Influence of elevated ozone concentration on methanotrophic bacterial communities in soil under field condition

Y.Z. Huang^{a,*}, M. Zhong^b

^a Agro-Environmental Protection Institute, Ministry of Agriculture, Tianjin 300191, China

^b Department of Soil Sciences, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

HIGHLIGHTS

- O₃ stress can influence the potential methane oxidation rate (PMOR).
- O₃ influenced the potential methane production rate (PMPR) in soil.
- O₃ and the soil depth affected the gene *pmoA* of total methanotrophic bacteria.
- O₃ and the soil depth affected the type I and type II methanotrophic bacteria.
- O₃ influenced the community structure of the methanotrophic bacteria in soil.

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ABSTRACT

The open top chamber (OTC) method was used in combination with real-time quantitative PCR and terminal restriction fragment length polymorphism (T-RFLP) techniques in the wheat field to study the influence of different levels of O₃ concentrations (ambient air filtered by activated carbons, 40 ppb, 80 ppb and 120 ppb) on the quantity and community structure of methanotrophic bacteria. O₃ stress can influence the potential methane oxidation rate (PMOR) and potential methane production rate (PMPR) in the farmland soil. O₃ treatment of 40 ppb improved significantly the 16S rRNA gene copy number in the total methanotrophic bacteria *pmoA*, and type I and type II methanotrophic bacteria in the soil depth of 0–20 cm. When the O₃ concentration reached 120 ppb, the 16S rRNA gene copy number in the total methanotrophic bacteria *pmoA* and type I methanotrophic bacteria decreased significantly as compared to the control treatment in 10–20 cm layer. The 16S rRNA gene copy number of total methanotrophic bacteria *pmoA* and type I and type II methanotrophic bacteria were influenced by different O₃ concentration and soil depth. The T-RFLP analysis indicated that O₃ stress influenced significantly the community structure of the methanotrophic bacteria in soil, causing potential threat to the diversity of methanotrophic bacteria. It seems to imply that the rise of O₃ concentration could produce an impact on the carbon cycling and the methane emission of the wheat field soil by changing the community structure and diversity of methanotrophic bacteria, which then influences the global climate change.

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1. Introduction

Methane is an important greenhouse gas, with a contribution of about 25% to the global warming (Thompson et al., 1992). The methane concentration in the global atmosphere has increased from 715 ppb before the industrialization to the present 1800 ppb (Shindell et al., 2009). The microbes are involved in the methane

cycling in two ways, methanogenesis and methane oxidation (Edwards et al., 1998; Raghoebarsing et al., 2005). In natural soil, methanogens and other bacteria form a special syntrophism, by which the biomass is continuously degraded and the terminal electrons are accepted to produce methane. A large part of the methane is oxidized by the methanotrophic bacteria before being released to the atmosphere from soil and water (Le Mer and Roger, 2001).

Methanotrophic bacteria are a group of microbes that rely on methane as the sole carbon source and energy. It oxidizes methane into methanol under the action of methane monooxygenase

* Corresponding author.

E-mail address: hyz@rcees.ac.cn (Y.Z. Huang).

(MMO), and the latter is subsequently oxidized into formaldehyde by methanoldehydrogenase (MDH). Formaldehyde is oxidized into formic acid by formaldehyde dehydrogenase (FADH), and under the effects of formate dehydrogenase (FDH), formic acid is oxidized into CO₂ (Hanson and Hanson, 1996; Madigan et al., 2009). The methanotrophic bacteria that participate in this oxidation process are divided into three types: type I, type II and type X methanotrophic bacteria (Hanson and Hanson, 1996). The methane consumed by the oxidation by methanotrophic bacteria in the soil takes up approximately 10% of the total methane consumption in the atmosphere (Duxbury and Mosier, 1993). Therefore, the methanotrophic bacteria play a crucial role in the carbon cycling of terrestrial ecosystems and in the global atmospheric methane balance (Duxbury and Mosier, 1993).

In the molecular ecological studies of methanotrophic bacteria, the available target genes include the 16S rRNA gene and some functional genes encoding the important enzymes involved in the methane oxidation (Zheng, 2009). Gene *pmoA*, which encodes particulate methane monooxygenase (pMMO), can be used to detect all the methanotrophic bacteria other than *Methylocella*; gene *mmoX* specifically encodes soluble methane monooxygenase (sMMO); gene *mxoF* is the gene encoding methanol dehydrogenase present in most Gram-negative methanotrophic bacteria. The 16S rRNA gene can be used to distinguish type I from type II methanotrophic bacteria.

