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ORIGINAL ARTICLE

Shifts of functional gene representation in wheat rhizosphere microbial communities under elevated ozone

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Although the influence of ozone (O_3) on plants has been well studied in agroecosystems, little is known about the effect of elevated O_3 (eO_3) on soil microbial functional communities. Here, we used a comprehensive functional gene array (GeoChip 3.0) to investigate the functional composition, and structure of rhizosphere microbial communities of Yannong 19 (O_3-sensitive) and Yangmai 16 (O_3-relatively sensitive) wheat (*Triticum aestivum* L.) cultivars under eO_3. Compared with ambient O_3 (aO_3), eO_3 led to an increase in soil pH and total carbon (C) percentages in grain and straw of wheat plants, and reduced grain weight and soil dissolved organic carbon (DOC). Based on GeoChip hybridization signal intensities, although the overall functional structure of rhizosphere microbial communities did not significantly change by eO_3 or cultivars, the results showed that the abundance of specific functional genes involved in C fixation and degradation, nitrogen (N) fixation, and sulfite reduction did significantly ($P<0.05$) alter in response to eO_3 and/or wheat cultivars. Also, Yannong 19 appeared to harbor microbial functional communities in the rhizosphere more sensitive in response to eO_3 than Yangmai 16. Additionally, canonical correspondence analysis suggested that the functional structure of microbial community involved in C cycling was largely shaped by soil and plant properties including pH, DOC, microbial biomass C, C/N ratio and grain weight. This study provides new insight into our understanding of the influence of eO_3 and wheat cultivars on soil microbial communities.

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Introduction

Ozone (O_3) is an atmospheric gas that has been rising due to human activity and industrial development. For agriculture systems, O_3 is considered as the most phytotoxic air pollutant potentially suppressing plant growth, productivity and yield (Schoene *et al.*, 2004; Ainsworth, 2008; Booker *et al.*, 2009), and more importantly, it may substantially impact below-ground functional processes, such as root growth and carbon (C) allocation (Morgan *et al.*, 2003; Rämö *et al.*, 2006; Feng and Kobayashi, 2009; Wittig *et al.*, 2009; Betzelberger *et al.*, 2010). It is expected that such effects will be much more rapid and have significant impacts on plant productivity, soil C and nitrogen (N) dynamics, and ecosystem functioning if anthropogenic activities leading to ozone formation continue unabated in the future (IPCC, 2007).

Wheat is the second largest food crop with an annual production of >650 million metric tons and harvested area of over 200 million hectares worldwide (Zhu *et al.*, 2011). In the Yangtze River Delta region of China, O_3 pollution resulted in about 10% yield loss of wheat in 1999 as predicted from results of local open-top chamber studies and monitoring data (Feng *et al.*, 2003). Currently, the mean O_3 concentrations (July–October) range from 38 to 46 ppb in the Yangtze River Delta of China (Wang *et al.*, 2006), and it is predicted that the average O_3 concentration will increase from 42 to 63 ppb by the

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end of this century, which could drive a further 10% decrease in yield for soybean, wheat and rice, and 20% for bean according to meta-analysis conducted by Feng and Kobayashi (2009). One of the strategies for reducing such negative O_3 effects on wheat yield is to screen cultivars that are better adapted to eO_3 conditions (Barnes *et al.*, 1990; Pleijel *et al.*, 2006). Recently, some O_3-relatively sensitive wheat cultivars have been reported in China, which may avoid yield reduction in a high O_3 environment (Cao *et al.*, 2009; Zhu *et al.*, 2011).

As soil systems depend on nutrient inputs from plants and nutrient cycling of soil microorganisms, a change in nutrient flux due to eO_3 could also alter structural and functional aspects of soil microbial biodiversity (Andersen, 2003). In recent years, most studies on the effect of eO_3 on rhizosphere microbial communities were largely based on open-top chambers, and the results mainly focused on the changes of microbial biomass and structure by phospholipid fatty acid analysis, denaturing gradient gel electrophoresis methods (Kasurinen *et al.*, 2005; Chung *et al.*, 2006; Kanerva *et al.*, 2008), and/or single-strand conformation polymorphism method (Dohrmann and Tebbe, 2005). Those studies indicated that eO_3 had little or significant effects on soil microbial communities with different plants/ecosystems. However, there are only few reports about effects of eO_3 on soil microbial functional processes (Larson *et al.*, 2002; Phillips *et al.*, 2002; Zak *et al.*, 2007; Chen *et al.*, 2009). It is important to comprehensively examine the effect of eO_3 on the functional composition, structure and metabolic potential of rhizosphere microbial communities.

Functional gene microarray-based technology (for example, GeoChip) has become a routine molecular tool to analyze the functional composition, structure and dynamics of microbial communities from a variety of ecosystems (He *et al.*, 2012a, c). For example, GeoChip 3.0 was used to examine how elevated CO_2 affected soil microbial communities, and the results showed that the functional composition, structure and metabolic potential of soil microbial communities were shifted, which was significantly correlated with soil C and N contents and plant productivity (He *et al.*, 2010b). Also, it was applied to examine the effects of global warming (temperature) on soil microbial communities (Zhou *et al.*, 2012), and to profile arsenic-contaminated soil microbial communities (Xiong *et al.*, 2010) and rhizosphere microbial communities of *Candidatus* Liberibacter asiaticus infected citrus trees (Trivedi *et al.*, 2012). All results demonstrate that GeoChip is a robust and high-throughput tool to specifically, sensitively and quantitatively profile microbial communities and link their structure with environmental factors and ecosystem functioning.

In this study, we hypothesized that changes in plant and soil properties resulting from eO_3 would alter the functional diversity, composition, structure and metabolic potential of rhizosphere microbial communities, and such effects would vary with wheat cultivars. To test those hypotheses, our objectives were (i) to examine the effect of eO_3 on rhizosphere microbial functional genes of Yannong 19 (O_3-sensitive) and Yangmai 16 (O_3-relatively sensitive) wheat cultivars; (ii) to distinguish whether there were differential responses between those two wheat cultivars; and (iii) to understand the correlation between the functional structure of rhizosphere microbial communities and the soil and plant properties under eO_3. The study was conducted on a free-air ozone enrichment (O_3-FACE) experimental site located in a suburb of Jiangdu City, China. GeoChip 3.0 was used to determine the gene abundance from GeoChip hybridization signal intensities. Although the overall functional structure of rhizosphere microbial communities did not significantly change under eO_3 or between cultivars, the results showed that eO_3 did alter the structure of functional genes involved in C cycling and change the abundance of some key functional genes involved in C, N and sulfur (S) cycling, which appeared to be cultivar dependent. This study provides new insights into our understanding of the eO_3 and wheat cultivars effect on rhizosphere microbial communities.

