Elevated Ozone Changed Soil Microbial Community in a Rice Paddy

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Although the environmental impact of ozone (O_3) has been widely investigated, there have been few studies about the effect of O_3 on underground processes, especially soil microbial communities. This study aimed to investigate O_3 effects on the soil microbial community structure and function in a rice paddy field, China. There were three treatments within open-top chambers: charcoal-filtered air (CF as a check treatment), CF plus 100 $nL O_3 L^{-1}$ (O_3-1), and CF plus 150 $nL O_3 L^{-1}$ (O_3-2). Soil samples were collected in August, September, and October to assess microbial biomass C, community-level physiological profiles (CLPPs), and phospholipid fatty acids (PLFAs) of soil microbes. The results showed that soil microbial biomass under elevated O_3 concentration was statistically significantly decreased compared with the check treatment in October. Soil microbial functional indices including average well color development, richness, and Shannon diversity were significantly decreased by elevated O_3 in September. Principal components analysis of CLPP data showed that elevated O_3 significantly changed the C sources utilization pattern of microbes in September (Principle Component 1, $P < 0.05$). During all growing stages, elevated O_3 had significant effects on soil microbial composition expressed as PLFA percentage, which indicated that PLFA was more sensitive than CLPP for study of the microbial community. The stress indicators, including the ratio of saturated to unsaturated fatty acids and the ratio of cyclopropyl fatty acids to their precursors, such as $cy17:0$ to $16:1\omega7c$, also increased under elevated O_3 exposure.

Abbreviations: AWCD, average well color development; CF, charcoal-filtered air; CLPP, community-level physiological profiles; OTC, open-top chamber; PLFA, phospholipid fatty acid.

Ozone is a photochemical oxidant that occurs naturally in the earth's troposphere; its background concentrations are thought to range between 25 and 50 $nL L^{-1}$ (USEPA, 1996). At some sites, its concentration has dramatically increased during the last century. High levels of O_3 usually accompany industrialization and urbanization, especially in the mega-cities of developing countries where environmental standards have not been adequately established (Ariyaphanphitak et al., 2005; Tingey et al., 1994). China has undergone rapid industrialization and urbanization. The increase in energy consumption from fossil fuels used in motor vehicles will increase the local levels of tropospheric O_3 . Information on ground-level O_3 concentrations in China is limited, but the available data suggest that O_3 concentrations can attain potentially damaging levels; concentrations exceed the Chinese National Air Quality Standard (based on U.S. standards) of 80 $nL L^{-1}$ for many hours during the summer (Zheng et al., 1998).

Crop losses from high ambient O_3 levels have been reported for several agronomically important crop species (Heck et al., 1984). Many studies have shown that high ambient O_3 significantly reduces wheat (*Triticum aestivum* L.), rice, soybean [*Glycine max* (L.) Merr.], and potato (*Solanum tuberosum* L.) yields (Chen et al., 2008; Feng and Kobayashi, 2009; Feng et al., 2008; Fuhrer et al., 1989; Kobayashi et al., 1995; Kress and Miller, 1985; Ollerenshaw et al., 1999; Pleijel et al., 1991; Wang et al., 2007). There are few comprehensive studies, however, on the effects of O_3 on the structural or functional components of soil food webs.

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Elevated O_3 has the potential to alter soil microbial communities through its influence on plant litter production (Larson et al., 2002), by decreasing photosynthesis (Coleman et al., 1995; Pye, 1988), and net C gain (Reich et al., 1990; Skärby et al., 1987). Some research has shown that a high concentration of O_3 has a greater impact on root growth than on foliage or stem growth (Blum and Tingey, 1967; Cooley and Manning, 1987; Hogsett et al., 1985). Elevated O_3 can also modify the biochemical composition of fine roots, causing starch and soluble sugar concentrations to decline following exposure to elevated O_3 (Andersen et al., 1991). Findlay et al. (1996) reported that elevated O_3 reduced litter quality and rates of decay. The types and amounts of organic root exudates can be changed when the shoots are exposed to a high level of O_3 and this change may affect rhizosphere microbial activity, potentially altering the ecological and nutrient dynamics in the rhizosphere (McCrary and Andersen, 2000). Therefore, the degree to which elevated O_3 modifies the production and chemistry of root and litter quality should affect soil microorganisms because these plant tissues are the primary substrate for microbial metabolism in the soil.

