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Distinct responses of soil microbial communities to elevated CO₂ and O₃ in a soybean agro-ecosystem

Zhili He^{1,7}, Jinbo Xiong^{1,2,7}, Angela D Kent³, Ye Deng¹, Kai Xue¹, Gejiao Wang⁴, Liyou Wu¹, Joy D Van Nostrand¹ and Jizhong Zhou^{1,5,6}

¹Institute for Environmental Genomics and Department of Microbiology and Plant Biology, the University of Oklahoma, Norman, OK, USA; ²Faculty of Marine Sciences, Ningbo University, Ningbo, China; ³Department of Natural Resources and Environmental Sciences, University of Illinois at Urbana-Champaign, Urbana, IL, USA; ⁴State Key Laboratory of Agricultural Microbiology, College of Life Science and Technology, Huazhong Agricultural University, Wuhan, China; ⁵State Key Joint Laboratory of Environment Simulation and Pollution Control, School of Environment, Tsinghua University, Beijing, China and ⁶Earth Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, USA

The concentrations of atmospheric carbon dioxide (CO₂) and tropospheric ozone (O₃) have been rising due to human activities. However, little is known about how such increases influence soil microbial communities. We hypothesized that elevated CO₂ (eCO₂) and elevated O₃ (eO₃) would significantly affect the functional composition, structure and metabolic potential of soil microbial communities, and that various functional groups would respond to such atmospheric changes differentially. To test these hypotheses, we analyzed 96 soil samples from a soybean free-air CO₂ enrichment (SoyFACE) experimental site using a comprehensive functional gene microarray (GeoChip 3.0). The results showed the overall functional composition and structure of soil microbial communities shifted under eCO₂, eO₃ or eCO₂+eO₃. Key functional genes involved in carbon fixation and degradation, nitrogen fixation, denitrification and methane metabolism were stimulated under eCO₂, whereas those involved in N fixation, denitrification and N mineralization were suppressed under eO₃, resulting in the fact that the abundance of some eO₃-suppressed genes was promoted to ambient, or eCO₂-induced levels by the interaction of eCO₂+eO₃. Such effects appeared distinct for each treatment and significantly correlated with soil properties and soybean yield. Overall, our analysis suggests possible mechanisms of microbial responses to global atmospheric change factors through the stimulation of C and N cycling by eCO₂, the inhibition of N functional processes by eO₃ and the interaction by eCO₂ and eO₃. This study provides new insights into our understanding of microbial functional processes in response to global atmospheric change in soybean agro-ecosystems.

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Introduction

Because of fossil fuel combustion and land-use changes, the global atmospheric carbon dioxide (CO₂) concentration has increased by more than 30% since the industrial revolution, and is projected to reach 700 p.p.m. by 2100 (IPCC, 2007). In addition, the average O₃ concentration is

expected to increase by 1–2% per year and reach 70 p.p.b. by 2100 (IPCC, 2007; Sitch *et al.*, 2007). Such increases will be more rapid and have significant impacts on plant productivity, soil carbon and nitrogen dynamics and ecosystem functioning if anthropological activities continue unabated in the future (IPCC, 2007). Soybean, one of the largest food crops with an annual world production of >250 million metric tons in 2012 (http://www.soystats.com/2012/page_30.htm), is sensitive to O₃ (Morgan *et al.*, 2003). The productivity/yield of soybean largely depends on the ecosystem functional processes, such as nutrient cycling (for example, carbon and nitrogen fixation, nitrification, denitrification and methane cycling) and residue decomposition governed by microbial

Correspondence: Z He or J Zhou, Institute for Environmental Genomics and Department of Microbiology and Plant Biology, University of Oklahoma, 101 David L Boren Blvd., Norman, OK 73019, USA.

E-mail: zhili.he@ou.edu or jzhou@ou.edu

⁷These authors contributed equally to this work.

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communities and their dynamics (Meriles *et al.*, 2009). Therefore, toward sustainable agro-ecosystems, it is necessary to understand the effect of elevated CO₂ (eCO₂) and elevated O₃ (eO₃) on the functional diversity, composition, structure, metabolic potential and dynamics of soil microbial communities and their linkages with ecosystem functioning.

CO₂ fertilization effects are well established by increased plant photosynthesis, growth, resource allocation and altered ecosystem functions (Reich *et al.*, 2001; Ainsworth *et al.*, 2002; Ainsworth and Long, 2005; Luo *et al.*, 2006; Reich, 2009; Lindroth, 2010; Drake *et al.*, 2011; van Groenigen *et al.*, 2011; Zak *et al.*, 2011; Biswas *et al.*, 2013; Kumari *et al.*, 2013; Twine *et al.*, 2013), but the magnitude of eCO₂ stimulation may be constrained by the nitrogen (N) supply (Zak *et al.*, 2003; Reich *et al.*, 2006) because of progressive N limitation under eCO₂ (Luo *et al.*, 2004; Reich *et al.*, 2006). In contrast, O₃ is a phytotoxic compound potentially suppressing crop yields and reducing above-ground plant growth, and more importantly, it may reduce below-ground functional processes, such as root growth, carbon (C) allocation and soil N status (Morgan *et al.*, 2003; Feng and Kobayashi, 2009; Betzelberger *et al.*, 2010; Lindroth, 2010; Zak *et al.*, 2012; Biswas *et al.*, 2013; Kumari *et al.*, 2013). Under eCO₂ and eO₃, it has been suggested that CO₂ might alleviate the negative effects of O₃ on the above-ground processes by a decrease of O₃ flux into leaves and by an increase of available photosynthates that may be used for detoxification processes (Allen, 1990; McKee *et al.*, 1997; Volin *et al.*, 1998). However, the impact of eCO₂ and/or eO₃ on the functional diversity, composition, structure and function of soil microbial communities is poorly understood. For example, no detectable effects of eCO₂ on microbial community structure, microbial activity, potential soil N mineralization or nitrification were observed at a sweetgum free-air CO₂ enrichment (FACE) experiment in TN, USA (Austin *et al.*, 2009), whereas in a no-till wheat-soybean rotation agro-ecosystem, the community composition and structure significantly affected by eCO₂ but not by eO₃ or eCO₂ + eO₃ (Cheng *et al.*, 2011). Recently, more studies suggest that eCO₂ and/or eO₃ significantly alter microbial community composition, structure, functional potential/activity, interaction network and/or dynamics (Lesaulnier *et al.*, 2008; Blagodatskaya *et al.*, 2010; Drigo *et al.*, 2010; Feng *et al.*, 2010; Zhou *et al.*, 2010, 2011; He *et al.*, 2010b, 2012b; Deng *et al.*, 2012; Drigo *et al.*, 2013; Li *et al.*, 2013). In addition, it has been shown that the response of soil microbial communities to global change factors may be directly or indirectly mediated by plant genotypes/cultivars, the diversity of plant assemblages and/or other environmental factors (Talhelm *et al.*, 2009; Singh *et al.*, 2010; Drigo *et al.*, 2013; Li *et al.*, 2013). In addition, the effect of multiple global change factors

(for example, eCO₂, eO₃, warming and precipitation) and their interactions on microbial communities might be highly uncertain (Castro *et al.*, 2010; Gutknecht *et al.*, 2012). Therefore, it is important to comprehensively examine the effect of eCO₂ and eO₃ on soil microbial communities.

