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Impact of Elevated O_3 on Soil Microbial Community Function Under Wheat Crop

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Abstract This study was initiated to explore the effects of ozone (O_3) exposure on potted wheat roots and soil microbial community function. Three treatments were performed: (1) Air with daily averaged O_3 concentration of 4–10 ppb (control situation, CK), (2) Air plus 8 h averaged O_3 concentration of 76.1 ppb (O_3-1), and (3) Air plus 8 h averaged O_3 concentration of 118.8 ppb (O_3-2). In treatments with elevated O_3 concentration (O_3-1 and O_3-2), the root and shoot biomass were reduced by 25% and 18%, respectively, compared to the control treatment (CK). On the other hand, root activity was significantly reduced by 58% and 90.8% in the O_3-1 and O_3-2 treatments, respectively, compared to CK. The soil microbial biomass was significantly reduced only in the highest O_3 concentration (O_3-2 treatment) in the rhizosphere soil. Soil microbial community composition was assessed under O_3 stress based on the changes in the sole carbon source utilization profiles of soil microbial communities using the Biolog™ system. Principal component analysis showed that there was significant discrimination in the sole-carbon source utilization pattern of soil microbial communities among the O_3 treatments in rhizosphere soil; however, there was none in the bulk soil. In rhizosphere soil, the functional richness of the soil microbial community was reduced by 27% and 38% in O_3-1 and O_3-2 treatments, respectively, compared to CK. O_3-2 treatment remarkably decreased the Shannon diversity index of soil microbial community function in rhizosphere soil, but the O_3-1 treatment did not. In the dominant microorganisms using carbon sources of carbohydrates and amino acids groups were significantly reduced by an elevated O_3 concentration in the rhizosphere soil. Our study shows that the elevated ozone levels may alter microbial community function in rhizosphere soil but not in the bulk soil. Hence, this suggests that O_3 effects on soil microbes are caused by O_3 detriments on the plant, but not by the O_3 direct effects on the soil microbes.

Keywords Microbial community · Microbial functional diversity · Ozone · Root · Wheat

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1 Introduction

Ozone (O_3) impact has been widely investigated because it is a highly active oxidant pollutant detrimental

to human health and crop yield. Although the increasing trend in O_3 concentration has leveled off or has been slightly negative in the USA and Europe (IPCC 2007), tropospheric O_3 is still rising in developing countries due to rapid industrialization and urbanization (Ariyaphanphitak et al. 2005; David et al. 1994). In China, higher O_3 concentration has been reported to have reached more than 150 ppb in some developed regions, such as the Beijing–Tian Region, Yangtze Delta, and Pearl Delta (Shao et al. 2006). Field open-top chamber (Zheng 2006), ethylenediurea (EDU) monitoring (Wang et al. 2007) and crop modeling (Yao et al. 2007) all showed that such high ambient O_3 concentration has a significant impact on crop yields.

Elevated O_3 does not only cause the loss of crop yield; it can moreover significantly reduce the allocation of assimilates into the root. The reduction in root biomass would influence the entry of organic matters into the soil. In recent years, considerable attention has been paid on the effect of elevated O_3 in the soil. The open-top chamber experiments with wheat and soybean have shown that total soil organic matter does not vary consistently with changes in O_3 concentration (Islam et al. 2000). In a 4-year FACE (Free air concentration enrichment) experiment with aspen stand and mixed aspen–birch stand in Rhinelander, Wisconsin, it was found that elevated O_3 reduced the total soil carbon formation (Loya et al. 2003). Soil respiration decreased beneath the trembling aspen (Coleman et al. 1996), *Pinus taeda* L. seedlings (Edwards 1991), and wheat and soybean (Islam et al. 2000) after being exposed to O_3 treatments. In contrast, soil respiration increased under O_3 stress in the experiment with silver birch (*Betula pendula* Roth) (Kasurinen et al. 2005). These inconsistent results make it difficult to assess the impact of elevated O_3 concentration on C cycle processes that critically influence plant growth as well as climate change (Andersen and Rygiewicz 1995).