In the few cases where soil microbial communities have been examined, O_3 has led to changes in bacterial and fungal biomass, and in some cases changes in soil enzyme activity (Andersen, 2003). A 4-yr FACE (free air concentration enrichment) experiment within a trembling aspen (*Populus tremuloides* Michx.) stand and a mixed aspen–paper birch (*Betula papyrifera* Marshall) stand in Rhinelander, WI, found that elevated O_3 reduced the total soil C formation (Loya et al., 2003). Soil respiration decreased beneath 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 an experiment with silver birch (*Betula pendula* Roth) (Kasurinen et al., 2005). Soil microbial community function measured by CLPPs was affected after wheat was exposed to elevated O_3 (Chen et al., 2009). The PLFA method showed that fungal PLFA was reduced and bacterial PLFA was increased underneath plants exposed to elevated O_3 compared with ambient O_3 (Phillips et al., 2002). After a meadow was exposed to elevated O_3 for 3 yr, bacterial, actinobacterial, and fungal PLFA and the fungal/bacterial PLFA ratio all were decreased, but mycorrhizal PLFA did not respond to elevated O_3 (Kanerva et al., 2008). On the contrary, denaturing gradient gel electrophoresis analyses of 16S rRNA showed that O_3 stress had a negligible effect on the structural diversity of the bacterial community in rhizospheres (Dohrmann and Tebbe, 2005). These inconsistent results make it difficult to assess the impact of elevated O_3 concentration on C cycling processes that critically influence plant growth as well as climate change (Andersen and Rygielwicz, 1995).

Rice is one of the two most important crops in China. In recent years, the tropospheric O_3 concentration has been reported to reach critical levels for damage to rice growth in developed regions of China, such as the Yangtze River delta and the Pearl River delta (Chen et al., 2008; Feng et al., 2003). Field open-top

chamber experiments showed that rice yield would be reduced under elevated O_3 concentration (Xie et al., 2006). In this study, we aimed to assess the impacts of elevated O_3 concentrations on the soil microbial community structure and function using the CLPP and PLFA methods.

MATERIALS AND METHODS

Site Description

The experimental site was located at Shuangqiao Farm (31°53' N, 121°18' E) in Jiaxing City, Zhejiang Province. The site is about 100 km from Shanghai (the biggest city in China). In this region, the annual average temperature and precipitation are 15.5°C and 1 199 mm, respectively (Yin et al., 2004). The soil is paddy soil developed after a long period of growing rice. The texture is clay. The soil pH is 5.36, organic matter content is 3.4%, and total N is 0.2%. The prevailing crops are wheat and rice in rotation.

Crop Exposure System

Open-top chambers (OTCs) (2.2 m high and 2 m in diameter), consisting of an octagonal aluminum frame covered by transparent film, were used for the exposure studies. They have an innovative improved O_3 distribution system. A rotatable transparent pipe with many small holes (10-mm diameter at 10-cm intervals) releases either CF or ozone above the crop canopy. It is driven by either CF or ozone + CF from a centrifugal blower. The O_3 was generated from pure O_2 by high-voltage electric discharge (Yuyao Shenglete Co., Zhejiang, China). A series of solenoid valves, linked online with a programmable logic controller (PLC K80S, LG, Seoul, South Korea), were used to control gas meters to provide O_2 based on the established relationship between the O_3 concentration within the OTCs and the O_2 volume. The O_3 concentrations within the OTCs were monitored by an O_3 analyzer (EC9810B, Knoxfield, VIC, Australia). A 21X datalogger (Campbell Scientific, Logan, UT) was connected to store O_3 concentration data and to monitor the temperature within the OTCs using constantan-copper thermocouples. The performance of the OTC has been assessed in the field. The O_3 concentration monitoring has shown that O_3 concentrations were uniformly distributed vertically and horizontally within the OTC. The air exchanges between inside and outside of the OTC at about two to three times per minute. The difference in temperature between the inside and outside of the OTC was <1.87°C (Zheng et al., 2007).

