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ORIGINAL ARTICLE

Distinct responses of soil microbial communities to elevated CO_2 and O_3 in a soybean agro-ecosystem

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The concentrations of atmospheric carbon dioxide (CO_2) and tropospheric ozone (O_3) have been rising due to human activities. However, little is known about how such increases influence soil microbial communities. We hypothesized that elevated CO_2 (eCO_2) and elevated O_3 (eO_3) 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 CO_2 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 eCO_2, eO_3 or $eCO_2 + eO_3$. Key functional genes involved in carbon fixation and degradation, nitrogen fixation, denitrification and methane metabolism were stimulated under eCO_2, whereas those involved in N fixation, denitrification and N mineralization were suppressed under eO_3, resulting in the fact that the abundance of some eO_3-supressed genes was promoted to ambient, or eCO_2-induced levels by the interaction of $eCO_2 + eO_3$. 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 eCO_2, the inhibition of N functional processes by eO_3 and the interaction by eCO_2 and eO_3. 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 (CO_2) 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 O_3 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 O_3 (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

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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 CO_2 (eCO_2) and elevated O_3 (eO_3) on the functional diversity, composition, structure, metabolic potential and dynamics of soil microbial communities and their linkages with ecosystem functioning.

CO_2 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 eCO_2 stimulation may be constrained by the nitrogen (N) supply (Zak *et al.*, 2003; Reich *et al.*, 2006) because of progressive N limitation under eCO_2 (Luo *et al.*, 2004; Reich *et al.*, 2006). In contrast, O_3 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 eCO_2 and eO_3, it has been suggested that CO_2 might alleviate the negative effects of O_3 on the above-ground processes by a decrease of O_3 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 eCO_2 and/or eO_3 on the functional diversity, composition, structure and function of soil microbial communities is poorly understood. For example, no detectable effects of eCO_2 on microbial community structure, microbial activity, potential soil N mineralization or nitrification were observed at a sweetgum free-air CO_2 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 eCO_2 but not by eO_3 or $eCO_2 + eO_3$ (Cheng *et al.*, 2011). Recently, more studies suggest that eCO_2 and/or eO_3 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 comunities 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, eCO_2, eO_3, warming and precipitation) and their interactions on microbial communities might be highly uncertain (Castro *et al.*, 2010; Gutknecht *et al.*, 2012). Therefore, it is imporant to comprehensively examine the effect of eCO_2 and eO_3 on soil micorbial 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 micorbial 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 eCO_2 and/or eO_3. 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 eCO_2 and eO_3 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 eCO_2 with indeterminate and nodulating soybean. The results showed the functional composition, structure and metabolic potential of microbial communities significantly altered under eCO_2, eO_3 and $eCO_2 + eO_3$, 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. CO_2 and ~37.9 p.p.b. O_3); (ii) eCO_2 (~550 p.p.m.); (iii) eO_3 (~ 61.3 p.p.b.); and (iv) eCO_2+eO_3 (~550 p.p.m. CO_2 and ~61.3 p.p.b. O_3). 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 eCO_2 on seed yield production before the time of our sampling, soybean yield data from 2004 to 2006 were averaged. Soil NO_3-N and NH_4-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 eCO_2, eO_3 and eCO_2+eO_3 on crop yield and soil properties

Historical soybean yields were significantly ($P<0.05$) greater by ~14% and ~12% under eCO_2 and eCO_2+eO_3, respectively, whereas eO_3 only caused an insignificant decrease (~2.3%) in soybean yield (Table 1). Concentrations of soil nitrate (NO_3-N), ammonium (NH_4-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, NO_3-N was significantly ($P<0.05$) higher in the subsoil under eO_3 and eCO_2+eO_3 compared with ambient or eCO_2 samples but not significantly different between ambient and eCO_2, or eO_3 and eCO_2+eO_3. Second, NH_4-N was significantly ($P<0.05$) lower in the surface soil under eO_3 compared with ambient but not significantly different among eCO_2, eO_3 and eCO_2+eO_3. Third, NO_3-N was significantly ($P<0.05$) different between two soil depths under ambient, eCO_2 or eO_3 despite no significant difference under eCO_2+eO_3. 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 eCO_2 and/or eO_3 affected crop yield and soil N status.

