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RESEARCH ARTICLE

Conversion of methane-derived carbon and microbial community in enrichment cultures in response to O_2 availability

Xiao-Meng Wei[1] · Ruo He[1] · Min Chen[1] · Yao Su[1] · Ruo-Chan Ma[1]

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Abstract Methanotrophs not only play an important role in mitigating CH_4 emissions from the environment, but also provide a large quantity of CH_4-derived carbon to their habitats. In this study, the distribution of CH_4-derived carbon and microbial community was investigated in a consortium enriched at three O_2 tensions, i.e., the initial O_2 concentrations of 2.5 % (LO-2), 5 % (LO-1), and 21 % (*v*/*v*) (HO). The results showed that compared with the O_2-limiting environments (2.5 and 5 %), more CH_4-derived carbon was converted into CO_2 and biomass under the O_2 sufficient condition (21 %). Besides biomass and CO_2, a high conversion efficiency of CH_4-derived carbon to dissolved organic carbon was detected in the cultures, especially in LO-2. Quantitative PCR and Miseq sequencing both showed that the abundance of methanotroph increased with the increasing O_2 concentrations. Type II methanotroph *Methylocystis* dominated in the enrichment cultures, accounting for 54.8, 48.1, and 36.9 % of the total bacterial 16S rRNA gene sequencing reads in HO, LO-1, and LO-2, respectively. Methylotrophs, mainly including *Methylophilus*, *Methylovorus*, *Hyphomicrobium*, and *Methylobacillus*, were also abundant in the cultures. Compared with the O_2 sufficient condition (21 %), higher microbial biodiversity (i.e., higher Simpson and lower Shannon indexes) was detected in LO-2 enriched at the initial O_2 concentration of 2.5 %. These findings indicated that compared with the O_2 sufficient condition, more CH_4-derived carbon was exuded into the environments and promoted the growth of non-methanotrophic microbes in O_2-limiting environments.

Keywords CH_4 oxidation · Methanotrophs · Microbial community · CH_4-derived carbon · O_2 concentration

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✉ Ruo He
heruo@zju.edu.cn

[1] Department of Environmental Engineering, Zhejiang University, Hangzhou 310058, China

Introduction

Aerobic methanotrophs in both aerobic and microaerobic environments act as sinks for CH_4 (Segers 1998; Wagner et al. 2003; Ho et al. 2015), which is the second most important greenhouse gas after CO_2, contributing 18 % to the total radiative forcing of long-lived greenhouse gases (IPCC 2007). Aerobic methanotrophs are a unique group of methylotrophic bacteria, which utilize CH_4 as sole carbon and energy source, and mainly belong to *Proteobacteria* (Hanson and Hanson 1996). Based on their cell morphology, ultrastructure, phylogeny, and metabolic pathways, methanotrophs in *Proteobacteria* can be generally classified into two groups: type I (including *Methylomonas*, *Methylomicrobium*, *Methylobacter*, *Methylosarcina*, *Methylosoma*, *Methylovulum*, *Methylosphaera*, *Crenothrix*, *Clonothrix*, *Methylococcus*, *Methylocaldum*, *Methylogaea*, *Methyloglobulus*, *Methylohalobius*, and *Methylothermus*) and type II (including the genera *Methylocystis*, *Methylosinus*, *Methylocella*, *Methylocapsa*, and *Methyloferula*) (Vorobev et al. 2011; Bodelier et al. 2013; Deutzmann et al. 2014). In addition, methanotrophs have been described in *Verrucomicrobia* phylum (Dunfield et al. 2007; Pol et al. 2007; Islam et al. 2008).

Methanotrophs not only can oxidize CH_4 and mitigate CH_4 emissions from the environments, but also provide a large quantity of CH_4-derived carbon to their habitat. Roslev et al. (1997) reported that 31–43 % of the oxidized atmospheric CH_4 was assimilated into microbial biomass. Roslev and Iversen (1999) found that the conversion efficiency of the CH_4-derived carbon into microbial biomass ranged from 20 % in agricultural soils to 54 % in forest soils of Vietnam by incubating soil samples with $^{14}CH_4$ at near atmospheric concentrations (<5 ppm CH_4). An average of 31 % of CH_4-derived carbon was assimilated into microbial biomass in Arctic lake sediment by the analysis of ^{13}C-phospholipid fatty acids (He et al. 2015). Besides assimilating CH_4 into the biomass, methanotrophs can produce extracellular polymeric substances (EPS) to prevent formaldehyde accumulation in case of carbon excess or lack of nutrients (Schloss et al. 2009). Additionally, methanotrophs also can excrete organic matters such as methanol and formaldehyde into the environment in the presence of mutations or inhibitors, such as NaCl, EDTA, and HCOONa, or gas composition (He et al. 2012b; Kalyuzhnaya et al. 2013). These CH_4-derived carbon can flow into microbial food web and higher trophic levels via direct grazing on methanotrophs, exudation and consumption of biomolecules and the cycling of CO_2, and finally influence the community structure and function of system (Murase and Frenzel 2008; Kalyuzhnaya et al. 2013; He et al. 2015).

Available O_2 as a substrate in aerobic CH_4 oxidation is a key factor affecting aerobic methanotrophic community and the fate of CH_4-derived carbon. Type I and type II methanotrophs both persist under different O_2 and CH_4 concentrations (Kong et al. 2013; Hernandez et al. 2015). Type I methanotrophs are also reported to dominate in environments with low CH_4 and high O_2 concentrations, while type II methanotrophs have propensity to grow in high CH_4 and low O_2 concentration environment (Amaral and Knowles 1995). Type II methanotrophs have been reported to be active under the CH_4 concentrations lower than 500 ppmv (Knief et al. 2006). In the two CH_4 assimilation pathways of methanotrophs (i.e., the ribulose monophosphate (RuMP) pathway of type I methanotrophs and serine pathway of type II methanotrophs), RuMP pathway is more efficient (Schloss et al. 2009). Type I and type II methanotrophs both can produce EPS from CH_4 oxidation. Compared with the serine pathway, type I methanotrophs produce a higher EPS of 25–28 % by the RuMP pathway (Malashenko et al. 2001). In O_2-limiting environments, type I methanotroph *Methylomicrobium alcaliphilum* 20Z is found to participate in a novel form of fermentation, driving the conversion of CH_4 to excreted products and hydrogen (Kalyuzhnaya et al. 2013), which in turn could be used by non-methanotrophs (Roslev and King 1995). In the environment such as landfill cover soils and non-wetland agricultural soils, since O_2 is mainly supplied by air diffusion, O_2 concentrations present a gradient distribution, i.e., a near atmospheric O_2 concentration at the surface, and microaerobic and anaerobic conditions in deep layers. Thus, an understanding about how O_2 affecting the production and excretion of methanotrophic exometabolites is helpful to reveal the microbial community and function of the environment.