Before the experiment, a homogenous field was selected and divided into 2- by 2-m plots by inserting plastic film to the 80-cm depth at the boundary of each plot to prevent lateral flow of water between plots. All OTCs were established 2 m apart from each other and the land between the OTCs was planted in rice the same as within the OTCs. The OTCs were placed on the experimental field in a completely randomized design. There were three treatments: CF (check or control), and CF plus the addition of diurnally changing O_3 at two levels. In the O_3 -1 treatment, the target O_3 concentrations were 50 nL L⁻¹ from 0900 to 1000 h, 100 nL L⁻¹ from 1000 to 1200 h, 150 nL L⁻¹ from 1200 to 1400 h, 100 nL L⁻¹ from 1400 to 1600 h, and 50 nL L⁻¹ from 1600 to 1700 h. In the O_3 -2 treatment, the same O_3 diurnal variation pattern was applied with different O_3 concentrations: 100, 150, 200, 150, and 100 nL L⁻¹ for 0900 to 1000, 1000 to 1200, 1200 to 1400, 1400 to

1600, and 1600 to 1700 h, respectively. Nine chambers were used to yield three replicates for each of the three O₃ treatments.

On 19 May 2006, rice ('3694-Fan') seeds were sown in the field; the seedlings were transplanted into the experimental plots on 20 June. Crop management was the same as local practice. Open-top chambers were set on the field on 10 July. The daily O₃ exposure began on 16 July when the rice was at the three-leaf stage and continued to 10 October when the rice was mature, except for rainy days. The mean O₃ concentrations were 0, 100, and 150 nL L⁻¹, and the accumulated O₃ concentrations above 40 nL L⁻¹ were 0, 23,000, and 39,000 nL L⁻¹ for the check, O₃-1, and O₃-2 treatments, respectively.

During the rice growing season, the climate was hot and humid. The mean monthly air temperature and relative humidity were 29.13 ± 2.94°C and 76.64 ± 13.16%, 29.21 ± 2.78°C and 74.44 ± 13.22%, 22.63 ± 3.64°C and 80.13 ± 14.00%, and 20.97 ± 3.20°C and 77.96 ± 16.78% in Jul., August, September, and October, respectively, in 2006. The soil moisture was always in the saturated condition because of flooding of the paddy.

Soil Sampling

The soils were sampled on 5 August (the jointing stage of rice), 10 September (flowering stage), and 8 October (mature stage) from the surface layer (0–10 cm) within each OTC. A total of 10 cores of the surface soil was randomly sampled with a 2-cm auger in each OTC and were mixed as one composite sample for each OTC. Each treatment had three soil samples from three OTCs, and all the samples were then transported to the laboratory on ice and stored at 4°C for CLPPs and -20°C for PLFAs before analysis. The samples were passed through a <2-mm sieve and large pieces of plant material and soil animals were removed.

Microbial Biomass

The fumigation–extraction method (Vance et al., 1987) was used to determine the microbial biomass C of the soil samples. The K₂SO₄-extracted C content of the CHCl₃-treated and untreated soils was determined with an automated TOC analyzer (Liquid TOC, Elementar, Hanau, Germany). The microbial biomass was the difference in total organic C in extracts from the CHCl₃-treated and untreated soils.

Community-Level Physiological Profiles from Sole Carbon Source Utilization Tests

Community-level physiological profiles were analyzed by a Biolog system using sole C source utilization (Biolog Inc., Hayward, CA). Triplicate 10-g soil samples were suspended in 90 mL of sterile 0.85% NaCl solution and agitated for 30 min, then serially diluted to 10⁻³. The dilutions were used to inoculate Biolog GN plates, which were incubated in a dark chamber at a constant temperature of 25°C. After inoculation, the plates were scanned at 595 nm with a Biolog microplate reader at 12-h intervals for 240 h. Absorbance values for the wells containing C sources were blanked against the control well. Overall color development in the Biolog plates was expressed as average well color development (AWCD) (Garland and Mills, 1991). The AWCD areas (the accumulated AWCD with time) (Guckert et al., 1996) were calculated to assess the substrate utilization activity of the microbial community.