Materials and methods

Experimental site and sample collections

The experimental site is located in the suburb of Jiangdu City in Jiangsu province of China (32°35′ N, 119°42′ E), and the soil type is Shajiang Aquic Cambosols (Chinese Soil Taxonomy) (Li *et al.*, 2009) with a sandy-loamy texture, with 15 g kg^{-1} total C, 1.59 g kg^{-1} total N, pH 6.8, and 25.1% clay (<0.001 mm) and bulk density 1.2 g cm^{-3} at 0–15 cm depth (Zhu *et al.*, 2011). An experimental platform of O_3-FACE was established in 2007 over a rice-wheat rotation system, with rice transplanted in mid-June and harvested in middle-to-late October and winter wheat sown in early November and harvested in late May or early June of the next year. This study was conducted during the wheat growing season of 2010 (for example, November 2009 to June 2010) after three growth seasons with O_3 fumigation at three periods: 14 April to 22 May in 2007, 5 March to 26 May in 2008 and 1 March to 24 May in 2009, and their average concentrations of O_3 were 42 ppb for aO_3 and 53.4 ppb for eO_3 (Zhu *et al.*, 2011).

The O_3-FACE system has three O_3-FACE rings and three similar ambient rings. Three O_3-FACE replicate rings, each 14.5 m in diameter, were set randomly to continuously provide an eO_3 concentration of 60 ppb from 0900 h to 1800 h during 3 March and 31 May 2010, while three ambient (40 ppb) replicate rings, each with the same size, were set randomly within the same area. All of the rings were far enough apart to prevent O_3 from spilling over from one ring to another to avoid the influence of O_3 from the O_3-FACE rings on the

ambient plots. In each O_3-FACE or ambient ring, two winter wheat cultivars (Yannong 19 and Yangmai 16) were sown in November in each subplot of a ring. Previous studies showed different responses of Yannong 19 and Yangmai 16 to eO_3, and Yannong 19 (Y19) was referred to as O_3-sensitive wheat cultivar, and Yangmai 16 (Y16) as O_3-relatively sensitive cultivar (Cao *et al.*, 2009; Zhu *et al.*, 2011). A total of 12 rhizosphere and 12 corresponding bulk samples were taken from 2 cultivars of 6 rings at the ripening stage on 10 June 2010. For GeoChip analysis, rhizosphere soil was taken by collecting soil that adhered to plant roots after the plant had been removed from soil and shaken, and each sample was a composite from the roots of five randomly selected plants and then stored at $-20\,^{\circ}C$ until DNA extraction. For soil property analysis, soil cores (2.5-cm diameter at the depth of 0–15 cm) were taken from the corresponding site of plants from which the rhizosphere soil had been collected, and each soil sample was pooled from five soil cores and stored at -20 or $4\,^{\circ}C$ until further analyses.

Analysis of plant and soil properties

The total carbon (TC) and nitrogen (TN) of plant or soil were determined by a TruSpec CN Elemental Analyzer (Leco Corporation, St. Joseph, MI, USA). Dissolved organic carbon (DOC) was determined by a Multi N/C 3100 analyzer (Analytik Jena AG, Jena, Germany). Soil pH was determined with a glass electrode in 1:2.5 (soil:water) solution (w/v). Fifteen winter wheat plants from each subplot were harvested, and partitioned into grain and litter. Litter and grain samples were dried at $65\,^{\circ}C$ until a constant weight was obtained, and then weighed for 1000 kernel weight (grain weight) and individual plant weight (plant weight).

Nucleic acid extraction, purification, amplification and labeling

Community DNA of 12 rhizosphere soil samples was extracted by the freeze-grinding method (Zhou *et al.*, 1996). Extracted DNA was stored at $-80\,^{\circ}C$ until it was used. The quality of purified DNA was assessed by an ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) and the concentration of DNA was measured using a Quant-It PicoGreen kit (Invitrogen, Carlsbad, CA, USA) using a FLUOstar Optima (BMG Labtech, Jena, Germany). An aliquot of 100 ng DNA from each sample was amplified in triplicate using the TempliPhi kit (Amersham Biosciences, Piscataway, NJ, USA) in a modified buffer containing single strand binding protein ($200\,ng\,\mu l^{-1}$) and spermidine (0.04 mM) to increase the sensitivity of amplification at $30\,^{\circ}C$ for 3 h (Wu *et al.*, 2006). Amplified DNA ($\sim 3.0\,\mu g$) was mixed with 20 ml 2.5 × random primers (Invitrogen), heated to $99\,^{\circ}C$ for 5 min, and immediately placed on ice, then fluorescently labeled in a reaction solution containing 50 μM dATP, dCTP, dGTP, 20 μM dTTP (USB Corporation, Cleveland, OH, USA), 1 mM Cy5 dUTP (Amersham Pharmacia Biotech, Piscataway, NJ, USA),and 40 U of Klenow fragment (Invitrogen), incubating at $37\,^{\circ}C$ for 3 h. The labeled products were purified with a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA), and then dried down in a SpeedVac (Thermo Fisher Scientific Inc., Milford, MA, USA) for 45 min at $45\,^{\circ}C$.

Microarray hybridizations, scanning and array data processing

The fluorescently labeled DNA was suspended in the hybridization mix (50% formamide, 3 × SSC, 0.3% SDS, $0.7\,mg\,ml^{-1}$ herring sperm DNA), and 0.86 mM DTT incubated at $95\,^{\circ}C$ for 5 min, and then maintained at $60\,^{\circ}C$ until hybridization. Samples were hybridized with GeoChip 3.0 (He *et al.*, 2010a) on a MAUI Hybridization System (BioMicro Systems, Salt Lake City, UT, USA) at $42\,^{\circ}C$ for 12 h. Microarrays were scanned on a Pro ScanArray Microarray Scanner (Perkin-Elmer, Boston, MA, USA), and signal intensities of each spot were measured with ImaGene 6.0 (Biodiscovery, El Segundo, CA, USA). Empty and poor spots were removed before the signal intensities were normalized by the mean signal across the slide, and spots with signal-to-noise ratio (SNR = (signal mean − background mean)/background standard deviation) > 2.0 were used as the cutoff for positive spots for further analyses (He and Zhou, 2008). A gene was considered as positive if it was detected in at least 2 of 12 samples.