The aim of this study was to characterize effects of O_2 concentrations on distribution of CH_4-derived carbon and microbial community in the consortia enriched with CH_4. Aerobic methanotrophs in landfill cover soils were used as a model system. The conversion efficiency of CH_4-derived carbon to CO_2 (CE_{CO2}), biomass ($CE_{biomass}$), EPS (CE_{EPS}), and dissolved organic carbon (DOC) (CE_{DOC}) was estimated during the enrichment process. The difference of microbial community in the enriched consortia was compared using Illumina Miseq sequencing. Additionally, the abundance of methanotrophs in the enriched consortia was assessed by quantitative PCR (Q-PCR). Our results increase current understanding on the fate of CH_4-derived carbon to different O_2 tensions and its effect on the microbial community and function of the environment.

Materials and methods

Enrichment experiment

In this study, three materials were used as the enrichment sources: landfill cover soil, waste biocover, and landfilled waste. The landfill cover soil was collected from the depth of 0–20 cm in Dawuao landfill in Zhejiang Province (China). Waste biocover was collected from an organic waste treating-bioreactor in a village in Zhejiang Province (China). After air-dried, the soil samples were sieved through a 4 mm mesh. The surface landfilled waste was taken from a simulated landfill in the laboratory, which had been operated for more than 1 year and reached the stable stage.

Approximately 5 g of each material was mixed together and put into a 400 ml sterile serum bottle containing 80 ml of sterilized nitrate mineral salts medium (NMS). The composition of NMS was described by Graham et al. (1992). The serum bottle was shaken at 150 rpm for 1 h, and then the suspension was added to fresh sterile NMS to form the inoculum with an initial cell concentration measured at 600 nm (OD_{600} of 0.105 ± 0.002). Eighty milliliter of the inoculum was transferred into a new 400 ml sterile serum bottle. After that, the serum bottles were flushed with high purity N_2 (99.99 %) for ~5 min at the flow rate of about 200 ml min^{-1} and then sealed with butyl rubber stoppers. After a certain volume of gas was taken from the bottles, CH_4 and O_2 were injected to constitute the tested concentrations. The initial CH_4 concentration for all the treatments was 10 % (*v*/*v*). Considering that an O_2 concentrations of 5 % is enough to

sustain the activity of methanotrophs in waste biocover soil (Wang et al. 2011), three types of O_2 tensions, i.e., the initial O_2 concentrations of 21, 5, and 2.5 % (*v*/*v*), respectively, were conducted and labeled as HO, LO-1, and LO-2, respectively. Each treatment was operated in triplicate. All the serum bottles were incubated at 30 °C and 120 rpm. The incubation was run until the OD_{600} of the enrichment culture reached about 1.1. After the first incubation (phase I), the enrichment culture was immediately transferred to a new sterile serum bottle containing 80 ml fresh sterile NMS to achieve an initial OD_{600} of 0.017 ± 0.002 for the incubation of phase II. The transfer was repeated for three times in the same way as described above. Since the microbial growth rate differed with O_2 concentrations, the incubation time for the enrichment cultures was different in this study (i.e., 21.5, 31.5, and 44 days for HO, LO-1, and LO-2, respectively).

During the incubation of each phase, gas in the serum bottles was refreshed with high purity N_2 and then O_2 and CH_4 were resupplied to the initial concentrations each day. For LO-1 and LO-2, O_2 was resupplied to the initial concentrations every 12 h. During the incubation, the O_2 concentration was kept higher than 10 % (*v*/*v*) in HO, while it was above the detection limit (0.02 %, *v*/*v*) in LO-1 and LO-2. The main gas concentrations (i.e., O_2, CO_2, and CH_4) in the headspace of serum bottles were detected every 3–12 h and the cell growth of the culture was measured each day. At the end of the experiment, the enrichment culture were centrifuged at 6000 rpm for 10 min, and then washed with sterilized water twice for sequential DNA extraction and molecular analysis.

Kinetics test of CH_4 oxidation

At the end of the enrichment experiment, the cultures were harvested by centrifuging at 6000 rpm for 10 min for kinetics test of CH_4 oxidation at the initial O_2 concentrations of 21, 5, and 2.5 % (*v*/*v*). The pellet was washed twice with sterile deionized water to remove salts and other debris and resuspended in fresh sterile NMS to form the inoculum. Then, 15 ml of the inoculum with an initial OD_{600} of 0.523 ± 0.03 was put in 80 ml sterile serum bottles. After that, the serum bottles were flushed with high purity N_2 (99.99 %) for ~5 min at the flow rate of about 200 ml min^{-1} and then sealed with butyl rubber stoppers. After a certain volume of gas was taken from the bottles, CH_4 and O_2 were injected to obtain the initial CH_4 concentrations ranging from 0.5 to 30 % (*v*/*v*) and the initial O_2 concentrations of 21, 5, and 2.5 % (*v*/*v*) in the headspace, respectively. The serum bottles were all incubated at 30 °C and 120 rpm. The concentrations of CH_4 and O_2 in the headspaces of serum bottles were measured periodically over a 2–6 h time course. During the test, the O_2 concentration was kept higher than 10 % (*v*/*v*) in HO, while it was above the detection limit in LO-1 and LO-2.

CH_4-derived carbon distribution test

In this test, the inoculum was prepared as described in kinetics test of CH_4 oxidation. Fifteen milliliters of inoculum with an initial OD_{600} of 0.523 ± 0.03 was put in 80 ml sterile serum bottles. The initial gas composition and incubate condition of the serum bottles were the same as those in the enrichment experiment. Gas sample was taken periodically to analyze the concentrations of O_2, CH_4, and CO_2 in the headspace. During the whole test, the O_2 concentration in HO was kept higher than 10 % (*v*/*v*), while it was above the detection limit in LO-1 and LO-2. The incubation was continued until the CH_4 concentration in the headspace dropped to about 3 % (*v*/*v*). The incubation for all the three treatments was finished within 24 h. Then, the cultures were collected and used for biomass, EPS, and total organic carbon analysis immediately.

