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 ecosystems. Soil microbial communities, especially rhizospheric microorganisms, serve as bridges between plants and soil, are key drivers of the global bio-geochemical cycles of mineral elements. Much attention, however, has been given to characterize the responses of these communities to various scenarios of climate change, 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. 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 properties. 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, but usually stimulated soil respiration. Analyses of functional genes indicated that elevated CO₂ decreased the expression of genes involved in the C and N cycles, but Liu et al. 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 enrichment, the soil, or the vegetation type.

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 activity. Zhou et al. and Ma et al. 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. The effects of N deposition on soil microbial communities are complicated, and further studies are needed to understand the mechanisms and impacts of N deposition.

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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 them25, 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 ecosystems28, 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.,33 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 type38.

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 effects36. 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.

**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.

| 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      |
| H               | 0.674       | 0.826     | 0.544      | 0.717       | 0.499     | 0.436      |
| D               | 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      |

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 affect 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
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

### 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.

| 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 ± 0.006b |
| 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 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.**

| 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 |

**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.
Elevated CO₂ for 106 days in our study significantly increased plant responses from changes in substrate availability. Elevated CO₂ in our study did not have significant impact on N addition significantly increased soil respiration by 7.84% in grasslands. These contradictory results could be due to differences of microbial substrate availability.

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. Elevated CO₂ and N deposition can have profound impacts on soil respiration. A meta-analysis found that N addition 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 CO₂ and N addition. However, we did not find a significant 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.

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.

| Treatment | 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 | 2.78 ± 0.08| 0.93 ± 0.01 | 0.93 ± 0.00|
| 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|

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.
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.
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 theories. 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. 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. 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 CO$_2$ and N addition.** Rhizospheres are zones of higher microbial turnover and activity, because they are adjacent to plant roots. 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 soil. 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 CO$_2$ and N addition.

Rhizospheric effects can be affected by elevated CO$_2$ and N addition. The types and amounts of organic root exudates can be altered when plants are exposed to high levels of CO$_2$, and these changes may affect rhizospheric microbial activity and community composition. Lee et al. reported that rhizospheric microbes responded to elevated CO$_2$ more strongly than the microbes in bulk soil. Our results showed that elevated CO$_2$ significantly increased the rhizospheric effects of total PLFA, as expected, because rhizospheric microbial communities are sensitive to elevated CO$_2$. The rhizospheric effects of other microbial variables (such as bacterial PLFA, G$^+$ PLFA, and G$^−$ PLFA), however, responded weakly to elevated CO$_2$. 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 addition. Phillips and Fahey 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. 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., 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. NH$_4^+$, NO$_3^−$) may also have been significantly absorbed by the plants (Table 5), which may account for the lack of significant shifts in the rhizospheric effects.

Evaluating the effects of elevated CO$_2$ 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 CO$_2$ and N addition had significant effects on the soil microbial community, especially in the rhizospheric soil. Both elevated CO$_2$ 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 analysis. In future studies, these molecular methods may be able to provide a more comprehensive understanding of microbial responses to scenarios of global climate change.

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**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 CO$_2$ and no N added; AN1 ambient CO$_2$ and N supply at a rate of 2.5 g N m$^{-2}$ y$^{-1}$; AN2, ambient CO$_2$ and N supply at a rate of 5 g N m$^{-2}$ y$^{-1}$; EN0, elevated CO$_2$ and no N added; EN1, elevated CO$_2$ and N supply at a rate of 2.5 g N m$^{-2}$ y$^{-1}$; EN2, elevated CO$_2$ 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 CO$_2$ only significantly increased total and fungal PLFAs in the rhizosphere. Different lowercase letters indicate significant differences in rhizospheric effects between treatments. AN0, ambient CO$_2$ and no N added; AN1 ambient CO$_2$ and N supply at a rate of 2.5 g N m$^{-2}$ y$^{-1}$; AN2, ambient CO$_2$ and N supply at a rate of 5 g N m$^{-2}$ y$^{-1}$; EN0, elevated CO$_2$ and no N added; EN1, elevated CO$_2$ and N supply at a rate of 2.5 g N m$^{-2}$ y$^{-1}$; EN2, elevated CO$_2$ and N supply at a rate of 5 g N m$^{-2}$ y$^{-1}$.

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.
prior to the experiment. Germination rates were 90% when germinated on moist filter paper in Petri dishes at 25 °C.