The advances of metagenomic technologies such as high-throughput sequencing (Margulies *et al.*, 2005; Caporaso *et al.*, 2012; Loman *et al.*, 2012) and functional gene arrays (He *et al.*, 2007, 2010a, 2012a) have revolutionized our analysis of microbial communities. For example, GeoChip 3.0, containing about 28 000 probes and covering about 57 000 genes in 292 functional groups, such as those involved in biogeochemical cycling of C, N, sulfur (S) and phosphorus (P) (He *et al.*, 2010a), has been applied to analyze soil microbial communities from various experimental sites (He *et al.*, 2010b; Zhou *et al.*, 2012; Li *et al.*, 2013) and other habitats (He *et al.*, 2012c). All results have demonstrated that GeoChip is a powerful tool to study the functional diversity, composition, structure and metabolic potential of microbial communities and link the microbial community structure to ecosystem functioning.

In this study, we hypothesize: (i) that the functional composition and structure of the soil microbial community would alter via changes in soil C and N inputs and soil chemistry (Dijkstra *et al.*, 2005; Adair *et al.*, 2009); and (ii) that various microbial functional groups (for example, C fixers, C degraders, N fixers and denitrifiers) would respond differentially due to changes in nutritional groups of microorganisms, leading to microbial utilization of more complex organic matter and C economy by plants and microorganisms (Elhottova *et al.*, 1997) in response to eCO₂ and/or eO₃. To test these hypotheses, GeoChip 3.0 (He *et al.*, 2010a) was used to detect functional genes and their associated populations. This study was conducted in a soybean FACE (SoyFACE) experimental site in Champaign, IL, USA. SoyFACE provides several advantages: (i) strong background knowledge about the effect of eCO₂ and eO₃ on soybean physiology, growth, yield and stress responses (Ainsworth *et al.*, 2002; Morgan *et al.*, 2003; Ainsworth and Long, 2005; Morgan *et al.*, 2005; Rogers *et al.*, 2009; Betzelberger *et al.*, 2010); (ii) eliminating effects of plant diversity on soil microbial communities; and (iii) minimizing progressive N limitation at eCO₂ with indeterminate and nodulating soybean. The results showed the functional composition, structure and metabolic potential of microbial communities significantly altered under eCO₂, eO₃ and eCO₂ + eO₃, and such changes appeared distinct for each treatment and significantly correlated with soil properties and soybean yield. This study has important implications for microbial responses to global change in soybean agro-ecosystems.

Materials and methods

This is a summary description of experimental site, sampling and methods used in this study. More detailed information is provided in Supporting Information A.

Site description and sampling

This study was conducted at the SoyFACE experimental site in Champaign, IL, USA (40°02'N, 88°14'W) (<http://www.soyface.uiuc.edu/index.htm>) in 2008. The 32-h SoyFACE experiment was established on a farmland that had been cultivated with an annual rotation of soybean, *Glycine max* (L.) Merr. and corn, *Zea mays* L. for more than 25 years, and the soil at the site is a Drummer fine-silty, mixed, mesic Typic Endoaquoll, typical of wet, dark-colored 'prairie soils' in northern and central Illinois (Pujol Pereira *et al.*, 2011). More soil background properties, including soil pH, moisture, Bray P, K, Ca and Mg were previously documented (Peralta and Wander, 2008). SoyFACE aims to discover the effects of atmospheric change on the agronomy, productivity and ecology of Midwestern agro-ecosystems planted in a typical corn-soy rotation. The experiment was a randomized complete block design ($n = 4$) with each block containing four treatments: (i) ambient (with ~400 p.p.m. CO₂ and ~37.9 p.p.b. O₃); (ii) eCO₂ (~550 p.p.m.); (iii) eO₃ (~61.3 p.p.b.); and (iv) eCO₂ + eO₃ (~550 p.p.m. CO₂ and ~61.3 p.p.b. O₃). A total of 96 soil samples were collected in 2008 October from four soybean (*Glycine max* Merr.) plots under each of four treatments at both surface soil (0–5 cm) and subsoil (5–15 cm) layers with 48 samples for each soil layer, and 12 samples (three subsamples for each plot) for each treatment. All soil samples were immediately transferred to the laboratory and stored at –80 °C or 4 °C until DNA extraction or soil property analyses.

Crop yield and soil property analysis

Annual crop yield and soil property analyses were collected for each plot as previously described (Twine *et al.*, 2013). To estimate the historical effects of eCO₂ on seed yield production before the time of our sampling, soybean yield data from 2004 to 2006 were averaged. Soil NO₃-N and NH₄-N were extracted with 1.0 M KCl solution and quantified by a Flow Injection Autoanalyzer. Soil organic C and total N were determined using a LECO Truspec dry combustion carbon analyzer.

DNA extraction, amplification, target preparation and microarray hybridization

For each sample, soil microbial community DNA was extracted and purified as described previously (Zhou *et al.*, 1996), and all DNA samples met

the criteria: 260 nm/280 nm > 1.70, and 260 nm/230 nm > 1.8. Each purified DNA (50 ng) was first amplified using whole community genomic amplification (Wu *et al.*, 2006), and 3.0 µg of amplified DNA was labeled and hybridized with GeoChip 3.0 on an HS4800 Pro Hybridization Station (Tecan US, Durham, NC, USA) at 42 °C for 16 h (He *et al.*, 2010b).

GeoChip imaging, data processing and statistical analysis

Hybridized GeoChips were analyzed as previously described (He and Zhou, 2008; He *et al.*, 2010b). A gene with a minimum of three positives out of 12 replicates for each treatment was considered positive and used for further statistical analyses. Permutational multivariate analysis of variance (PERMANOVA) was used to evaluate the contribution of various factors to microbial community variations with the Adonis function, and to partition sums of squares from a centroid based on a Bray-Curtis dissimilarity matrix implemented in R (R Development Core Team, 2011). Significance tests among four treatments were analyzed by multi-way analysis of variance (Geladi, 1989). Detrended correspondence analysis was used to determine the overall change in microbial community structures (He *et al.*, 2010b).