Sampling and analysis

Gas samples (100 μl) were periodically withdrawn to detect the concentrations of O_2, CO_2, and CH_4 by a gas chromatography (GC) equipped with thermal conductivity detector (TCD) and flame ionization detector (FID) as described by Wang et al. (2011). CH_4 oxidation rate was assessed from the zero-order decrease in CH_4 concentration in the headspace (Wang et al. 2011). The average cell amount (dry weight at 105 °C) in the period of zero-order decrease in CH_4 concentration was used to normalize CH_4 oxidation activity. For CH_4-derived carbon distribution test, after the culture was centrifuged at 6000 rpm (4 °C) for 10 mins, the pellet was collected for EPS extraction and the supernatant was filtered through a 0.22 μm fiber filter to remove the residual cells for TOC measurement using a multi N/C analyzer (3100 Analytik Jena, Jena, Germany) (Visco et al. 2004).

The pellet obtained above was washed twice with sterile deionized water and resuspended in sterile deionized water. Then, EPS was extracted as described by Kang and Zhu (2013). The exopolysaccharide (ECPS) and extracellular protein (ECP) were measured by the phenol–sulfuric acid method (Gerhardt et al. 1994) and the Bradford method (Bradford 1976), respectively.

DNA extraction and Q-PCR analysis

At the end of the enrichment experiment, the cultures of HO, LO-1, and LO-2 were harvested for total DNA extraction using E.Z.N.A.TM Soil DNA Kit (Omega Bio-Tek, Inc., Norcross, USA). Three replicates of each culture were used and the extracted DNA was quantified using the Nanodrop ND-1000 spectrophotometer. The DNA from each replicate was used for Q-PCR analysis.

Q-PCR analysis of *pmoA* and bacterial 16S rRNA gene was run as described by Kong et al. (2013) and Swan et al. (2010) using the primer sets of A189f/mb661r and bac331f/bac797r, respectively. Q-PCR reaction was conducted in three replicates of 15 μl reactions containing 7.5 μl All-in-one qPCR Mix (GeneCopoeia, Inc., Rockville, MD, USA), 0.45 (for *pmoA*) or 0.9 (for bacterial 16S rRNA gene) μl primer (10 μM) and 1 μl template. Thermal cycler conditions were as follows: an initial stage at 95 °C for 10 min; 40 cycles of 95 °C for 10 s, 58 °C for 30 s and 72 °C for 30 s for *pmoA*; and an initial stage at 95 °C for 10 min; 40 cycles of 95 °C for 15 s, 60 °C for 1 min and 72 °C for 45 s for 16S rRNA gene. Standards were made from 10-fold dilutions of linearized plasmids containing the same fragment of *pmoA* and bacterial 16S rRNA gene. A melting point analysis was performed to confirm that no unspecific PCR products were generated and analyzed. The detection limit of Q-PCR for *pmoA* and bacterial 16S rRNA gene was ~10^2 copies per reaction. The abundance of *pmoA* and bacterial 16S rRNA was expressed as copies mg^{-1} dry weight (copies mg^{-1}).

16S rRNA gene sequencing

The DNA from each replicate was mixed in equal amounts to compose the DNA sample of each culture for bacterial 16S rRNA gene amplicon sequencing. PCR amplification of the V4 hypervariable regions of bacterial 16S rRNA gene for Illumina Miseq sequencing was amplified using the primers 520 F (5′-AYTGGGYDTAAAGNG-3′) and 802R (5′-TACNVGGGTATCTAATCC-3′). PCR reactions were conducted in a 25-ml PCR mixture containing final concentrations of 1 × Q5 PCR buffer, 1× GC high enhancer, 200 μM dNTPs, 400 nM each primer, 0.05 U Q5 DNA polymerase and ~4 ng template DNA. Thermal cycler conditions were as follows: initial denaturation at 98 °C for 30 s, 25 cycles of 30 s at 98 °C, 30 s at 50 °C, 30 s at 72 °C, and a final elongation step for 5 min at 72 °C. PCR product was evaluated by electrophoresis in 2 % agarose gel and purified, before sequencing by Illumina Miseq in Shanghai Personalbio Biotechnology (Shanghai, China).

The raw reads were firstly treated for quality control. If the reads contained more than one ambiguous base (N) and low-quality sequences (average Q <20) in any paired reads, they were removed from the dataset before pairing. The pair reads with the length of above 150 bp and the overlapping regions of more than 10 bp were merged using FLASH program (version 1.2.7, http://ccb.jhu.edu/software/FLASH/) (Magoc and Salzberg 2011). Subsequently, the reads were filtered using Qiime (version 1.7.0, http://qiime.org/) (Caporaso et al. 2010) and chimera check was conducted using Mothur program (version 1.31.2, http://www.mothur.org/) (Edgar 2010; Schloss et al. 2009). The operational taxonomic units (OTUs) of the remaining high quality reads were performed with a cutoff of 97 % identity using Qiime program. The representative sequence from each OTU was assigned at different taxonomic levels using RDP-classifier (Cole et al. 2009) and BLAST (Altschul et al. 1990). Based on the OTUs of the samples, Shannon and Simpson diversity indices were conducted using Mothur program. The heatmap based on the hierarchical clustering method of the three samples was generated using heatmap.2 program.

The sequences obtained in this study were deposited in the Sequence Read Archive at the NCBI under the accession number of SRP058404.

Results

Cell growth and CH_4 oxidation activity

In the incubation of phase I, the cultures presented a lag period of 4–5 days, during which there was no significant difference in the cell concentrations among HO, LO-1, and LO-2 ($P = 0.076$–0.903) (Fig. 1). After the lag period, the cell growth of the enrichment culture showed a positive correlation with the O_2 concentrations. It took 3.5–4.5 days to reach the OD_{600} of ~1.1 in HO in the phases II, III, and IV, while it took about 6 and 10 days to reach the OD_{600} of ~1.1 in LO-1 and LO-2, respectively. Compared with the incubation of phase I, the cell grew more quickly in the later phases (phases II, III, and IV).