Due to rapid population growth, economic development and energy demand, people have to recklessly exploit and utilize fossil fuels and nitrogen fertilizers, which increases the O₃ concentration in the surface layer. The surface O₃ pollution has become one of the most challenging environmental issues (Selin et al., 2009). The average O₃ concentration in the troposphere rose from 38 ppb before the industrial revolution to 50 ppb in 2000. It is predicted that by 2050, the average O₃ concentration in the surface layer will be as high as 70 ppb, and will reach 80 ppb by 2100, much higher than 40 ppb, which is the critical value of damage to plants (Fiscus et al., 2005; Sitch et al., 2007). In the Yangtze River Delta region and Beijing, the maximum surface O₃ concentration has reached over 150 ppb (Shao et al., 2006; Zhang, 2010).

The O₃ pollution has damaging effects to plants (Avnery et al., 2013), causing the reduction of crop yield and huge economic loss (Feng et al., 2003; Zhu et al., 2011). The influence of O₃ stress on terrestrial ecosystems is not limited to the aboveground part. O₃ stress also affects the reactions and underground carbon cycling. It is reported that the rise of atmospheric O₃ concentration inhibits the growth of plant roots, reduces the root biomass and the microbial activity (Kasurinen et al., 2005; Chen et al., 2009). Reddy et al. (1995) studied the impact of O₃ stress on the soil enzymes of *Pinus taeda*. They found that when the O₃ concentration was 160 ppb, it inhibited significantly the activity of the phosphatase in the soil; when the concentration reached 240 ppb, it significantly inhibited the activity of soil arylsulfatase. The high O₃ concentration reduced significantly the microbial biomass carbon in the farmland soil, but had little influence on the soil organic carbon content (Islam et al., 2000).

Recently, it was found that the O₃ stress reduced significantly the methane emission in the farmland soil (Bhatia et al., 2011; Zheng et al., 2011), however the microbial mechanics that regulates the soil methane emission under O₃ stress has not been reported. Our hypothesis is that the O₃ stress influences the soil methane emission by exerting impact on the structure and functions of the microbial communities that participate in the soil carbon cycling in the farmland (such as methanotrophic bacteria). This study investigated the influence of O₃ concentration increase on the structure and functions of soil methanotrophic bacteria by the open top chamber (OTC) method in the winter wheat field. The

molecular biotechnologies such as real-time quantitative PCR and T-RLFP were used to reveal the changing characteristics of the soil methanotrophic bacteria and the mechanism driving the dynamics of the methane emission by microbes under the O₃ stress in the field.

2. Materials and methods

2.1. Experiment site

The experiment site was located in the Seed Management Station of Changping, Beijing (40°12'N, 116°8'E). The station is located in the northwest Beijing with continental monsoon climate and four distinct seasons. The annual average rainfall is 550.3 mm. The annual average temperature is 11.8 °C. The fundamental physico-chemical properties of soil are as follows: organic matter contents 16.4 g kg⁻¹; total nitrogen 0.9 g kg⁻¹; available phosphorus 38.1 mg kg⁻¹; available potassium 102.1 mg kg⁻¹; pH value 8.3.

2.2. Plant material

The variety used in the experiment was *Triticum aestivum* L. Beinong 9549, provided by Beijing Agriculture College. The seeds were sowed on September 28, 2009. Before sowing, the compost was applied. On April 26, 2010, urea was applied (225 kg ha⁻¹). The field management was coherent to that of the local farms during the entire growth season of winter wheat.