Soil carbon and respiration strongly rely on organic decomposition which is mediated by microorganisms. Soil microbe plays a very important role in the ecosystem as a decomposer, and makes up of a part of the soil carbon pool. O_3 induced changes in substrate inputs from plant roots would influence soil microorganisms because the inputs are valuable energy sources for cellular metabolism. The effects of elevated O_3 on soil microbial biomass, structure and function would influence the nutrient supply to plants and worsen the impacts of O_3 on plants. Microbial biomass is the indicator most investigated with respect to the soil's response to O_3 stress. Elevated O_3 has been observed to reduce soil microbial biomass (Islam et al. 2000). In the experiment by Scagel and Andersen (1997), the effect of O_3 on soil microbial biomass was not linear: at low O_3 level, the total fungal and bacterial biomass increased. In contrast, total fungal and bacterial at high O_3 level decreased compared with those of controls without O_3 application. The response of the microbial community structure to O_3 varied with experimental methods. The phospholipid fatty acid (PLFA) method showed that fungal PLFA was reduced and bacterial PLFA was increased underneath plants exposed to elevated O_3 compared to ambient O_3 (Phillips et al. 2002). On the contrary, the denaturing gradient gel electrophoresis analyses of 16S rRNA showed that O_3 stress had a very negligible effect on the structural diversity of the bacterial community in rhizospheres (Dorhmann and Tebbe 2005).

Other than the PLFA and 16S rRNA methods, the Biolog Microplate method which is based on sole carbon source utilization pattern has been widely used in assessing microbial function diversity since it was introduced by Garland and Mills (1991). This method is simple and economical. Given these advantages, this study uses the Biolog Microplate Method to assess the changes that occur in the soil microbial community when wheat is exposed to elevated O_3.

The O_3 effects on soil are initiated by plant root activities, and the rhizosphere connecting root and soil change in response to O_3 stress. This study aims to assess the effects of O_3 on microbial community function in rhizosphere and bulk soil under wheat.

2 Materials and Methods

2.1 Crop Managements

After having been sterilized with 10% H_2O_2 solution for 5 min and washed thoroughly with deionized water, wheat (*Triticum aestivum L.* Jia 002) seeds were immersed in water at 25°C in the dark for 48 h. In order to separate the rhizosphere and bulk soils, the treated seeds were set into special nylon net bags filled with soil passed 2 mm sieve. Thereafter, the nylon bag was buried in PVC pot full of loam soil passed 2 mm

sieve. The soil pH was 5.36, organic matter content was 3.4%, total carbon was 1.9%, and total nitrogen was 0.2%. In addition, the soil moisture was 13.7%. Before sowing, fertilizers were applied at the following rates: 0.428 g urea, 0.323 g·$CaHPO_4 \cdot 2H_2O$ and 0.247 g·K_2SO_4.per kg soil. The pots were kept in plant growth chambers at a temperature of 20–25°C at daytime, and 15–20°C at nighttime. The relative humidity was 50–85%; light intensity at plant height 220 μmol m^{-2} s^{-1} over the waveband 400–700 nm. After 3 weeks, the pots were transferred into open-top chambers (OTC, 1.2-m-length, 1.2-m-width, and 2.1-m-height) which were specially designed for air pollutants fumigation experiments. During the growth period the wheat were watered daily with deionized water.