Results

Effects of eCO₂, eO₃ and eCO₂ + eO₃ on crop yield and soil properties

Historical soybean yields were significantly ($P < 0.05$) greater by ~14% and ~12% under eCO₂ and eCO₂ + eO₃, respectively, whereas eO₃ only caused an insignificant decrease (~2.3%) in soybean yield (Table 1). Concentrations of soil nitrate (NO₃-N), ammonium (NH₄-N) and total nitrogen (TN) as well as soil total carbon (TC) and the TC/TN ratio (C/N) showed three different trends in both surface soil and subsoil samples. First, NO₃-N was significantly ($P < 0.05$) higher in the subsoil under eO₃ and eCO₂ + eO₃ compared with ambient or eCO₂ samples but not significantly different between ambient and eCO₂, or eO₃ and eCO₂ + eO₃. Second, NH₄-N was significantly ($P < 0.05$) lower in the surface soil under eO₃ compared with ambient but not significantly different among eCO₂, eO₃ and eCO₂ + eO₃. Third, NO₃-N was significantly ($P < 0.05$) different between two soil depths under ambient, eCO₂ or eO₃ despite no significant difference under eCO₂ + eO₃. However, no significant changes were seen in TN, TC or C/N among different treatments or between two soil depths (Table 1). These results indicated that eCO₂ and/or eO₃ affected crop yield and soil N status.

Table 1 Effects of eCO₂, eO₃ and eCO₂ + eO₃ on crop yield and soil properties at both surface soil and subsoil

	Relative yield (%) ^a	Depth (cm)	NO ₃ -N (lb/a)	NH ₄ -N (lb/a)	TN (%)	TC (%)	C/N
Ambient	100.00 ^b	0–5	18.1 ± 8.16 ^A	8.0 ± 1.43 ^A	0.20 ± 0.021 ^A	2.6 ± 0.34 ^A	12.5 ± 0.86 ^A
		5–15	11.6 ± 1.73 ^b	8.9 ± 4.00 ^a	0.19 ± 0.021 ^a	2.4 ± 0.39 ^a	12.4 ± 0.89 ^a
		<i>P</i>	0.0131	0.4707	0.2559	0.1942	0.7822
eCO ₂	113.97 ^a	0–5	17.2 ± 4.73 ^A	7.4 ± 1.84 ^{AB}	0.21 ± 0.031 ^A	2.7 ± 0.44 ^A	12.9 ± 0.56 ^A
		5–15	12.6 ± 2.85 ^b	6.8 ± 1.25 ^a	0.22 ± 0.040 ^a	2.8 ± 0.69 ^a	12.7 ± 4.00 ^a
		<i>P</i>	0.0086	0.3603	0.5008	0.6762	0.8654
eO ₃	97.67 ^b	0–5	20.3 ± 7.88 ^A	6.2 ± 1.53 ^B	0.22 ± 0.056 ^A	2.8 ± 0.87 ^A	12.6 ± 0.88 ^A
		5–15	14.8 ± 2.92 ^a	7.0 ± 1.25 ^a	0.21 ± 0.033 ^a	2.6 ± 0.41 ^a	12.8 ± 1.10 ^a
		<i>P</i>	0.0336	0.1747	0.5994	0.4789	0.6277
eCO ₂ + eO ₃	112.03 ^a	0–5	17.1 ± 5.26 ^A	7.5 ± 1.47 ^{AB}	0.20 ± 0.009 ^A	2.5 ± 0.23 ^A	12.5 ± 0.79 ^A
		5–15	16.5 ± 4.67 ^a	7.8 ± 1.82 ^a	0.21 ± 0.039 ^a	2.6 ± 0.57 ^a	12.5 ± 0.84 ^a
		<i>P</i>	0.7704	0.6612	0.3961	0.5787	1.0000

Abbreviations: ANOVA, analysis of variance; eCO₂, elevated CO₂; eO₃, elevated O₃; TN, total nitrogen; TC, total carbon; C/N, TC/TN ratio. Soil variables from each depth were analyzed separately and significances among four treatments (ambient, eCO₂, eO₃ and eCO₂ + eO₃) were tested by ANOVA at the $P < 0.05$ level. A and B indicate significant changes among treatments for surface soils, and a and b for subsoils and crop yield. Significances between two soil depths were performed by the Student *t*-test and *P*-values are given Bold face indicates significantly ($P < 0.05$) changed correlations.

^aCrop yield data are the average of four plots in the previous two years (2004 and 2006) for each treatment.

Effects of block, depth, CO₂ and eO₃ on soil microbial communities

To assess whether block, soil depth, CO₂ and O₃ as well as their combinations affect soil microbial communities, Adonis analysis (Anderson, 2001) of all detected genes showed that these factors and their combinations significantly ($P < 0.01$) impacted soil microbial communities with about 53% of the total variation explained by this model, suggesting that soil microbial community structure was shaped by all three treatments with eCO₂ (3.5%) as the main factor, followed by eO₃ (3.1%) and eCO₂ + eO₃ (2.5%). Interestingly, a relatively strong block effect (5.2%) was observed, which explained even more total variance than those treatments: CO₂, O₃ or CO₂ + O₃ (Supplementary Table S1). As depths significantly ($P < 0.01$) affected microbial communities, further analyses were performed with two separate depths: surface soil (0–5 cm) and subsoil (5–15 cm).

Overall responses of soil microbial communities to eCO₂, eO₃ and eCO₂ + eO₃

To examine the effect of eCO₂ and eO₃ on the functional diversity, composition and structure of soil microbial communities, 96 soil samples (48 from each soil depth) of four plots (each with three subsamples) from each of three treatments (eCO₂, eO₃ and eCO₂ + eO₃) and the control (ambient) were analyzed by GeoChip 3.0. Detrended correspondence analysis of all detected functional genes showed that samples from all three treatments harbored gene assemblages distinct from ambient, and this was observed for both surface soil (Figure 1a) and subsoil (Figure 1b) samples. Samples from all four FACE treatments harbored distinct gene assemblages in the subsoil; however, the assemblages appeared less distinct under eO₃ and under eCO₂ + eO₃ in the surface soil (Figure 1).

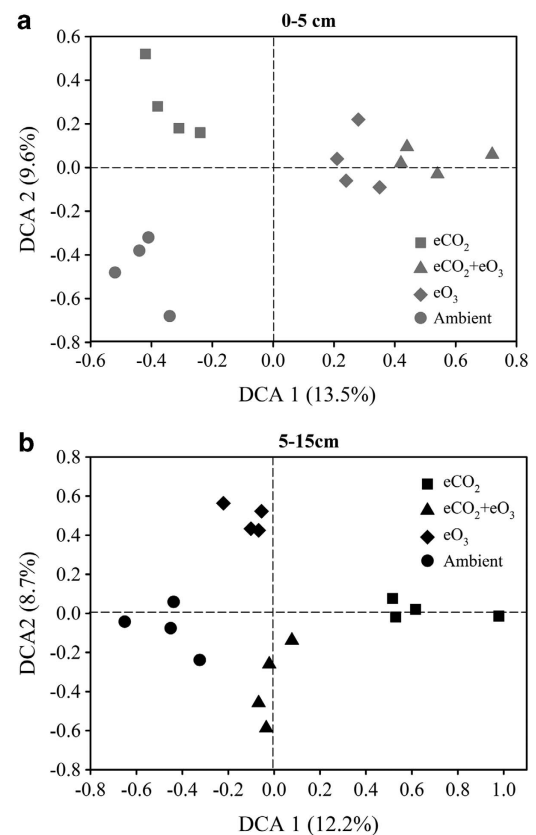


Figure 1 Detrended corresponding analysis (DCA) of three treatments and control samples in the surface soil (a) and in the subsoil (b) with four plots for each condition (three subsamples from each plot were combined).