2.3. Ozone fumigation

In-situ ozone fumigation experiment was carried out on winter wheat with the self-made open-top fumigation system (Fig. 1, Huang et al., 2012). Four O₃ treatments were set up: the ambient air filtered by activated carbon (CK), 40 ppb, 80 ppb and 120 ppb. 3 replicates were set for each treatment. O₃ fumigation on winter wheat began from April 5, 2010. The fumigation lasted for 9 h (8:00–17:00) every day and stopped on June 12. The duration was 50 days. On June 13, 2010 (during the grain-filling stage of the winter wheat), the soil samples were collected. The 5-point mixing method was used for sampling. In every experimental plot, the fresh soil at the depths of 0–10, 10–20 and 20–40 cm was collected respectively with an earth auger 2 cm in diameter. The gravels and root residues were removed. The samples were mixed separately, put into sterile sampling bags for cold storage and taken back to the laboratory. The samples were sieved through the filter of 2.0 mm. Part of the soil samples were preserved in refrigerator at –80 °C and 4 °C. The test of soil properties and total DNA extraction were conducted. The remaining soil samples were freeze dried and

Fig. 1. Simulation experiment of ozone stress on winter wheat grown in the field.

sieved through the filter of 1.0 mm, for the determination of basic soil physical and chemical properties and nutrient contents.

2.4. Potential methane oxidation rate (PMOR) and potential methane production rate (PMPR) analysis

The PMOR was measured by reference to the method of McDonald et al. (1996), and the PMPR analysis referenced to the method of Wang et al. (2007). The PMOR and PMPR were both expressed as $\mu\text{g g}^{-1} \text{h}^{-1}$.

2.5. DNA extraction

DNA was extracted from 0.5 g fresh soil using the FastDNA SPIN Kit (Bio 101, Inc., USA) following the manufacturer's instructions. Extracted DNA was separated on a 1% agarose gel. The concentration was determined using a ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE), and DNA was stored at -20°C before use.

2.6. Quantitative analysis of the methanotrophs by real-time PCR

The functional gene *pmoA* was chosen as the target gene to quantify methanotrophic bacteria. The specific primers of the 16S rRNA gene in methanotrophic bacteria were selected to distinguish between type I and type II methanotrophic bacteria. The primers A189 (Bodrossy et al., 1997)/mb661 (Knief et al., 2006) were used to quantify the *pmoA* gene copy number in the total methanotrophic bacteria. The primers MB10γ/533r and MB9α/533r were used to quantify the 16S rRNA gene copy number of type I and type II methanotrophic bacteria (Henckel et al., 1999; Bodelier et al., 2000). The primer sequences are shown in Table 1. The reaction concentration was 200 nM. The PCR primers were synthesized by Invitrogen, Life Technologies, Beijing, China. For all the PCR reactions, the SYBR®Premix Ex Taq™ (TaKaRa, Biotechnology) tool kit was used. The 0.4 mg ml^{-1} bovine serum albumin (BSA) and 1 μl DNA templates were added to the reactions. The quantitative PCR apparatus was iCycler iQ5 (Bio-Rad, USA). The reaction procedures are shown in Table 1. The data analysis was performed by iCycler (version 1.0.1384.0 CR). The clones of the tested soil samples were adopted for the quantification of the *pmoA* gene of total methanotrophic bacteria and the 16S rRNA genes of type I and type II methanotrophic bacteria.

2.7. *pmoA*-based T-RFLP analysis

The primers used were A189 (Bodrossy et al., 1997) and mb661 (Knief et al., 2006). Their sequences are listed in Table 1. The 5' end of the forward primer was labeled with fluorescent dye 6-carboxyfluorescein (6-FAM). The PCR reaction system was 50 μl , including 5 μl 10 × PCR Ex buffer (Mg^{2+} plus), 4 μl 2.5 mM dNTPs, 0.5 μl Ex Taq polymerase (5 U μl^{-1} , Takara, Biotechnology, Japan), 1 μl of forward and backward primers, respectively, 1 μl BSA (20 mg mL^{-1}), 2 μl DNA template ($\sim 10 \text{ ng } \mu\text{l}^{-1}$), sterilized double-distilled water 35.5 μl . The amplification reaction was conducted

in eppendorf thermocycler. The procedures were 5 min of pre-degeneration at 95°C ; 10 cycles of "Touchdown" procedure; degeneration at 94°C for 45 s; the renaturation temperature started from 62°C ; after each thermal cycle, the temperature was lowered by 1.0°C until 52°C ; the renaturation duration was 1 min; the extension was conducted at 72°C for 1 min. After the "Touchdown" procedure, 30 cycles of isothermal amplification were conducted. The renaturation temperature was 56°C . With other conditions constant, the extension was conducted again for 10 min.

