Stimulation of soil respiration by elevated CO$_2$ is enhanced under nitrogen limitation in a decade-long grassland study

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Whether and how CO$_2$ and nitrogen (N) availability interact to influence carbon (C) cycling processes such as soil respiration remains a question of considerable uncertainty in projecting future C-climate feedbacks, which are strongly influenced by multiple global change drivers, including elevated atmospheric CO$_2$ concentrations (eCO$_2$) and increased N deposition. However, because decades of research on the responses of ecosystems to eCO$_2$ and N enrichment have been done largely independently, their interactive effects on soil respiratory CO$_2$ efflux remain unresolved. Here, we show that in a multifactor free-air CO$_2$ enrichment experiment, BioCON (Biodiversity, CO$_2$, and N deposition) in Minnesota, the positive response of soil respiration to eCO$_2$ gradually strengthened at ambient (low) N supply but not enriched (high) N supply for the 12-y experimental period from 1998 to 2009. In contrast to earlier years, eCO$_2$ stimulated soil respiration twice as much at low than at high N supply from 2006 to 2009. In parallel, microbial C degradation genes were significantly boosted by eCO$_2$ at low but not high N supply. Incorporating those functional genes into a coupled C–N ecosystem model reduced model parameter uncertainty and improved the projections of the effects of different CO$_2$ and N levels on soil respiration. If our observed results generalize to other ecosystems, they imply widely positive effects of eCO$_2$ on soil respiration even in infertile systems.

Elevation of atmospheric CO$_2$ concentrations, owing to fossil fuel combustion and land-use changes, represents one of the greatest scientific and political concerns of the 21st century (1). Carbon (C) movement into the atmosphere annually from soils (i.e., soil CO$_2$ efflux or soil respiration) is much larger than annual C emissions from fossil fuel combustion (2), and thus even small changes in soil respiration could have significant impacts on the pace of change in atmospheric CO$_2$. Numerous studies have demonstrated that elevated CO$_2$ (eCO$_2$) has a direct stimulatory effect on rates of plant photosynthesis (3), and an indirect positive effect on soil respiration, which typically includes autotrophic respiration from plant roots and heterotrophic respiration from microbial decomposition of litter and soil organic matter (SOM). The eCO$_2$ stimulatory effect on soil respiration is commonly attributed to the following three mutually nonexclusive mechanisms from the actions of plants and microorganisms (4–7): enhanced root respiration associated with greater belowground biomass, enhanced microbial decomposition of fresh C due to greater supply of foliar and root-derived labile soil C, and increased microbial priming of old SOM fueled by this increased supply of labile soil C (4, 5). The stimulation of soil respiration by eCO$_2$ (7, 8) has the potential to greatly accelerate the future rate of increase in atmospheric CO$_2$ concentrations unless matched by an offsetting increase in net C uptake. Human activities have also increased nitrogen (N) deposition to natural ecosystems (9). N enrichment is a growing concern because it disturbs N-cycle processes in many ecosystems (9). Various studies have suggested that N addition can either increase (10, 11) or reduce (12–15) soil CO$_2$ flux, while other studies have suggested that N addition does not influence soil CO$_2$ flux (16, 17), depending on ecosystem type and season of the year.

Significance

The magnitude of CO$_2$ efflux from soils (resulting from autotrophic and heterotrophic respiration) is one of the largest uncertainties in projecting future carbon-climate feedbacks. Despite research over several decades, the magnitude, direction, and duration of such feedbacks and their underlying microbial mechanisms are poorly understood, especially in the context of potentially interacting global environmental changes. In a decade-long experiment examining the interactive effects of CO$_2$ and N enrichment, N limitation strengthened the stimulatory effects of elevated CO$_2$ on soil respiration, primarily via N mining during the decomposition of more recalcitrant organic compounds. This study also provides a strategy for integrating genomics information into ecosystem and Earth system models to improve carbon-cycle predictions.


The authors declare no competing interest.