Similarly, such trends were also observed for comparisons between each treatment (eCO₂, eO₃ or eCO₂ + eO₃) with ambient with 12 individual samples (Supplementary Figure S1), which were further confirmed by three statistical methods, ANOISM and adonis and Multi-Response Permutation

Procedures (Supplementary Table S2). Generally, each community was significantly ($P < 0.05$) different from others except eCO₂ + O₃ vs ambient in the surface soil ($P = 0.066$ for adonis, and $P = 0.052$ for MRPP) and eO₃ vs ambient and eCO₂ + O₃ vs ambient ($P > 0.05$) in the subsoil (Supplementary Table S2). The results suggest that the functional composition and structure of microbial communities significantly changed under eCO₂, eO₃ and eCO₂ + eO₃, which is consistent with our PERMANOVA analysis above and that such effects appeared to be distinct for each treatment.

Effects of eCO₂ and eO₃ on key functional genes and processes

To further understand the effect of eCO₂ and eO₃ on specific functional processes of soil microbial communities, key genes involved in C, N, S and P cycling were further examined below.

C fixation genes. A total of 365, 450, 391 and 420 probes had positive signals under ambient, eCO₂, eO₃ and eCO₂ + eO₃, respectively, in the surface soil, and 453, 432, 426 and 411 probes, respectively, in the subsoil, which are involved in C fixation, methane metabolism and C degradation (Supplementary Table S3). For C fixation, genes involved in CODH (carbon monoxide dehydrogenase), Pcc/Acc (propionyl-CoA/acetyl-CoA carboxylase) and Rubisco (Ribulose-1,5-bisphosphate carboxylase oxygenase) pathways were detected. Under eCO₂ and eCO₂ + eO₃, the signal intensities of Rubisco genes in the surface soil and CODH genes in the subsoil significantly ($P < 0.05$) increased (Figure 2a), suggesting a potentially increased in microbial C fixation. Under eO₃, the signal intensity of Pcc/Acc genes significantly ($P < 0.05$) decreased in the surface soil despite no significant differences for Rubisco or CODH genes or all three genes in the subsoil (Figure 2a). Further analysis of all detected Rubisco gene sequences showed that all four forms of Rubisco genes were detected, but most of them belonged to Form I, a major form for CO₂ fixation (Supplementary Figure S2). In the surface soil, a total of 52 sequences were detected with 35 clustered into Form I, and examples include gi22415761 from *Synechocystis trididemni*, gi91690340 from *Burkholderia xenovorans* LB400, gi148254105 from *Bradyrhizobium* sp. and gi91802339 from *Nitrobacter hamburgensis* X14 (Supplementary Figure S2A). In addition, 59 Rubisco gene sequences were detected in the subsoil samples, and similar results were observed (Supplementary Figure S2B). The results indicate that eCO₂ and eCO₂ + eO₃ may potentially lead to an increased C fixation, and that eO₃ may cause a decreased C fixation in SoyFACE ecosystems.

C degradation genes. The pattern of signal intensities for functional genes involved in C degradation changed in response to all three treatments. In the

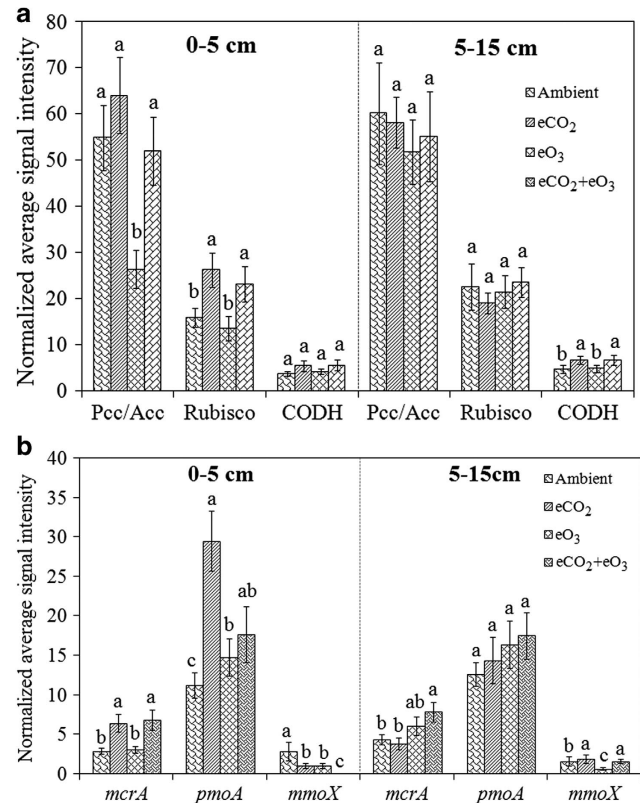


Figure 2 Normalized signal intensities of detected genes involved in C fixation pathways (a) and methane metabolism (b) in both surface soil (0–5 cm) and subsoil (5–15 cm) samples. Rubisco: ribulose-1,5-bisphosphate carboxylase/oxygenase; CODH: carbon monoxide dehydrogenase; Pcc/Acc: propionyl-CoA/acetyl-CoA carboxylase; *mcrA*: the alpha-subunit of methyl coenzyme M reductase for methane production; *pmoA*: particulate methane monooxygenase for methane oxidation; *mmoX*: methane monooxygenase for methane oxidation. All data are presented as mean ± s.e. Significance among the treatments was analyzed by multi-way analysis of variance (ANOVA). The significance of a, b and c is at $P < 0.05$ level.

surface soil, eCO₂ either increased or had no effects on the abundance of detected C degradation genes. For example, the abundance of most C degradation genes significantly ($P < 0.05$) increased under eCO₂, including those encoding amylase, glucoamylase, pullulanase, fungal arabinofuranosidase, xylanase, endoglucanase, acetylglucosaminidase and exochitinase for labile C degradation, and those encoding lignin peroxidase, manganese peroxidase and phenol oxidase for recalcitrant C degradation (Figure 3). Under eO₃, the abundance of most C degradation genes, especially those for recalcitrant C remained unchanged except with significant ($P < 0.05$) increases for fungal arabinofuranosidase and endoglucanase, and significant decreases for xylanase, cellobiase and exochitinase (Figure 3). Under eCO₂ + eO₃, only genes for glucoamylase, pullulanase and fungal arabinofuranosidase significantly ($P < 0.05$) increased, and there were no significant changes for other C degradation genes detected (Figure 3). For the subsoil samples, very few significant changes were observed for C degradation

