1.53 ± 0.19	1.58 ± 0.01	0.19 ± 0.02	0.20 ± 0.02	0.53 ± 0.03a	0.56 ± 0.01a

Table 2. Soil organic carbon (SOC), total nitrogen (TN), and total phosphorus (TP) contents (g kg^{-1}) in the rhizosphere and bulk soil in the treatments. Different letters within a column indicate significant differences between treatments.

Indicator	C	N	C × N
BR	0.282	<0.001	0.175
SIR	0.339	0.601	0.875
Total PLFA	0.010	0.108	0.476
Bacterial PLFA	0.173	0.410	0.864
Fungal PLFA	0.057	0.365	0.685
G ⁺ PLFA	0.921	0.633	0.337
G ⁻ PLFA	0.051	0.186	0.120
Fungal/bacterial PLFA	0.140	0.557	0.497
G ⁺ /G ⁻ PLFA	0.118	0.134	0.851

Table 3. *P* values for the effects of elevated CO₂ (C), N addition (N), and their interaction on rhizospheric effects. Significant *P* values are highlighted in bold.

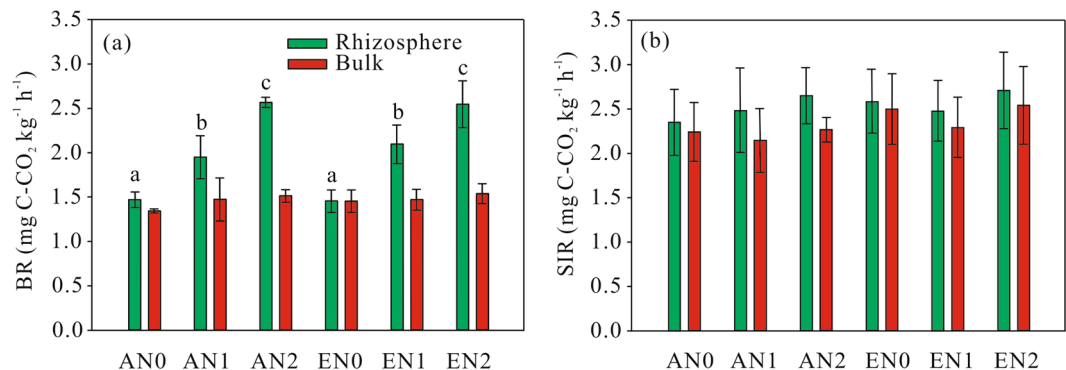


Figure 1. BR (a) and SIR (b) in the rhizosphere and bulk soil in the treatments. Different lowercase letters indicate significant differences between treatments in the rhizosphere. AN0, ambient CO₂ and no N added; AN1, ambient CO₂ and N supply at a rate of 2.5 g N m⁻² y⁻¹; AN2, ambient CO₂ and N supply at a rate of 5 g N m⁻² y⁻¹; EN0, elevated CO₂ and no N added; EN1, elevated CO₂ and N supply at a rate of 2.5 g N m⁻² y⁻¹; EN2, elevated CO₂ and N supply at a rate of 5 g N m⁻² y⁻¹.

BR in the rhizosphere ($P < 0.001$). BR for the rhizosphere was 74.7 and 101.2% higher in N2 than N0 at ambient and elevated CO₂, respectively (Fig. 1). BR was significantly higher in the rhizosphere than the bulk soil in all six treatments, and this positive rhizospheric effect was significantly affected by N addition ($P < 0.001$; Table 3, Fig. 2a). SIR was similar in the rhizosphere and bulk soil, indicating no rhizospheric effect (Figs 1b and 2b).

CLPP analysis. A two-way ANOVA indicated that neither elevated CO₂ nor N addition had significant effect on AWCD, *H*, or *D* (Table 1). Elevated CO₂ and N addition did not have interactive effect on the functional diversity of the soil microbial communities in either the rhizosphere or the bulk soil (Table 4).

PLFA analysis. A two-way ANOVA indicated that elevated CO₂ had significant effect on total and fungal PLFAs in the rhizosphere (Table 1). Compared with AN2, EN2 significantly increased total PLFA in the rhizospheric soil, and fungal PLFA was significantly higher in EN1 than AN1 in the rhizospheric soil (Fig. 3). N addition significantly increased total, bacterial, fungal, G⁺, and G⁻ PLFAs in both the rhizosphere and bulk soil (Table 1, Fig. 3). A principal component analysis of the PLFA data indicated that the first two components, PC1