The soil organic C content at field capacity (FC) and the wilting point were 20.0 and 4.0%, respectively. The soil organic C content was determined via the dry combustion method. The available N content was determined colorimetrically after wet digestion with H2SO4. TN content was determined by the semimicro Kjeldahl method after digestion by H2SO4 and dichromate and concentrated sulfuric acid. The pH value of the soil solution was measured with a pH meter.

The experiment began on 1 August 2014 after the seedlings were thinned to three per pot. The pots were transplanted at 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 μmol mol−1 until the end of the experiment. An automatic control system was used to adjust the CO2 concentration based on the CO2 levels in the chambers. The N-addition treatments (0, 2.5, and 5 g N m−2) were supplied in three split applications during the experiment, with a frequency of every 15 days. The 0, 2.5, and 5 g N m−2 treatments received an equal volume of deionized water.

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**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 substrates\(^{66}\). 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):

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

\[
H = - \frac{\sum_{i=1}^{n} p_i \ln p_i}{\sum_{i=1}^{n} p_i}
\]

\[
D = 1 - \frac{\sum_{i=1}^{n} (p_i)^2}{\sum_{i=1}^{n} p_i}
\]

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 ChemStation 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\(^+\)) bacteria\(^ {68}\), monounsaturated and cyclopropyl 17:0 and 19:0 fatty acids for Gram-negative (G\(^-\)) bacteria\(^ {69}\), and 18:2w6c for fungi\(^ {70}\). 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).

**References**

1. van der Putten, W. H., Bradford, M. A., Brinkman, E. P., van de Voorde, T. F. J. & Veen, G. T. Where, when and how plant-soil feedback matters in a changing world. *Funct. Ecol.** 30, 1109–1121 (2016)*.

2. Larsen, J., Jaramillo-Lopez, P., Najera-Rincon, M. & Gonzalez-Esquivel, C. E. Biotic interactions in the rhizosphere in relation to plant and soil nutrient dynamics. *J. Soil Sci. Plant Nutr.* 15, 449–463 (2015).

3. Blankinship, J. C., Niklaus, P. A. & Hungate, B. A. A meta-analysis of responses of soil biota to global change. *Oecologia** 165, 553–565 (2011)*.

4. Lee, S. H. & Kang, H. Elevated CO₂ causes a change in microbial communities of rhizosphere and bulk soil of salt marsh system. *Appl. Soil Ecol.* 108, 307–314 (2016).

5. Xue, S. et al. Effects of elevated CO₂ and drought on the microbial biomass and enzymatic activities in the rhizospheres of two grass species in Chinese loess soil. *Geserma* 286, 25–34 (2017).

6. Freedman, Z. B., Romanowicz, K. J., Upchurch, R. A. & Zak, D. R. Differential responses of total and active soil microbial communities to long-term experimental N deposition. *Soil Biol. Biochem.* 90, 275–282 (2015).

7. Grover, M., Maheshwari, M., Desai, S., Gopinath, K. A. & Venkateswarlu, B. Elevated CO₂: Plant associated microorganisms and carbon sequestration. *Appl. Soil Ecol.* 95, 73–85 (2015).

8. Kassem, I. I., Joshi, P., Sigler, V., Heckathorn, S. & Wang, Q. Effect of elevated CO₂ and drought on soil microbial communities associated with Andropogon gerardii. *J. Integr. Plant Biol.* 50, 1406–1415 (2008).
9. Austin, E. E., Castro, H. F., Cooper, K., Schadt, C. W. & Classen, A. T. Assessment of 10 years of CO₂ fumigation on soil microbial communities and function in a sweetgum plantation. *Soil Biol. Biochem.* 41, 514–520 (2009).

10. Guenet, B. et al. The impact of long-term CO₂ enrichment and moisture levels on soil microbial community structure and enzyme activities. *Geoderma* 170, 331–336 (2012).

11. Hagedorn, F. et al. Nine years of CO₂ enrichment at the alpine treeline stimulates soil respiration but does not alter soil microbial communities. *Soil Biol. Biochem.* 57, 390–400 (2013).

12. Duan, B. L. et al. Long-term responses of plant growth, soil microbial communities and soil enzyme activities to elevated CO₂ and neighbouring plants. *Agr. Forest Meteorol.* 213, 91–101 (2015).