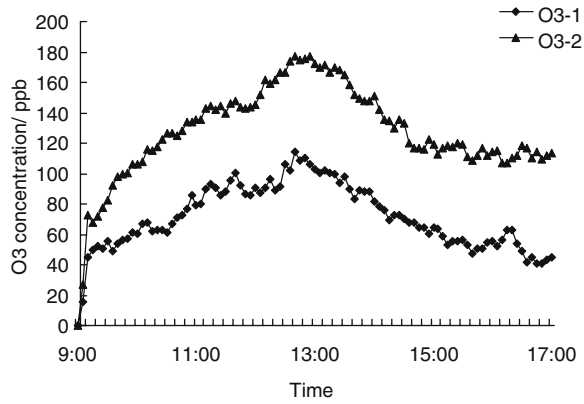


Fig. 1 Actual ozone diurnal pattern within OTC monitored by O_3 analyzer

2.2 Experimental Design

Three treatments were used namely CK, O_3-1 and O_3-2. A total of 24 pots were repeated for each treatment. In CK, the OTC was aerated with charcoal filtered air (<10 ppb) as the control. Elevated O_3 concentrations (O_3-1 and O_3-2 treatments) were achieved by mixed charcoal filtered air with O_3 generated by electric discharge (ozone generator, QHG-1, Yuyao, China) from pure oxygen. O_3 concentrations within the chambers were monitored by O_3 analyzer (ML9810B, Eco-Tech, Australia). In O_3-1 and O_3-2 treatment, the OTCs were fumigated from 9:00 to 17:00 with targets of 8 h mean concentration of 75 ppb and peak of 110 ppb, and mean 110 ppb and peak 170 ppb, respectively. The diurnal pattern of O_3 concentration within OTC was achieved by mass controller (Shenye, Beijing, China) which regulated oxygen volume into the ozone generator (Fig. 1). Table 1 shows the 8 h mean O_3 concentration, total accumulated O_3 concentration and the accumulated exposure over a concentration threshold of 40 ppb ozone based on hourly averages (AOT40, Fuhrer et al. 1997).

After 98 days from germination, the wheat was harvested to immediately determine the height as well as the biomass of the roots and the shoot after drying at 80°C for 72 h. The root:shoot ratio was calculated on a weight basis.

2.3 Root Activity Measurement

Root activity was measured by TTC (triphenyl tetrazolium chloride) method (Chen 2003) and expressed by the amount of TPF (triphenyl formazan) formed. In brief, 0.5 g fresh root was immersed in 10 ml of equally mixed solution of 0.4% TTC and phosphate buffer, and kept in the dark at 37°C for 2 h. Subsequently, 2 ml of 1 M H_2SO_4 was added to stop the reaction with the root. The root was dried with filter paper and then extracted with ethyl acetate. The red extractant was transferred into the volumetric flask to reach 10 ml by adding ethyl acetate. The amount of TPF produced by the metabolic activity of the root tissue (TTC reduced to TPF) over the incubation period was quantified by spectrophotometer based on the relationship between TPF concentration and light absorbance at 485 nm.

2.4 Soil Microorganism Assessment

A small fraction of root-free soil (the layer 5–10 mm close to the nylon screen) can be considered a part of the rhizosphere soil where water-soluble rhizodeposits are diffused (Helel and Sauerbeck 1984). This physical approach of separating rhizosphere from bulk soil is more preferable than shaking procedures because it is more precise (Yevdokimov et al. 2006). Hence, the soil within 5 mm of the nylon net was taken as a rhizosphere soil sample, and the soil beyond 5 mm from the nylon net was categorized as a bulk soil sample. Soil microbial biomass and community functions were assessed immediately after the samples were obtained.

The fumigation–extraction method (Vance et al. 1987) was used to determine soil microbial biomass-C. The content of K_2SO_4-extracted C from the $CHCl_3$-

Table 1 8 h mean ozone concentration, total ozone dose and AOT40 recorded during wheat growth period

Treatment	8 h mean ppb	Total O_3 ppm·h	AOT40 ppm·h
CK	8	0.5	0
O_3-1	76	41.4	21.4
O_3-2	119	65.8	44.1

treated and untreated soils was determined by an automated TOC analyzer (Liqui TOC, Elementa, Germany).