Microbial community functional richness was estimated as the total number of wells with absorbance >0.1. Microbial community function diversity was calculated as the Shannon–Wiener diversity index $H' = -\sum(P_i \log P_i)$, where P_i is the proportion of total microbial metabolic capability (blanked absorbance values of wells in this study) on the i th C source. We used the absorbance of microplates read at 96 h after inoculation because 90% of the wells had turned positive within 96 h.

Ester-Linked Phospholipid Fatty Acid Analysis

Phospholipid fatty acid analyses were performed using a modified Bligh and Dyer method (Frostegård et al., 1993). Briefly, triplicate 4-g subsamples of freeze-dried soil were extracted in a one-phase mixture consisting of chloroform, methanol, and phosphate buffer (1:2:0.8 v/v/v). After splitting the extracts into two phases by adding chloroform and the buffer, the lipid-containing phase was dried under a stream of N₂. The lipid material was fractionated on columns containing silicic acid into neutral and glyco- and phospholipid-containing polar lipids (Frostegård et al., 1991). The phospholipid fraction was dried under a stream of N₂ and saved for preparation of fatty acid methyl esters. The phospholipids were subjected to a mild alkaline methanolysis (Dowling et al., 1986), and the resulting fatty acid methyl esters were analyzed on a gas chromatograph equipped with a mass spectrometer (GC-MS, HP6890/MSD5973, Agilent Technologies, Santa Clara, CA).

Known amounts of methyl nonadecanoate (19:0) was added before analysis by GC-MS. The total PLFA was calculated with 19:0 as the internal standard.

Fatty acids are designated in terms of the total number of C atoms: number of double bonds, followed by the position of the double bond from the methyl end of the molecule. *Cis* and *trans* configurations are indicated by *c* and *t*, respectively. The prefixes *a* and *i* indicate anteiso and iso branching, *br* indicates an unknown methyl branching position, 10Me indicates a methyl group on the 10th C atom from the carboxyl end of the molecule, and *cy* refers to cyclopropane fatty acids.

The stress indicators can be assessed by ratios of *cy*17:0/16:1 ω 7*c* and saturated fatty acids/unsaturated fatty acids (Bossio and Scow, 1998; Reichardt et al., 1997).

Statistical Analyses

Ozone effects on the microbial biomass C, AWCD, Biolog richness, and Shannon–Wiener diversity were tested by one-way ANOVA with a LSD post hoc test using SPSS 11.5 (SPSS Inc., Chicago, IL). The Biolog absorbance data and PLFA data (expressed as mol%) were analyzed by principal component analysis (PCA). The ratios of *cy*17:0/16:1 ω 7*c* and saturated fatty acids/unsaturated fatty acids were used as stress indicators to assess the O₃ effect by a one-way ANOVA with a LSD post hoc test.

RESULTS

Microbial Biomass Carbon

In the check and O₃-1 treatments, microbial biomass C decreased at first and then increased from August to October. Microbial biomass C was lowest in September and highest in October (Table 1). In the O₃-2 treatment, however, microbial biomass C decreased from August to September and October. In

Table 1. Effect of different levels of elevated O₃ on soil microbial biomass C at different sampling times assessed by one-way ANOVA with LSD.

Treatment†	Microbial biomass C		
	Aug.	Sept.	Oct.
	mg C kg ⁻¹ dry soil		
Check	690 ± 140 a‡	379 ± 16 a	793 ± 78 a
O ₃ -1	595 ± 65 a	410 ± 6.2 a	684 ± 74 a
O ₃ -2	540 ± 58 a	439 ± 37a	164 ± 64 b

† O₃-1 had lower levels of O₃ exposure and O₃-2 had higher levels.
‡ Means ± SE (*n* = 3); different letters within the column indicate significant differences among treatments (*P* < 0.05).