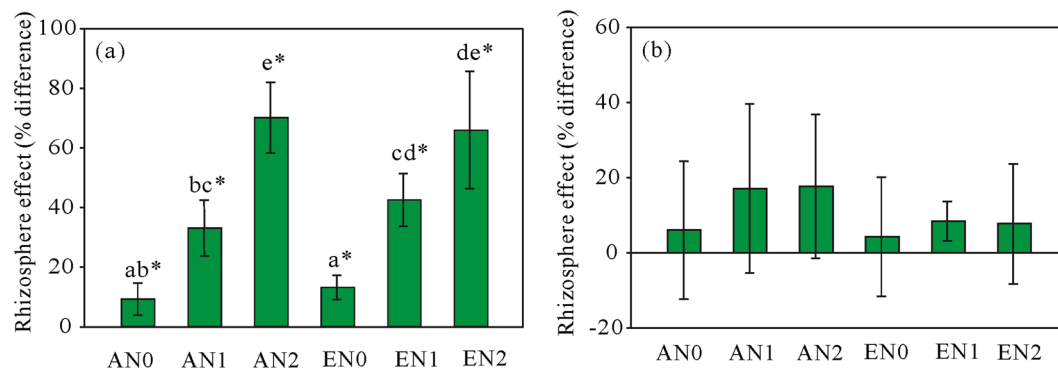


Figure 2. Rhizospheric effects for BR (a) and SIR (b) in the treatments. *Above each bar indicates significant difference between the rhizosphere and bulk soil. Different lowercase letters indicate significant differences in rhizospheric effects between treatments. AN0, ambient CO₂ and no N added; AN1, ambient CO₂ and N supply at a rate of 2.5 g N m⁻² y⁻¹; AN2, ambient CO₂ and N supply at a rate of 5 g N m⁻² y⁻¹; EN0, elevated CO₂ and no N added; EN1, elevated CO₂ and N supply at a rate of 2.5 g N m⁻² y⁻¹; EN2, elevated CO₂ and N supply at a rate of 5 g N m⁻² y⁻¹.

Treatment	AWCD		<i>H</i>		<i>D</i>	
	Rhizosphere	Bulk soil	Rhizosphere	Bulk soil	Rhizosphere	Bulk soil
AN0	0.24 ± 0.01	0.27 ± 0.02	2.76 ± 0.01	2.73 ± 0.02	0.93 ± 0.00	0.92 ± 0.00
AN1	0.23 ± 0.02	0.30 ± 0.02	2.74 ± 0.05	2.79 ± 0.03	0.93 ± 0.00	0.93 ± 0.00
AN2	0.24 ± 0.01	0.29 ± 0.01	2.79 ± 0.07	2.76 ± 0.05	0.93 ± 0.01	0.93 ± 0.00
EN0	0.26 ± 0.02	0.29 ± 0.01	2.75 ± 0.07	2.77 ± 0.04	0.93 ± 0.01	0.93 ± 0.00
EN1	0.25 ± 0.05	0.29 ± 0.02	2.81 ± 0.12	2.77 ± 0.08	0.93 ± 0.01	0.93 ± 0.01
EN2	0.27 ± 0.04	0.28 ± 0.02	2.78 ± 0.09	2.71 ± 0.10	0.93 ± 0.01	0.92 ± 0.01

Table 4. Average well color development (AWCD) and functional diversity (Shannon index (*H*), and Simpson index (*D*)) of the soil microbial communities in the treatments in the rhizosphere and bulk soil.

and PC2, accounted for 81.44 and 1.71% of the variance, respectively (Fig. 4). The PLFA patterns differed significantly between the rhizosphere and bulk soil along PC1. Elevated CO₂ and N addition did not have significant interactive effect on the composition of microbial PLFAs (Table 1).

Total, bacterial, G⁺, and G⁺/G⁻ PLFAs were significantly higher in the rhizosphere than bulk soil (Fig. 5). The positive rhizospheric effect (variables were higher in the rhizosphere than the bulk soil) for total PLFA was significantly increased only by elevated CO₂ (Table 3). The positive rhizospheric effects for bacterial, G⁺, and G⁺/G⁻ PLFAs were not affected by either elevated CO₂ or N addition.