The PCR purity and yield were tested by 1% agarose gel electrophoresis. The *pmoA* gene fragments were purified by gel slice with Wizard SV Gel and PCR Clean-Up System (Promega, USA). The procedures were referred to the instruction. Finally, the products were dissolved in 40 μl sterilized deionized water. The restriction endonuclease Msp I was used for the enzyme digestion of the PCR products. The reaction lasted for 6 h at 37°C ; then denaturation was conducted at 95°C for 3 min. The products were cooled on ice immediately. Genescan was conducted on the enzyme digestion products after purification with the 3130xl Genetic Analyzer (Applied Biosystems, USA). The T-RFLP analysis was conducted by using GeneMapper (Applied Biosystems, USA). According to the detection range of the standard sample GeneScan™ 1000 ROX™ Size Standard (Applied Biosystems), the fragments with length less than 500 bp were selected for the analysis. The ratio of every peak height to the total peak height was calculated. The peak with a height ratio less than 0.05% was not included in the data analysis.

2.8. Cloning and sequencing

The primers used in the establishment of cloning library were the same as those in the real-time fluorescence quantitative PCR. The PCR system and procedures were the same as those of the PCR reactions in the T-RFLP analysis. The total soil DNA of the four duplicates of every treatment was mixed and then used as the templates for PCR amplification. The purity and yield of the products were determined with 1% agarose gel electrophoresis. The *pmoA* and *mcrA* gene fragments obtained from PCR were purified by Wizard SV Gel and PCR Clean-Up System (Promega, USA). The procedures were conducted by following the instruction of the toolkit. Then, the product was eluted with 50 μl sterilized water. The purified DNA fragments were connected to the vector by pGEM-T Easy toolkit (Promega, Corp., Madison, Wis., USA) and then transferred to the competent *Escherichia coli* cells (JM109) (TaKaRa), which were coated in the LB (Luria–Bertani) media containing Ampicillin/IPTG/X-Gal for culture at 37°C for 16–24 h. A certain amount of white clones were selected randomly. With direct amplification, the exogenous fragments were amplified with the primer T7/SP6 of pGEM-T Easy Vector. The clones carrying the inserted fragments were tested with electrophoresis. The corresponding clone liquid was sequenced.

2.9. Phylogenetic analysis

The sequencing analysis of the target gene fragments was

Table 1
PCR primers used in this study.

Target gene	Primer set	Sequence (5'–3')	Thermal profile	Reference
<i>pmoA</i>	A189	GGNGACTGGGACTTCTGG	95°C for 2 min followed by 40 cycles of 1 min at 94°C ,	Bodrossy et al., 1997; Knief et al., 2006
	mb661	CCGGMGCAACGTCYTTACC	1 min at 56°C and 45 s at 72°C , plate read at 83°C	
16S rRNA-Type I	MB10γ	AAGCGGGGATCTTCGGACC	95°C for 2 min followed by 36 cycles of 1 min at 95°C ,	Henckel et al., 1999; Bodelier et al., 2000
	533r	TTACCGCGGCTGCTGGCAC	1 min at 60°C , plate read at 83°C	
16S rRNA-Type II	MB9α	GTTCGGAATAACTCAGGG	95°C for 2 min followed by 36 cycles of 1 min at 95°C ,	Henckel et al., 1999; Bodelier et al., 2000
	533r	TTACCGCGGCTGCTGGCAC	1 min at 60°C , plate read at 83°C	

performed by using DNASTar and DNAMAN version 4.0. Then the sequence with the right length was submitted to the NCBI database (<http://www.ncbi.nlm.nih.gov/>). It was compared with the sequences in the database with the Blast method. The sequence with the highest similarity was downloaded as the reference sequence of the phylogenetic tree. The multiple sequence comparison was conducted with the ClustalW alignment method in MEGA 4.0. The neighbor joining method was used for the phylogenetic analysis. The confidence level of the branches of the phylogenetic tree was tested with Bootstrap analysis. The test was repeated for 1000 times. The obtained sequences were submitted to the GenBank database under accession numbers KF015205–KF015221.

2.10. Diversity indices

The Shannon diversity index H and Evenness index E were used to calculate the band pattern diversity of methanotrophs based on the following equations:

$$H = - \sum p_i \ln p_i = - \sum (N_i/N) \ln(N_i/N)$$

$$E = H/H_{\max} = H/\ln S$$

where N_i is the abundance of the i th ribotype, N is the total abundance of all ribotypes in the sample (lane of T-RF gels) and S is the number of ribotypes.