Table 1 Effects of eCO_2, eO_3 and $eCO_2 + eO_3$ 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}
		P	**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}
		P	**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}
		P	**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}
		P	0.7704	0.6612	0.3961	0.5787	1.0000

Abbreviations: ANOVA, analysis of variance; eCO_2, elevated CO_2; eO_3, elevated O_3; 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, eCO_2, eO_3 and $eCO_2 + eO_3$) 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.
[a]Crop yield data are the average of four plots in the previous two years (2004 and 2006) for each treatment.

Effects of block, depth, CO_2 and eO_3 on soil microbial communities

To assess whether block, soil depth, CO_2 and O_3 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 eCO_2 (3.5%) as the main factor, followed by eO_3 (3.1%) and $eCO_2 + eO_3$ (2.5%). Interestingly, a relatively strong block effect (5.2%) was observed, which explained even more total variance than those treatments: CO_2, O_3 or $CO_2 + O_3$ (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 eCO_2, eO_3 and $eCO_2 + eO_3$

To examine the effect of eCO_2 and eO_3 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 (eCO_2, eO_3 and $eCO_2 + eO_3$) 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 eO_3 and under $eCO_2 + eO_3$ in the surface soil (Figure 1).

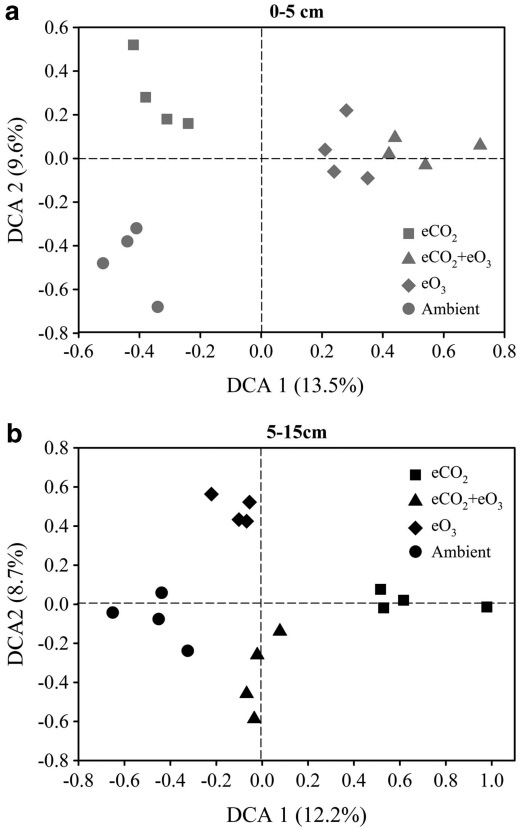

Figure 1 Detrended corresponding analysis (DCA) of three treatments and control samples in the surface soil (**a**) and in the subsoil (**b**) with four plots for each condition (three subsamples from each plot were combined).

Similarly, such trends were also observed for comparisons between each treatment (eCO_2, eO_3 or $eCO_2 + eO_3$) 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 eCO_2+O_3 vs ambient in the surface soil ($P=0.066$ for adonis, and $P=0.052$ for MRPP) and eO_3 vs ambient and eCO_2+O_3 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 eCO_2, eO_3 and eCO_2+eO_3, which is consistent with our PERMANOVA analysis above and that such effects appeared to be distinct for each treatment.

Effects of eCO_2 and eO_3 on key functional genes and processes

To further understand the effect of eCO_2 and eO_3 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, eCO_2, eO_3 and eCO_2+eO_3, 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 eCO_2 and eCO_2+eO_3, 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 eO_3, 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 CO_2 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 eCO_2 and eCO_2+eO_3 may potentially lead to an increased C fixation, and that eO_3 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 surface soil, eCO_2 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 eCO_2, 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 eO_3, 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 eCO_2+eO_3, 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