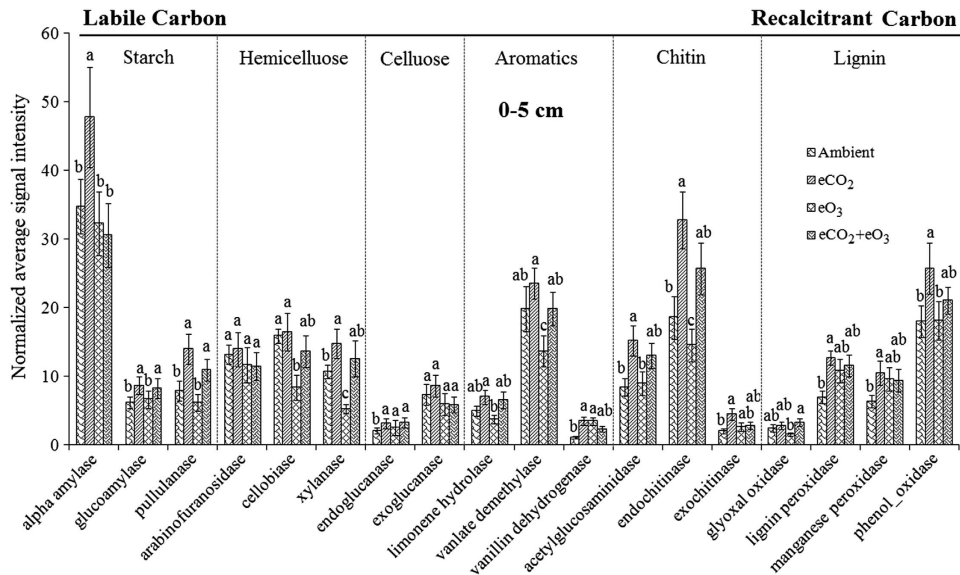


Figure 3 Effects of eCO₂, eO₃ and eCO₂ + eO₃ on carbon degradation genes detected in the surface soil (0–5 cm). The complexity of carbon is presented in the order from labile to recalcitrant. All data are presented as mean ± s.e. Significance among the treatments was analyzed by multi-way ANOVA. The significance of a and b is at $P < 0.05$ level.

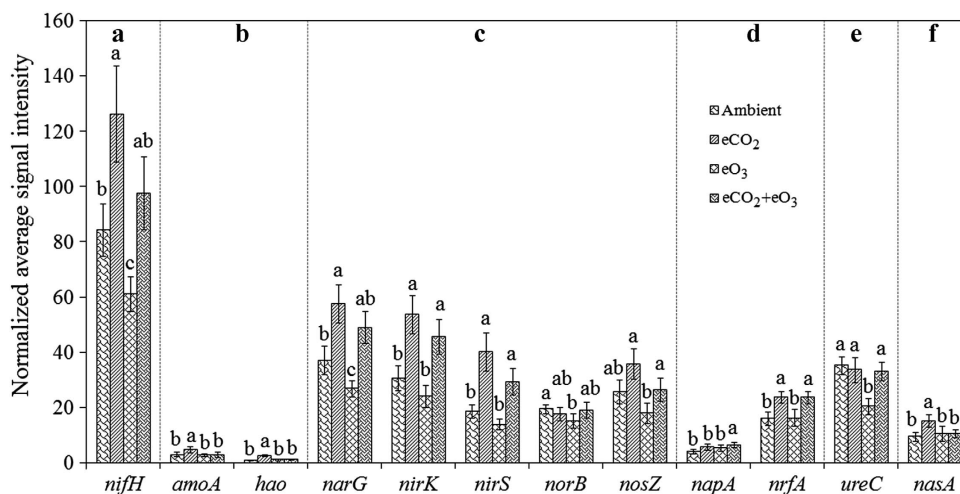


Figure 4 Normalized signal intensities of key N cycling genes under eCO₂, eO₃ and eCO₂ + eO₃ and control conditions in the surface soil (0–5 cm). (a) N₂ fixation, *nifH* encoding nitrogenase; (b) Nitrification, *amoA* encoding ammonia monooxygenase; (c) Denitrification, including *narG* for nitrate reductase, *nirS* and *nirK* for nitrite reductase, *norB* for nitric oxide reductase and *nosZ* for nitrous oxide reductase; (d) Dissimilatory N reduction to ammonium, including *napA* for nitrate reductase and *nrfA* for c-type cytochrome nitrite reductase; (e). Ammonification, including *gdh* for glutamate dehydrogenase and *ureC* encoding urease; (f) Assimilatory N reduction, *nasA* encoding nitrate reductase. All data are presented as mean ± s.e. Significance among the treatments was analyzed by multi-way ANOVA. The significance of a, b and c is at $P < 0.05$ level.

genes (Supplementary Figure S3). The results indicate that degradation of both labile and recalcitrant C might increase under eCO₂ with fewer changes under eCO₂ + eO₃, whereas the abundance of C degradation genes largely remained unchanged under eO₃.

N cycling genes. A total of 434, 531, 436 and 441 genes involved in N fixation, nitrification, ammonification, denitrification, dissimilatory N reduction and assimilatory N reduction were detected under ambient, eCO₂, eO₃ and eCO₂ + eO₃, respectively, in the surface soil, and 536, 483, 503 and 480,

respectively, in the subsoil (Supplementary Table S3). In the surface soil, the abundance of 186 detected *nifH* genes was significantly higher ($P < 0.05$) under eCO₂, lower ($P < 0.05$) under eO₃ and unchanged under eCO₂ + eO₃ compared with ambient (Figure 4, Supplementary Figure S4A). Among four defined *nifH* clusters, only Cluster II and III genes were significantly ($P < 0.05$) greater under eCO₂, most of the detected Cluster II and III genes were closely related to known microorganisms, such as *Rhizobium*, *Azospirillum*, *Methanococcus*, *Desulfovibrio*, *Methanosarcina* and *Bradyrhizobium* species (Supplementary Figure S4A). In the subsoil, although

similar clusters were formed with 217 *nifH* sequences detected, no significant differences were observed among three treatments and ambient in each cluster (Supplementary Figure S4B).

eCO₂ significantly ($P < 0.05$) increased the abundance of *narG*, *nirS* and *nirK* related to denitrification, and the abundances of *nirS* and *nirK* also significantly ($P < 0.05$) increased under eCO₂ + eO₃. Although the abundance of *norB* and *nosZ* genes remained unaffected under both eCO₂ and eCO₂ + eO₃, the abundance of *narG* and *norB* genes were significantly ($P < 0.05$) decreased under eO₃. In addition, the abundance of *amoA*, *nrfA* and *nasA* increased under eCO₂, and *ureC* decreased under eO₃ (Figure 4). In addition, fewer significant changes in the abundance of N cycling genes were observed in the subsoil (Supplementary Figure S5) than in the surface soil. These results indicated that eCO₂ might stimulate N cycling by a general increase in the abundance of N cycling genes, and that eO₃ might inhibit N cycling by a general decrease in the abundance of N cycling genes, especially in the surface soil.