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The stimulation of soil respiration by eCO₂ also could be strongly influenced by variability in ambient soil N availability and the rate of atmospheric N deposition (18). However, studies that have explored the interactive effects of eCO₂ and N on soil respiration are extremely scarce. For instance, an open-top study of young subtropical tree seedlings in contrasting eCO₂ and N treatments in transplanted soil found that response to eCO₂ was enhanced by high levels of N addition (10 g m⁻² yr⁻¹) in the earliest 2 y but unaffected by the same N supply in the subsequent year (19, 20). A free-air enrichment study in perennial grasslands also found no interaction between eCO₂ and N addition treatments over the first 2 y of the study (21). Given that many questions about such potential interactions remain unresolved (22), here we report on 12 y of results in that same grassland study, assessing whether interactions develop and, if so, what underlying mechanisms might drive them.

It is well known that N availability alters many aspects of ecosystems (12, 23, 24) and thus could hypothetically influence responses of soil respiration to eCO₂. Three potentially off-setting and interrelated mechanisms have been proposed. First, N limitation could affect belowground productivity and thus root respiration. For example, if N limitation constrains plant canopy development and the stimulatory effect of eCO₂ on photosynthesis, and thus limits total productivity belowground, root respiration will decline (24). On the other hand, in the same N limitation constraint on canopy development combined with stimulatory effects of eCO₂ on photosynthesis could increase plant investment of N in nutrient-absorbing systems (25, 26), favoring N allocation to roots at the expense of aboveground biomass. Such a shift in allocation could increase root respiration (27). Second, changes in root detrital production and exudation of labile C into soils can influence substrate supply that fuels soil microbial activity and heterotrophic respiration. Third, the supply of labile C into soils can influence decomposition of SOM through the priming effect, which would also influence soil heterotrophic respiration (28). Under N limitation, greater photosynthesis caused by eCO₂ could stimulate mining of N from SOM, and thus soil heterotrophic respiration, through enhanced priming mechanisms (29).

Although various studies indicate that N availability plays critical roles in mediating soil respiration (10–17, 23, 30, 31), divergent results are observed: positive (10, 11, 23), neutral (16, 17, 30), or negative (15, 18, 21, 31). Thus, the magnitude and duration of the CO₂ enhancement of soil respiration and its underlying mechanisms remain elusive, particularly under field settings. In addition, recent modeling efforts demonstrated the importance of understanding microbial C decomposition for more confidently extrapolating soil C cycling processes (32, 33). However, to date, it remains uncertain whether and how microbial processes influence the responses of terrestrial ecosystems to eCO₂ and N deposition and how best to incorporate information regarding microbial responses to eCO₂ and N into climate-C models for better simulation and prediction (32, 34, 35).

Herein, we report results from a well-replicated long-term (12 y at the time of sampling) CO₂ × N experiment, BioCON (Biodiversity, CO₂, and N deposition) (24), to elucidate the interactive effects of eCO₂ and N enrichment on soil respiration and their underlying mechanisms. From 1998 to 2009, we measured soil CO₂ efflux and other biochemical processes on 256 plots containing different numbers (1, 4, 9, or 16 species) and combinations (C₃ and C₄ grasses, forbs, and legumes) of perennial plant species at ambient CO₂ (aCO₂) or eCO₂ (+180 ppm) with either ambient N supply (aN) or enriched N supply (eN, i.e., +4 g N m⁻² yr⁻¹). Hereafter, we refer to these four treatment combinations as aCO₂-aN, eCO₂-aN, aCO₂-eN, and eCO₂-eN. The contrasting high versus low levels of N supply in this study was a rough proxy for a part of the worldwide range of N supply rates in soils as well as for times or places with low versus high N deposition (24). Thus, we posit that the results are relevant to understanding the potentially different responses to eCO₂ of both low versus high N fertility soils and contexts with low versus high N deposition. In 2009, we also assessed responses of microbial community functional gene structure to eCO₂ and N enrichment to gain insights into microbial regulation of soil respiration. In addition, we incorporated microbial functional trait information into ecosystem models to explore means of better prediction of C cycling. Our overarching hypothesis is that N limitation would accelerate the stimulatory effects of eCO₂ on soil respiration, primarily via microbial N mining mechanisms. We further explored the possibility that microbial functional trait information would greatly help to constrain the uncertainty of model parameters and hence significantly improve confidence in model simulations and predictions.

Results and Discussion

N Modulation of the Stimulatory Effect of eCO₂ on Soil Respiration.