2.11. Statistical analysis

The copy number obtained in the quantitative test was statistically analyzed after the logarithmic conversion. The Duncan's test of the one-way ANOVA in SPSS 16.0 was used to calculate the significant difference ($p < 0.05$). The correlation was analyzed with the Correlation-Bivariate; multivariate ANOVA was conducted to determine the influence level of the environmental factors. CANOCO for Windows 4.5 was used for the T-RFLP analysis.

3. Results

3.1. Effects of O_3 on methanotrophic communities in soil

Compared with the control treatment, the PMORs of 0–10 cm and 10–20 cm layers were both decreased under O_3 stress (Fig. 2). When O_3 concentration was over 80 ppb, the PMOR of 0–10 cm layer was also decreased significantly as compared to the CK treatment ($P < 0.05$). There was not significant difference of PMOR of 20–40 cm layer between all treatments.

The PMPR of 0–10 cm layer was higher than the control treatment under O_3 stress (Fig. 2). At the O_3 concentration of 40 ppb, the PMPR reached the maximum ($0.15 \mu\text{g CH}_4 \text{ h}^{-1} \text{ g}^{-1} \text{ dry soil}$). There was not significantly different PMPR of 0–10 cm layer between treatments. The similar changing tendency of PMPR of 10–20 cm layer was showed in Fig. 2. There was significant difference of PMPR of 10–20 cm layer between O_3 stress and control treatment ($P < 0.05$). The PMPR of 20–40 cm layer was not significantly different under every O_3 concentration. With the increase of O_3 concentration, the PMPR of 20–40 cm layer decreased.

3.2. Effects of O_3 on methanotrophic abundance indicated by type I and II

Compared with the control, the 16S rRNA gene numbers of type I methanotrophic bacteria in the three depths of winter wheat field soil were increased significantly under low (40 ppb) and medium

Fig. 2. Effect of ozone on potential methane oxidation rate (PMOR) and potential methane production rate (PMPR) in winter wheat field soil.

(80 ppb) O_3 concentrations ($P < 0.05$). This indicated that low and medium O_3 stress stimulated the growth of type I methanotrophic bacteria. Under high O_3 concentration (120 ppb), the 16S rRNA gene numbers of type I methanotrophic bacteria decreased significantly, especially at the depth of 10–20 cm (Fig. 3). The variance analysis of the experimental data indicated that the O_3 concentration and soil depth significantly influenced the 16S rRNA gene numbers of type I methanotrophic bacteria, especially O_3 concentration had a greater influence.

The 16S rRNA gene number of type II methanotrophic bacteria in 0–10 cm layer (Fig. 3) was higher under O_3 stress than the control treatment. When the O_3 concentration was low (40 ppb), the gene copy number reached the maximum. The changes of the 16S rRNA gene number of type II methanotrophic bacteria under O_3 stress in 10–20 cm layer were similar to that of the 0–10 cm layer. When the O_3 concentration was 40 ppb, the 16S rRNA gene number of type II methanotrophic bacteria of this layer reached the maximum ($5.56 \times 10^7 \text{ copies g}^{-1} \text{ dry soil}$). There was significant difference of the 16S rRNA gene number of type II methanotrophic bacteria of 10–20 cm layer between O_3 stress and control treatment ($P < 0.05$). The type II methanotrophic bacteria number in 20–40 cm layer was significantly lower under every O_3 concentration than that of the control treatment ($P < 0.05$). The variance analysis indicated that the soil depth and the O_3 concentration significantly influenced the number of type II methanotrophic bacteria.

3.3. Effects of O_3 on methanotrophic abundance indicated by *pmoA*

The *pmoA* gene numbers of the total methanotrophic bacteria in

Fig. 3. Quantification of 16S rRNA gene copy numbers of Type I methanotrophs (M-Type I: a), Type II methanotrophs (M-Type II: b) and *pmoA* gene copy numbers of total methanotrophs (M-*pmoA*: c) in winter wheat field soil under different ozone concentrations. The different letters above bars indicate significant differences between different ozone concentrations at $p < 0.05$.