Greenhouse gas emission genes. The emission of greenhouse gases (for example, CH₄ and N₂O) was reported to be increased under eCO₂ (van Groenigen *et al.*, 2011). GeoChip 3.0 mainly targets *mcrA* for CH₄ generation, *pmoA* and *mmoX* for CH₄ oxidation (Figure 2b), *norB* for N₂O production and *nosZ* for N₂O reduction (Figure 4c). In the surface soil, the *mcrA* abundance was significantly ($P < 0.05$) higher under eCO₂ and eCO₂ + eO₃, whereas eO₃ did not substantially affect the *mcrA* abundance compared with ambient (Figure 2b). In the subsoil, a significant ($P < 0.05$) increase in the *mcrA* abundance was observed only under eCO₂ + eO₃ without significant differences detected under eCO₂ or eO₃ (Figure 2b). In addition, the *norB* abundance significantly ($P < 0.05$) decreased under eO₃, but no significant changes were seen under eCO₂ or eCO₂ + eO₃ in the surface soil (Figure 4c) or in the subsoil (Supplementary Figure S5C). The results indicated that eCO₂ and/or eCO₂ + eO₃ had the potential to stimulate CH₄ emission and that eO₃ could potentially decrease N₂O emission.

P and S cycling genes. Under eCO₂, the abundance of exopolyphosphatase (Ppx) and polyphosphate kinase (Ppk) genes significantly ($P < 0.05$) increased in the surface soil but not in the subsoil. Under eCO₂ + eO₃, only the Ppx gene abundance significantly ($P < 0.05$) increased, and there were no significant changes under eO₃ (Supplementary Figure S6A). Similarly, for S cycling, an increase in the signal intensity of *dsrA* and *sox* was only observed at eCO₂ in the surface soil, and there were no significant changes under eO₃ or eCO₂ + eO₃ (Supplementary Figure S6B). These results suggest that eCO₂ may enhance both P and S cycling in the

surface soil, but eO₃ and eCO₂ + eO₃ had little impact, especially in the subsoil.

Relationships between the community structure and soil properties or crop yield

To link the microbial community structure and soil properties and crop yield, correlation analyses were performed by the Mantel test. The signal intensity of all detected genes did not show significant ($P > 0.05$) correlations with soil properties (for example, NO₃-N, NH₄-N, TC, TN and C/N) or crop yield in the surface soil (Table 2), or subsoil (Supplementary Table S4) samples. However, further analysis of relationships between individual functional genes detected in the surface soil and soil properties and crop yield indicated that 36 functional gene families had significant correlations with soil properties (26 families) or crop yield (16 families), including those involved in C degradation, N cycling and bioremediation of aromatics, herbicides and pesticides (Table 2). For example, genes involved in C degradation (fungal arabinofuranosidase, cellobiase and glucoamylase genes) and denitrification (*norB* and *nosZ*) were significantly ($P < 0.05$) correlated with crop yield. In addition, genes involved in recalcitrant C degradation (*mnp*), N reduction (*nasA*) and aromatics degradation (*bclA*, *dfbA* and *tphA*) were significantly ($P < 0.05$) correlated with both soil properties and crop yield. In addition, there were significant correlations between genes involved in N reduction (*nrfA*) or biodegradation of aromatics (for example, *amiE*, *arhA*, *benD*, *bphA*, *cymA* and *pheA*) and soil variables (Table 2). It is noted that most of the aromatic compounds are the products of lignin degradation, which are also related to C degradation. Similarly, 30 functional gene families showed significant correlations with soil properties (25 families) or crop yield (7 families) in the subsoil (Supplementary Table S4). These results suggested that the microbial community functional structure was significantly correlated with soil C and N dynamics, and crop yield.

Discussion

Understanding the response of soil microbial communities of terrestrial ecosystems to atmospheric changes is necessary to predict future global change. In this study, we comprehensively examined functional responses of soil microbial communities to eCO₂, eO₃ and eCO₂ + eO₃ using GeoChip technology. The results showed that the functional composition and structure of soil microbial communities shifted, and accordingly, the abundance of key functional genes for C fixation and degradation, N fixation, denitrification, greenhouse gas emission and N mineralization significantly changed under eCO₂, eO₃ and/or eCO₂ + eO₃. Such changes were distinct for each treatment and significantly

Table 2 Correlations (*P*-values) between soil properties or plant yield and signal intensities of functional genes by Mantel analysis of surface (0–5 cm) soil samples

<i>Gene/enzyme</i>	<i>Functional process</i>	<i>NO₃-N</i>	<i>NH₄-N</i>	<i>TN</i>	<i>TC</i>	<i>C/N</i>	<i>Yield</i>
All detected		0.091	0.272	0.293	0.431	0.275	0.126
<i>ara</i> (fungi)	C degradation	0.201	0.619	0.064	0.063	0.700	0.018
Cellobiase	C degradation	0.543	0.077	0.780	0.753	0.781	0.026
Endochitinase	C degradation	0.603	0.605	0.863	0.539	0.014	0.421
Glucoamylase	C degradation	0.648	0.935	0.301	0.193	0.295	0.023
<i>mnp</i>	C degradation	0.503	0.637	0.031	0.064	0.538	0.025
<i>nasA</i>	N reduction	0.372	0.662	0.047	0.032	0.252	0.000
<i>norB</i>	Denitrification	0.278	0.687	0.189	0.076	0.129	0.028
<i>nosZ</i>	Denitrification	0.643	0.302	0.090	0.108	0.065	0.044
<i>nrfA</i>	N reduction	0.595	0.619	0.606	0.359	0.028	0.358
<i>cnrC</i>	Cobalt and nickel	0.611	0.354	0.491	0.519	0.625	0.017
<i>cueO</i>	Copper	0.481	0.727	0.051	0.022	0.183	0.062
<i>metC</i>	Mercury	0.008	0.049	0.885	0.942	0.370	0.771
<i>terD</i>	Tellurium	0.901	0.579	0.104	0.039	0.337	0.169
<i>amiE</i>	Aromatics	0.241	0.045	0.884	0.824	0.311	0.911
<i>arhA</i>	Aromatics	0.003	0.060	0.483	0.516	0.592	0.380
<i>bclA</i>	Aromatics	0.203	0.365	0.049	0.072	0.811	0.021
<i>benD</i>	Aromatics	0.015	0.073	0.988	0.975	0.463	0.983
<i>bphA</i>	Aromatics	0.382	0.042	0.916	0.827	0.541	0.414
<i>cymA</i>	Aromatics	0.550	0.617	0.685	0.240	0.025	0.626
<i>dfbA</i>	Aromatics	0.244	0.655	0.068	0.027	0.054	0.025
<i>hcaACD</i>	Aromatics	0.164	0.047	0.798	0.756	0.861	0.969
<i>hdnO</i>	Aromatics	0.005	0.129	0.559	0.684	0.574	0.692
<i>nagG</i>	Aromatics	0.464	0.585	0.093	0.073	0.164	0.027
<i>nbaC</i>	Aromatics	0.327	0.312	0.053	0.059	0.155	0.042
<i>phdCI</i>	Aromatics	0.920	0.580	0.954	0.828	0.026	0.156
<i>pheA</i>	Aromatics	0.474	0.112	0.022	0.108	0.597	0.267
<i>tmoABE</i>	Aromatics	0.787	0.923	0.666	0.372	0.029	0.261
<i>tphA</i>	Aromatics	0.444	0.688	0.283	0.186	0.016	0.003
<i>tutFDG</i>	Aromatics	0.021	0.019	0.428	0.676	0.784	0.876
<i>xylC</i>	Aromatics	0.548	0.968	0.094	0.156	0.851	0.046
<i>mauAB</i>	Herbicides	0.262	0.390	0.066	0.054	0.630	0.044
<i>pcpB</i>	Herbicides	0.163	0.033	0.991	0.998	0.760	0.792
<i>chnE</i>	Hydrocarbons	0.587	0.986	0.086	0.034	0.213	0.276
<i>cpnA</i>	Hydrocarbons	0.046	0.246	0.488	0.269	0.011	0.963
<i>xamO</i>	Hydrocarbons	0.902	0.663	0.193	0.135	0.360	0.016
<i>adpB</i>	Pesticides	0.229	0.005	0.810	0.928	0.529	0.424