August and September, there was no significant difference in microbial biomass C among the three O₃ treatments. In October, microbial biomass C decreased with increasing O₃ concentration and was significantly lower in the O₃-2 treatment than in the other two treatments (Table 1).

Community-Level Physiological Profiles Microbial Activity and Diversity

The metabolic functions, including the Shannon–Wiener diversity and the richness of microorganisms in the rice paddy soil decreased gradually from August to October. At the earlier exposure days in August and the later growth stage in October, O₃ had no significant effects on AWCD area (Fig. 1) or diversity index (Table 2). In September, the O₃-2 treatment significantly decreased microbial activity (AWCD area, Fig. 1) and the microbial functional diversity and richness indices (Table 2).

Principal Component Analysis

Principal component analyses were conducted to assess the microbial community functional diversity in rice paddy soil under different O₃ treatments at different growth stages. In August, the first principal component (PC1) explained 22% of the variance and very clearly distinguished the O₃ treatments (*F* = 8.25, *P* ≤ 0.05; Fig. 2A). Bromosuccinic acid strongly (*r* > 0.8) correlated positively to PC1, whereas glycyl-L-bromosuccinic glutamic acid and D-glucose-6-phosphate were strongly (*r* < -0.8) negatively correlated to PC1. The second principal component (PC2) could explain 18% of the variance but did not distinguish the treatments. The substrates positively correlated to PC2 included citric acid, D-saccharic acid, urocanic acid, and α-glycerol phosphate; formic acid and thymidine were strongly negatively correlated to PC2.

In September, PC1 explained 36% of the variance and very clearly distinguished the O₃ treatments (*F* = 64, *P* ≤ 0.01; Fig.

Table 2. Effects of different levels of elevated O₃ on soil microbial community functional richness and diversity indices in rice paddy assessed by one-way ANOVA with LSD.

Treatment†	Shannon–Wiener diversity index			Richness index		
	Aug.	Sept.	Oct.	Aug.	Sept.	Oct.
Check	1.81 ± 0.03 a‡	1.55 ± 0.09 a	1.30 ± 0.21 a	75.5 ± 3.50 a	48.0 ± 8.44 a	27.0 ± 4.24 a
O ₃ -1	1.82 ± 0.01 a	1.37 ± 0.10 ab	1.33 ± 0.03 a	79.7 ± 1.45 a	36.2 ± 10.62 ab	24.0 ± 5.66 ab
O ₃ -2	1.85 ± 0.01 a	1.21 ± 0.03 b	1.21 ± 0.08 a	84.0 ± 1.00 a	17.0 ± 1.53 b	12.5 ± 0.71 b

† O₃-1 had lower levels of O₃ exposure and O₃-2 had higher levels.
‡ Means ± SE (*n* = 3); different letters within a column indicate significant differences among treatments (*P* < 0.05).

Fig. 1. Average well color development (AWCD) area in Biolog microplates after incubation of rice paddy soil under elevated O₃ exposure; O₃-1 had lower levels of O₃ exposure, O₃-2 had higher levels, and CK is the check or control. Bars with different letters indicate significant differences (*P* < 0.05).

2B). The compounds that were strongly (*r* > 0.8) positively correlated to PC1 were six carbohydrates, five carboxylic acids, two amino acids, one miscellaneous, and one polymer compound; PC2 could explain 21% of the variance but did not distinguish the treatments (Fig. 2B). Four carbohydrates, three amides and amines, two miscellaneous, a polymer, and a carboxylic acid were strongly positively (*r* > 0.8) correlated to PC2. There were no substrates that were strongly negatively (*r* < -0.8) correlated to either PC1 or PC2.

In October, PC1 and PC2 explained 29 and 25% of the variance, respectively, and there were some substrates positively correlated to PCs although neither of the two factors distinguished the treatments (Fig. 2C).

Phospholipid Fatty Acids

A total of 18 PLFAs with 11 to 20 carbons were detected in the rice paddy soil samples covering three treatments and three different sampling times. The PLFAs detected included saturated, monounsaturated, branched, and cyclopropyl fatty acids. The most abundant PLFAs were i16:0, a17:0, 15:0, a15:0, and cy19:0; together they amounted to 68% of the total PLFAs.