13. Baronti, S., Tognetti, R., Lanini, G. M., Tonon, G. & Raschi, A. Soil respiration and microbial activity in a Mediterranean grassland exposed to Free Air CO₂ Enrichment (FACE). *Community Ecol.* 9, 65–73 (2008).

14. He, Z. L. et al. The phylogenetic composition and structure of soil microbial communities shifts in response to elevated carbon dioxide. *ISME J.* 6, 259–272 (2012).

15. Butterfly, C. R. et al. Long-term effects of elevated CO₂ on carbon and nitrogen functional capacity of microbial communities in three contrasting soils. *Soil Biol. Biochem.* 97, 157–167 (2016).

16. Liu, Y. et al. Short-term response of nitrifier communities and potential nitrification activity to elevated CO₂ and temperature interaction in a Chinese paddy field. *Appl. Soil Ecol.* 96, 88–98 (2015).

17. Zheng, J. Q. et al. Microbial activity in a temperate forest soil as affected by elevated atmospheric CO₂. *Pedosphere* 20, 427–435 (2010).

18. Das, S., Bhattacharyya, P. & Adhya, T. K. Interaction effects of elevated CO₂ and temperature on microbial biomass and enzyme activities in tropical rice soils. *Environ. Monit. Assess.* 182, 555–569 (2011).

19. Song, Y. Y. et al. Short-term effects of nitrogen addition and vegetation removal on soil chemical and biological properties in a freshwater marsh in Sanjiang Plain, Northeast China. *Catal. 104*, 263–271 (2013).

20. Zhou, X. et al. Effects of 44 years of chronic nitrogen fertilization on the soil nitrifying community of permanent grassland. *Soil Biol. Biochem.* 91, 76–83 (2015).

21. Ma, W. B. et al. Response of microbial functional groups involved in soil N cycle to N, P and NP fertilization in Tibetan alpine meadows. *Soil Biol. Biochem.* 101, 195–206 (2016).

22. Li, F. L., Liu, M. & Li, Z. P. Changes in soil microbial biomass and functional diversity with a nitrogen gradient in soil columns. *Appl. Soil Ecol.* 64, 1–6 (2013).

23. Chen, D., Lan, Z. C., Hu, S. J. & Yai, Y. F. Effects of nitrogen enrichment on belowground communities in grassland: Relative role of soil nitrogen availability vs. soil acidification. *Soil Biol. Biochem.* 89, 99–108 (2015).

24. Contosta, A. R., Frey, S. B. & Cooper, A. B. Soil microbial communities vary as much over time as with chronic warming and nitrogen additions. *Soil Biol. Biochem.* 88, 19–24 (2015).

25. He, Y. T. et al. Effects of nitrogen fertilization on soil microbial biomass and community functional diversity in temperate grassland in Inner Mongolia, China. *Clean-Soil Air Water* 41, 1216–1221 (2013).

26. Sun, S. et al. Response of bacterial community to simulated nitrogen deposition in soils and a unique relationship between plant species and soil bacteria in the Songnen grassland in Northeastern China. *J. Soil Sc. Plant Nutr.* 14, 565–580 (2014).

27. Franklin, J., Serra-Diaz, J. M., Syphard, A. D. & Regan, H. M. Global change and terrestrial plant community dynamics. *P. Natl. Acad. Sci. USA* 113, 3725–3734 (2016).

28. Lee, S. H., Kim, S. Y., Ding, W. X. & Kang, H. Impact of elevated CO₂ and N addition on bacteria, fungi, and archaea in a marsh ecosystem with various types of plants. *Appl. Microbiol. Biotechnol.* 99, 5295–5305 (2015).

29. Liu, J. X. et al. CO₂ enrichment and N addition increase nutrient loss from decomposing leaf litter in subtropical model forest ecosystems. *Sci. Rep.* 5, 7952 (2015).

30. Deng, Q. et al. Seasonal responses of soil respiration to elevated CO₂ and N addition in subtropical forest ecosystems in southern China. *Ecol. Eng.* 61, 65–73 (2013).

31. Deng, Q. et al. Responses of soil respiration to elevated carbon dioxide and nitrogen addition in young subtropical forest ecosystems in China. *Biogeosciences* 7, 315–328 (2010).