0–10 cm layer increased significantly under low O_3 concentration (40 ppb) as compared to the control treatment ($P < 0.05$); while under high O_3 concentration (120 ppb), the *pmoA* gene copy number decreased (Fig. 3). The *pmoA* gene number in 10–20 cm layer was significantly decreased under 120 ppb O_3 treatment as compared to the control treatment. The *pmoA* gene copy number under this O_3 concentration was only 3.89×10^7 copies g^{-1} dw, 56.8%, lower than the control treatment. In the 20–40 cm layer, every O_3 treatment had an increased *pmoA* gene number. When the O_3 concentration was 40 ppb, the *pmoA* gene number reached the maximum (8.02×10^7 copies g^{-1} dw). The variance analysis results indicated that the O_3 concentration and the soil depth both had significant influence on the *pmoA* gene number of the total methanotrophic bacteria.

Based on the *pmoA* gene cloning and sequencing, the methanotrophic bacterial community structure was analyzed by T-RFLP. There were 8 terminal restriction fragments, 78 bp, 225bp, 243bp, 348 bp, 373 bp, 436 bp, 445 bp and 504 bp, in the pattern. In every sample, 78 bp, 225bp, 243bp, 348 bp, 373 bp, 436 bp fragments were detected. The sum of their relative abundances represented over 91.0% of the total methanotrophic bacteria (Fig. 4). The phylogenetic tree of *pmoA* gene indicated that the dominant

Fig. 4. Community structure of methanotrophs in winter wheat field soil under different ozone treatments, (a) 0–10 cm soil depth, (b) 10–20 cm soil depth, (c) 20–40 cm soil depth.

methanotrophic bacteria in the rhizosphere soil of winter wheat were type I and type II (Methylocystis, Methylosinus, Methylocapsa), with high diversity (Fig. 5). The analysis of the T-RFLP pattern and cloning and sequencing results indicated that only the matching sequence of T-RF 445 bps with low relative abundance was not found in the cloning library. The methanotrophic bacteria corresponding to 78 bp, 225 bp, 373 bp, 436 bp and 504 bp fragments belonged to type I methanotrophic bacteria; while those of 348 bp and 243 bp belonged to type II.

Under different O_3 treatments, the 78 bp fragment was the most abundant, followed by 225 bp and 243 bp fragments. The structure of methanotrophic bacterial community in different soil depths showed different changes with the increasing O_3 concentration. In 0–20 cm layer, methanotrophic bacteria corresponding to T-RF 504 bp were not detected; in 20–40 cm layer, T-RF 445 bp was not detected. In 0–10 cm layer, the relative abundance of the T-RF 78 bp decreased when the O_3 concentration was 40 ppb; then the relative abundance increased with increasing the O_3 concentration. In this layer, the relative abundances of T-RF 243 bp and 373 bp fragments increased with the increase of O_3 concentration, however those of T-RF 436 bp and 445 bp decreased.

The relative abundance of T-RF 78 bp in the 10–20 cm layer reached the maximum at the O_3 concentration of 40 ppb, and then decreased with the increase of O_3 concentration. Its relative

Fig. 5. Phylogenetic tree of representative *pmoA* gene clone sequences retrieved from rhizosphere soil of winter wheat.

abundance reached the minimum when the O_3 concentration was 120 ppb. In this layer, the relative abundance of T-RF 445 bp increased with the increase of O_3 concentration; while those of T-RF 348 bp, 436 bp and 373 bp decreased within the O_3 concentration range of 40–80 ppb. The relative abundance of T-RF 78 bp in the 20–40 cm layer was decreased under 40 and 80 ppb O_3 concentrations, but increased under 120 ppb. In this layer, no T-RF 504 bp was detected in the CK treatment without O_3 fumigation. Under 40–120 ppb O_3 stress, T-RF 504 bp was detected.

Table 2
Shannon diversity and evenness of soil methanotrophic community in O_3 treatments.