Soil CO₂ efflux was measured ca. biweekly during the growing season (May to August) from 1998 to 2009. Overall, significantly (P < 0.01) higher soil respiration was observed at eCO₂ than aCO₂ at both low and high N supply (Fig. 1A), indicating that eCO₂ stimulated soil respiration, consistent with previous reports (6, 7). Along with significant main effects of CO₂, N, and plant species diversity as individual treatments, there were significant CO₂ × N (P = 0.03; Table 1) and CO₂ × N × year (P = 0.05) interactive effects on soil respiration, indicating that the stimulatory effect of eCO₂ on soil respiration was modulated by N supply and that this interaction varied with time. Although the effect of eCO₂ varied with plant diversity (P = 0.01 for the CO₂ × plant diversity interaction; Table 1), the CO₂ × N interaction was independent of plant diversity (P = 0.83 for the three-way interaction of CO₂ × N × plant diversity; Table 1).

To better identify the timing of the shift in the responses of soil respiration to eCO₂ at contrasting N supplies, four commonly used change-point tests—Petitt’s test, Buishand range test, Buishand U test, and standard normal homogeneity test (SI Appendix, Table S1)—were used. Our results indicated that 2005 was the breakpoint when the N influence on the stimulatory effects of eCO₂ on soil respiration significantly changed (SI Appendix, Table S1). Therefore, we have divided the whole experimental period into two phases: phase I from 1998 to 2005 and phase II from 2006 to 2009 (see Materials and Methods for details). Using this breakpoint, the CO₂ × N interactive effects on soil respiration significantly differed between these two phases, as indicated by a significant three-way interaction, CO₂ × N × phase, on soil respiration (P = 0.02; SI Appendix, Table S2). In phase I, eCO₂ significantly (P < 0.01) stimulated mean soil respiration regardless of N level (+22% vs. +24% at low and high N, respectively, Fig. 1B; P = 0.07 for the CO₂ × N interaction, SI Appendix, Table S3). In contrast, the CO₂ × N interaction became significant (P < 0.01; SI Appendix, Table S3) in phase II, and eCO₂ stimulated mean soil respiration by 40% at low N supply but by only 19% at high N supply (Fig. 1C). These results indicate that long-term N limitation strengthened the stimulatory effects of eCO₂ on soil respiration as the experiment proceeded.

Conceptually, the changing interactive effects of N and eCO₂ on soil respiration between phase I and phase II are most likely due to soil processes, plant characteristics, and microbial community structure (21, 34, 36–40). Similar to soil respiration, significant (P < 0.01) CO₂ × N × phase interactions were observed for soil net N mineralization rate and aboveground plant N concentration, but not for other soil and plant variables (SI Appendix, Table S2), indicating that there were temporal shifts in CO₂ × N effects on those two variables. By examining the CO₂ × N effect per year from 1998 to 2009, we found that the CO₂ × N effect on soil respiration was significantly correlated with that on soil net N mineralization rate (P = 0.05), aboveground plant N
concentration ($P = 0.04$), and aboveground plant C/N ratio ($P = 0.03$) (SI Appendix, Table S4). Further analysis revealed that eCO$_2$ had no effect on net N mineralization rate at both N supplies in phase I but significantly increased the mineralization rate at high, but not low N supply, in phase II (SI Appendix, Fig. S1A and B). In addition, aboveground plant N concentration was 8% lower at low than high N supply in phase I but was 20% lower in phase II (SI Appendix, Fig. S1 C and D). These data suggest that soil and plant N availability became more limited at low than high N supply as the time proceeded. The progressive N limitation could lead to less C allocation by plants to grow but more labile C inputs by eCO$_2$ at low N supply (41), stimulating SOM decomposition and soil respiration. Collectively, the more positive soil respiration response to eCO$_2$ at lower than higher N supply in phase II is probably related, at least in part, to the N-mediated phase shift of soil and plant N dynamics in response to eCO$_2$. Similarly, microbes play important roles in regulating the interactive effects of CO$_2$ and N on soil respiration, as discussed in the following section.