In the incubation of phase I, the CH_4 oxidation rate (MOR) was low in the first several days, and then increased rapidly and reached the peak between days 6 and 9 in the three treatments. No significant difference was observed in the maximum MOR between HO (8.29 μmol mg^{-1} h^{-1}) and LO-1 (7.26 μmol mg^{-1} h^{-1}) ($P = 0.078$). However, the maximum MOR in LO-2 (5.77 μmol mg^{-1} h^{-1}) was significantly lower than in HO and LO-1 ($P = 0.001$–0.014). In the last three phases, the MORs in all the three treatments increased sharply as soon as the incubations started and the maximum values were achieved after 1–2 days of incubation. After the peak value, the MORs in the incubation of each phase decreased and dropped to a low level of 2.84–4.81, 1.20–2.18, 1.19–1.98 μmol mg^{-1} h^{-1} in HO, LO-1, and LO-2, respectively, which might be attributable to a shortage of nutrients in the media. Compared with the O_2-limiting environments (2.5 and 5 %), CH_4 was consumed more rapidly in the culture enriched under the O_2 sufficient condition (21 %).

CH_4 oxidation kinetics

To understand the CH_4 oxidation kinetics of the enrichment cultures in response of O_2 concentrations, the CH_4

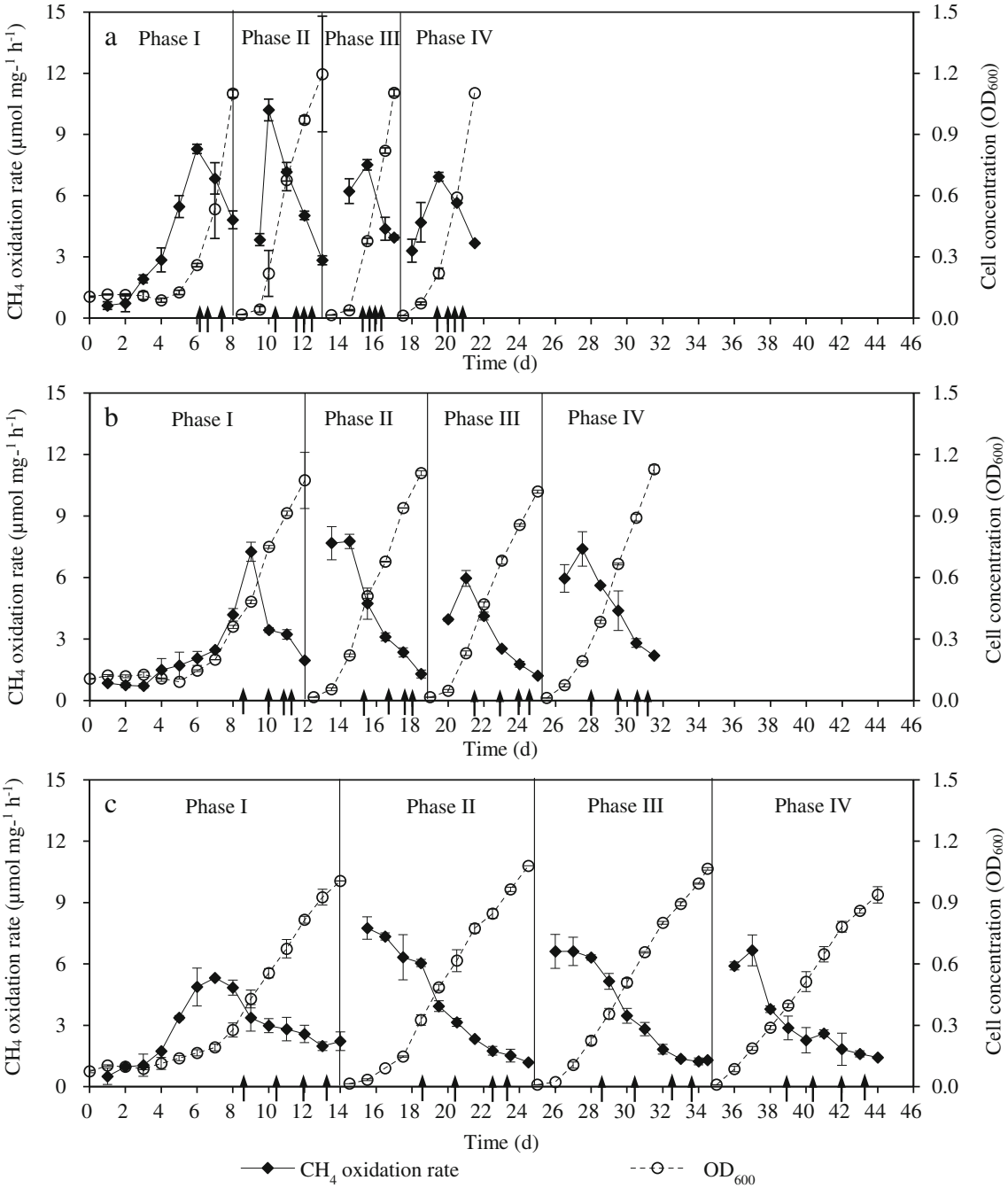

Fig. 1 Variation of CH_4 oxidation rate and cell concentration in the enrichment cultures of HO (the initial O_2 concentration = 21 %) (**a**), LO-1 (the initial O_2 concentration = 5 %) (**b**), and LO-2 (the initial O_2 concentration = 2.5 %) (**c**) with time. Data are means ± standard deviation ($n = 3$). The *arrows* in the figure show that the serum bottles were flushed with high purity N_2 and the initial concentrations of CH_4 and O_2 were re-established

oxidation activity of the three cultures collected at the end of phase IV was determined at the initial O_2 concentrations of 2.5, 5, and 21 %, respectively. The CH_4 oxidation activity of the three cultures obtained from the CH_4 concentrations between 0.5 and 30 % were fitted by Monod model (Eq. 1). The values of the correlation coefficient ($R^2 = 0.97$–0.99) showed that the Monod model had a good fit for the CH_4 oxidation kinetics of the enrichment cultures (Fig. S1 and Table 1).

$$\mu = \mu_{\max} \frac{[S]}{K_s + [S]} \quad (1)$$

Table 1 CH_4 oxidation kinetics of the enrichment cultures at different O_2 concentrations ($n = 3$)

Enrichment culture*	Initial O_2 concentration					
	21 %		5 %		2.5 %	
	μ_{max}	K_s	μ_{max}	K_s	μ_{max}	K_s
HO	25.823 A/A**	0.1167 a/a	10.83 a/B	0.0252 a/b	5.232 a/B	0.0086 ab/b
LO-1	15.900 b/a	0.045 b/a	14.13 b/a	0.0415 a/a	6.951 b/B	0.0151 a/b
LO-2	13.8 b/a	0.0271 b/a	11.4 a/ab	0.0171 b/a	7.928 c/b	0.0122 ac/a

*HO enriched at the initial O_2 concentration of 21 %; LO-1 enrichment at the initial O_2 concentration of 5 %, and LO-2 enriched at the initial O_2 concentration of 2.5 %