Principal Component Analysis

In August, 47% of the total variance in the data set was accounted for by PC1 and 22% by PC2, and both components clearly discriminated the PLFA profiles among different O₃ treatments (*F* = 37, *P* ≤ 0.001 for PC1; *F* = 14, *P* ≤ 0.01 for PC2; Fig. 3A). The PLFAs that were strongly positively correlated to PC1 were three branched fatty acids and a saturated and a monounsaturated fatty acid; 14:0 was significantly negatively correlated to PC1. The PLFAs 11:0 and i14:0 were assigned the two largest positive coefficients and three saturated fatty acids (16:0,

17:0, and 20:0) were assigned the most negative coefficients to PC2 (Fig. 3A).

In September, PC1 captured 64% of the total variance and significantly discriminated the PLFA profiles of the different O₃ treatments ($F = 71.6$, $P \leq 0.001$; Fig. 3B). A number of branched fatty acids and a saturated fatty acid were positively correlated to PC1, and three saturated fatty acids (including 16:0, 17:0, and 20:0) and two monounsaturated fatty acids (18:1 ω 12t and 16:1 ω 7c) were strongly negatively correlated to PC1. The second principal component captured 12% of the variance and did not discriminate among the PLFA profiles. For PC2, 15:0 and cy19:0 were assigned the largest positive and negative coefficients, respectively (Fig. 3B).

In October, 53% of the total variance in the data set was accounted for by PC1 and 30% by PC2 (Fig. 3C). The PLFA profiles of the different O₃ treatments were discriminated by both PC1 ($F = 622$, $P \leq 0.001$) and PC2 ($F = 124$, $P \leq 0.001$). Two saturated fatty acids (C15 and C18) and three terminally (C15, C16, and C17) and one mid-chain (C19) branched saturated fatty acids and cy17:0 were strongly positively correlated to PC1. Two saturated fatty acids (16:0 and 17:0) were significantly negatively correlated to PC1. Three fatty acids including 11:0, 14:0 and i14:0 were assigned the three largest positive coefficients and two C-16 fatty acids (16:0 and 16:1 ω 7c) were assigned the two largest negative coefficients to PC2 (Fig. 3C).

Stress Indicators

The ratios of saturated to unsaturated fatty acids and cyclopropyl acids to their acyclic precursors were used as stress indicators in this study. The unsaturated

Fig. 2. Biplots of soil microbial community function generated by principal component (PC) analysis of Biolog data under different O₃ treatments at different sampling times: (A) August, (B) September, and (C) October. Bars indicate standard errors of factor scores in principle component analysis. AA, acetic acid; ALA, alanine; ASN, asparagine; ASP, aspartic acid; BRO, bromosuccinic acid; BUT, hydroxybutyric acid; CisAco, *cis*-aconitic acid; CIT, citric acid; α-GP, α-glycerol phosphate; D-CEL, D-cellobiose; DEX, dextrin; D-G6P, D-glucose- 6-Phosphate; D-GAL, D-galactonic acid lactone; D-GLC, D-gluconic acid; D-MAN, D-mannitol; D-MEL, D-melibiose; D-PSI, D-psicose; D-SAC, D-saccharic acid; FOR, formic acid; GalNAc, N-acetyl-D-glucose-6-phosphate-galactosamine; GAM, glucuronamide; GASP, glycyl-L-aspartic acid; GBG, glycyl-L-bromosuccinic glutamic acid; GCO, glycogen; GlcNAc, N-acetyl-D-glucosamine; GLU, glutamic acid; GLY, glycerol; ITA, itaconic acid; L-AAM, L-alaninamide; L-AG, L-alanyl-glycine; L-ARA, L-arabinose; LEU, leucine; L-Fuc, L-fucose; L-RHA, L-rhamnose; MAL, malonic acid; MEA, 2-aminoethanol; MLT, maltose; ORN, Ornithine; PAME, pyruvic acid methyl ester; PGLU, pyroglutamic acid; PHE, phenylalanine; PRO, proline; PRP, propionic acid; PUT, putrescine; QUI, quinic acid; SA, succinic acid; SEB, sebatic acid; SER, serine; SUA, succinamic acid; SUC, sucrose; T-40, Tween 40; URO, urocanic acid; XLT, xylitol; α-CyD, α-cyclodextrin; α-D-GLU, α-D-glucose; α-D-LAC, α-D-lactose; α-KET, α-ketobutyric acid; α-KG, α-ketoglutaric acid.