Statistical analyses

All data were analyzed through a general linear model for split-plot design to determine the effects of O_3 concentration (ambient vs. elevated), different wheat cultivars (Y19 vs. Y16) and their interactions. Diversity indices were calculated as previously described (He *et al.*, 2010b). Permutational multivariate analysis of variance (adonis) was based on Euclidean distance matrices to partition differences among different treatments using permutations (999 times), and the significance test was based on pseudo-*F* ratio (Oksanen *et al.*, 2010). Mantel and partial Mantel analyses were used to link the functional structure of microbial communities with plant and soil variables (He *et al.*, 2010b). All statistical analyses were performed by the Vegan package in R (Dixon, 2003). Detrended correspondence analysis (DCA) and canonical correspondence analysis (CCA) were performed using CANOCO for Windows version 4.5 (Biometris—Plant Research International, Wageningen, The Netherlands).

Results

Effects of eO_3 on soil and plant properties

To understand whether eO_3 affects soil and wheat properties, 5 soil variables and 10 plant variables

were measured (Supplementary Table S1). ANOVA results showed that soil pH and the TC in both grain (TC-grain) and straw (TC-straw) were significantly ($P<0.05$) increased under eO_3, while DOC and grain weight were significantly ($P<0.05$) decreased under eO_3. Also, the decreases in DOC and grain weight were significantly ($P<0.05$) different between Yannong 19 and Yangmai 16 cultivars with an average of 15.4% decrease for the Yangmai 16 cultivar and an average of 17.4% decrease for the Yannong 19 cultivar (Figure 1). However, microbial biomass C (MBC), TC, TN, C/N ratio (CNR), total N in grain (TN-grain) and straw (TN-straw), grain and straw CNRs (CNR-grain, CNR-straw), panicle length, plant weight and root-straw weight did not significantly change at eO_3 (Supplementary Table S1). In addition, no significant effects were observed for the interaction of O_3 and cultivar (Supplementary Table S1; Figure 1). Such changes in soil and plant properties may affect the functional composition and structure of rhizosphere microbial communities, which was further examined by GeoChip 3.0 in this study.

Overall review of soil microbial functional structure under eO_3

A total of 3691 genes were detected by GeoChip 3.0 in at least 2 out of 12 samples, including 244 genes derived from fungi, and 131 from archaea. The number of genes detected for individual samples ranged from 1485 to 2858, but no significant differences in the number of detected genes were observed between aO_3 and eO_3 samples or between both cultivars. Similarly, the Shannon index (H′) and the Simpson's reciprocal index (1/D) were not significantly different between aO_3 and eO_3 or between both cultivars. However, the Simpson's evenness was higher at aO_3 for both cultivars than at eO_3, at which this index was also higher in the Yangmai 16 cultivar than in the Yannong 19 cultivar (Supplementary Table S2). Adonis analysis of abundances of all detected genes showed that O_3, cultivar and their combination had little effect upon the overall functional structure of rhizosphere microbial community (Table 1). Also, DCA of all detected genes, or subsets of fungal or archaeal genes indicated that aO_3 and eO_3 samples or Yannong 19 and Yangmai 16 samples could not be well separated, though eO_3 samples appeared to be more closely clustered together than aO_3 samples (Figure 2). In addition, the ratios of fungi to bacteria, and archaea to bacteria were significantly decreased and increased, respectively, at eO_3 for the Yannong 19 cultivar only, while no significant changes were seen for the Yangmai 16 cultivar (Supplementary Figure S1). Therefore, all these results indicated that the overall functional diversity and structure of rhizosphere microbial communities appeared not to be significantly altered by eO_3.

Relationships between functional structure and environmental variables

To explore possible linkages between the functional structure of rhizosphere microbial communities and environmental factors, including pH, MBC, DOC, TC, TN, CNR, TC-grain, TN-grain, CNR-grain, TC-straw, TN-straw, CNR-straw, panicle length, grain weight, plant weight and straw-root weight (Supplementary Table S1), we analyzed GeoChip data and those environmental variables by Mantel or partial Mantel tests and CCA. First, Mantel analysis of all environmental factors and the signal intensity of all detected genes showed significant ($P<0.05$) correlations between 11 individual functional genes

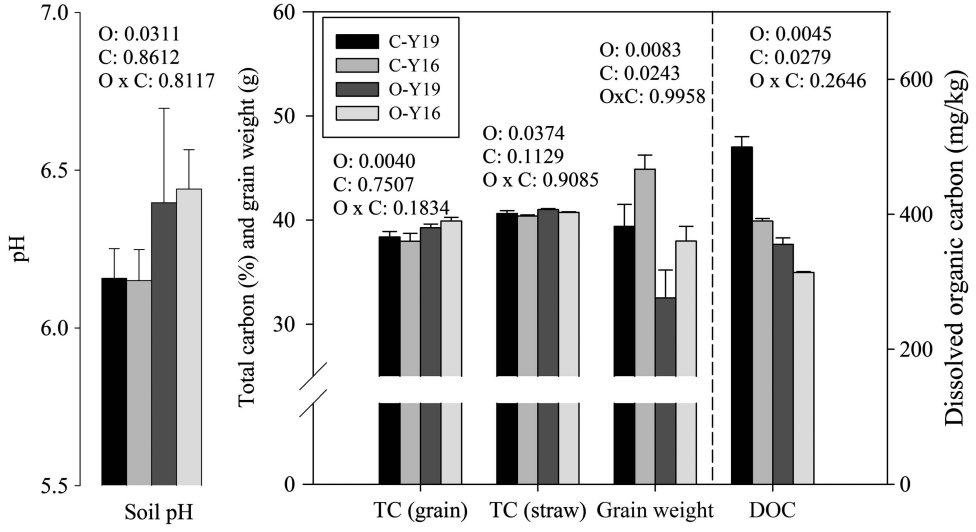

Figure 1 Significantly changed soil and plant properties under eO_3 and with Yannong 19 and Yangmai 16 wheat cultivars. C-Y19: Yannong 19 (O_3-sensitive) cultivar under control conditions; C-Y16: Yangmai 16 (O_3-relatively sensitive) cultivar under control conditions; O-Y19: Yannong 19 cultivar under elevated O_3 conditions; O-Y16: Yangmai 16 cultivar under elevated O_3 conditions. *P*-values shown in the figure are based on split-plot ANOVA (O: O_3; C: cultivar; O × C: O_3 × cultivar).