32. Haase, S., Philippot, L., Neumann, G., Marhan, S. & Kandel, E. Local response of bacterial densities and enzyme activities to elevated atmospheric CO₂ and different N supply in the rhizosphere of *Phaseolus vulgaris* L. *Soil Biol. Biochem.* 40, 1225–1234 (2008).

33. Ultra, V. U. Jr., Han, S. H. & Kim, D. H. Soil properties and microbial functional structure in the rhizosphere of *Punensdensiflora* (S. et Z.) exposed to elevated atmospheric carbon dioxide. *J. Forest Res.* 18, 149–158 (2013).

34. Phillips, R. P. & Fahey, T. J. Tree species and mycorrhizal associations influence the magnitude of rhizosphere effects. *Ecology* 87, 1302–1303 (2006).

35. Kuziyakov, Y. Review: Factors affecting rhizosphere priming effects. *J. Plant Nutr. Soil Sci.* 165, 382–396 (2002).

36. Zhu, B., Panke-Buisse, K. & Kao-Kniffin, J. Nitrogen fertilization has minimal influence on rhizosphere effects of smooth crabgrass (*Digitaria ischaemum*) and bermudagrass (*Cynodon dactylon*). *J. Plant Ecol.* 8, 390–400 (2015).

37. Liu, Z. P., Shao, M. A. & Wang, Y. Q. Spatial patterns of soil total nitrogen and soil total phosphorus across the entire loess plateau region of China. *Geoderma* 197-198, 67–78 (2013).

38. Wei, Y. et al. Preliminary estimate of the atmospheric nitrogen deposition in different ecological regions of Shaanxi province. *J. Agro-Environ.* Sci. 29, 795–800 (2010).

39. Han, X. W., Tsunekawa, A., Tsuibo, M. & Shao, H. B. Responses of plant-soil properties to increasing N deposition and implications for large-scale eco-restoration in the semiarid grassland of the northern Loess Plateau, China. *Ecol. Eng.* 60, 1–9 (2013).

40. Galloway, J. N. et al. Nitrogen cycles: past, present, and future. *Biogeochemistry* 70, 153–226 (2004).

41. Xiao, L., Liu, G. B., Zhang, J. Y., Yang, T. & Xue, S. Effects of elevated CO₂ drought stress and nitrogen deposition on photosynthesis light response curves of *Bothriochloa ischaemum*. *Acta Agric. Sc.* 24, 69–75 (2016).

42. Xiao, L., Liu, G. B., Li, P. & Xue, S. Effects of nitrogen addition and elevated CO₂ concentration on soil dissolved organic carbon and nitrogen in rhizosphere and non-rhizosphere of *Bothriochloa ischaemum*. *Chinese J. Appl. Ecol.* 26, 64–70 (2017).

43. Jia, X. X., Shao, M. A. & Wei, X. R. Responses of soil respiration to N addition, burning and clipping in temperate semiarid grassland in northern China. *Agr. Forest Meteorol.* 166-167, 32–40 (2012).

44. Gao, Y. H., Ma, G., Zeng, X. Y., Xu, S. Q. & Wang, D. X. Responses of microbial respiration to nitrogen addition in two alpine soils in the Qinghai-Tibetan Plateau. *J. Environ. Biol.* 35, 261–265 (2015).

45. Zhou, L. Y. et al. Different responses of soil respiration and its components to nitrogen addition among biomes: a meta-analysis. *Global Change Biol.* 20, 2332–2334 (2014).

46. Luo, Q. P. et al. The responses of soil respiration to nitrogen addition in a temperate grassland in northern China. *Sci. Total Environ.* 569-570, 1466–1477 (2016).

47. Zhang, C. P. et al. Effects of simulated nitrogen deposition on soil respiration components and their temperature sensitivities in a semiarid grassland. *Soil Biol. Biochem.* 75, 113–123 (2014).