fatty acids were detected in September and October, but O_3 significantly increased the ratio of saturated to unsaturated fatty acids only in October (Fig. 4). The ratio of cy17:0 to 16:1w7c was not affected by O_3 in August, but was significantly increased by the O_3 -2 treatment in September, and significantly increased by both O_3 -1 and O_3 -2 treatments in October (Fig. 5). This indicated that the stress of O_3 on PLFA increased with prolonged exposure.

DISCUSSION

In this study, the microbial biomass decreased from August to September and then increased to its maximum in October in the check and O_3 -1 treatments. This is the same pattern as in Bai et al. (2000), who found that the microbial biomass of a rice paddy soil first decreased and then increased from July to October. In the O_3 -2 treatment, the effect of O_3 on the soil microbial biomass was only obvious in October after a long period of exposure. A study on soil under ponderosa pine (*Pinus ponderosa* P. Lawson & C. Lawson) showed that the total soil microbial biomass increased at a low O_3 level and decreased at a high O_3 level compared with a control (Scagel and Andersen, 1997). Islam et al. (2000) reported that soil contained 17% less microbial biomass C under an elevated O_3 treatment relative to a control treatment, and Kasurinen et al. (2005) showed that O_3 had only slight effects on soil fungal biomass. These inconsistent results suggest that the uncertainty of O_3 effects on the soil microbial community might be related to species differences, exposure periods, and O_3 doses.

The CLPP by sole C source utilization tests is a simple and economical method to assess the metabolic function of a soil microbial community using Biolog microplates (Garland, 1997). In this study, at different sampling times that corresponded to different rice growth stages, microorganisms had different responses to elevated O_3 . Because the soil has different microbial communities at different growth stages of rice (Bai et al., 2000), the soil microbial community response to elevated O_3 could change with growth stage. In September, there were significant effects of O_3 on the AWCD area and the Shannon-Wiener diversity and richness indices among the three treatments. The substrates that strongly correlated to PC1 and PC2 were mainly carbohydrates, carboxylic acids, and amino acids. This was consis-

Fig. 3. Biplots of soil microbial community structure generated by principal component (PC) analysis of phospholipid fatty acid data under different O_3 treatments at different sampling times: (A) August, (B) September, and (C) October. Bars indicate standard errors of factor scores in principle component analysis.

tent with the results that the utilization of these three substrates was affected by high levels of O₃ in September. The strongest effects of elevated O₃ on microbial metabolic function occurred in September because then the rice was in the flowering stage when rice root growth reaches its peak. This is considered a key time for crop-induced changes in the soil microbial community (Reichardt et al., 1997).

Analysis of PLFA has proven to be a powerful tool for structural analysis of microbial communities (Buyer and Drinkwater, 1997). The method does not require growth in culture, so it is nonselective. It has hitherto been difficult to relate changes in PLFA patterns to certain groups of microorganisms, mainly because dominating PLFAs exist in wide ranges of taxa (Ratledge and Wilkinson, 1988). Different mixtures of soil microbes could be separated on the basis of their fatty acid profiles, however, by the use of multivariate statistical methods such as PCA (Haack et al., 1994). Principal component analysis and similar statistical techniques have also been a useful tool to indicate shifts in PLFA profiles due to different environmental disturbances (Bååth et al., 1992).