Table 1 Adonis analysis of the effect of eO_3 and cultivars on the functional structure of rhizosphere microbial communities based on the signal intensity of all detected genes by GeoChip 3.0

	F-*value*	P-*value*	R^2
O_3	0.7905	0.495	0.0805
Cultivar	0.5213	0.889	0.0531
O_3:cultivar	0.5047	0.904	0.0514

Abbreviation: eO_3, elevated ozone.

(for example, *aceA*, *aceB*, *nirK*, *ppk* and *dsrA*) and those soil variables, but none of those genes were significantly correlated with the plant variables (Table 2). Second, CCA was used to further explore what environmental factors largely shaped the functional structure of rhizosphere microbial communities. Although no significant correlations were found between the signal intensity of all detected functional genes and environmental variables selected, the detected C cycling genes were found to have a significant ($P<0.05$) relationship with the selected environmental variables, including soil pH, MBC, DOC, CNR, grain weight and O_3 concentration (mean concentrations for each plot during the wheat growth season) (Figure 3). Based on this model, a total of 60.7% of the total variance could be explained by the first two constrained axes, with the first axis explaining 48.4% and the second axis for 12.3%. Both the first canonical axis ($F=4.692$, $P=0.022$) and the sum of all canonical axes ($F=2.694$, $P=0.010$) were significant by the Monte Carlo test. The structure of C cycling genes was quite different between aO_3 and eO_3, which was well separated by the first axis, and positively correlated with DOC and negatively correlated with O_3 concentration, soil pH and MBC. The second axis was positively correlated with soil C/N and negatively with grain weight. The first axis indicated that DOC decreased, and pH and MBC increased following an exposure to eO_3 for both wheat cultivars, while MBC was higher in the Yangmai 16 cultivar than in the Yannong 19 cultivar. The second axis indicated grain weight decreased and soil C/N increased under eO_3 compared with aO_3 for both cultivars (Figure 3). The results indicated that the functional diversity and structure of microbial communities involved in C cycling was closely correlated with soil and plant properties, and largely shaped by O_3 concentration, soil pH, DOC, MBC and CNR as well as by grain weight.

Responses of functional genes involved in C, N, S and phosphorus (P) cycling under eO_3

Although eO_3 did not have significant effects on the overall microbial functional structure, ANOVA of all detected genes showed significant changes at the gene family level under eO_3. The abundances of 17 gene families were significantly ($P<0.05$) affected by eO_3 (*fhs* and *cor*C), by cultivars (*limEH*, *aclB*, *chrA*, *copA*, *nifH*, *pimF*, *pobA*, *dsrA* and carbon monoxide dehydrogenase (CODH), exoglucanase, aliphatic nitrilase and small multidrug resistance genes), or by their interaction (*lip*, *alkH* and *chnB*) (Supplementary Table S3). With a focus on functional genes involved in C, N, P and S cycling, some general trends of microbial community responses to eO_3 and cultivars were described in detail below.

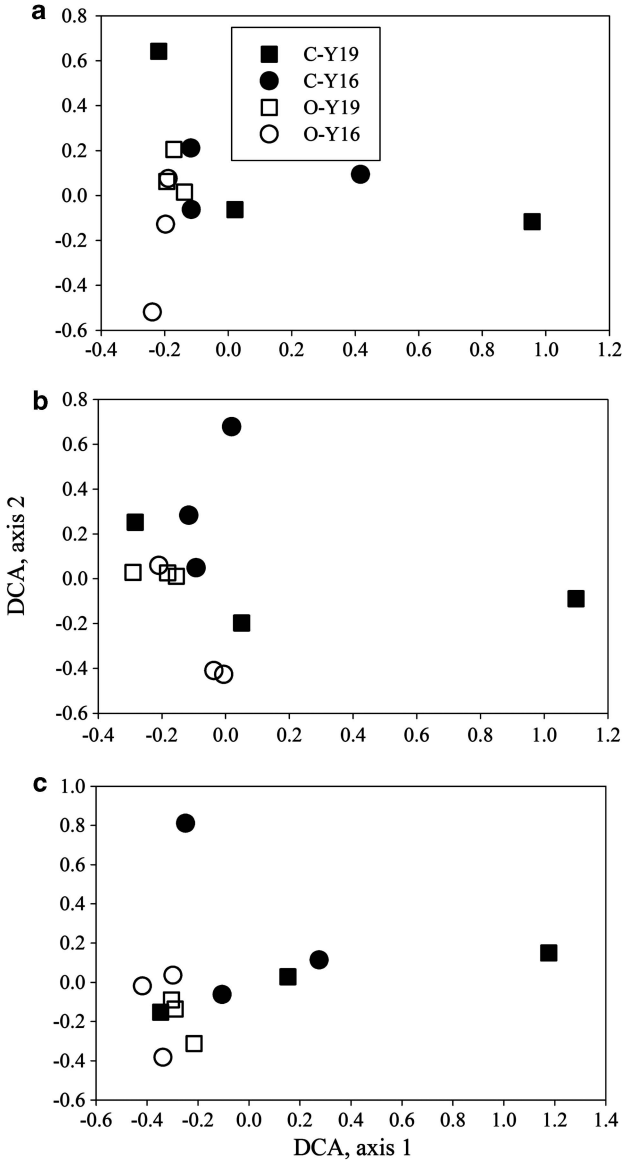

Figure 2 DCA of all detected genes (**a**), and subsets of genes derived from archaea (**b**) and fungi (**c**) based on hybridization signal intensities detected by GeoChip 3.0. For C-Y19, C-Y16, O-Y19 and O-Y16, please see Figure 1 for details.