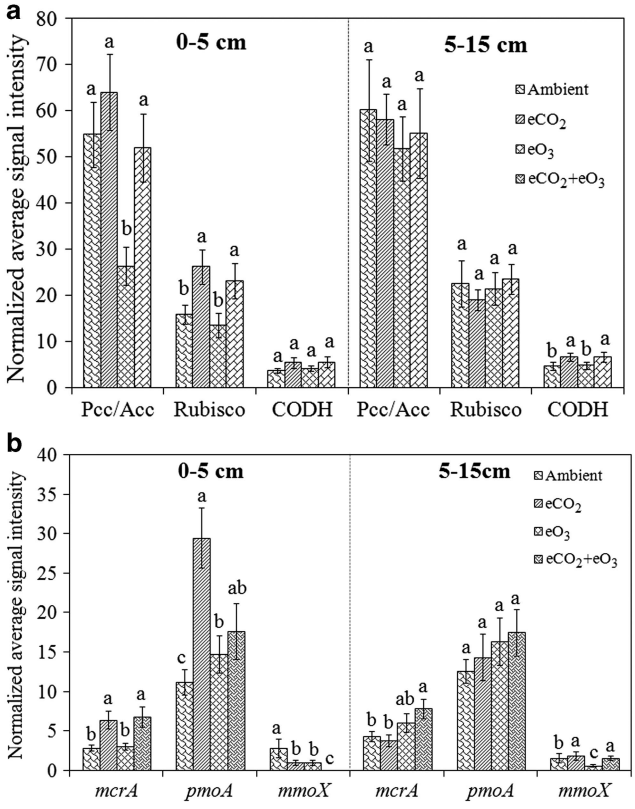

Figure 2 Normalized signal intensities of detected genes involved in C fixation pathways (**a**) and methane metabolism (**b**) in both surface soil (0–5 cm) and subsoil (5–15 cm) samples. Rubisco: ribulose-1,5-bisphosphate carboxylase/oxygenase; CODH: carbon monoxide dehydrogenase; Pcc/Acc: propionyl-CoA/acetyl-CoA 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.

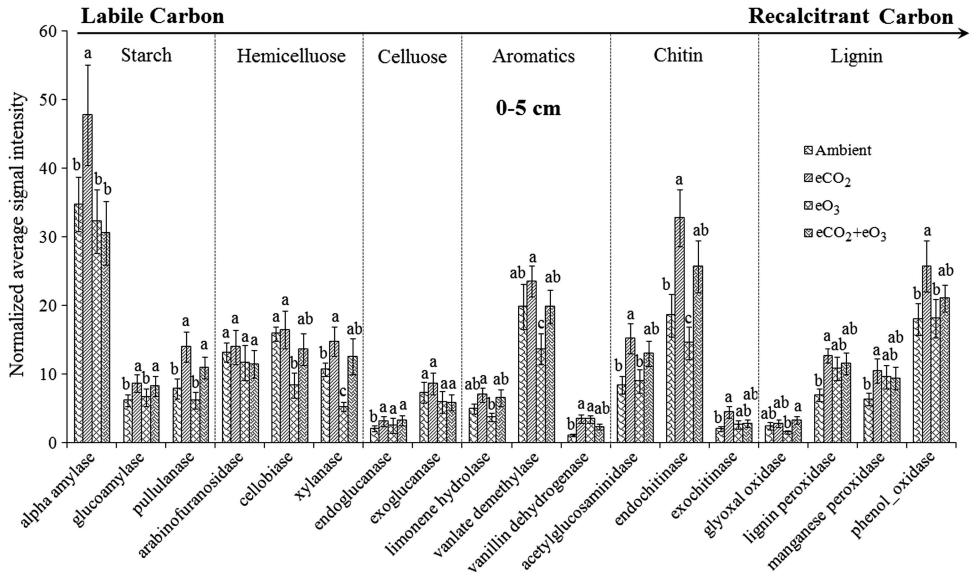

Figure 3 Effects of eCO_2, eO_3 and $eCO_2 + eO_3$ on carbon degradation genes detected in the surface soil (0–5 cm). The complexity of carbon is presented in the order from labile to recalcitrant. All data are presented as mean ± s.e. Significance among the treatments was analyzed by multi-way ANOVA. The significance of a and b is at $P<0.05$ level.