**The letters before and after slash shows the level of significance among data in the column and row, respectively; Different capital and small letters within the table refer to significant difference at 1 and 5 % level based on least significant difference (LSD) method, respectively

The μ_{max} and K_s in the Monod model increased with the increase of O_2 concentrations. When the initial O_2 concentration increased from 5 to 21 %, the μ_{max} and K_s in HO were increased by 2.4 and 4.8 times, respectively, while they were not obviously different in LO-1 ($P_{\mu max} = 0.064$; $P_{Ks} = 0.627$). When the initial O_2 concentration was increased from 2.5 to 5 % in HO, the CH_4 oxidation activity was significantly enhanced ($P_{\mu max} = 0.014$), but K_s was not significantly different ($P_{Ks} = 0.505$). The μ_{max} and K_s in LO-1 were both remarkably increased ($P_{\mu max} = 0.001$; $P_{Ks} = 0.028$) with the increase of the initial O_2 concentration from 2.5 to 5 %. As the initial O_2 concentration was increased from 2.5 to 21 %, the μ_{max} was significantly increased in LO-2 ($P_{5-21\ \%} = 0.033$; $P_{2.5-5\ \%} = 0.011$), while the K_s did not have a significant variation ($P = 0.144$–0.405). When the initial O_2 concentration was increased from 2.5 to 21 %, the μ_{max} was increased by about fivefold in HO, while it was increased by only 2.3 and 1.7 times in LO-1 and LO-2, respectively. This suggested that the culture enriched at the initial O_2 concentration of 2.5 % had a high affinity for O_2, after which CH_4 oxidation activity did not change much with the increase of O_2 concentrations.

Distribution of CH_4-derived carbon

At the end of phase IV, a test of CH_4-derived carbon distribution among CO_2, biomass, DOC, and EPS (including ECP and ECPS) in the cultures was conducted. Assuming that the formula for a microorganism is $C_5H_8O_2N$ and the carbon amount accounts for 50.4 % of biomass (Rittman and McCarty 2000), the $CE_{biomass}$ was calculated from the carbon amount of the biomass divided by the total carbon amount of CH_4 consumption. The standard compound for the analysis of ECPS and ECP concentrations was glucose ($C_6H_{12}O_6$) and bovine serum albumin ($C_{2932}H_{4618}N_{780}O_{898}S_{39}$), respectively. The carbon amount in ECPS and ECP was assumed to be the same as that in glucose and bovine serum albumin, which was 40 and 72 %, respectively. The result showed that the CH_4-derived carbon was mainly converted into CO_2 in all experiment treatments (Fig. 2). Compared to the O_2-limiting environments (2.5 and 5 %), higher CE_{CO2} and $CE_{biomass}$ were observed in the O_2 sufficient condition (21 %). Besides biomass and CO_2, a high CE_{DOC} was detected in the cultures with the values of 30.9, 15.5, and 5.5 % in LO-2, LO-1, and HO, respectively.

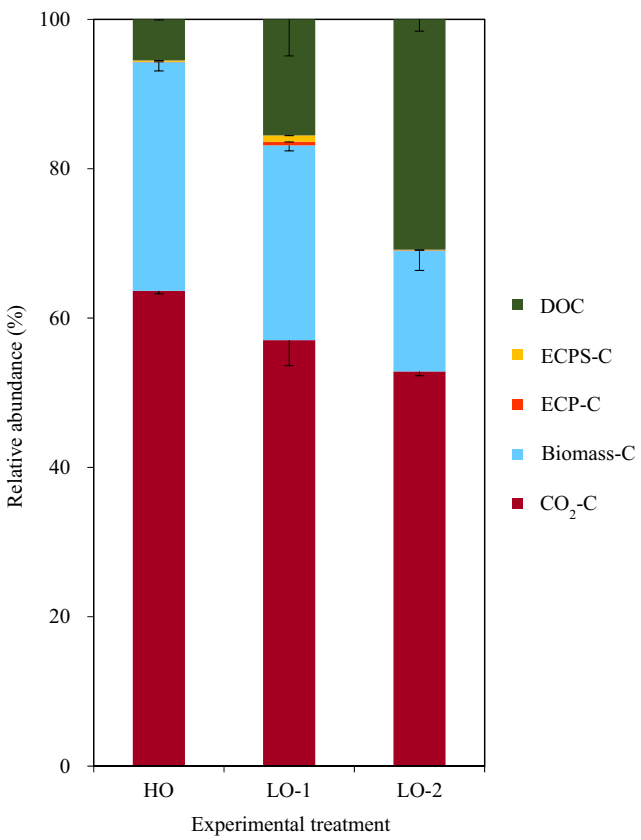

Fig. 2 The distribution of CH_4-derived carbon in the consortia of HO (the initial O_2 concentration = 21 %), LO-1 (the initial O_2 concentration = 5 %), and LO-2 (the initial O_2 concentration = 2.5 %) at the end of the enrichment. Data are means ± standard deviation ($n = 3$)

The CH_4-derived carbon converted to EPS was less than 1 %, which was mainly comprised of ECPS.

Abundance of methanotrophs

Aerobic methanotrophs oxidize CH_4 to CH_3OH using the enzyme methane monooxygenase (MMO) is the first step in the microbial oxidation of CH_4 to CO_2. There are two forms of MMO, soluble MMO (sMMO) and membrane-bound MMO (pMMO) (Hanson and Hanson 1996), of which pMMO is present in almost all known methanotrophs except for the genera *Methylocella* (Dedysh et al. 2000) and *Methyloferula* (Vorobev et al. 2011). The gene of *pmoA* coding for the β-subunit of the pMMO is widely used to identify methanotrophic community in various environmental samples (Radajewski et al. 2002; Qiu et al. 2008).

At the end of phase IV, the abundance of methanotrophs and bacteria was analyzed by *pmoA* and 16S rRNA genes, respectively, using Q-PCR technique. The result showed that the bacterial abundance was 1.7×10^9–1.76×10^9 copies mg^{-1} in the enrichment cultures (Fig. 3). There was no significant difference in the bacterial abundance per gram cell (dry weight) among the three treatments ($P = 0.804$–0.906). The abundance of *pmoA* was 5.28×10^8 copies mg^{-1} in HO, accounting for 30 % of the abundance of 16S rRNA gene, which was significantly higher than in LO-1 and LO-2 ($P = 0.009$–0.034).