C cycling genes. The abundances of four C cycling genes were significantly ($P<0.05$) changed by eO_3 (*fhs*), cultivars (CODH and exoglucanase genes), or eO_3 and cultivar (*lip*). The abundances of *fhs* were lower at eO_3 than at aO_3 for both cultivars (Figure 4b); the abundances of CODH and

Table 2 Significantly correlated functional genes involved in C, N, S and P cycling with soil and/or plant properties analyzed by partial Mantel analysis

Gene or enzyme	*Functional process*	*Soil with plant as control*		*Plant with soil as control*	
		r-*value*	P-*value*	r-*value*	P-*value*
aceA	C degradation	0.357	**0.025**	0.017	0.446
aceB		0.326	**0.044**	−0.017	0.536
CODH		0.316	**0.039**	0.123	0.209
Phenol oxidase		0.352	**0.037**	0.121	0.240
pcc	C fixation	0.326	**0.031**	0.010	0.462
nasA	Assimilatory N reduction	0.362	**0.022**	0.075	0.306
nirB		0.355	**0.040**	0.069	0.308
nirK	Denitrification	0.366	**0.020**	0.052	0.348
norB		0.434	**0.017**	0.278	0.089
ppk	P utilization	0.421	**0.026**	0.118	0.236
dsrA	Sulfite reduction	0.405	**0.010**	0.254	0.056

Abbreviations: C, carbon; CODH, carbon monoxide dehydrogenase; N, nitrogen; P, phosphorus.
Bold values are significant, with $P<0.05$.

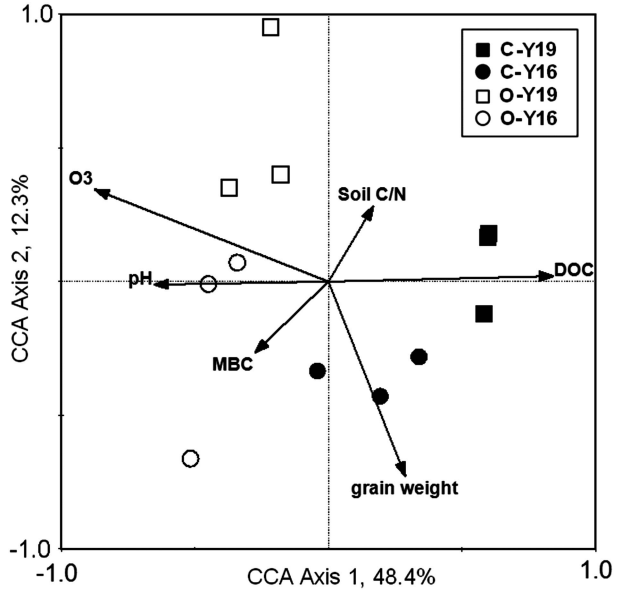

Figure 3 CCA of GeoChip hybridization signal intensities of detected functional genes involved in C cycling and environmental variables, including O_3, DOC, MBC, soil pH, soil C/N and grain weight. For C-Y19, C-Y16, O-Y19 and O-Y16, please see Figure 1 for details.

exoglucanase genes were higher in Yangmai 16 than in Yannong 19 (Figures 4a and c); under eO_3, a significant decrease for Yannong 19 and a significant increase for Yangmai 16 were observed for *lip* (Figure 4d). Also, the abundance of C cycling genes generally decreased at eO_3, but the abundances of a few genes increased, including *mcrA*, and mannanase and xylanase genes for both cultivars, *amyX*, *nplT* and *lip* for Yangmai 16, and *pcc*, *aceA/B*, bacterial *ara*, and CODH and phenol oxidase genes for Yannong 19 (Table 3). In addition, for the rhizosphere microbial communities, the abundances of C cycling genes were generally higher in Yangmai 16 than in Yannong 19 under both aO_3 and eO_3 conditions (Table 3). The results generally indicated that the C cycling process of rhizosphere microbial communities from both Yannong 19 and Yangmai 16 cultivars might be inhibited by eO_3, but functional genes of microbial communities from Yangmai 16 maintained relatively high abundances, indicating that rhizosphere microbial communities of Yangmai 16 (O_3-relatively sensitive) may be also more tolerant under eO_3 conditions.

N cycling genes. The abundances of most N cycling genes generally decreased or remained unchanged for microbial communities of Yangmai 16 but increased for Yannong 19 at eO_3; however, the abundances of N cycling genes were generally higher in Yangmai 16 than those in Yannong 19 (Supplementary Table S4). For example, *nifH* was detected as the only significantly ($P<0.05$) changed gene in the N cycle in this study. Compared with aO_3, the percentage changes were −6.1% and 18.6% for Yangmai 16 and Yannong 19, respectively, at eO_3, while those percentage changes of Yangmai 16 over Yannong 19 were 2.9% and 30.0% at eO_3 and aO_3, respectively, indicating a higher abundance for Yangmai 16 than for Yannong 19 at both aO_3 and eO_3 conditions (Supplementary Table S4; Figure 4e). These results indicated that eO_3 might affect N cycling processes (for example, N fixation) of microbial communities, and that the rhizosphere microbial community of Yannong 19 (O_3-sensitive) cultivar may be also more sensitive under eO_3 than that from the Yangmai 16 (O_3- relatively sensitive) cultivar.

P and S cycling genes. The changes in abundance of most P and S cycling genes were similar to those for N cycling genes with a general increase for Yannong 19, and a decrease for Yangmai 16 at eO_3. *dsrA* was detected as the only significantly ($P<0.05$) changed gene in S cycling and no significantly changed genes were detected for P utilization in this study. Compared with aO_3, the percentage changes of *dsrA* were −6.7% and 25.9% for Yangmai 16 and