**Roles of Microbiological Processes.** The stimulation of soil respiration by eCO$_2$ might be caused by changes in heterotrophic microbial processes and/or root-associated autotrophic processes (26). However, partitioning soil respiration into autotrophic and heterotrophic respiration is generally difficult (42). Thus, we used root biomass as a proxy to determine whether autotrophic respiration was a major component of our observed soil efflux interaction over time, given certain assumptions and caveats (43, 44). Root respiration is driven by a number of factors, including current soil temperature, prior soil temperature (which could drive acclimation), tissue N concentration, and soil water (45–48), as well as root biomass (43). Several of these factors (e.g., soil temperature, soil moisture, and root N concentration) showed no significant difference between eCO$_2$ and aCO$_2$ plots at both low and high N supply (SI Appendix, Table S5). Hence, although translating root biomass into absolute values of simulated soil respiration is challenging, assuming that root biomass is a reliable measure of relative differences in autotrophic respiration seems sound.

To evaluate whether root biomass mirrored the shifting N effect on eCO$_2$ stimulation of soil respiration, we examined its responses to CO$_2$ and plant N. In phase I, eCO$_2$ stimulated root biomass to similar extents at low (11%) and high N (14%) supply (SI Appendix, Fig. SIE), which might partially account for the parallel responses of soil respiration to eCO$_2$ at low and high N supply (Fig. 1B). In contrast, live root biomass was stimulated more by eCO$_2$ at high N (22%) than low N (14%) supply in phase II (SI Appendix, Fig. S1F), whereas soil respiration was stimulated less by eCO$_2$ at high N (19%) than at low N (40%) supply (Fig. 1C). Thus, live root biomass and associated autotrophic respiration responses likely were not the main drivers of the shifting responses of soil respiration to CO$_2$ and N treatments, as mentioned above (SI Appendix, Table S4).

To examine the potential importance of different microbial processes in explaining the phase shift in CO$_2$ × N interactive effects on soil respiration, we analyzed the composition and abundance of microbial functional genes for soil samples collected in 2009 using GeoChip (49). GeoChip is a generic microarray targeting hundreds of functional gene categories important to biogeochemical, ecological, and bioremediation processes. As predicted, the functional community structure was significantly shifted by CO$_2$, N, and plant diversity treatments (SI Appendix, Table S6). All functional gene categories involved in C degradation and N cycling showed significant ($P \leq 0.05$) or marginally significant ($P \leq 0.10$) correlations across plots with mean soil CO$_2$ efflux in phase II (SI Appendix, Table S7), but none of them did so in phase I ($P > 0.10$). Thus, microbial communities could play an important role in mediating the phase shift of N-induced differences in the soil respiration response to eCO$_2$.

Directly relevant to questions of CO$_2$ × N interactive effects on soil CO$_2$ efflux in phase II, many microbial genes involved in C degradation and N cycling were significantly stimulated or suppressed by eCO$_2$, but in different ways at low than at high N supply (Fig. 2). In general, at low N supply, most genes related to C degradation and N cycling were stimulated by eCO$_2$ (Fig. 2A), whereas at high N supply most were slightly suppressed (Fig. 2B). Among those genes, antagonistic CO$_2$ × N effects, whereby the

### Table 1. The main and interactive effects of CO$_2$, N, and plant diversity (PD) on soil CO$_2$ efflux measured from 1998 to 2009 based on repeated-measures mixed model across 296 plots

<table>
<thead>
<tr>
<th></th>
<th>$F$</th>
<th>$p$</th>
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<tbody>
<tr>
<td>CO$_2$</td>
<td>763.33</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>N</td>
<td>59.56</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>PD</td>
<td>692.89</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Year</td>
<td>410.76</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CO$_2$ × N</td>
<td>4.63</td>
<td>0.03</td>
</tr>
<tr>
<td>CO$_2$ × PD</td>
<td>13.99</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>N × PD</td>
<td>2.34</td>
<td>0.12</td>
</tr>
<tr>
<td>CO$_2$ × year</td>
<td>9.02</td>
<td>0.01</td>
</tr>
<tr>
<td>N × year</td>
<td>15.69</td>
<td>0.01</td>
</tr>
<tr>
<td>PD × year</td>
<td>4.32</td>
<td>0.03</td>
</tr>
<tr>
<td>CO$_2$ × N × PD</td>
<td>0.04</td>
<td>0.83</td>
</tr>
<tr>
<td>CO$_2$ × N × year</td>
<td>3.73</td>
<td>0.05</td>
</tr>
<tr>
<td>CO$_2$ × PD × year</td>
<td>3.02</td>
<td>0.08</td>
</tr>
<tr>
<td>N × PD × year</td>
<td>0.16</td>
<td>0.69</td>
</tr>
<tr>
<td>CO$_2$ × N × PD × year</td>
<td>0.51</td>
<td>0.47</td>
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Significant ($P < 0.05$) effects are bolded.