Soil depths/cm	Ozone treatment	H	E
0–10	CK	1.04 ± 0.05b	0.53 ± 0.03b
	40 ppb	1.35 ± 0.03a	0.70 ± 0.01a
	80 ppb	1.12 ± 0.03b	0.54 ± 0.02b
	120 ppb	0.90 ± 0.02c	0.46 ± 0.03b
10–20	CK	1.16 ± 0.03b	0.60 ± 0.02b
	40 ppb	0.95 ± 0.05c	0.49 ± 0.02c
	80 ppb	1.01 ± 0.02c	0.63 ± 0.04 ab
	120 ppb	1.52 ± 0.06a	0.73 ± 0.03a
20–40	CK	1.14 ± 0.04b	0.64 ± 0.04a
	40 ppb	1.13 ± 0.03b	0.70 ± 0.03a
	80 ppb	1.36 ± 0.06a	0.70 ± 0.03a
	120 ppb	0.88 ± 0.03c	0.49 ± 0.02b

The diversity index is a comprehensive measure of the abundance and the evenness. The Shannon index (H) and the evenness index (E) of the methanotrophic bacteria are shown in Table 2. It can be seen that the soil depth had greater impact on the diversity index and evenness index. The H and E values in the 0–10 cm layer reached the maximum when O_3 concentration was 40 ppb. The H and E values in the 10–20 cm layer reached the maximum when O_3 concentration was 120 ppb. The H and E values in the 20–40 cm layer reached the maximum when O_3 concentration was 80 ppb and the minimum when O_3 concentration was 120 ppb. In the 10–20 cm and 20–40 cm layers, the E values of the methanotrophic bacteria were in significant correlation with the O_3 concentration. The correlation coefficients r were 0.693 and -0.587 , respectively ($n = 12$, $p < 0.05$).

4. Discussion

4.1. The influence of O_3 concentration increase on PMOR and PMPR

Potential methane production rate (PMOR) is the comprehensive phenomenal characteristic of the methanotrophic bacterial communities, methane-oxidizing activity and their influential factors. The comprehensive research on PMOR and soil methanotrophic bacterial community could help understand the influencing mechanism of the rising O_3 concentration on the methane cycling

from the biological perspective. This study indicated that under O₃ treatments, PMOR in 0–20 cm layer was lower than that of CK treatment, while in the 20–40 cm layer, the difference was not significant. The analysis of PMOR and O₃ AOT40 (accumulated exposure over a threshold of 40 ppb) values showed negative correlation between the two indicators ($r = -0.493$, $p < 0.01$, $n = 36$). As it is hard for O₃ to penetrate the soil, its influence on soil methane oxidation is indirect (McCool and Menge, 1983). The increase of O₃ concentration will disturb the photosynthesis of plants, causing the drop of plant biomass and changing the distribution of the root exudates (McCool and Menge, 1983). Thus, the content of mineral elements and organic elements may change, resulting in the change of soil methanotrophic bacterial activity and PMOR. The correlation analysis between soil PMOR and PMPR indicated negative correlation ($r = -0.288$, $p < 0.05$, $n = 36$). This showed that the soil methane-oxidizing process and the methane production process are mutually interfering. In this research, the *pmoA* gene number of soil total methanotrophic bacteria, the numbers of type I and type II methanotrophic bacteria were not in significant correlation with PMOR, indicating that PMOR is not determined by the number of methanotrophic bacteria.

PMPR is generally associated with the activity of soil methanotrophic bacteria (Yavitt et al., 1988). The research on the influence of different O₃ concentrations on the PMPR of winter wheat field soil indicated that the soil PMPR increased as at deeper depth without O₃ fumigation. After the increase of O₃ concentration, this tendency was not observed. The rising O₃ concentration would cause the change of the soil microbial biomass carbon (Sinsabaugh et al., 2002), plant roots (Renaud et al., 1997), soil respiration (Edwards, 1991) and soil temperature (Mosier, 1998), therefore affecting the activity of soil methanogens. In this research, O₃ increased PMPR at 0–20 cm layer of wheat soil. With the deepening of the soil depth, especially in the 20–40 cm layer, PMPR decreased as the O₃ concentration increased. This might be related to the influence of O₃ stress on plant root growth and root exudates (Renaud et al., 1997). Some researches have found that the O₃ stress could significantly lower the methane emission in farmland soil (Bhatia et al., 2011; Zheng et al., 2011). Its mechanism can be explained by the influence of O₃ stress on soil PMOR, PMPR and soil microbial activity.