Microbial community function was analyzed by Biolog system using sole-carbon-source-utilization (Biolog, Inc., Hayward, CA). Triplicate 10 g soil sample was suspended in 90 ml 0.85% sterile NaCl solution and vibrated for 30 min. It was then serially diluted to 10^{-3}. The dilution was inoculated in Biolog GN-plate in a dark chamber at a constant temperature of 25°C. After inoculation, the inoculated plates were scanned at 595 nm with a Biolog microplate reader at 8 h intervals for 240 h. The absorbance values for the wells containing carbon sources were designated as vacant against the control well. Overall color development in Biolog plates was expressed as average well color development (AWCD) (Garland and Mills 1991). To assess the substrate utilization pattern of the microbial community, the AWCD area (the accumulated AWCD with time, Guckert et al. 1996) for each carbon substrate group (polymers, carbohydrates, carboxylic acids, amides/amines, amino acids and miscellaneous) was also calculated (Benizri and Amiaud 2005).

The richness of the microbial community function was assumed as the total number of wells with absorbance of over 0.1. Microbial community function diversity was calculated as Shannon–Weinner diversity index (H') as $H' = -\sum (Pi \times \log Pi)$, where Pi was the proportion of total microbial metabolic capability (blanked absorbance values of well in this study) on a particular carbon source. At this point, we used the absorbance of microplate read at 96 h after the incubation because 90% of the wells have turned positive within 96 h.

2.5 Statistical Analyses

One-way ANOVA was used to determine statistically significant differences in root growth and microbial assays among treatments. The LSD for 95% confidence interval ($LSD_{0.05}$) was used for multiple comparisons. On the basis of the covariance matrix, principal component analysis (PCA) was used to distinguish the soil microbial community's carbon substrate utilization pattern among the various treatments.

3 Results

3.1 Wheat Biomass and Root Activity

The elevated O_3 significantly reduced wheat height as well as the root and shoot biomass. However, there were no significant differences in height and biomass between the O_3-1 and O_3-2 treatments (Table 2). Relative to CK treatment, elevated O_3 decreased root and shoot biomass by 25% and 18%, respectively (Table 2). No significant difference was found in the root:shoot ratio between the O_3 treatments and the control. As compared to CK treatment, root activity was significantly reduced by 58.9% and 91.1% in the O_3-1 and O_3-2 treatments, respectively (Table 2).

3.2 Soil Microbial Biomass

Relative to CK, there was a slight increase in the soil microbial biomass in the O_3-1 treatment. In contrast in the O_3-2 treatment, it was reduced by 8.7% and 0.4% for rhizosphere and bulk soil, respectively (Table 3). Compared to the control treatment, the O_3-2 treatment significantly decreased the microbial

Table 2 Height, biomass and root activity of wheat under different ozone treatments

Treatments	Plant height (cm)	Root biomass (g/plant)	Shoot biomass (g/plant)	Root:shoot	Root activity ($mg \cdot g^{-1}\ h^{-1}$)
CK	55.6±1.82a	0.078±0.0015a	0.61±0.025a	0.13±0.005	168±1.52a
O_3-1	51.3±0.62b	0.058±0.0035b	0.50±0.031b	0.12±0.020	69±0.35b
O_3-2	51.8±1.60b	0.059±0.0035b	0.50±0.031b	0.12±0.020	15±0.35c

Means±SE (n=3). Different letter within the column indicates significant differences among treatments ($P \leq 0.05$)

Table 3 Microbial biomass in rhizosphere and bulk soil under different ozone treatments

Treatments	Microbial biomass (mg C·kg^{-1} dry soil)	
	Rhizosphere soil	Bulk soil
CK	263.3±11.4a	264.8±9.1
O_3-1	270.2±4.9a	269.5±6.4
O_3-2	240.3±0.5b	263.7±2.2

Means±SE (n=3). Different letter within the column indicates significant differences among treatments (P≤0.05).

biomass in the rhizosphere soil, and O_3 had no effects on the microbial biomass in the bulk soil (P≤0.05).

3.3 Soil Microbial Community Function

3.3.1 Average Well Color Development (AWCD)

In all treatments, the AWCD in rhizosphere soil was higher than that in the bulk soil (Fig. 2), suggesting that rhizosphere soil had higher microbial metabolic capability than the bulk soil. Elevated O_3 significantly decreased the AWCD in the rhizosphere soil, although it had no such effect in the bulk soil (Fig. 2).