Microbial community in the enrichment cultures

At the end of the enrichment experiment, DNA was extracted from the harvested cells for sequencing of total bacterial 16S rRNA gene. Rarefaction curves of the sequencing reads (at 3 % sequencing dissimilarity) showed that a diverse bacterial consortium was presented in the enrichment cultures (Fig. 4). The number of OTUs was 472 in LO-2, which was about twofold higher than in HO (241 OTUs) and LO-1 (174 OTUs). As the initial O_2 concentrations increased from 2.5 to 21 %, an increase in the index of Simpson and a decrease in the index of Shannon were observed in the microbes of the cultures.

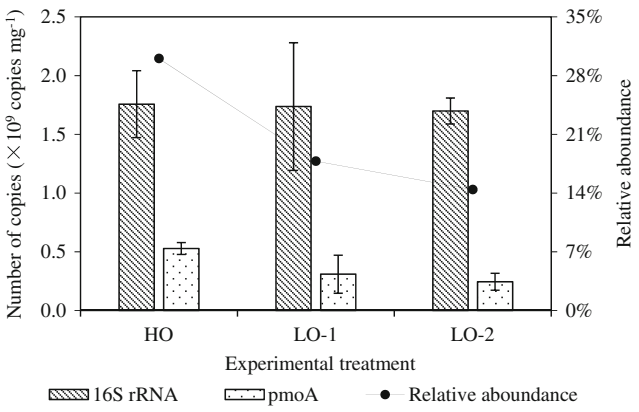

Fig. 3 Q-PCR analysis of bacterial 16S rRNA gene and *pmoA* in the cultures of HO (the initial O_2 concentration = 21 %), LO-1 (the initial O_2 concentration = 5 %), and LO-2 (the initial O_2 concentration = 2.5 %) at the end of the enrichment. Data are means ± standard deviation ($n = 3$)

Phylogenetic affiliations of the sequencing reads showed that members of the phyla *Proteobacteria, Bacteroidetes* and *Firmicutes* predominated in the three enrichment cultures, accounting for 98.9–99.7 % of the total 16S rRNA gene sequencing reads (Fig. 4c). Of them, *Proteobacteria* was the most abundant phylum in the enrichment cultures, especially in HO with the relative abundance of 94.5 %. The relative abundance of *Bacteroidetes* was 12.8–14.4 % in LO-1 and LO-2, while it was 5 % in HO. Compared with the high O_2 concentrations of 5 and 21 % (LO-1 and HO), members of *Firmicutes* were more abundant in the O_2-limiting environment (LO-2) with the relative abundance of 2 %.

Type II methanotroph *Methylocystis* dominated in the enrichment cultures, accounting for 54.8, 48.1, and 36.9 % of the total 16S rRNA gene sequencing reads in HO, LO-1, and LO-2, respectively. Other type II methanotrophs *Methylocapsa*, *Methylocella* and *Methylovirgula* were also detected in the three treatments with less than 10 sequencing reads. Additionally, type I methanotroph *Methylococcus* was detected in LO-1 with 15 sequencing reads.

In addition to methanotrophs, methylotrophic bacteria, mainly including *Methylophilus*, *Methylovorus*, *Hyphomicrobium*, and *Methylobacillus*, were also abundant in the cultures. Among them, *Methylophilus* was the most abundant methylotroph in all the treatments with the relative abundance of 22.7–26.3 %. The relative abundances of *Methylovorus*, *Hyphomicrobium*, and *Methylobacillus* were 2.48, 0.38, and 0.28 %, respectively, in HO, while they were less than 0.1 % in LO-1 and LO-2.

The enriched microbial community varied with the O_2 concentrations. The hierarchically clustered heatmap analysis based on the bacterial community profiles at the genera and family levels, which accounted for 95.8, 97.7, and 93.8 % of the total 16S rRNA gene sequencing reads in HO, LO-1, and LO-2, respectively, showed that the bacterial communities at the initial O_2 concentrations of 2.5 and 5 % clustered firstly together, and then they clustered with that at the high O_2 concentration of 21 % (Fig. 5). This showed that the major microbes in the cultures enriched at the initial O_2 concentrations of 2.5 and 5 % were more similar than at the initial O_2 concentration of 21 %.

Discussion

Among the cultures enriched at the three O_2 tensions, i.e., the initial O_2 concentrations of 2.5, 5, and 21 % in this study, the conversion efficiency of CH_4-derived carbon to CO_2 and