Discussion

Characteristics of the soil microbial communities under elevated CO₂ and N addition. Soil respiration is an important part of the global C cycle and the largest component of C flux from terrestrial ecosystems to the atmosphere^{43,44}. Elevated CO₂ and N deposition can have profound impacts on soil respiration³⁰. A meta-analysis found that N addition significantly increased soil respiration by 7.84% in grasslands⁴⁵. N addition in our study significantly increased BR in the rhizosphere. This result was consistent with those by Luo *et al.*⁴⁶ and Zhang *et al.*⁴⁷, who found that N application significantly increased soil respiration, which they attributed to N-induced increases in plant growth, especially root biomass. We also found that N addition significantly increased root biomass (Table 5). Soil respiration is generally sensitive to elevated CO₂⁴⁸. Baronti *et al.*¹³ and Liu *et al.*⁴⁹ observed increased soil respiration under elevated CO₂. A meta-analysis by De Graaff *et al.*⁵⁰ found that soil respiration increased by 17.1% under elevated CO₂ and suggested that these increases could be due to microbial responses from changes in substrate availability. Elevated CO₂ in our study did not have significant impact on BR in either the rhizosphere or bulk soil, suggesting that 106 days elevated CO₂ might not significantly increase substrate availability to the community, although elevated CO₂ significantly increased root biomass (Table 5).

The response of the soil microbial community to elevated CO₂ and N deposition depends on many factors, such as plant species, soil temperature and water content, and especially nutrient availability^{23,51}. Previous studies have reported that elevated CO₂ or N addition increased, decreased, or had no significant impact on community structure^{7,15,16,26,28,52}. These contradictory results could be due to differences of microbial substrate availability under different scenarios of climate change. Elevated CO₂ usually indirectly affects microbial communities by altering root biomass and exudation. Elevated CO₂ for 106 days in our study significantly increased plant biomass, and elevated CO₂ and N addition had significant interactive effect on plant root biomass, so total and fungal PLFAs significantly increased in the rhizosphere in the treatments with both elevated CO₂ and N addition. N addition directly increased soil N content and may have indirectly improved nutrient availability by changing