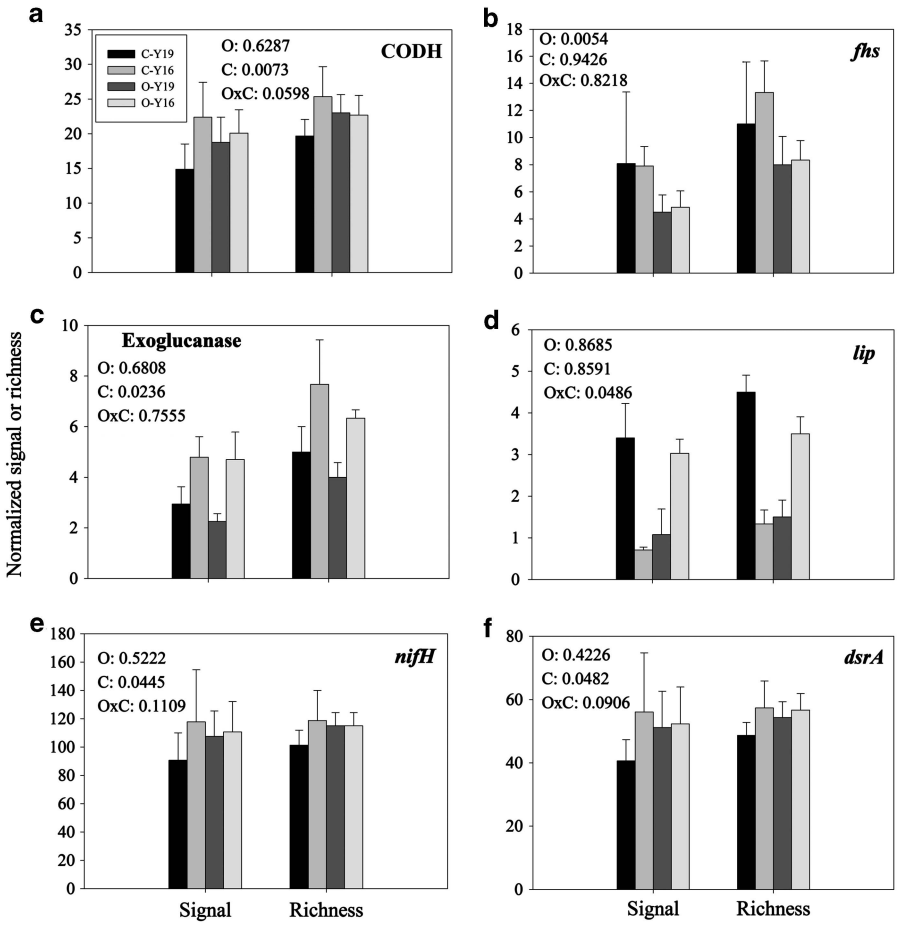

Figure 4 Representatives of key functional genes significantly affected by eO_3 and/or wheat cultivars via ANOVA. (**a**) CODH; (**b**) *fhs* encoding formyltetrahydrofolate synthetase; (**c**) Exoglucanase; (**d**) *lip*; (**e**) *nifH*; (**f**) *dsrA*. Details of C-Y19, C-Y16, O-Y19 and O-Y16 are described in Figure 1. *P*-values shown in the figure are based on split-plot ANOVA (O: O_3; C: cultivar; O × C: O_3 × cultivar).

Yannong 19, respectively, at eO_3, while the percentages of changes for Yangmai 16 over Yannong 19 were 2.3% and 38.0% at eO_3 and aO_3, respectively (Supplementary Table S5; Figure 4f). Similarly to N cycling genes, these results indicated that eO_3 might modify S and P cycling with Yannong 19 (O_3-sensitive) cultivar microbial communities being more sensitive under eO_3.

gyrB. GeoChip 3.0 also targets *gyrB* (He *et al.*, 2010a), a phylogenetic marker to examine the phylogenetic diversity, composition and structure of microbial communities. A total of 195 *gyrB* probes showed positive signals, but the number or signal intensity of detected genes was not significantly different between aO_3 and eO_3, or between two cultivars (Supplementary Figure S2A), and nor did DCA of all detected *gyrB* genes show a clear separation by aO_3 and eO_3, or two cultivars (Supplementary Figure S2B). The results indicated that the phylogenetic diversity and structure of the rhizosphere microbial communities was not significantly impacted by eO_3 or by wheat cultivars, which was generally consistent with our analyses of functional genes above.

Discussion

Understanding the response of soil microbial communities to eO_3 is essential for establishing sustainable agroecosystems in an eO_3 environment. In this study, we examined the functional composition and structure of rhizosphere microbial communities of Yannong 19 and Yangmai 16 wheat cultivars under eO_3. Our results indicated that the overall microbial functional diversity or structure was not significantly affected by eO_3, but the structure of functional genes involved in C cycling altered, and significantly changed functional genes involved in C, N and S cycling were identified. Also, the abundance of functional genes generally decreased, indicating an inhibitory effect of eO_3 on soil microbial communities. In addition, the Yannong 19 (O_3-sensitive) cultivar appears to harbor rhizosphere microbial communities more sensitive under eO_3 than the Yangmai 16 cultivar.

Table 3 Effects of elevated O_3 and wheat cultivars on abundances of key functional genes involved in C cycling analyzed by ANOVA. Only genes with >3 probes detected were shown

Gene or enzyme	*Functional process*	*O_3 (%)*[a]		*Cultivar (%)*[b]		P-*value*[c]		
		Y19	*Y16*	*aO_3*	*eO_3*	*O_3*	*Cultivar*	*O_3:cultivar*
CODH	C fixation	25.98	−10.24	50.25	7.05	0.6287	**0.0073**	0.0598
pcc		10.54	−15.88	36.83	4.12	0.6759	0.1223	0.2280
Rubisco		−5.21	−16.74	21.70	6.90	0.4621	0.4169	0.6630
fhs	Acetogenesis	−44.37	−38.62	−2.25	7.85	**0.0054**	0.9426	0.8218
aceA	Glyoxylate cycle	22.50	−13.07	43.92	2.13	0.9152	0.1802	0.2340
aceB		5.90	−8.45	24.39	7.52	0.8213	0.1135	0.4216
amyA	Starch degradation	−6.81	−11.87	14.80	8.57	0.3071	0.2547	0.7335
amyX		−8.24	124.84	−58.02	2.87	0.2292	0.7732	0.7298
Glucoamylase		−33.45	−6.77	−12.92	21.99	0.4231	0.9721	0.5746
nplT		−44.24	27.04	−37.11	43.30	0.6793	0.8439	0.3539
pulA		−16.23	−0.91	0.32	18.67	0.6362	0.6594	0.6724
Cellobiase	Hemi-cellulose degradation	−17.60	−7.36	4.09	17.03	0.6117	0.7158	0.8416
Mannanase		29.91	112.68	62.12	165.41	0.1579	0.0675	0.3079
ara-bacteria		10.35	−10.73	12.28	−9.17	0.9730	0.9656	0.6549
ara-fungi		−3.67	−9.40	3.64	−2.53	0.7180	0.9742	0.8700
xylA		−48.45	−21.45	−22.94	17.41	0.0567	0.6811	0.3479
Xylanase		20.18	6.12	26.61	11.80	0.5302	0.3593	0.7800
Endoglucanase	Cellulose degradation	−22.09	−13.52	8.29	20.21	0.4212	0.5986	0.8704
Exoglucanase		−23.22	−1.97	63.17	108.31	0.6806	**0.0236**	0.7555
vanA	Aromatics degradation	12.84	−19.23	29.16	−7.54	0.7606	0.5999	0.3388
NAG*	Chitin degradation	−24.27	−16.72	−6.83	2.46	0.2560	0.8875	0.8043
Endochitinase		−17.45	−7.24	3.47	16.27	0.2640	0.4491	0.6556
glx	Lignin degradation	−38.68	−34.61	32.03	40.78	0.1337	0.3092	0.9001
lip		−68.33	327.27	−79.14	181.42	0.8685	0.8591	**0.0486**
mnp		−86.30	−57.76	−11.15	173.85	0.0539	0.8953	0.2185
Phenol oxidase		16.21	−11.16	23.71	−5.43	0.9536	0.6727	0.4663
mcrA	Methanogenesis	19.73	34.46	29.98	45.97	0.4396	0.3089	0.7638
pmoA	CH_4 oxidation	−23.20	−9.22	28.92	52.38	0.4147	0.1085	0.7924