3.3.2 Principal Component Analysis

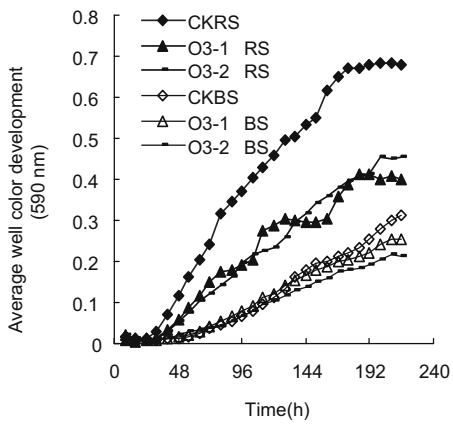
Principal component analyses were conducted to determine the microbial community functions of wheat soil under different O_3 treatments. For rhizosphere soil, PC1 and PC2 explained 31% and 28.6% of the variances of AWCD, respectively. There was clear discrimination in the metabolic capability of the soil microbial community between O_3 treatments (Fig. 3a). For the bulk soil, the PC1 and PC2 could only account for 18.3% and 15.5% of the AWCD variance, respectively. There was no clear discrimination between the soil microbial community function for the treatments (Fig. 3b).

3.3.3 Microbial Community Functional Richness and Diversity

In comparison to the CK treatment, soil microbial community functional richness in the rhizosphere soil were significantly reduced by 27% and 38% in O_3-1 and O_3-2 treatments, respectively (Table 4). However, the Shannon diversity was reduced significantly only in the O_3-2 treatment compared to the CK and O_3-1 treatments. Furthermore, there were no significant differences in the bulk soil's microbial community functional richness and diversity among treatments.

3.3.4 Carbon Substrate Utilization Pattern

The impacts of O_3 on soil microbial community function can be assessed by differences in the microbial carbon source utilization pattern. By using the GN-Plate Biology system, six groups of carbon sources can be identified. In this experiment, the microbial potential metabolic capability using six groups of carbon sources was lower in rhizosphere



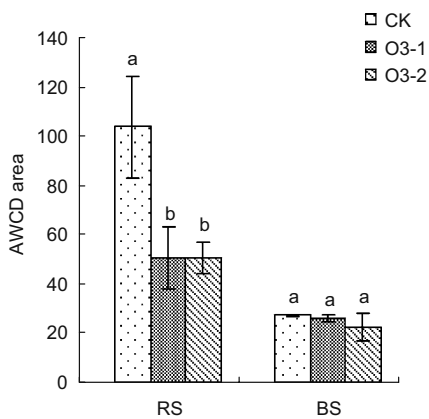


Fig. 2 Average well color development (*AWCD*) and AWCD area in Biolog microplates after incubation of rhizosphere and bulk soil beneath wheat exposed to ozone treatments. *CKRS*, rhizosphere soil in CK treatment; *O_3-1RS*, rhizosphere soil in O_3-1 treatment; *O_3-2 RS*, rhizosphere soil in O_3-2 treatment; *CKBS*, bulk soil in CK treatment; *O_3-1 BS*, bulk soil in O_3-1 treatment; *O_3-2 BS*, bulk soil in O_3-2 treatment. *Bars with different letters* indicate significant difference (P≤0.05)