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combined CO2 and N effect on functional gene abundance was less than additive, were dominant (67%) (SI Appendix, Table S8), but no synergistic interactive effects were observed (50). Additionally, to summarize gene responses across all 14 assessed gene categories (in addition to those in Fig. 2A and B), we determined the percentage of the significantly shifted genes (for each function) that increased versus decreased at eCO2 at each of the two N supply rates. A markedly greater percentage (59%) of affected genes were stimulated by eCO2 at low than at high N supply (Fig. 2C vs. Fig. 2D; P = 0.04 for CO2 × N effect on the relative abundance of those genes; SI Appendix, Table S6). Altogether, the changes in various functional gene abundances suggest enhanced microbial decomposition response to eCO2 at low N supply. These results are consistent with the above experimental observations that the effects of eCO2 on soil respiration in phase II were more enhanced at low N than at high N supply.

In parallel with changes in overall community functions, CO2 and N showed antagonistically interactive effects on a variety of bacterial genes (26% of the bacterial genes on the arrays) related to C degradation and N cycling, which were significantly (P < 0.05) stimulated by eCO2 at low N supply but were suppressed by eCO2 at high N supply (SI Appendix, Table S9). However, only three fungal genes (15%) related to C degradation were antagonistically affected by CO2 and N; while most of the fungal genes (85%) showed similar responses to eCO2 at the two N supplies. The results suggest that high N supply suppressed the eCO2 effect on bacterial functional capacity, thus potentially shifting the microbial community toward relatively higher fungal capacity.

Two major competing, but nonexclusive, theories have been proposed to explain the mechanisms underlying the impacts of N on eCO2-induced microbial decomposition of SOM (23). Herein, we identify which ones may be at work in BioCON. The “stoichiometric decomposition” theory posits that microbial activity (e.g., decomposition and respiration) will be highest when the stoichiometry of substrates matches that of microbial demand and C and N colimit decomposition (51). Accordingly, soil respiration will be stimulated more by eCO2 at high than at low N supply (SI Appendix, Table S10). This is because with higher substrate C/N ratios at eCO2 and low N supply microbes are unable to meet their N demand, which may suppress microbial C decomposition rates and disfavor rapidly growing microbes (r-strategists) that primarily use labile C. In contrast, the “microbial N mining” theory asserts that, at low N availability, microbes use labile C as an energy source to decompose recalcitrant SOM to acquire N, accelerating microbial decomposition of SOM and favoring genes involved in recalcitrant C degradation (slow-growing k-strategists) (SI Appendix, Table S10) (52).

Data from BioCON in phase II are more consistent with the microbial N limitation and N mining theory, eCO2 significantly increased soil net N mineralization at high, but not low, N supply (SI Appendix, Fig. S1B) and the aboveground plant N concentration and total plant N pool were considerably less under low than high N supply (SI Appendix, Fig. S1D and H). Those results suggest limited N availability at low N supply may not have met microbial N demand, and hence microbial C decomposition was stimulated to acquire N. As a likely result, most genes involved in C and N cycling were stimulated by eCO2 at low N supply (Fig. 2A), in contrast to their suppression by eCO2 at high N supply (Fig. 2B). Alternatively, eCO2 weakly (P = 0.08) decreased soil C/N ratio at low but not high N supply (SI Appendix, Fig. S1J). As microbial C content relative to N is one to two orders of magnitude lower than that of plants (51), a decreased soil substrate C/N ratio may relieve nutrient limitation and promote substrate-induced microbial respiration (53), echoing the stoichiometric decomposition theory. It should be noted that N addition could reduce soil respiration (12–15) by suppressing microbial decomposition via both N mining and substrate stoichiometry, which are time-dependent and may take a long time to appear. This could be one of the main reasons that the N-induced suppression of the stimulatory effects of eCO2 on soil respiration was more obvious in phase II.