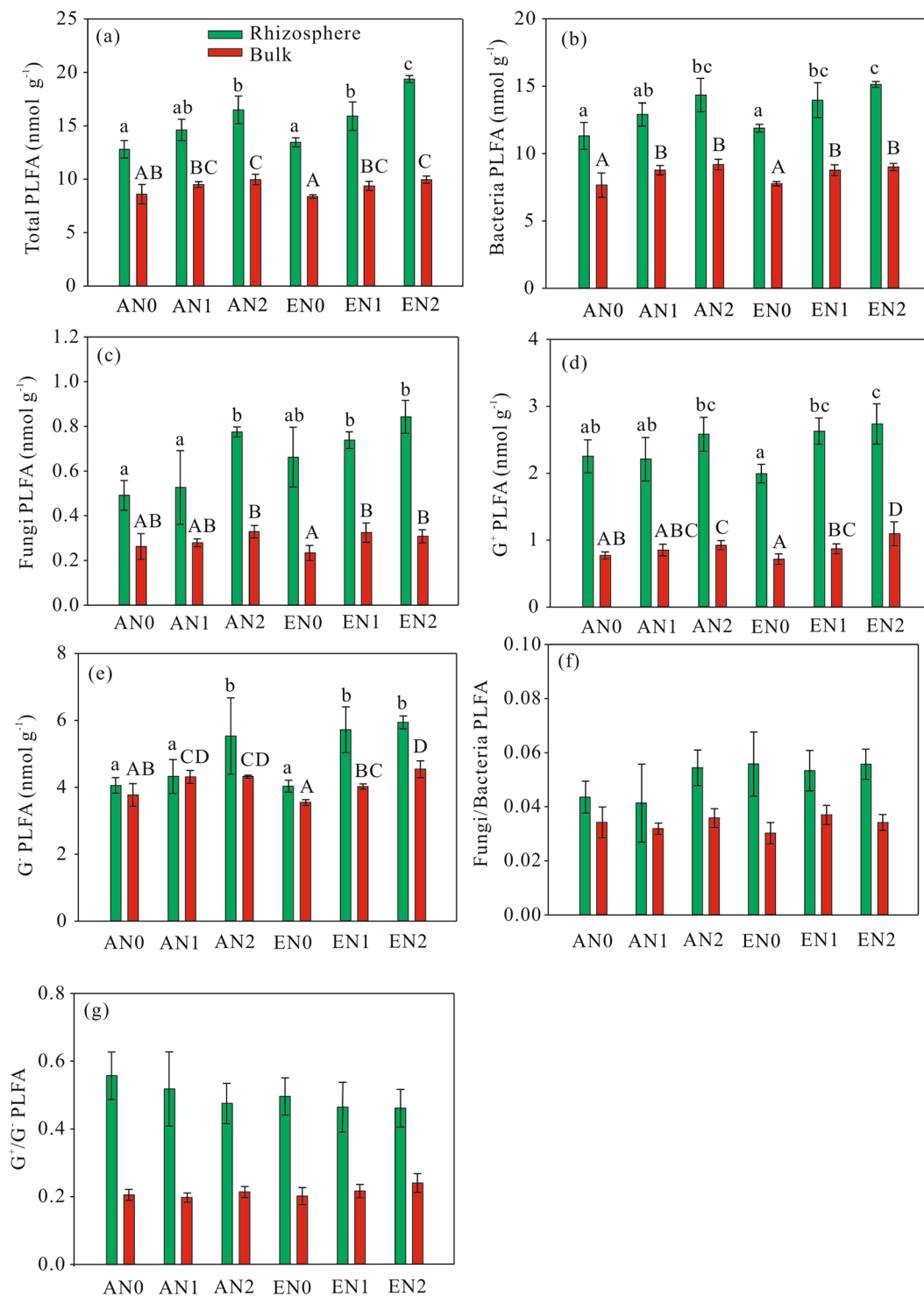


Figure 3. Total PLFA (a), bacterial PLFA (b), fungal PLFA (c), G⁺ PLFA (d), G⁻ PLFA (e), fungal/bacterial PLFA (f), and G⁺/G⁻ PLFA (g) in the rhizosphere and bulk soil in the treatments. Different lowercase letters indicate significant differences between treatments in the rhizosphere, and different uppercase letters indicate significant differences between treatments in the bulk soil. AN0, ambient CO₂ and no N added; AN1, ambient CO₂ and N supply at a rate of 2.5 g N m⁻² y⁻¹; AN2, ambient CO₂ and N supply at a rate of 5 g N m⁻² y⁻¹; EN0, elevated CO₂ and no N added; EN1, elevated CO₂ and N supply at a rate of 2.5 g N m⁻² y⁻¹; EN2, elevated CO₂ and N supply at a rate of 5 g N m⁻² y⁻¹.

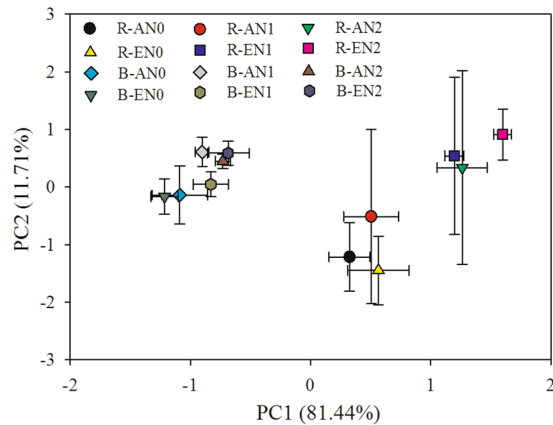


Figure 4. Principal component (PC) analysis of the PLFAs in the rhizosphere and bulk soil in the treatments. R, rhizospheric soil; B, bulk soil; AN0, ambient CO₂ and no N added; AN1, ambient CO₂ and N supply at a rate of 2.5 g N m⁻² y⁻¹; AN2, ambient CO₂ and N supply at a rate of 5 g N m⁻² y⁻¹; EN0, elevated CO₂ and no N added; EN1, elevated CO₂ and N supply at a rate of 2.5 g N m⁻² y⁻¹; EN2, elevated CO₂ and N supply at a rate of 5 g N m⁻² y⁻¹.

the conditions of plant growth (Table 5). The effects of N enrichment on the communities are associated with soil critical N loads or N saturation theories⁵³. These theories propose that the effect of N enrichment on ecosystem functions would switch from stimulation to inhibition when the ecosystem reaches a critical N loading or saturation level^{23, 54, 55}. The N-saturation theories suggest that low N additions usually increase soil microbial biomass and microbial diversity and that high N addition would decrease them^{25, 26}. Plant growth on the Loess Plateau is restricted by soil N content, and N addition in our study significantly increased soil microbial biomass and shifted the community structure.

Rhizospheric effects under elevated CO₂ and N addition. Rhizospheres are zones of higher microbial turnover and activity, because they are adjacent to plant roots⁵⁶. Many important aspects of plant-soil interactions are mediated by rhizospheric processes, including nutrient acquisition and root colonization by rhizospheric microorganisms. Microbial activity is higher and more diverse in rhizospheres than bulk soil³³. We also found that BR and total, bacterial, G⁺, and G⁺/G⁻ PLFAs were significantly higher in the rhizosphere than bulk soil. The higher G⁺/G⁻ PLFAs in the rhizosphere than the bulk soil indicated that the rhizosphere communities were more heterotrophic via increases in C inputs when the plants were exposed to elevated CO₂ and N addition^{57, 58}.