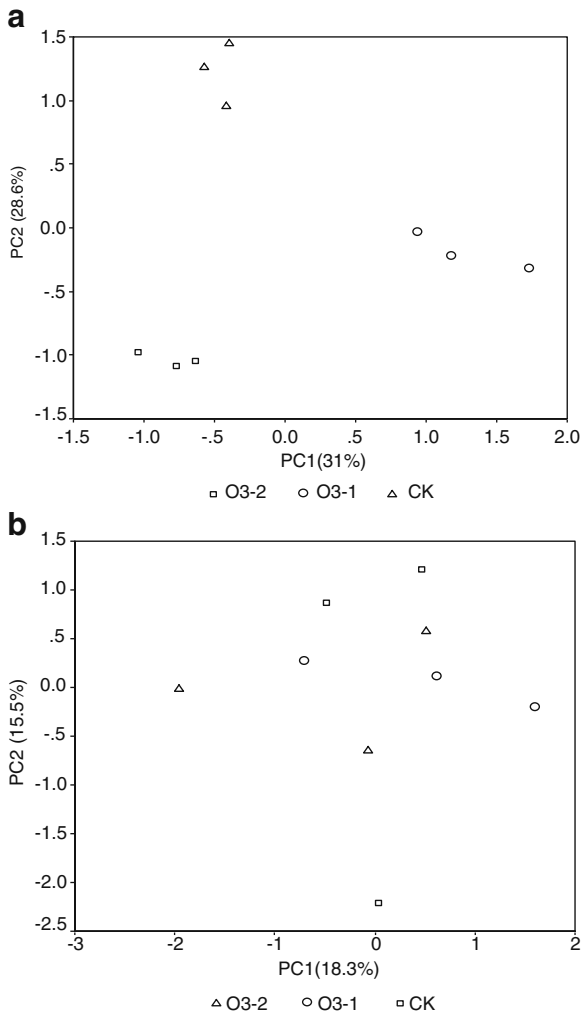


Fig. 3 Ordination plot of soil microbial community function generated by principal components analysis (PCA) of BIOLOG data under different ozone treatments in (**a**) rhizosphere soil and (**b**) bulk soil

soil under elevated O_3 treatments than the CK (Fig. 4a). These reductions were significant in rhizosphere soil that used carbon sources of carbohydrates, carboxylic acid and amino acids (Fig. 4a). In bulk soil, elevated O_3 impacts varied with the carbon sources group and there was no statistically significant difference between CK, O_3-1 and O_3-2 (Fig. 4b).

It was obvious that polymers and amino acids were the most dominant metabolized carbon sources in the rhizosphere soil (Fig. 4a). They were followed by carbohydrate and carboxylic acids in the CK treatment. In O_3-1 treatment, the microbial community utilized carboxylic acids the most, followed by polymers and carbohydrates. In O_3-2 treatment, the communities utilized mostly polymers and carboxylic acids. In the bulk soil (Fig. 4b), the microbial community using carbon sources of carboxylic acids and amino acids was dominant and little influenced by O_3 exposure.

4 Discussion

Elevated ozone reduces plant photosynthesis and carbon fixation, thus resulting in carbon-limiting stresses (Reiling and Davison 1995; Cooley and Manning 1987). The carbon-limiting stresses caused by O_3 impact could have a rapid and significant effect on root growth, because roots are often dependent on current photosynthate for their structural development (Andersen 2003). In this study, significant reduction in root biomass as well as above-ground biomass was observed under elevated O_3 levels (Table 2). Although the root:shoot ratio was decreased under elevated O_3, this decrease was not significant in our experiment. Likewise, we were not able to observe any impact of the elevated O_3 concentration on the root:shoot ratio for wheat (McCrady and Andersen 2000), ponderosa pine (Scagel and Andersen 1997) and alfalfa (Renaud et al. 1997).

Under elevated O_3 exposure, wheat root activity was significantly reduced, suggesting that carbohydrate was less available for root development under O_3 stress rather than a direct effect of O_3 on tissue

Table 4 Effects of ozone on soil microbial community functional richness and diversity

Treatments	Rhizosphere soil		Bulk soil	
	Richness Index	Shannon–Weinner Diversity Index	Richness Index	Shannon–Weinner Diversity Index
CK	41.7±0.88a	1.56±0.07a	10.3±0.3	1.21±0.13
O_3-1	30.3±2.33b	1.56±0.06a	12.3±1.3	1.30±0.08
O_3-2	25.0±1.53b	1.37±0.03b	11.7±2.6	1.13±0.17

Means±SE (n=3). Different letter within the column indicates significant differences among treatments ($P \leq 0.05$)