Decomposition Modeling Enabled by Microbial Functional Traits. As demonstrated above, microbial functional community structure likely plays an important role in mediating responses of soil respiration to eCO2 and N availability. Such information is a prerequisite for predicting how the soil microbial community and associated functions respond to multiple global change factors. The next urgent need is to translate such conceptual understanding into an ecosystem model-based quantitative framework.
because process-based microbial-explicit ecosystem models can provide mechanistic insights, integration, and scenario testing not available from or possible with experiments (54). In this regard, microbial-explicit ecosystem models will enable us to mechanistically simulate large-scale experiments that would be too costly to establish in reality and predict their future dynamics. However, a grand challenge in ecology is how to integrate microbial functional traits into ecosystem models to improve their performance and predictive ability (55).

To address the above challenge, we incorporated the GeoChip-detected microbial functional genes into the C–N coupled microbial-enzyme decomposition (MEND) model (SI Appendix, Fig. S2 and Tables S1–S15). We used tMEND to denote the MEND model parameterized with traditional observations such as soil CO2 efflux and mineral N concentrations. For comparison, gMEND refers to the MEND model calibrated with additional GeoChip-based microbial functional gene abundance data (Fig. 3B and SI Appendix, Fig. S3A). We compared the results of these two microbial models (tMEND, gMEND) plus a third model, the nonmicrobial C-only terrestrial ecosystem (TECO) model (SI Appendix, Fig. S2B). In addition to the best fit between observed and simulated soil CO2 efflux and mineral N (NH4+ and NO3−) concentrations, we constrained the model by achieving the highest goodness of fit between MEND-modeled relative changes in enzyme concentrations and GeoChip-detected relative changes in oxidative and hydrolytic gene abundances in response to eCO2 (SI Appendix, Table S11).

The eCO2-induced changes in hydrolytic and oxidative genes observed by GeoChip were consistent with changes simulated by gMEND but not tMEND (Fig. 3A). Also, the parameter uncertainty (i.e., coefficient of variation) of gMEND was considerably reduced compared to both tMEND (by 35%) and the nonmicrobial C-only TECO model (by 86%; Fig. 3B). As a result, the gMEND model was able to simulate the observed soil CO2 efflux at aCO2−aN relatively well (R2 = 0.61; Fig. 3C). In addition, the gMEND model that had been calibrated only with the data at aCO2−aN was further validated against independent datasets from the other three CO2 and N treatments. The performance was almost as good as model calibration for ambient conditions (5% less variance explained on average) (R2 = 0.53 to 0.59; Fig. 3D). In contrast, the TECO model explained considerably less variation in observed soil respiration at the other three treatment combinations (R2 = 0.35 to 0.44; Fig. 3D) than at ambient conditions (explaining about 16% less of the variance). These differences suggest that gMEND better adjusts for CO2 and N effects than TECO. Finally, gMEND-simulated ammonium and nitrate concentrations also agreed fairly well with the observations (SI Appendix, Fig. S3B). Altogether, the above results suggested that the gMEND model can capture the dynamics of soil CO2 efflux reasonably well, comparable to or better than several previously field modeling studies (56, 57).

We further estimated eCO2-induced soil C loss via heterotrophic respiration. Our simulations showed that eCO2 would cause 38% and 20% more heterotrophic respiration at low and high N supply (Fig. 3E), respectively, and that enriched N would lead to 18% and 2% more heterotrophic respiration at aCO2 and eCO2 (Fig. 3E), respectively. We then asked what the implications might be if such results were general for grasslands globally. Applying our results to the world’s grasslands based on the International Geosphere-Biosphere Program classification scheme and the estimated annual soil respiration from grasslands between 2001 and 2009 (58), eCO2 (+180 ppm) alone would increase heterotrophic respiration by 1.6 ± 0.1 Pg C·yr−1 whereas enriched N (+4 g N·m−2·yr−1) alone would increase heterotrophic respiration by 0.8 ± 0.2 Pg C·yr−1. However, combined eCO2 and enriched N would increase heterotrophic respiration by 1.7 ± 0.2 Pg C·yr−1 across global grasslands, 29% less than the additive effects of eCO2 and enriched N alone. Thus, interactions noted herein could be significant globally.