In this study, Gram-positive bacteria, indicated as a15:0, i16:0, a17:0 and 18:0(10Me) (Bossio and Scow, 1998; Kelly et al., 2003), were the main microorganisms detected using the PLFA method. At different sampling times, different microbial community compositions and different responses to elevated O₃ were detected. In August and October, PCA analysis of PLFA profiles clearly discriminated three clusters of microbial composition: control, low O₃ treatment, and high O₃ treatment. In September, however, the microbial composition expressed as PLFAs was only discriminated into two clusters: lower O₃ levels (including the control and the O₃-1 treatment) and high O₃ level. Ozone decreased some PLFAs and increased others. In August, Gram-positive bacteria indicators, including i16:0 and 18:0 (10Me) (Yao et al., 2000), decreased and saturated fatty acids 14:0 and 17:0 increased after O₃ fumigation. In September, Gram-positive bacteria indicators (Yao et al., 2000), including i14:0 and a17:0, increased and saturated fatty acids, which were nonspecific indicators (C16, C17, and C20), decreased after O₃ fumigation. In October, some monounsaturated fatty acids such as 16:1 ω 7c, 18:1 ω 9c and cy17:0, which were indicators of Gram-negative bacteria (Nakamura et al., 2003), decreased and Gram-positive bacteria, indicated by i14:0 and a17:0, increased under elevated O₃ treatments. Across the three sampling times, there was only one common fatty acid, 18:1 ω 9c, which was strongly correlated to PCs and whose abundance was affected significantly by O₃. This monounsaturated fatty acid, indicative of Gram-negative bacteria, first decreased, then increased, and then decreased under elevated O₃ at different sampling times. The PLFA profiles showed that O₃ changed the composition of the soil microbial community throughout the whole growing season, and in different stages the effects were different. Because the rice was growing in an inundated environment throughout almost all stages, the anaerobic bacteria community is the key community that should be considered. The indicative PLFAs of anaerobic bacteria, including i16:0 and a17:0, had different responses to

Fig. 4. Increased stress indicator (the ratio of saturated to unsaturated fatty acids) in rice paddy soil under elevated O₃ exposure; O3-1 had lower levels of O₃ exposure, O3-2 had higher levels, and CK is the check or control. Bars with different letters indicate significant difference ($P < 0.05$).

elevated O₃ in different stages. In August, the abundance of these two PLFAs decreased with increasing O₃ concentration, whereas in September their abundance increased with increasing O₃ concentration, and in October their abundance first increased and then decreased with increasing O₃ concentration.

The stress indicators, the ratios of saturated to unsaturated fatty acids and cyclopropyl fatty acids to their precursors such as cy17:0/16:1 ω 7c, are increased by different environmental stresses like starvation and desiccation (Kieft et al., 1994) and metal contamination (Frostegård et al., 1996). These stress indicators were found to be increased at elevated O₃ concentration. This indicated that O₃ caused stress to the soil microbial community in the rice paddy and the stress was worse with prolonged exposure to O₃.

CONCLUSIONS

The response of the belowground component of the ecosystem, including roots and soil microorganisms, to elevated O₃ is less investigated than the aboveground component, although underground components play critical roles in biogeochemical

Fig. 5. Elevated O₃ exposure induced changes in a stress indicator (the ratio of 16:1 ω 7c to cy17:0) in rice paddy soil; O3-1 had lower levels of O₃ exposure, O3-2 had higher levels, and CK is the check or control. Bars with different letters indicate significant difference ($P < 0.05$).

cycles and there might be a cumulative effect of O₃ stress on the ecosystem. Using the OTC method established in a rice paddy, the effects of elevated O₃ on the soil microbial community were assessed. The changes in the soil microbial biomass under an elevated O₃ concentration were statistically significant only at the last sampling. Community-level physiological profiles from sole C source utilization analyzed by the Biolog microbial identification system showed that the soil microbial activity and diversity were significantly decreased by elevated O₃ at the flowering stage in September. Although there were temporal differences in the response of the soil microbial community to elevated O₃ exposure in the rice paddy, the C utilization patterns of the microbial community were statistically significantly altered by elevated O₃. Ester-induced phospholipid fatty acid analyses showed that the elevated O₃ significantly altered the composition of the soil microbial community. This implies that O₃ significantly affected the microbial community structure and function, and indicates that elevated O₃ would result in more serious changes in organic matter decomposition, C and N cycling, and plant nutrients.

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