Rhizospheric effects can be affected by elevated CO₂ and N addition^{36, 59}. The types and amounts of organic root exudates can be altered when plants are exposed to high levels of CO₂, and these changes may affect rhizospheric microbial activity and community composition. Lee *et al.*⁴ reported that rhizospheric microbes responded to elevated CO₂ more strongly than the microbes in bulk soil. Our results showed that elevated CO₂ significantly increased the rhizospheric effects of total PLFA, as expected, because rhizospheric microbial communities are sensitive to elevated CO₂. The rhizospheric effects of other microbial variables (such as bacterial PLFA, G⁺ PLFA, and G⁻ PLFA), however, responded weakly to elevated CO₂. The impact of N addition on rhizospheric effects can be affected by many factors, such as plant species, soil type, soil chemical properties, and the amount and duration of N addition³⁵. Phillips and Fahey⁶⁰ found that N fertilization had a positive, negative, or no impact on rhizospheric effects for trees, depending on the tree species and soil variables. Ai *et al.*⁶¹ reported that long-term inorganic N addition reduced rhizospheric effects in a wheat-maize rotation system. N addition in our study did not significantly affect the rhizospheric effects for the soil variables, except BR, consistent with the results by Zhu *et al.*³⁶, who also reported that N fertilization had minimal influence on the rhizospheric effects of two grass species. N is the limiting factor for plant growth on the Loess Plateau, and N addition in our study significantly increased plant total biomass and root biomass, so the root-derived C inputs to the soil (e.g. root exudates) may have been significantly affected by the N addition, and the available substrates in the rhizosphere (e.g. NH₄⁺, NO₃⁻) may also have been significantly absorbed by the plants (Table 5), which may account for the lack of significant shifts in the rhizospheric effects^{34, 62}.

Evaluating the effects of elevated CO₂ and N deposition on soil microorganisms in the rhizosphere and bulk soil is challenging because of their high diversity. Our PLFA analysis found that elevated CO₂ and N addition had significant effects on the soil microbial community, especially in the rhizospheric soil. Both elevated CO₂ and N addition contributed little to the changes in the magnitude of the rhizospheric effects, perhaps due to the low resolution of PLFAs for classifying soil microbial communities. As science and technology have developed, especially in the last decade, high-throughput molecular technologies have been developed for characterizing microbial communities, including high-throughput DNA/RNA sequencing, PhyloChio, GeoChip, mass spectrometry-based proteomics for community analysis, and metabolite analysis⁶³. In future studies, these molecular methods may be able to provide a more comprehensive understanding of microbial responses to scenarios of global climate change.