Although our modeling results via calibration (Fig. 3A–C) and validation (Fig. 3D) indicated that the gMEND could encapsulate the dynamics of soil CO2 efflux fairly well, about 40% of the variation was not captured, likely for two primary reasons. First, various experimental measurements such as gross primary productivity, soil CO2 efflux, temperature, moisture, and microbial traits were highly variable and some were uncertain, which could contribute to the discrepancy between model simulations and experimental observations. Second, the MEND model used in

![Fig. 3. Model simulations. (A) Comparison of eCO2-induced percent changes of hydrolytic and oxidative enzymes observed by GeoChip to the simulated effects by gMEND and traditional MEND without gene information (tMEND) at low N supply. The GeoChip data were obtained from the samples from 2009. (B) Parameter uncertainty quantified by the coefficient of variation (CV) for the nonmicrobial C-only TECO, tMEND, and gMEND models; the bars show mean CV of 10 calibrated parameters represented by dots. (C) Model calibration with the soil respiration (R0, 1998 to 2009) at aCO2−aN. (D) Model validations were performed using R0 at eCO2−aN, aCO2−aN, and eCO2−enN for gMEND and TECO. (E) Percent changes of gMEND-simulated heterotrophic respiration (R0) between different CO2 and N levels. The error bars represent SEs. P values of the permutation t test are labeled as **P < 0.01.](https://www.pnas.org/doi/abs/10.1073/pnas.2013292117)
this study does not consider the differential roles of diverse microbial communities (e.g., bacteria and saprotrophic and mycorrhizal fungi) in regulating C–N cycling in response to eCO2 and enriched N supply owing to our poor understanding of these processes (8). Incorporating additional biological processes and their interactions into the MEND model may improve the modeling of soil CO2 efflux and its response to environmental change (8). Nevertheless, this study demonstrates the feasibility of integrating massive omics information into ecosystem models for better predictions of the soil C response to eCO2 and enriched N.

Conclusions

We found that the positive effect of eCO2 on soil respiration at low N supply was greater in years 9 to 12 than in years 1 to 8 of a long-term experiment and that changes in microbial functional traits, such as functional genes involved in C and N cycling processes, as well as temporal shifts in soil and plant N availability, likely underlie this dynamic. These findings would, if general, have important implications for predicting the responses of ecosystems to future environmental changes. For example, because N limitation is widespread in natural ecosystems, considerable stimulation of soil respiration in response to rising CO2 concentration might occur. Pervasive N deposition due to anthropogenic activities could offset, at least partially, the stimulation of soil respiration by elevated atmospheric CO2, and thus could weaken the positive feedback between the terrestrial C cycle and climate change. Our study also shows that whether microbially mediated feedback to rising CO2 concentrations and climate change is positive or negative depends on microbial functional groups and whether their associated functions are stimulated by eCO2, suggesting the necessity of integrating microbial functional traits into climate-C models for better prediction (34, 55). As expected, incorporating those functional genes into a coupled C–N ecosystem model substantially reduced model parameter uncertainty and improved the prediction of soil respiration in response to eCO2 and enriched N supply. Although further model development, calibration, and validation of a microbially enabled model will require rigorous benchmarking with observations, this study serves as a step forward to mechanistically assimilate microbial functional traits into climate-C cycle modeling.

Materials and Methods

Experimental Design and Sampling. The BioCON experiment contains 296 main plots with a fully factorial 2 × 2 × 4 combinations of three treatments: CO2 (ambient vs. +180 ppm), N deposition (ambient vs. +4 g N m–2 y–1), and plant diversity (1, 4, 9, or 16 species) (59). Plots were established with di- main plots with a fully factorial 2 × 2 × 4 combinations of three treatments: CO2 (ambient vs. +180 ppm), N deposition (ambient vs. +4 g N m–2 y–1), and plant diversity (1, 4, 9, or 16 species) (59). Plots were established with di-
eCO2 at low or high N supply in every month of the growing season. The N influence was then calculated as RR at high N supply minus RR at low N supply, representing the CO2 × N interaction. The annual mean value of the N influence was calculated for each year. Four commonly used change-point tests, including Buishand range test, Buishand U test, standard normal homogeneity test, and Pettitt's test, were performed on the annual mean values of the N influence. Because no soils were collected for microbial analysis in phase I, most of the statistics-based mechanistic analyses were focused on phase II.