Abbreviations: TN, total nitrogen; TC, total carbon; C/N, TC/TN ratio. Bold face indicates significantly (*P*<0.05) changed correlations.

correlated with soil properties and soybean yield. Overall, our analyses suggest possible mechanisms of microbial responses to global atmospheric changes through the stimulation of C and N cycling by eCO₂, the inhibition of N functional processes by eO₃ and their interactive effects by eCO₂ and eO₃. This study has important implications for microbial responses and feedbacks to global change and their impacts on crop productivity and ecosystem functioning in soybean agro-ecosystems.

Our core hypothesis is that eCO₂, eO₃ and eCO₂ + eO₃ would significantly affect the functional composition and structure of soil microbial communities largely by indirect effects, such as altered C and N inputs into soil and soil microenvironments (Ainsworth *et al.*, 2002; Dijkstra *et al.*, 2005; Adair *et al.*, 2009; Feng *et al.*, 2010). Several previous studies showed that the microbial community composition and structure was affected by eCO₂ (Feng *et al.*, 2010; He *et al.*, 2010b, 2012b; Deng *et al.*, 2012; Hayden *et al.*, 2012), eO₃ and/or eCO₂ + eO₃ (Phillips *et al.*, 2002; Kasurinen *et al.*,

2005; Kanerva *et al.*, 2008). For example, a previous study at the BioCON site showed that eCO₂ increased plant and microbial biomass, soil pH and moisture and significantly shifted the functional and phylogenetic/taxonomic composition, structure and network interactions of soil microbial communities (Zhou *et al.*, 2010; He *et al.*, 2010b, 2012b; Zhou *et al.*, 2011; Deng *et al.*, 2012). In this study, the results support this hypothesis demonstrated by adonis and Detrended correspondence analysis analyses of all detected functional genes. As soybean yield increased under eCO₂ and eCO₂ + eO₃, it is expected that C and N input into soil increase and this along with available C and N and other factors (for example, soil moisture, pH and food-web interactions) may affect microbial responses either singly or in combination, leading to changes in the microbial community structure (Zak *et al.*, 2000). However, two recent studies showed that eO₃ did not alter the overall microbial community structure (Cheng *et al.*, 2011; Li *et al.*, 2013), which are not consistent with our

observations in this study. Such differences may be due to different O₃ levels, different replicates used, different exposure time and/or different soil chemistry in those sites. Indeed, in this study, we observed that NH₄-N decreases in the surface soil and NO₃-N increases in the subsoil despite no significant changes in total soil N. In addition, other eCO₂-induced changes in plants like root exudate may shift the soil microbial community structure (Phillips *et al.*, 2011), although they were not measured in this study. It should be noted that a randomized complete block design is used in the SoyFACE experiment and a relatively strong block effect was observed. The spatial variations in many environmental variables that may affect soil microbial communities have the potential to be responsible for the block effect, such as soil pH, sunshine angle and plant growth. Most importantly, the treatment effects by CO₂ and O₃ were still significant via analysis of variance analysis, implying consistent impacts of CO₂ and O₃ on the soil microbial community across all environmental heterogeneous conditions tested in this study. The results also appeared to be generally consistent with a previous study at the Duke Forest FACE site, showing that spatial factor could explain 20% of the variation in the microbial community structure and CO₂ or N treatment for less than 3% of the variation (Ge *et al.*, 2010).

With the change in the microbial community structure under global changes factors, one of most important questions is whether the change in the soil microbial community structure affects microbial functional processes and ecosystem services, such as C and N dynamics. In this study, we found three distinct patterns for microbial responses and feedbacks to eCO₂ and eO₃: (i) the stimulation of C and N cycling genes by eCO₂, (ii) the inhibition of N fixation, denitrification and N mineralization by eO₃ and (iii) an interactive effects by eCO₂ + eO₃. The results generally support one of our hypotheses that various microbial functional groups (for example, C fixers, C degraders, N₂ fixers and denitrifiers) would differentially respond to eCO₂ and eO₃.

The first scenario is how microbial communities modify their functional processes by eCO₂. Previous studies showed inconsistent responses of soil C and/or N to eCO₂ with positive (Carney *et al.*, 2007; Heimann and Reichstein, 2008), negative (Jastrow *et al.*, 2005) or no significant effects (Hungate *et al.*, 2009). A previous study at the BioCON, a grassland ecosystem showed that key genes involved in C fixation and labile C degradation were stimulated under eCO₂ but there were no significant changes for recalcitrant C degradation genes (He *et al.*, 2010b). This is generally consistent with the current study using the same GeoChip technology (GeoChip 3.0) except that the abundances of lignin degradation genes encoding lignin peroxidase, manganese peroxidase and phenol oxidase were also significantly increased under eCO₂. Such differences may be

ecosystem-dependent. A previous study showed that as soybean yield and root biomass increased under eCO₂, soil organic matter turnover was accelerated but soil moisture and nutrients were not limiting factors at the SoyFACE (Peralta and Wander, 2008). Compared with grassland ecosystems, the soybean agro-ecosystem is N-rich and has competitive advantages over non-leguminous species at eCO₂ by capitalizing on eCO₂ benefits (for example, increasing N fixation) and limiting deleterious eCO₂ effects (Rogers *et al.*, 2009). Therefore, with more labile C inputs into soil via litter and root exudation, N cycling may be enhanced in soybean agrosystems. Indeed, the present study showed that the abundances of most N cycling genes (for example, *nifH*, *amoA*, *narG*, *nirS*, *nirK*, *nrfA* and *nasA*) significantly increased at eCO₂. On the other hand, a meta-analysis suggests that soil N may modulate soil C cycling at eCO₂, and that eCO₂-induced soil C inputs are generally offset by increased heterotrophic respiration, resulting in no significant changes in soil C content (Dieleman *et al.*, 2010), which appeared to be the case for this study. The results point toward a possible positive microbial feedback to eCO₂, although further investigation is necessary.