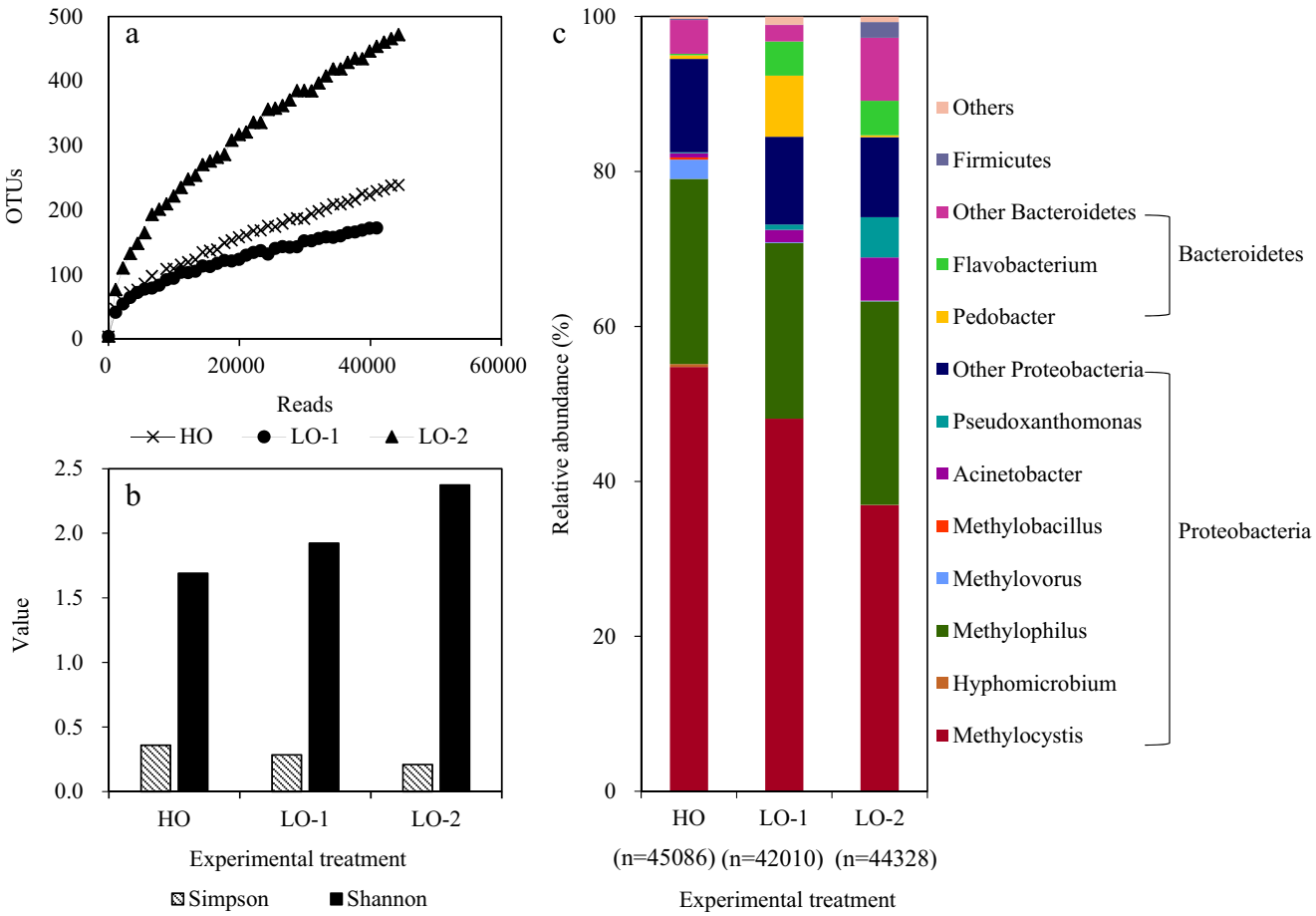

Fig. 4 Rarefaction curves for the numbers of sequencing reads and operational taxonomic units (OTUs) (at 3 % sequencing dissimilarity) (**a**), diversity indices (**b**) and microbial community (**c**) of the enrichment cultures of HO (the initial O_2 concentration = 21 %), LO-1 (the initial O_2 concentration = 5 %) and LO-2 (the initial O_2 concentration = 2.5 %) at the end of the enrichment

biomass was highest under the O_2 sufficient condition (21 %) (Fig. 2). However, more CH_4-derived carbon was exuded into the environments under the O_2-limiting conditions (5 and 2.5 %) relative to the O_2 sufficient condition (21 %). Similar result was obtained by Kalyuzhnaya et al. (2013) showing that extracellular carbon mainly formate and acetate accounted for 40–50 % of the total CH_4 consumption in the pure culture of type I methanotroph *Methylomicrobium alcaliphilum* 20Z under the O_2-limiting condition (5 %, *v*/*v*), due to CH_4 metabolism in the form of fermentation with little biomass synthesis and considerable amount of extracellular organics under the O_2-limited environments. In this study, type II methanotroph *Methylocystis* predominated in the enrichment cultures, while type I methanotroph *Methylomicrobium alcaliphilum* 20Z was used as a model microorganism in the study of Kalyuzhnaya et al. (2013), which suggested that the exudation of organic compounds might not be restricted to type I methanotrophs under the O_2-limiting environment.

Q-PCR and Miseq sequencing both showed that the abundance of non-methanotrophic microbes decreased with the increasing O_2 concentrations. Higher microbial richness was detected in the O_2-limiting environment (2.5 %) than at the initial O_2 concentrations conditions of 5 and 21 %. It might be attributed to that the CH_4-derived carbon was converted to excreted products (Kalyuzhnaya et al. 2013) into the cultures, and thus promoted the growth of non-methanotrophic microbes in the O_2-limiting environments.

Members of the phyla *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* were abundant in the three enrichment cultures (Fig. 4). Similar results were observed in stable isotope probing experiment that *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* were the major microbes to obtain CH_4-derived carbon in the process of CH_4 oxidation (Neufeld et al. 2007; He et al. 2012a). Compared with the incubation under the high O_2 concentrations of 5 and 21 %, low O_2 concentrations of 2.5 % could better promote the growth of members of *Bacteroidetes* and *Firmicutes* in CH_4 oxidation, likely due to a high exudation of organic compounds by methanotrophs at the O_2 concentration of 2.5 %, which provided nutrient for the growth of non-methanotrophs.

Type II methanotroph *Methylocystis* dominated in the enrichment cultures, accounting for 36.9–54.8 % of the relative