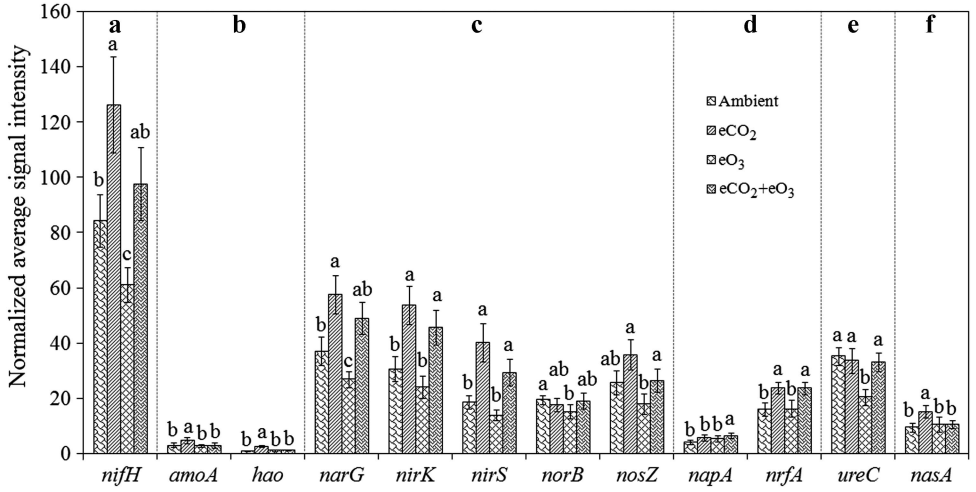

Figure 4 Normalized signal intensities of key N cycling genes under eCO_2, eO_3 and $eCO_2 + eO_3$ and control conditions in the surface soil (0–5 cm). (**a**) N_2 fixation, *nifH* encoding nitrogenase; (**b**) Nitrification, *amoA* encoding ammonia monooxygenase; (**c**) Denitrification, including *narG* 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 eCO_2 with fewer changes under $eCO_2 + eO_3$, whereas the abundance of C degradation genes largely remained unchanged under eO_3.

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, eCO_2, eO_3 and $eCO_2 + eO_3$, 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 eCO_2, lower ($P<0.05$) under eO_3 and unchanged under $eCO_2 + eO_3$ 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 eCO_2, 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).

eCO_2 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 eCO_2+eO_3. Although the abundance of *norB* and *nosZ* genes remained unaffected under both eCO_2 and eCO_2+eO_3, the abundance of *narG* and *norB* genes were significantly ($P<0.05$) decreased under eO_3. In addition, the abundance of *amoA*, *nrfA* and *nasA* increased under eCO_2, and *ureC* decreased under eO_3 (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 eCO_2 might stimulate N cycling by a general increase in the abundance of N cycling genes, and that eO_3 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, CH_4 and N_2O) was reported to be increased under eCO_2 (van Groenigen *et al.*, 2011). GeoChip 3.0 mainly targets *mcrA* for CH_4 generation, *pmoA* and *mmoX* for CH_4 oxidation (Figure 2b), *norB* for N_2O production and *nosZ* for N_2O reduction (Figure 4c). In the surface soil, the *mcrA* abundance was significantly ($P<0.05$) higher under eCO_2 and eCO_2+eO_3, whereas eO_3 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 eCO_2+eO_3 without significant differences detected under eCO_2 or eO_3 (Figure 2b). In addition, the *norB* abundance significantly ($P<0.05$) decreased under eO_3, but no significant changes were seen under eCO_2 or eCO_2+eO_3 in the surface soil (Figure 4c) or in the subsoil (Supplementary Figure S5C). The results indicated that eCO_2 and/or eCO_2+eO_3 had the potential to stimulate CH_4 emission and that eO_3 could potentially decrease N_2O emission.

P and S cycling genes. Under eCO_2, 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 eCO_2+eO_3, only the Ppx gene abundance significantly ($P<0.05$) increased, and there were no significant changes under eO_3 (Supplementary Figure S6A). Similarly, for S cycling, an increase in the signal intensity of *dsrA* and *sox* was only observed at eCO_2 in the surface soil, and there were no significant changes under eO_3 or eCO_2+eO_3 (Supplementary Figure S6B). These results suggest that eCO_2 may enhance both P and S cycling in the surface soil, but eO_3 and eCO_2+eO_3 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, NO_3-N, NH_4-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 eCO_2, eO_3 and eCO_2+eCO_3 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 eCO_2, eO_3 and/or eCO_2+eCO_3. 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