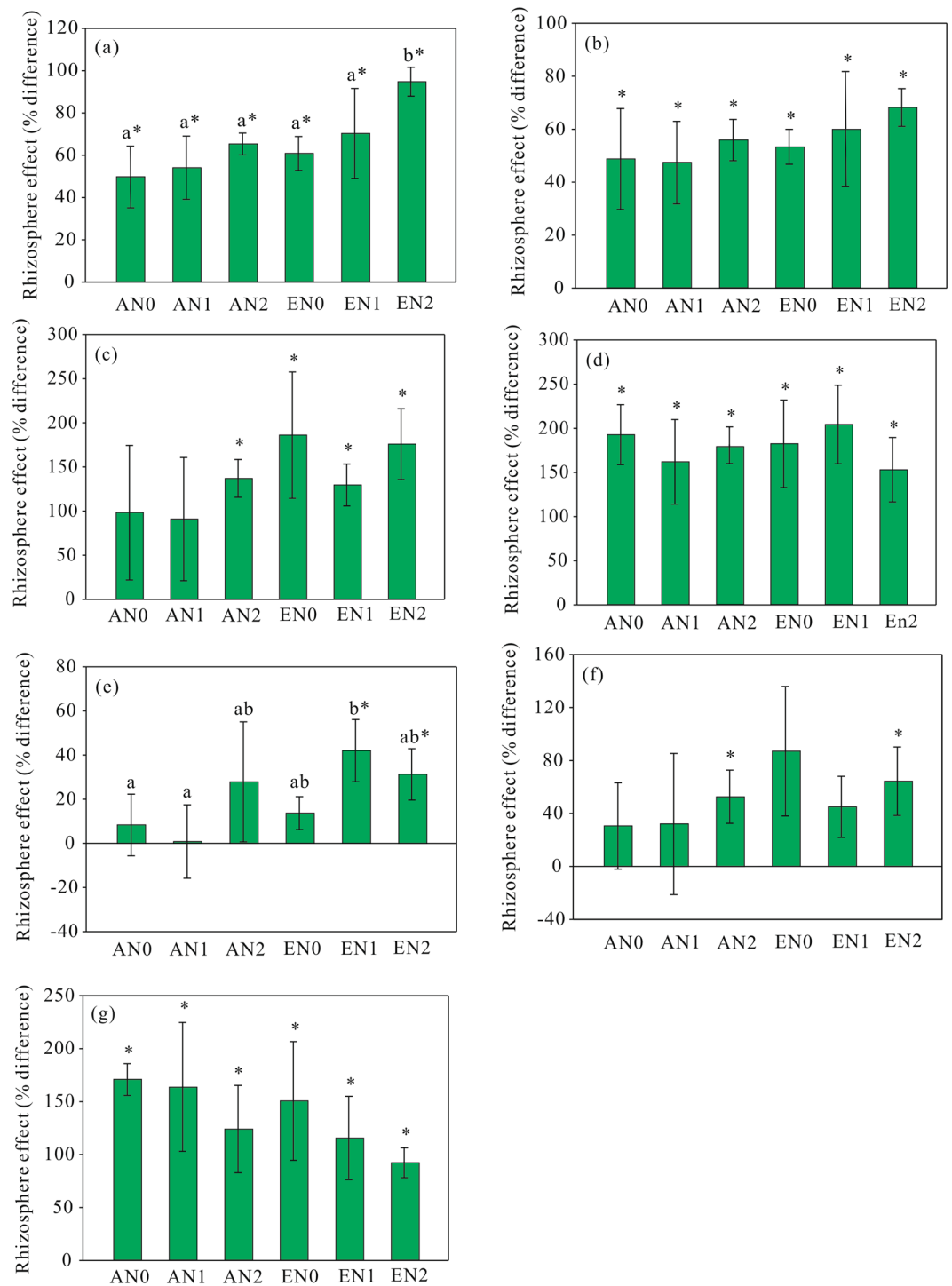


Figure 5. Rhizospheric effects for total PLFA (a), bacterial PLFA (b), fungal PLFA (c), G⁺ PLFA (d), G⁻ PLFA (e), fungal/bacterial PLFA (f), and G⁺/G⁻ PLFA (g) in the treatments. * Above each bar indicates significant difference between the rhizosphere and bulk soil. Different lowercase letters indicate significant differences in rhizospheric effects between treatments. AN0, ambient CO₂ and no N added; AN1, ambient CO₂ and N supply at a rate of 2.5 g N m⁻² y⁻¹; AN2, ambient CO₂ and N supply at a rate of 5 g N m⁻² y⁻¹; EN0, elevated CO₂ and no N added; EN1, elevated CO₂ and N supply at a rate of 2.5 g N m⁻² y⁻¹; EN2, elevated CO₂ and N supply at a rate of 5 g N m⁻² y⁻¹.

Conclusions

N addition significantly increased BR in the rhizosphere and increased total, bacterial, fungal, G⁺, and G⁻ PLFAs in both the rhizosphere and bulk soil, but elevated CO₂ only significantly increased total and fungal PLFAs in the

Treatment	Total biomass (mg)	Shoot biomass (mg)	Root biomass (mg)	Root:shoot ratio	Nitrogen concentration (mg g ⁻¹)	Nitrogen content (mg pot ⁻¹)
AN0	466.67 ± 51.32a	280.87 ± 23.58a	185.8 ± 27.83a	0.66 ± 0.05a	4.75 ± 0.32a	2.21 ± 0.11a
AN1	1013.33 ± 247.86b	581.22 ± 153.55b	432.11 ± 97.7a	0.75 ± 0.08ab	5.37 ± 0.38b	5.46 ± 1.31b
AN2	2716.67 ± 358.38d	1473.87 ± 110d	1242.79 ± 249.93c	0.84 ± 0.11bc	5.31 ± 0.44ab	14.42 ± 1.83d
EN0	686.67 ± 75.06ab	406.55 ± 68.04ab	280.12 ± 18.14a	0.70 ± 0.10ab	4.77 ± 0.12a	3.28 ± 0.44a
EN1	1733.33 ± 94.52c	879.59 ± 54.56c	853.74 ± 41.76b	0.97 ± 0.03c	5.27 ± 0.34ab	9.12 ± 0.51c
EN2	4103.33 ± 290.23e	1966.89 ± 285.25e	2136.44 ± 178.17d	1.10 ± 0.19d	5.40 ± 0.26b	22.18 ± 1.73e
P values of two-way ANOVAs						
C	<0.001	<0.001	<0.001	0.005	0.969	<0.001
N	<0.001	<0.001	<0.001	0.002	0.009	<0.001
C × N	<0.001	0.130	0.002	0.201	0.841	<0.001