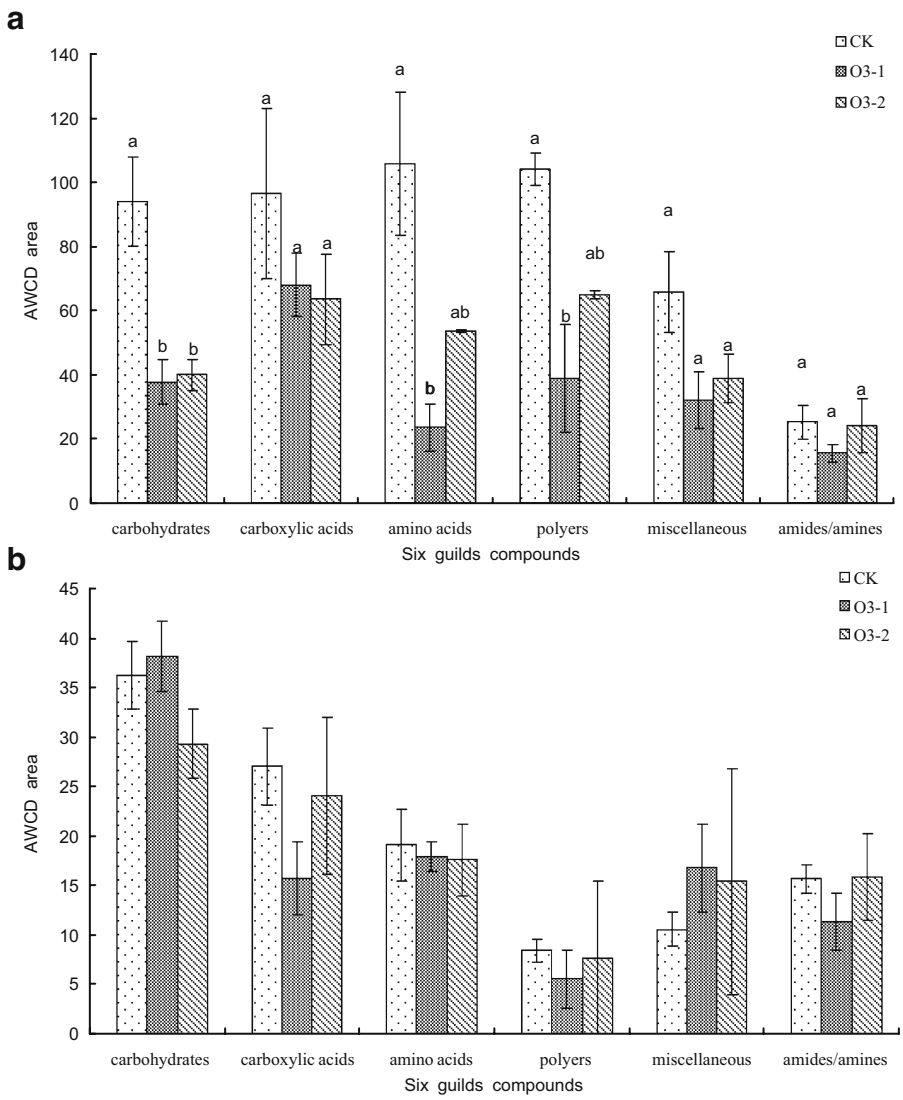


Fig. 4 AWCD area of different carbon sources in rhizosphere soil (**a**) and bulk soil (**b**). *Bars with different letters* indicate significant difference ($P \leq 0.05$)

metabolism *per se* (Stroo et al. 1988; Blum and Tingey 1977). The O_3 induced reduction in root activity which would influence the root's ability to absorb nutrition and water, thus altering nutrient dynamics and cycling in the plant–soil system.