4.2. Influence of O₃ on the abundance of methanotrophic bacteria

In the CK treatment without O₃ fumigation, the *pmoA* gene copy number in the 10–20 cm layer was much higher than that in the other two layers. The low concentration (40 ppb) of O₃ could significantly increase the *pmoA* gene copy number of methanotrophic bacteria and the 16S rRNA gene number of type I and type II methanotrophic bacteria in the 0–20 cm layer. Under high concentration (120 ppb), the three gene numbers were significantly lower than those of the CK treatment. The correlation analysis between O₃ AOT40 value and the abundance of methanotrophic bacteria indicated that the O₃ concentration showed significantly negative correlations with *pmoA* gene number ($r = -0.396$, $p < 0.01$, $n = 36$), and the 16S rRNA gene numbers of type I ($r = -0.422$, $p < 0.01$, $n = 36$) and type II ($r = -0.379$, $p < 0.01$, $n = 36$). Thus, the rise of O₃ concentration had an inhibitory effect on the growth and proliferation of methanotrophic bacteria. The possible reasons are (1) CH₄ oxidation involves the aerobic microbes. The sufficient supply of O₂ is important to the growth of methanotrophic bacteria and CH₄ oxidation. Elevated O₃ concentration would lead to the rise of CO₂ production and O₂ consumption of plants (Scagel and Andersen, 1997). Thus, the abundance of the soil methanotrophic bacteria would decrease (McLain et al., 2002). (2) The ammonia oxidizing bacteria and the methanotrophic bacteria in the soil shared several common substrates

(Holmes and Costello, 1995). The rise of O₃ concentration could cause the decrease of soil ammonium nitrogen and nitrate nitrogen (Li et al., 2010). The ammonium oxidizing bacteria and the methanotrophic bacteria compete for the substrates such as nitrogen (Bedard and Krause, 2007), causing the decrease of the abundance of methanotrophic bacteria.

In this research, the 16S rRNA gene numbers of type I and type II methanotrophic bacteria were in significant positive correlation with *pmoA* gene copy number, indicating that the quantification results of the 16S rRNA gene of methanotrophic bacteria was basically consistent with those of the *pmoA* gene. In all treatments, the number of type I bacteria was significantly higher than that of Type II. The methane oxidizing process is as follows (Hanson and Hanson, 1996; Madigan et al., 2009): first, methane is oxidized to methanol by type I methanotrophic bacteria under the action of soluble methane monooxygenase (sMMO), or by type II bacteria under the action of particulate methane monooxygenase (pMMO) and sMMO; then, methanol is oxidized to formaldehyde and CO₂ by type I bacteria via ribulose monophosphate pathway (RuMP) or by type II bacteria via the serine pathway. The RuMP of type I methanotrophic bacteria is more efficient than that of the serine pathway of type II bacteria. This could be one of the reasons for the higher number of type I bacteria than type II in winter wheat field soil.

4.3. Influence of O₃ on the structure and diversity of methanotrophic bacterial community

The T-RFLP analysis of the *pmoA* gene of methanotrophic bacteria indicated that the relative abundance of type I was higher than that of type II. This was coherent to the quantitative PCR results of 16S rRNA gene of methanotrophic bacteria. The T-RFLP analysis on *pmoA* gene and the phylogenetic tree results indicated that the diversity of type I bacteria was significantly higher than that of Type II bacteria. This conformed to the results of many previous studies (Henckel et al., 2000; Eller and Frenzel, 2001). This research found that no T-RF 504 bp was detected in the 0–20 layer, but fragment T-RF 445 bp was detected. This phenomenon was not observed in the 20–40 layer. The methanotrophic bacterial population corresponding to T-RF 504 bp is phylogenetically closer to *Methylobacterium methanica* (G-) (Fig. 5). Bacteria of this type are widely found in the sediments of lakes, water and rivers, as well as swamps (Yun et al., 2013).

The Shannon index (H) and the evenness index (E) are both used to represent the diversity of the soil microbial community. In this research, the H and E values of the soil methanotrophic bacterial community. Especially, the E values in the 10–20 cm and 20–40 cm layers were in significant correlation with O₃ concentration. This indicated that the rise of O₃ concentration could influence the diversity and evenness of soil methanotrophic bacteria.

In conclusion, O₃ stress could influence the gene copy number of total methanotrophic bacteria *pmoA*, 16S rRNA gene numbers of type I and type 2 methanotrophic bacteria at different depths of winter wheat field soil. Thus, the structure and diversity of soil methanotrophic bacterial community are further affected. The high concentration of O₃ inhibits the growth and proliferation of soil methanotrophic bacteria. The rise of O₃ concentration would cause the changes of PMOR and PMPR, thereby indirectly influencing the carbon cycling and methane emission in the field soil.

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