The second scenario is microbial responses to eO₃. An early study showed that N concentration in soybean plants was not affected by eO₃, but N fixation decreased due to a reduced photosynthate translocation to nodules (Pausch *et al.*, 1996), whereas a recent study at the SoyFACE indicated that eO₃ could lead to an increase in soil N availability in both bulk and rhizosphere soils by reducing the mineralization rates of plant-derived residues (Pujol Pereira *et al.*, 2011). In addition, a previous study with soil enzyme activity analysis showed that 1,4-β-glucosidase activity was suppressed, but there were no significant changes for 1,4-β-N-acetylglucosaminidase and other C degradation enzymes under O₃ (Larson *et al.*, 2002), and another study of wheat rhizosphere microbial communities at an O₃-FACE site identified a decreased abundance of *fhs* for acetogenesis, but most C and N cycling genes remained unchanged under eO₃ (Li *et al.*, 2013). The present study showed that under eO₃, the abundances of almost all key C and N cycling genes remained unchanged, or significantly decreased (for example, *Pcc/Acc*, xylanase, cellobiase, endochitinase, *nifH*, *narG*, *norB* and *ureC*), and accordingly, we found significantly higher NO₃-N in the subsoil and significantly lower NH₄-N in the surface soil compared with ambient. Therefore, based on our results and current knowledge, a simple conceptual model is constructed to summarize microbial N cycling and N dynamics in response to eO₃ (Figure 5). Under eO₃, plant residue was input into the surface soil and mineralized (for example, *ureC*) to NH₄⁺, which also came from microbial N fixation (for example, *nifH*). NH₄⁺ could mobilize in soil and/or be used by plants and

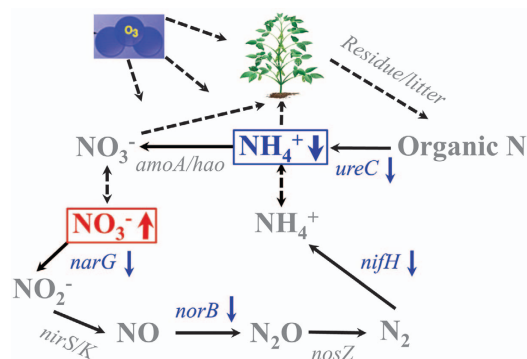


Figure 5 A conceptual model for microbial N cycling in response to eO₃. Red color indicates an increase in NO₃⁻ in the subsoil; blue color indicates decreases in NH₄⁺ in the surface soil or abundances of N cycling genes; gray indicates those parameters were not measured or were not changed under eO₃.

microorganisms. The abundances of both *nifH* and *ureC* significantly decreased, resulting in a decrease of NH₄-N in the surface soil. NH₄⁺ was transformed to NO₃ by nitrification (for example, *amoA* and *hao*), which could mobilize in soil or/and be used by plants and microorganisms. NO₃ was then transformed to NO₂⁻, NO, N₂O and N₂ by denitrification (for example, *narG*, *nirS/K*, *norB* and *nosZ*). As the *narG* and *norB* abundances significantly decreased at eO₃, denitrification, especially the first step (NO₃ to NO₂⁻ by *narG*) was probably inhibited, leading to an increased NO₃ in the subsoil. This conceptual model may suggest a possible negative microbial feedback to eO₃ in this soybean agro-ecosystem.

The third scenario is how eCO₂ and eO₃ interact to affect microbial functional processes and ecosystem functioning. It has been proposed that eO₃-induced negative effects on soil microbial communities may be ameliorated by eCO₂, and a couple of mechanisms are considered. First, under eCO₂, the uptake of O₃ by plants may be reduced (Allen, 1990; McKee *et al.*, 2000) due to reduced stomatal conductance (Wittig *et al.*, 2009), which may reduce the loss of plant productivity. Second, an increase of available C, especially labile C and root exudation under eCO₂ may help plants and microorganisms detoxify and activate cell repair processes (Allen, 1990; McKee *et al.*, 1997). Although there were no significant interactions between eCO₂ and eO₃ in plant responses to eCO₂ and eO₃ (Valkama *et al.*, 2007), previous studies showed that eO₃ eliminated eCO₂-induced effects, such as increased C inputs and microbial enzyme activity in soil (Larson *et al.*, 2002; Phillips *et al.*, 2002), leading to a negative feedback on soil N availability under CO₂ + O₃ (Holmes *et al.*, 2006). In this study, under eCO₂ and eO₃, we found that eCO₂ promoted abundances of some eO₃-suppressed genes to ambient, or even to eCO₂-induced levels. For example, the abundances of four eO₃-inhibited C cycling genes (*Pcc/Acc*, xylanase, cellobiase and

endochitinase genes) exhibited ambient/eCO₂ levels under eCO₂ + eO₃. In addition, the abundances of four eO₃-suppressed N cycling genes (*nifH*, *narG*, *norB* and *ureC*) were returned to ambient or promoted to eCO₂ levels under eCO₂ + eO₃. In addition, a few C and N cycling genes (for example, *pula*, *napA*, *nirK* and *nirS*) showing no significant differences under eO₃ were significantly increased under eCO₂ + eO₃. The results suggest that the interactive effects by eCO₂ + eO₃ may be highly uncertain, largely depending on their concentrations, exposure time, soil properties, and ecosystems.

The results from all three scenarios showed that the change in soil microbial community structure affected microbial functional processes and ecosystem services, and such effects appeared distinct for each treatment. Some key functional genes involved in C and N cycling were identified, but their attributions to soil C and N dynamics remain unknown, warranting further investigation by high-throughput sequencing and other metagenomics approaches, such as stable isotope probing, metatranscriptomics and metaproteomics. Also, we analyzed microbial communities from bulk soil in this study, and it will be interesting to analyze the response of rhizosphere microbial communities including arbuscular mycorrhizal fungi to eCO₂ and eO₃. In addition, we only examined one time point (late vegetation) in this study, and a future study may focus on the temporal dynamics of soil microbial communities in response to elevated greenhouse gases. Finally, it is necessary to understand the interaction of C and N cycling in soil microbial communities under different global change factors in the future.

Conflict of Interest

The authors declare no conflict of interest.

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