Table 5. Effects of elevated CO₂ concentration (C) and N addition (N) on plant biomass and nitrogen content. Different letters within a column indicate significant differences ($P < 0.05$) based on Duncan's multiple range test. Significant P values are highlighted in bold. AN0, ambient CO₂ and no N added; AN1 ambient CO₂ and N supply at a rate of 2.5 g N m⁻² y⁻¹; AN2, ambient CO₂ and N supply at a rate of 5 g N m⁻² y⁻¹; EN0, elevated CO₂ and no N added; EN1, elevated CO₂ and N supply at a rate of 2.5 g N m⁻² y⁻¹; EN2, elevated CO₂ and N supply at a rate of 5 g N m⁻² y⁻¹.

rhizosphere when combined with N addition. These results demonstrated that N addition had a larger impact on the soil microbial communities than elevated CO₂. Contrary to our second hypothesis, the rhizospheric effects of soil microbial variables were not significantly affected by elevated CO₂ and N addition. These results suggest that the rhizospheres of *B. ischaemum* exert a more important control of community composition and structure than short-term elevated CO₂ and N addition.

Materials and Methods

Plant species and soil used in the experiment. Seeds of *B. ischaemum* were collected in autumn 2013 from the natural grasslands at the Ansai Research Station (ARS) of the Institute of Soil and Water Conservation, Chinese Academy of Sciences (CAS), on the Loess Plateau of China (36°51'30"N, 109°19'23"E, 1068–1309 m a.s.l.). The rates of seed germination were >90% when germinated on moist filter paper in Petri dishes at 25°C prior to the experiment.

A sandy-loam soil collected from the upper 20 cm of a farmland at ARS was used for this study. Soil gravimetric water content at field capacity (FC) and the wilting point were 20.0 and 4.0%, respectively. The soil organic C (SOC), total N (TN), and total phosphorus (TP) contents were 1.50, 0.21, and 0.57 g kg⁻¹, respectively.

Experimental design and sample collection. Each plastic pot (20 cm high, 15 cm in diameter) was separated vertically into two concentric zones, a central root zone and a root-free zone, by 25-μm nylon mesh bags (20 cm high, 9 cm in diameter) buried in the centers of the pots, enabling the passage of water and nutrients but not roots. The seeds of *B. ischaemum* were sown in the mesh bags on 1 June 2014. The soil-water content was maintained above 80% FC during the entire experiment.

The experiment began on 1 August 2014 after the seedlings were thinned to three per pot. The pots were transferred to two closed climate-controlled chambers (AGC-D001P, Qiushi Corp., Hangzhou, China) programmed at 13 h of light (28°C, relative humidity (RH) of 50%, 300 μ (photons) m⁻²s⁻¹) from 7:30 to 20:30 and 11 h of dark (22°C, RH of 55%). The CO₂ concentrations in the two chambers were maintained at 400 (ambient) and 800 (elevated) μmol mol⁻¹ until the end of the experiment. An automatic control system was used to adjust the CO₂ to the desired concentration in each chamber by regulating the influx rate of pure CO₂ to the air blower. Each chamber housed three N-addition treatments (0 (control), 2.5, and 5 g N m⁻² y⁻¹). The N-addition treatments began on 18 August 2014. For each pot, NH₄NO₃ was dissolved in deionized water and then added to the pot soil, except for the control treatment where an equal volume of deionized water was added. N was added a total of six times during the experiment, with a frequency of every 15 days. The 0, 2.5, and 5 g N m⁻² y⁻¹ treatments received 0, 0.021, and 0.042 g NH₄NO₃, respectively, each time. All pots were weighed daily at 18:00, and water was added via plastic pipes to maintain soil-water contents above 80% FC. A total of six treatments were thus tested: ambient CO₂ but no N added (AN0), ambient CO₂ and 2.5 g N m⁻² y⁻¹ (AN1), ambient CO₂ and 5 g N m⁻² y⁻¹ (AN2), elevated CO₂ but no N added (EN0), elevated CO₂ and 2.5 g N m⁻² y⁻¹ (EN1), and elevated CO₂ and 5 g N m⁻² y⁻¹ (EN2). Each treatment had five replicates.

The experiment was completed on 15 November 2014. The soils of the root zone (adhering to the roots in the mesh bag) and the root-free zone (>1.5 cm outside the mesh bag) were collected and sieved through a 2-mm mesh. One subsample of each type of soil was air-dried, crushed, and passed through a 0.25-mm mesh for the determination of chemical properties, another subsample was stored at 4°C for respiration and Biolog analysis, and a third subsample was stored at -20°C for PLFA analysis.

Soil chemical properties. SOC content was determined by wet digestion with a mixture of potassium dichromate and concentrated sulfuric acid, TN content was determined by the semimicro Kjeldahl method after digestion by H₂SO₄, and TP content was determined colorimetrically after wet digestion with H₂SO₄ + HClO₄.