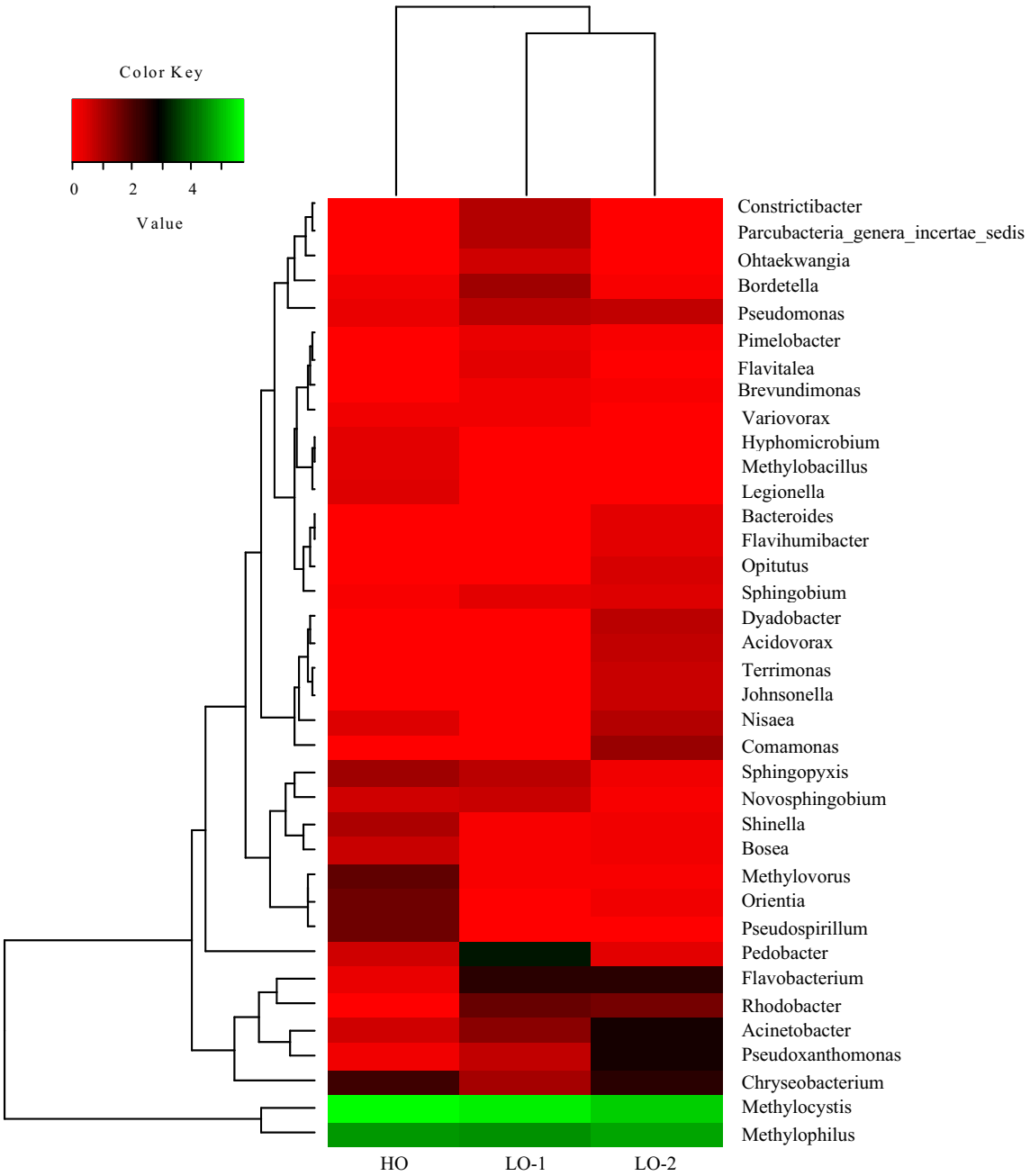

Fig. 5 Hierarchically clustered heatmap of the major bacterial families (not including sequences being classified into genera) and genera of HO (the initial O_2 concentration = 21 %), LO-1 (the initial O_2 concentration = 5 %), and LO-2 (the initial O_2 concentration = 2.5 %). The values in heatmap represent the standard relative abundance of each bacteria calculated by the formula of $\log_2 (1 + x)$ (x represents the relative abundance of bacteria in the total 16S rRNA gene sequencing reads). The standard relative abundance of each bacteria are depicted by color intensity with the legend at the upper left corner of the figure

abundance of the total 16S rRNA gene. Type II methanotrophs have been reported to be dominant in high CH_4 and low O_2 concentration environment (Amaral and Knowles 1995). However, a high abundance of type II methanotrophs is also observed in various environments and the enrichment cultures at near atmospheric O_2 concentrations (He et al. 2012c; Kong et al. 2013). This might be attributed to that besides O_2 concentration, methanotrophic community was also affected by many environmental factors such as CH_4 concentrations, pH, and temperatures (Hanson and Hanson 1996; Scheutz et al. 2009; Semrau et al. 2010; Ho et al. 2013).

In addition to methanotrophs, methylotrophs, mainly including *Methylophilus*, *Methylovorus*, *Hyphomicrobium*, and *Methylobacillus*, were also observed to be abundant in the enrichment cultures. The high abundance of methylotrophs,

especially *Methylophilus*, was also detected in the enrichment cultures of water column collected from arctic lakes (He et al. 2012c) and sediment from lake Washington (Hernandez et al. 2015; Oshkin et al. 2015). Stable isotope probing analysis also showed that an unexpectedly high abundance of methylotrophs was active in utilizing CH_4-derived carbon in various environments (Cébron et al. 2007; Qiu et al. 2008; Martineau et al. 2010; He et al. 2012b). Methanotrophs usually oxidize CH_4 completely to CO_2, with methanol, formaldehyde, and formate produced as intermediates, which occurs intracellularly and no methanol is released extracellularly (Corder et al. 1986). However, the interruption of the enzymatic reactions has been reported to produce excess extracellular methanol by manipulation of the environmental conditions or mutation (Corder et al. 1986; Lee et al. 2004). The high abundance of methylotrophs in the cultures might be related with extracellular methanol. Further studies such as methanol concentration should be taken into account to better understand the role of methylotrophs in the flow of CH_4-derived carbon.

In this study, the abundance of methanotroph was lower in LO-1 and LO-2 than in HO (Fig. 3), but the CH_4 oxidation rate was higher in LO-1 and LO-2 than in HO at the initial O_2 concentration of 2.5 and 5 % (Fig. S1). It suggested a higher cell-specific activity at the low O_2 concentrations. This might be attributed to that methanotrophs enriched at low O_2 concentrations might be well adapt to the O_2-limiting environments, likely due to the difference of gene expression of methanotrophs (Luesken et al. 2012). Additionally, the non-methanotrophic microbes might play an important role in CH_4 oxidation in the enrichment cultures due to removal of toxic intermediates of CH_4 oxidation (e.g., formaldehyde) and secretions of growth factors including vitamins (e.g., cobalamin) (Hanson and Hanson 1996). Methanotrophs have been reported to interact with other organisms such as invertebrates, plants, algae, and heterotrophic bacteria (Stock et al. 2013). Co-cultivation with heterotrophs could stimulate or inhibit the growth of methanotrophs, depending largely on the species of the heterotrophs (Stock et al. 2013). A high richness of heterotrophs has also been observed to enhance the activity of methanotrophs in co-cultures (Ho et al. 2014). The distribution of CH_4-derived carbon among diverse bacterial populations also has been hypothesized to be likely responsible for CH_4 oxidation, rather than a single type of microbe (Oshkin et al. 2015). Thus, the interaction of methanotrophs and non-methanotrophic microbes could not be neglected in understanding the CH_4 oxidation capacity in the environment.

In conclusion, O_2 tensions could affect the distribution of CH_4-derived carbon and microbial community participating in the metabolism of CH_4-derived carbon. Compared with the O_2-limiting environments, more CH_4-derived carbon was converted into CO_2 and biomass under the O_2 sufficient condition. However, a higher amount of DOC was exuded into the environment and thus led to a higher microbia richness and biodiversity in the O_2-limiting environments than under the O_2 sufficient condition. These results indicated that more attention should be paid to the flow of CH_4-derived carbon in microbial community to understand the role of methanotrophs and the relationship between methanotrophs and the non-methanotrophic microbes in the environment and their response to the variation of O_2 tension.

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