For each year from 1998 to 2009, data points of soil CO2 efflux (micro-moles per mule2 per second) that were higher than mean plus 1.96 SDs or lower than mean minus 1.96 SDs of all data points in a plot were regarded as outliers and removed before the analysis (65). By doing this, we reduced the within-plot variation in soil CO2 efflux measurements to enhance the statistical power. We used the same approach to identifying and excluding outliers for other soil and plant variables, including soil net N mineralization rate (milligrams per kilogram per day), soil temperature (degrees Celsius), soil moisture, soil pH, soil C:N ratio, plant N concentration (percent), plant C:N ratio, plant biomass (grams per meter2) and plant N pool (grams per meter2). Net N mineralization data in 2008 were contaminated and thus were not included in the analysis (41). The significance of CO2 × N effects and CO2 × N × phase effects on soil CO2 efflux, soil, and plant variables was tested using repeated-measures mixed models following the previous method (66). The CO2 × N effects (N influence on the eCO2 effect) on each of the soil and plant variables and on soil CO2 efflux were calculated per year from 1998 to 2009, then relationships between CO2 × N effects on soil/plant variables and on soil CO2 efflux were examined using Pearson correlation.

The eCO2 effects on soil and plant variables as well as microbial functional genes at low and high N supply were calculated based on Eqs. 1 and 2:

\[
e_{\text{CO2 effect}} = \frac{e_{\text{CO2}} - a_{\text{CO2}}}{\text{N supply}} \times 100\% \tag{1}
\]

\[
e_{\text{CO2 effect-thick}} = \frac{e_{\text{CO2}} - a_{\text{CO2}}}{\text{N supply}} \times 100\% \tag{2}
\]

where \( e_{\text{CO2}} \) and \( a_{\text{CO2}} \) represent mean of soil CO2 efflux, soil variables, plant variables, or the relative abundance of microbial functional genes in eCO2-N, eCO2-N, aCO2-N, and aCO2-N plots, respectively. Permutation testing was conducted to examine the significance of the eCO2 effect on plant and soil properties at both low and high N supply (67). At the low or high N supply, the significance of eCO2 effect on the abundance of each functional gene (total abundance of all probes of this gene; SI Appendix, Table S8) was examined by response ratio with 95% confidence intervals of gene abundance differences between eCO2 and aCO2 plots. We also examined the eCO2 effect on the abundance of each gene probe by response ratio. Of all significantly changed probes of an individual gene, we calculated the percentage of stimulated and suppressed probes by eCO2. Then, we calculated the averaged percentages of stimulated and suppressed probes across genes in different gene categories for C cycling, including starch, hemicellulose, cellulose, chitin, pectin, aromatics and lignin degradation, gene categories for N cycling, including assimilatory/dissimilatory N reduction, denitrification, ammonification, nitrification, and N fixation as well as gene categories for phosphorus (P) cycling, including P fixation and P utilization.

To determine the direction (additive, synergistic, or antagonistic) of interactive effects of CO2 and N addition, we compared the observed effects (OEs, i.e., combined eCO2 and enriched N effects) and the expected effects (EEs), that is, additive effects of eCO2 alone and enriched N alone (50). For each functional gene, OE was calculated as follows: 100% × (expected EE) + 100% × (expected eCO2). The interactive effects are additive when OE is not different from EE. Interactive effects are synergistic if OE is significantly higher than EE or antagonistic if OE is significantly lower than EE. The significance of the interactive CO2 and N effect on each functional gene was tested by the permutational multivariate analysis of variance (Adonis) using the abundance matrix of this microbial functional gene.

Data Availability. Genomic microarray data have been deposited in Gene Expression Omnibus (accession no. GSE98512).

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