Microbial respiration rate. The rate of soil microbial respiration was determined by CO₂ emission^{64,65}. The basal respiration (BR) was determined by placing 10 g of each soil sample, moistened to 50–60% of the field capacity, in a hermetically sealed flask equipped with a rubber septum for gas sampling. The samples were then incubated at 28 °C for 7 days under aerobic conditions, and the CO₂ released was measured 0.5, 1, 2, 3, 4, 5, 6, 7 days after incubation with an infrared gas analyzer (QGS-08B, Befen-Ruili Analytical Instrument Co. Ltd., Beijing, China). Soil samples were amended with 0.6% (w/w) glucose before incubation for determining substrate-induced respiration (SIR). The BR and SIR data are expressed as μg CO₂ g⁻¹ dw h⁻¹.

Community-level physiological profile (CLPP). The CLPPs of the soil microbial communities were assessed using Biolog EcoPlates (Biolog, Hayward, USA) containing three replicates of 31 unique C substrates⁶⁶. Briefly, soil samples were serially diluted to 10⁻³ suspensions in a sterile solution of 0.85% NaCl. The diluted suspensions were added to the Biolog EcoPlates, and all plates were incubated at 25 °C for one week. Color development was measured as optical density (OD) at 590 nm every 24 h using Microlog Rel 4.2 (Biolog, Hayward, USA). Negative optical densities or those under 0.06 were set to zero. The final OD of each well at 72 h was used to calculate the average well color development (AWCD), Shannon index (*H*), and Simpson index (*D*):

$$AWCD = \sum_{i=1}^n \frac{(x_i - c)}{31} \quad (1)$$

$$H = - \sum_{i=1}^n p_i (\ln p_i) \quad (2)$$

$$D = 1 - \sum_{i=1}^n (p_i)^2 \quad (3)$$

where x_i is the optical density measured at 590 nm for substrate in the EcoPlates, c is the OD of the control well, 31 is the number of C sources, p_i is the ratio of the absorbance in each well to the sum of absorbance for all wells, and n is the total number of C sources.

PLFAs. The method for phospholipid extraction was adapted from Buyer *et al.*⁶⁷. Briefly, 3 g of lyophilized soil were placed in a 30-ml centrifuge tube with a Teflon-lined screw cap. The fatty acids were directly extracted from the soil twice by adding 3.6 ml of citrate buffer (pH 4.0), 4 ml of chloroform, and 8 ml of methanol. The PLFAs were separated from neutral and glycolipid fatty acids by solid-phase-extraction chromatography. After mild alkaline methanolysis, the PLFA samples were qualitatively and quantitatively analyzed using an Agilent 7890 gas chromatograph (Agilent Technologies, Santa Clara, USA) equipped with an autosampler, split-splitless injector, and flame ionization detector. The system was controlled with Agilent ChemiStation and MIDI Sherlock software (Microbial ID, Inc., Newark, USA). An external standard of 19:0 methyl ester was used for quantification.

We selected the following PLFA signatures to serve as indicators of specific microbial groups: iso- and anteiso-branched fatty acids for Gram-positive (G⁺) bacteria⁶⁸, monounsaturated and cyclopropyl 17:0 and 19:0 fatty acids for Gram-negative (G⁻) bacteria⁶⁹, and 18:2w6c for fungi⁷⁰. Total biomass was obtained by summing the concentrations of all fatty acids detected in each soil sample.

Statistical analysis. All results are expressed as means ± standard deviations. Rhizospheric effects were calculated as the percent difference between the rhizospheric and bulk-soil samples for each measured variable^{34,36}. Student's *t*-tests were used to compare the values between the rhizosphere and bulk soil to indicate the statistical significance of the calculated rhizospheric effect. SOC, TN, and TP contents and microbial functional diversity did not differ significantly between the rhizosphere and bulk soil, so we have not reported the rhizospheric effects for these variables. Two-way analyses of variance (ANOVAs) at a probability level of 0.05 were used to assess the effects of elevated CO₂, N addition, and their interaction on the biochemical properties of the rhizospheric and bulk soil and the rhizospheric effect for each variable. Means were compared using Duncan's multiple range test for significant differences ($P < 0.05$). A principal component analysis examined the PLFA community structure among the treatments. The above statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, USA).

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Author Contributions

The manuscript was reviewed and approved for publication by all authors. S.X. conceived and designed the experiments. L.X. performed the experiments, analyzed the data, drew the figures and wrote the paper. G.B.L. and P.L. revised the paper.

Additional Information

Competing Interests: The authors declare that they have no competing interests.

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