Carbon entering soil would be decreased due to the reduction in root biomass under O_3 stress. The soil microbial biomass could be influenced because carbon input is the energy source for soil microbes. Islam et al. (2000) reported that the soil had 17% less microbial biomass under wheat–soybean exposed to elevated O_3 concentration for 2 years compared to the control treatment. In our experiment, the microbial biomass was slightly increased in low O_3 level (O_3-1) but significantly decreased in high O_3 level (O_3-2) compared with CK treatment. This was the same in both the rhizosphere or bulk soil under wheat and consistent with the result of Scagel and Andersen (1997) on total soil fungal and bacteria biomass under ponderosa pine. In our study, the changes in soil microbial biomass were statistically significant in the rhizosphere soil and not statistically significant in the bulk soil. These inconsistencies might have been caused by the difference in the measurement methods and ozone regime that was applied. Further investigation is needed before a definite explanation can be obtained to account for these inconsistencies.

Sole-carbon-source-utilization of microbial community assessed by Biolog micro-plates can provide functional ability of microbe in soil (Garland 1997).

In this study, O_3 reduced the microbial ability to utilize carbon sources based on the value of average well color development (AWCD) (Fig. 2), especially in rhizosphere soil. This might have been caused by the decreased carbon exudates from the roots of O_3 exposed crop. Changes in root exudation might affect rhizosphere microbial activity, potentially altering the ecological and nutrient dynamics in the rhizosphere (Bardgett et al. 1999). It was reported that the exudation of organic compounds from the roots of the O_3-exposed plants declined due to reduced translocation of photosynthate to the roots. Consequently, the supply of nutrients to soil microorganisms could decrease, resulting in lower microbial metabolism (Edwards 1991). Differences in the microbial utilization of carbon sources suggests a change in substrate availability between CK and elevated O_3 treatments, because Biolog is capable of detecting changes in microbial communities' metabolic function as a result of actual carbon source availability (Grayston et al. 1998). Our results on carbon substrate utilization pattern show that microorganisms in rhizosphere soil use fewer carbohydrate and amino acids under elevated O_3, which may be attributable to the less amounts of carbohydrate and amino acid compounds exuded from the root of ozone-exposed plants. McCool and Menge (1983) found that O_3 treatment decreased the amount of sugars and amino acids in root exudates.

It is without doubt that the responses of microbial biomass and community function are more sensitive in rhizosphere soil than bulk soil. Soil microbial biomass under O_3-2 treatment decreased by 8.7% in rhizosphere soil while it only decreased by 0.47% in bulk soil (Table 3). Under elevated O_3-2 treatment, microbial functional richness and Shannon diversity indices in rhizosphere soil were decreased by 40% and 12.2%, respectively. The PCA showed that there was significant difference in the carbon source utilization pattern of rhizosphere soil among different treatments (Fig. 3a), while there was no difference in bulk soil (Fig. 3b). We can infer that the impact of O_3 on soil microbial community function is limited in space and significantly occurs in rhizosphere soil other than in bulk soil.

5 Conclusion

Few studies have been conducted on the response of the below-ground components of the ecosystem, including root and soil microorganism under elevated O_3 exposure compared to above-ground components, although the below-ground components play critical roles in biogeochemical cycles, and might experience the accumulative effect of O_3 stress on the ecosystem. This experiment showed that root biomass and activity were reduced by elevated O_3 concentration, thus, affecting the water and nutrient supplies of crop production. Changes in soil microbial biomass under elevated O_3 concentration were statistically significant in rhizosphere, while it was not statistically significant in bulk soil. Based on soil microbial utilization pattern of sole-carbon source measured by Biolog microbial identification system, soil microbial community functions in rhizosphere soil were significantly decreased by the elevated O_3 in terms of AWCD, richness and Shannon diversity indices. There is no significant alteration in microbial community functions found in bulk soil under elevated O_3 exposure. In this study, there is no particular O_3 effect on the microbes in the bulk soil. This strongly suggests that the effects detected in the rhizosphere microbial community came about due to the ozone effects on the plant, not as direct effects of ozone on the rhizosphere microbes. However, there are still many uncertainties in soil microbial community response to O_3 stress. Long term experiments should be carried on in order to know if the O_3 impacts on the soil are accumulative.

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