48. Selsted, M. B. et al. Soil respiration is stimulated by elevated CO₂ and reduced by summer drought: three years of measurements in a multifactor ecosystem manipulation experiment in a temperate heathland (CLIMAIT). *Global Change Biol.* 18, 1216–1230 (2012).
49. Liu, Y. et al. Short-term responses of microbial community and functioning to experimental CO₂ enrichment and warming in a Chinese paddy field. Soil Biol. Biochem. 77, 58–68 (2014).
50. De Graaff, M. A., van Grooten, K. J., Six, J., Hungate, B. & van Kessel, C. Interactions between plant growth and soil nutrient cycling under elevated CO₂: a meta-analysis. Global Change Biol. 12, 2077–2091 (2006).
51. Das, S., Bhattacharyya, P. & Adhya, T. K. Impact of elevated CO₂ flooding, and temperature interaction on heterotrophic nitrogen fixation in tropical rice soils. Biol. Fert. Soils 47, 25–30 (2011).
52. Long, X. E., Chen, C. R., Xu, Z. H., Oren, R. & He, J. Z. Abundance and community structure of ammonia-oxidizing bacteria and archaea in a temperate forest ecosystem under ten-years elevated CO₂. Soil Biol. Biochem. 46, 163–171 (2012).
53. Shen, R. C., Xu, M., Chi, Y. G., Yu, S. & Wan, S. Q. Soil microbial responses to experimental warming and nitrogen addition in a temperate steppe of northern China. Pedosphere 24, 427–436 (2014).
54. Bowman, W. D., Murgel, J., Blett, T. & Porter, E. Nitrogen critical loads for alpine vegetation and soils in Rocky Mountain National Park. J. Environ. Manage. 103, 165–171 (2012).
55. Chen, H. et al. Nitrogen saturation in humid tropical forests after 6 years of nitrogen and phosphorus addition: hypothesis testing. Funct. Ecol. 30, 305–313 (2016).
56. Zhang, Y. X., Ruyter-Spira, C. & Bouwmeester, H. J. Engineering the plant rhizosphere. Curr. Opin. Biotech. 32, 136–142 (2013).
57. Tscherko, D., Hammesfahr, U., Marx, M. C. & Kandeler, E. Shifts in rhizosphere microbial communities and enzyme activity of Poa alpina across alpine chronosequence. Soil Biol. Biochem. 36, 1685–1689 (2004).
58. Xiao, L., Liu, G. B., Xue, S. & Zhang, C. Soil microbial community composition during natural recovery in the Loess Plateau, China. J. Integr. Agr. 12, 1872–1883 (2013).
59. Jia, X. & Zhou, C. J. Effects of long-term elevated CO₂ on rhizosphere and bulk soil bacterial community structure in Pinus sylvestrisforms seedlings fields. Adv. Mater. Res. 343–344, 351–356 (2012).
60. Phillips, R. P. & Fahey, T. J. The influence of soil fertility on rhizosphere effects in northern hardwood forest soils. Soil Sci. Soc. Am. J. 72, 453–461 (2008).
61. Ai, C., Liang, G. Q., Sun, J. W., Wang, X. B. & Zhou, W. Responses of extracellular enzyme activities and microbial community in both the rhizosphere and bulk soil to long-term fertilization practices in a flavo-aquic soil. Geoderma 173–174, 330–338 (2012).
62. Cheng, W. X., Johnson, D. W. & Fu, S. L. Rhizosphere effects on decomposition: controls of plant species, phenology, and fertilization. Soil Sci. Soc. Am. J. 67, 1418–1427 (2003).
63. Zhou, J. Z. et al. High-throughput metagenomic technologies for complex microbial community analysis: open and closed formats. MBio 6, e02288–14 (2015).
64. Hueso, S., Hernandez, T. & Garcia, C. Resistance and resilience of the soil microbial biomass to severe drought in semiarid soils: The importance of organic amendments. Appl. Soil Ecol. 50, 27–36 (2011).
65. Liu, Y. Z. et al. Decline in topsoil microbial quotient, fungal abundance and C utilization efficiency of rice paddies under heavy metal pollution across South China. PLoS ONE 7, e38858 (2012).
66. Garland, J. L. Patterns of potential C resources utilization by rhizosphere communities. Soil Biol. Biochem. 28, 223–230 (1996).
67. Buyer, J. S., Teasdale, J. R., Roberts, D. P., Zasada, I. A. & Manul, J. E. Factors affecting soil microbial community structure in tomato cropping systems. Soil Biol. Biochem. 42, 831–841 (2010).
68. Zelles, L. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterization of microbial communities in soil: a review. Biol. Fert. Soils 29, 111–129 (1999).
69. Zelles, L. Phospholipid fatty acid profiles in selected members of soil microbial communities. Chemosphere 35, 275–294 (1997).
70. Frostegård, A. & Bååth, E. The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. Biol. Fert. Soils 22, 59–65 (1996).

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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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