Gene/enzyme	*Functional process*	*NO_3-N*	*NH_4-N*	*TN*	*TC*	*C/N*	*Yield*
All detected		0.091	0.272	0.293	0.431	0.275	0.126
ara (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**
mnp	C degradation	0.503	0.637	**0.031**	0.064	0.538	**0.025**
nasA	N reduction	0.372	0.662	**0.047**	**0.032**	0.252	**0.000**
norB	Denitrification	0.278	0.687	0.189	0.076	0.129	**0.028**
nosZ	Denitrification	0.643	0.302	0.090	0.108	0.065	**0.044**
nrfA	N reduction	0.595	0.619	0.606	0.359	**0.028**	0.358
cnrC	Cobalt and nickel	0.611	0.354	0.491	0.519	0.625	**0.017**
cueO	Copper	0.481	0.727	0.051	**0.022**	0.183	0.062
metC	Mercury	**0.008**	**0.049**	0.885	0.942	0.370	0.771
terD	Tellurium	0.901	0.579	0.104	**0.039**	0.337	0.169
amiE	Aromatics	0.241	**0.045**	0.884	0.824	0.311	0.911
arhA	Aromatics	**0.003**	0.060	0.483	0.516	0.592	0.380
bclA	Aromatics	0.203	0.365	**0.049**	0.072	0.811	**0.021**
benD	Aromatics	**0.015**	0.073	0.988	0.975	0.463	0.983
bphA	Aromatics	0.382	**0.042**	0.916	0.827	0.541	0.414
cymA	Aromatics	0.550	0.617	0.685	0.240	**0.025**	0.626
dfbA	Aromatics	0.244	0.655	0.068	**0.027**	0.054	**0.025**
hcaACD	Aromatics	0.164	**0.047**	0.798	0.756	0.861	0.969
hdnO	Aromatics	**0.005**	0.129	0.559	0.684	0.574	0.692
nagG	Aromatics	0.464	0.585	0.093	0.073	0.164	**0.027**
nbaC	Aromatics	0.327	0.312	0.053	0.059	0.155	**0.042**
phdCI	Aromatics	0.920	0.580	0.954	0.828	**0.026**	0.156
pheA	Aromatics	0.474	0.112	**0.022**	0.108	0.597	0.267
tmoABE	Aromatics	0.787	0.923	0.666	0.372	**0.029**	0.261
tphA	Aromatics	0.444	0.688	0.283	0.186	**0.016**	**0.003**
tutFDG	Aromatics	**0.021**	**0.019**	0.428	0.676	0.784	0.876
xylC	Aromatics	0.548	0.968	0.094	0.156	0.851	**0.046**
mauAB	Herbicides	0.262	0.390	0.066	0.054	0.630	**0.044**
pcpB	Herbicides	0.163	**0.033**	0.991	0.998	0.760	0.792
chnE	Hydrocarbons	0.587	0.986	0.086	**0.034**	0.213	0.276
cpnA	Hydrocarbons	**0.046**	0.246	0.488	0.269	**0.011**	0.963
xamO	Hydrocarbons	0.902	0.663	0.193	0.135	0.360	**0.016**
adpB	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 eCO_2, the inhibition of N functional processes by eO_3 and their interactive effects by eCO_2 and eO_3. 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 eCO_2, eO_3 and $eCO_2 + eO_3$ 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 eCO_2 (Feng *et al.*, 2010; He *et al.*, 2010b, 2012b; Deng *et al.*, 2012; Hayden *et al.*, 2012), eO_3 and/or $eCO_2 + eO_3$ (Phillips *et al.*, 2002; Kasurinen *et al.*, 2005; Kanerva *et al.*, 2008). For example, a previous study at the BioCON site showed that eCO_2 increased plant and microbial biomass, soil pH and moisture and significantly shifted the functional and phylogentic/taxonmoic composition, structure and network interactions of soil micorbial 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 eCO_2 and $eCO_2 + eO_3$, 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 eO_3 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 O_3 levels, different replicates used, different exposure time and/or different soil chemistry in those sites. Indeed, in this study, we observed that NH_4-N decreases in the surface soil and NO_3-N increases in the subsoil despite no significant changes in total soil N. In addition, other eO_3-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 CO_2 and O_3 were still significant via analysis of variance analysis, implying consistent impacts of CO_2 and O_3 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 CO_2 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 eCO_2 and eO_3: (i) the stimulation of C and N cycling genes by eCO_2, (ii) the inhibition of N fixation, denitrification and N mineralization by eO_3 and (iii) an interactive effects by $eCO_2 + eO_3$. The results generally support one of our hypotheses that various microbial functional groups (for example, C fixers, C degraders, N_2 fixers and denitrifiers) would differentially respond to eCO_2 and eO_3.

The first scenario is how microbial communities modify their functional processes by eCO_2. Previous studies showed inconsistent responses of soil C and/or N to eCO_2 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 eCO_2 but there were no signifcant 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 eCO_2. Such differences may be ecosystem-dependent. A previous study showed that as soybean yield and root biomass increased under eCO_2, 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 competive advantages over non-legumious species at eCO_2 by capitializing on eCO_2 benefits (for example, increaseing N fixation) and limiting deleterious eCO_2 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*) signifcantly increased at eCO_2. On the other hand, a meta-analysis suggests that soil N may modulate soil C cycling at eCO_2, and that eCO_2-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 eCO_2, although further investigation is necessary.