Abbreviations: aO_3, ambient ozone; C, carbon; eO_3, elevated ozone; CODH, carbon monoxide dehydrogenase; NAG*, *N*-acetyl-glucosaminidase.
[a]Percentage change by eO_3 was calculated using the following formula: $(eO_3 - aO_3) \times 100/aO_3$, where aO_3 and eO_3 were the average signal intensities of genes detected by GeoChip 3.0 at aO_3 or eO_3, respectively.
[b]Percentage change by cultivar is calculated using the following formula: $(cult_{Y16} - cult_{Y19}) \times 100/cult_{Y19}$ under aO_3 or eO_3 conditions.
[c]*P*-values based on ANOVA using the model: $Y \sim O_3 + cultivar + O_3$: cultivar.
Bold values are significant, with $P<0.05$.

One of our hypotheses is that eO_3 significantly affects the overall functional diversity and structure of rhizosphere microbial communities via the changes in plant properties and soil microenvironments (Biswas *et al.*, 2008; Feng *et al.*, 2008; Feng and Kobayashi, 2009; Zhu *et al.*, 2011). Previous studies showed that eO_3 decreased plant aboveground and root biomass and grain yield, altered litter and soil chemistry, and adjusted antioxidant capacity (Morgan *et al.*, 2003; Liu *et al.*, 2005; Rämö *et al.*, 2006; Betzelberger *et al.*, 2010), especially in wheat (Biswas *et al.*, 2008; Feng *et al.*, 2008; Feng and Kobayashi, 2009; Zhu *et al.*, 2011). However, only few studies have been focused on the effect of eO_3 on microbial community functions. For example, a significant discrimination of sole-carbon source utilization patterns was observed for the wheat rhizosphere soil but not for the bulk soil, and the functional diversity of the rhizosphere microbial communities was reduced under eO_3 (Chen *et al.*, 2009). Also, a few studies at the Aspen FACE site (Larson *et al.*, 2002; Phillips *et al.*, 2002; Chung *et al.*, 2006; Holmes *et al.*, 2006; Zak *et al.*, 2007) and with open-top chambers (Kasurinen *et al.*, 2005) or mesocosms (Kanerva *et al.*, 2008) indicated that eO_3 altered the functional diversity, structure and/or metabolic potential of soil microbial communities. In this study, our results did not fully support the above hypothesis that eO_3 would alter the overall functional diversity and structure of rhizosphere microbial communities. There are a few possibilities. First, the difference of concentrations between aO_3 (40 ppb) and eO_3 (60 ppb) was not large enough to cause significant changes in the overall soil microbial community diversity and structure. However, it should also be noted that although this increase in O_3 is relatively small, those differences identified in this study are highly relevant to the near future as well as for current years when O_3 concentration is relatively high. Second, the eO_3 exposure time may be still too short for soil microbial communities to respond to eO_3. Third, since DOC is largely composed of low molecular weight compounds (for example, organic acids, amino acids and sugars) with low pH, a decrease in DOC could lead to an increase in pH, but the overall effect of both factors may result no significant changes observed. Fourth, plant biomass or root biomass was not significantly changed between aO_3 and eO_3, or Yannong 19 and Yangmai 16 cultivars,

indicating that soil C inputs may remain unchanged. In addition, only three replicates were used in this study so that the statistical power may not be enough to distinguish subtle changes.

Although the overall microbial diversity and structure did not significantly change in response to eO_3, we did observe eO_3-induced and cultivar-specific changes for key functional genes involved in C, N and S cycling. For example, the abundance of *fhs* signficantly decreased at eO_3. Formyltetrahydrofolate synthetase encoded by *fhs* catalyzes a key step in the reductive acetogenesis pathway and produces acetate from C1 precursors (for example, CO_2). Acetogenesis is of great important to the global C cycle, producing ~10% of acetate annually in anaerobic habitats (Leaphart and Lovell, 2001). Actate is considered as an important substrate for many micoorganisms in soil, and a decrease of *fhs* abundance indicates a decrease of acetate production at eO_3, which may result in an increase in pH. Interestingly, the abundance of *lip* signifcantly decreased in Yannong 19, and signifcantly increased in Yangmai 16 at eO_3. Lignin peroxidase encoded by *lip* uses two substrates, 1,2-bis(3,4-dimethoxyphenyl)propane-1,3-diol and H_2O_2, and produces 3,4-dimethoxybenzaldehyde, 1-(3,4-dimethoxyphenyl)ethane-1,2-diol, and H_2O, where H_2O_2 is mainly responsible for plant adjustements in response to eO_3 (Heath, 2008). A significant increase of the *lip* abundance at eO_3 may be related to a reduction of H_2O_2 concentration in the rhizosphere, and faciliate detoxication of H_2O_2 in the rhizosphere and/or in plants. Also, more genes showed cultivar-specific changes at aO_3 or/and eO_3. For example, the abundances of genes encoding CODH, exoglucanase, NifH and DsrA were signifcantly higher in Yangmai 16 than those in Yannong 19 although they were not significantly different between aO_3 and eO_3. These results are generally consistent with previous studies upon enzyme activities of 1,4-β-glycosidase, 1,4-β-N-acetylglucosaminidase and other C degradation enzymes under eO_3 (Larson *et al.*, 2002; Chung *et al.*, 2006). Furthermore, the changes of functional gene abundances showed two clear patterns: (i) most C cycling genes decreased under eO_3 with a generally higher abundance for Yangmai 16 under both eO_3 and eO_3 and (ii) most N, S and P cycling genes increased for Yannong 19 and decreased for Yangmai 16 under eO_3 although a general higher abundance remained for Yangmai 16. Therefore, our results indicated that the abundances of some key genes signifcantly changed at eO_3 and/or between two cultivars, which may modify ecosystem functional processes, strongly supporting one of our hypotheses that eO_3 would affect the composition and metablic potential of rhizosphere microial commuities. In addition, this study identifies some important functional genes invovled in the reductive acetogenesis pathway (for example, *fhs*), lignin degradation (for example, *lip*) and N fixation (for example, *nifH*) in response to eO_3; thus, our future studies may focus on the diversity and quantification of those key genes by quantitative PCR and/or high-throughput sequencing approaches.