The second scenario is microbial responses to eO_3. An early study showed that N concentration in soybean plants was not affected by eO_3, 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 eO_3 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 O_3 (Larson *et al.*, 2002), and another study of wheat rhizosphere microbial communities at an O_3-FACE site identified a decreased abundance of *fhs* for acetogenesis, but most C and N cycling genes remained unchanged under eO_3 (Li *et al.*, 2013). The present study showed that under eO_3, 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 signicantly higher NO_3-N in the subsoil and signifcantly lower NH_4-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 eO_3 (Figure 5). Under eO_3, plant residue was input into the surface soil and mineralized (for example, *ureC*) to NH_4^+, which also came from microbial N fixation (for example, *nifH*). NH_4^+ could moblize in soil and/or be used by plants and

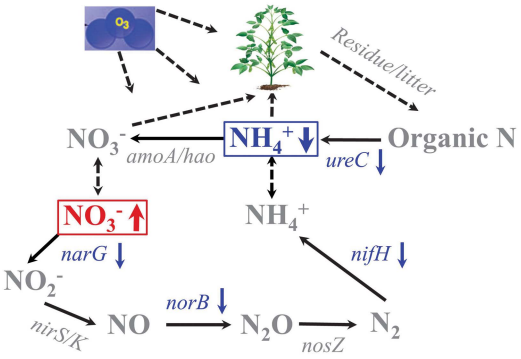

Figure 5 A conceptual model for microbial N cycling in response to eO_3. Red color indicates an increase in NO_3^- in the subsoil; blue color indicates decreases in NH_4^+ in the surface soil or abundances of N cycling genes; gray indicates those parameters were not measured or were not changed under eO_3.

microorganisms. The abundances of both *nifH* and *ureC* significantly decreased, resulting in a decrease of NH_4-N in the surface soil. NH_4^+ was transformed to NO_3^- by nitrification (for example,, *amoA* and *hao*), which could mobilize in soil or/ and be used by plants and microorganisms. NO_3^- was then transformed to NO_2^-, NO, N_2O and N_2 by denitrification (for example, *narG*, *nirS/K*, *norB* and *nosZ*). As the *narG* and *norB* abundances signficantly decreased at eO_3, denitrification, especially the first step (NO_3^- to NO_2^- by *narG*) was probably inhibited, leading to an increased NO_3^- in the subsoil. This conceptual model may suggest a possible negative microbial feedback to eO_3 in this soybean agro-ecosystem.

The third scenario is how eCO_2 and eO_3 interact to affect microbial functional processes and ecosystem functioning. It has been proposed that eO_3-induced negative effects on soil microbial communities may be ameliorated by eCO_2, and a couple of mechanisms are considered. First, under eCO_2, the uptake of O_3 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 eCO_2 may help plants and microorganisms detoxify and activate cell repair processes (Allen, 1990; McKee *et al.*, 1997). Although there were no significant interactions between eCO_2 and eO_3 in plant responses to eCO_2 and eO_3 (Valkama *et al.*, 2007), previous studies showed that eO_3 eliminated eCO_2-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 $CO_2 + O_3$ (Holmes *et al.*, 2006). In this study, under eCO_2 and eO_3, we found that eCO_2 promoted abundances of some eO_3-suppressed genes to ambient, or even to eCO_2-induced levels. For example, the abundances of four eO_3-inhibited C cycling genes (Pcc/Acc, xylanase, cellobiase and endochitinase genes) exhibited ambient/eCO_2 levels under $eCO_2 + eO_3$. In addition, the abundances of four eO_3-suppressed N cycling genes (*nifH*, *narG*, *norB* and *ureC*) were returned to ambient or promoted to eCO_2 levels under $eCO_2 + eO_3$. In addition, a few C and N cycling genes (for example, *pulA*, *napA*, *nirK* and *nirS*) showing no signifcant differences under eO_3 were significantly increased under $eCO_2 + eO_3$. The results suggest that the intereactive effects by $eCO_2 + eO_3$ 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, waranting further investigation by high-throughput sequencing and other metagenomics approaches, such as stable isotope probing, metatranscriptomics and meteproteomics. Also, we analyzed microbial communities from bulk soil in this study, and it will be interesting to analyze the response of rhizosphere micorbial communities including arbuscular mycorrhizal fungi to eCO_2 and eO_3. 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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