The microbial community diversity, structure and functional activity are also shaped by environmental factors, such as soil and plant properties. Previous studies indicated that soil pH and C inputs significantly affected soil microbial community diversity and structure (Fierer and Jackson, 2006; Aciego Pietri and Brookes, 2009). In this study, an increase in soil pH and a decrease in DOC were observed. Mantel analyses of all detected genes also showed that the abundances of many genes (for example, *aceA*, *aceB*, *pcc*, *nasA*, *nirK*, *ppk* and *dsrA*) involved in C, N, P and S cycling were significantly correlated with soil variables. Further CCA indicated that the structure of functional genes involved in C cycling could be largely shaped by soil pH, DOC, MBC, CNR and grain weight. Therefore, this study indicated that the microbial community structure might be shaped by soil environmental factors and plant properties.

Root exudates have an important role in plant–microbe interactions and shape the diversity, structure and function of soil, especially rhizosphere microbial communities (Bais *et al.*, 2006; Dennis *et al.*, 2010; Doornbos *et al.*, 2012). A previous study showed a degree of specificity in the interaction of wheat cultivars and fluorescent *Pseudomonas* species via analysis of *phlD* genes (Mazzola *et al.*, 2004). Also, a recent study with *Arabidopsis thaliana* suggested that root exudation differences could influence rhizosphere bacterial communities (Micallef *et al.*, 2009). Similarly, another study with different plants (wheat, maize, rape and barrel clover) demonstrated that plant species root exudates significantly shaped the rhizosphere bacterial community structures (Haichar *et al.*, 2008). In addition, artificial root exudate solutions were added to soil micrososms, and the results indicated that organic acids might have an important role in shaping soil bacterial communities (Shi *et al.*, 2011). However, it is unclear if root exudates shape the rhizosphere micorbial communities from Yannong 19 and Yangmai 16 cultivars, and further investigations are needed by a comprehesive profiling of root exudates and linking them with microbial functional structure and activity.

The rhizosphere microbial communities of Yannong 19 may be more sensitive than those from Yangmai 16 in response to eO_3. First, based on the number of significantly changed genes and the abundance changes of all detected genes, more shifts were found by cultivars than by O_3, or by their interaction. Second, many genes detected had higher abundances at eO_3 than at aO_3 for Yannong 19 rhizosphere microbial communities, while the abundances of detected genes in Yangmai 16 rhizosphere microbial communities remained largely unchanged. Third, CCA suggests that the signal intensity of detected genes involved in C cycling

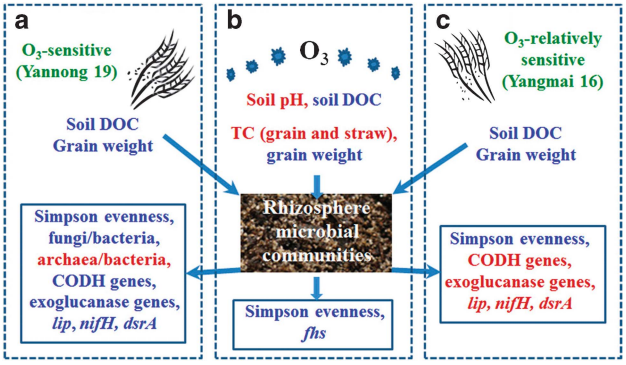

Figure 5 Summary of significant effects of eO_3 on plant and soil properties, and key microbial functional genes involved in C, N, S and P cycling (**b**), and cultivar-specific responses in Yannong 19 (**a**), and Yangmai 16 (**c**). In three rectangle boxes, the red and blue text is for significantly increased and decreased functional gene abundances, respectively. Soil, plant properties or functional genes without significant changes between aO_3 and eO_3, or between two cultivars are not shown.

had a positive correlation with MBC for Yangmai 16 rhizosphere samples, but a negative correlation for Yannong 19 rhizosphere samples, indicating that Yangmai 16 may be more favorable for soil microbial growth than Yannong 19. Therefore, our results suggested that the rhizosphere microbial communities from Yannong 19 may be also more sensitive in response to eO_3 than those from Yangmai 16. However, further investigations are necessary to understand the mechanism using different approaches, such as quantitative PCR or high-throughput sequencing analysis of 16S rRNA and/or key functional genes (for example, *fhs*, *nifH* and *lip*) identified in this study. It should be noted that two crops were grown at the FACE site per year with wheat being planted after rice, and rice was also exposed to eO_3 in the same ring/plot as wheat. Although rice is also sensitive to ozone, it is expected that rice has little effect on wheat rhizosphere microbial communities during the wheat growth season. Previous studies also suggest that the effect of O_3 on soil microbial communities largely came from wheat in the current growth season, especially after anthesis (Slaughter *et al.*, 1989; Pleijel *et al.*, 1998; Soja *et al.*, 2000).

In summary, this study indicates that continuously increasing O_3 may be a threat for sustainable production of wheat agroecosystems in the future (Figure 5). First, eO_3 significantly caused an increase in soil pH, total C concentration of straw and grain, and a decrease in soil DOC and grain weight. Second, although the overall rhizosphere microbial functional diversity and structure was not significantly affected by eO_3 or cultivars, the Simpson evenness and fungi/bacteria decreased, and many key microbial processes, especially C cycling showed a trend of inhibition under eO_3. Third, Yannong 19 appeared to harbor microbial communities more sensitive in response to eO_3 than Yangmai 16 (Figure 5). This study provides new insights into our understanding of the influence of eO_3 and wheat cultivars on soil microbial communities.

Conflict of Interest

The authors declare no conflict of interest.

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