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Methanotrophic community structure and activity under warming and grazing of alpine meadow on the Tibetan Plateau

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Abstract Knowledge about methanotrophs and their activities is important to understand the microbial mediation of the greenhouse gas CH_4 under climate change and human activities in terrestrial ecosystems. The effects of simulated warming and sheep grazing on methanotrophic abundance, community composition, and activity were studied in an alpine meadow soil on the Tibetan Plateau. There was high abundance of methanotrophs ($1.2–3.4\times10^8$ *pmoA* gene copies per gram of dry weight soil) assessed by real-time PCR, and warming significantly increased the abundance regardless of grazing. A total of 64 methanotrophic operational taxonomic units (OTUs) were obtained from 1,439 clone sequences, of these OTUs; 63 OTUs (98.4%) belonged to type I methanotrophs, and only one OTU was *Methylocystis* of type II methanotrophs. The methanotroph community composition and diversity were not apparently affected by the treatments. Warming and grazing significantly enhanced the potential CH_4 oxidation activity. There were significantly negative correlations between methanotrophic abundance and soil moisture and between methanotrophic abundance and NH_4–N content. The study suggests that type I methanotrophs, as the dominance, may play a key role in CH_4 oxidation, and the alpine meadow has great potential to consume more CH_4 under future warmer and grazing conditions on the Tibetan Plateau.

Keywords Clone library · Grazing · Methanotrophs · *pmoA* · Tibetan Plateau · Real-time PCR

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Introduction

Methane (CH_4) is the second most important greenhouse gas contributing roughly 20% to observed global warming (IPCC 2007). Oxidation of CH_4 in soil by the methane-oxidizing bacteria (methanotrophs) currently removes 30 Tg annually from the atmosphere, which equals 5.4% of the global CH_4 sink (IPCC 2007), and therefore play a critical role in the mitigation of global warming. Methanotrophs are widely distribute in various environments (e.g., McDonald et al. 2008; Op den Camp et al. 2009; Semrau et al. 2010), such as in paddy soils (Bodelier et al. 2000; Zheng et al. 2008), forest soils (Mohanty et al. 2007; Kolb 2009), landfill soils (Chen et al. 2007; Einola et al. 2007; Semrau 2011), grassland soils (Zhou et al. 2008; Abell et al. 2009), oil field soil (Zhang et al. 2010), and extreme thermoacidophilic environments (Dunfield et al. 2007; Pol et al. 2007; Islam et al. 2008).

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Methanotrophs are traditionally classified into type I (aerobic γ-Proteobacteria) and type II (aerobic α-Proteobacteria) groups (Hanson and Hanson 1996). Recently, thermoacidophilic methanotrophs, representing a distinct lineage within the bacterial phylum *Verrucomicrobia* were found in a mudpot in the Solfatara volcano in Italy, a steaming soil at Tikitere, New Zealand, and an acidic hot spring in Uzon Caldera, Kamchatka, Russia (Dunfield et al. 2007; Pol et al. 2007; Islam et al. 2008; Op den Camp et al. 2009). Methanotrophs have unique functional genes *pmoA* and *mmoX*, which encode subunits of particulate methane monooxygenase and soluble methane monooxygenase, respectively. Compared with *mmoX* gene, *pmoA* gene exists in all methanotrophs with the exceptions of *Methylocella silvestris* (Theisen et al. 2005) and *Methyloferula stellata* (Vorob'ev et al. 2011). Therefore, *pmoA* is a preferred gene and extensively used to detect methanotrophs in soils (Holmes et al. 1999; Mohanty et al. 2007; Zheng et al. 2008; Yrjälä et al. 2011). More recently, new *pmoA* primers were developed and successfully tested on the detection of anaerobic methanotrophs in freshwater samples (Luesken et al. 2011).

The distribution, diversity, and CH_4 oxidation activity of methanotrophs may be affected by many environmental factors (Le Mer and Roger 2001; Semrau et al. 2010), such as soil pH (Knief et al. 2003), temperature (King 1997; Mohanty et al. 2007; Singh et al. 2010), moisture (Börjesson et al. 1998; Einola et al. 2007), nitrogen availability (Bodelier et al. 2000; Semrau et al. 2010), land use (Menyailo et al. 2008; Zheng et al. 2010), and copper (Semrau et al. 2010). Particularly, increasing temperature is likely to have a more pronounced effect in alpine, arctic, and temperate ecosystems (Singh et al. 2010). Some previous studies indicated that temperature could alter the growth, activity, and community composition of methanotrophs both in rice and forest soils incubated at different temperatures between 5°C and 45°C for 40 days (Mohanty et al. 2007) and in permafrost-affected soils incubated at 0°C and 22°C for up to 11 weeks (Knoblauch et al. 2008). However, there was no apparent effect of temperature on methanotroph community and activity in an artificial column experiment (Urmann et al. 2009). To our knowledge, few studies concerned the effect of temperature on the community structure and activity of methanotrophs under field condition (Horz et al. 2005), in particular, under grazing with future warming.

Grazing, as the main land utilization patterns in grassland, would result in alteration to soil properties, such as nitrogen input (Yamulki et al. 1998), and to the nitrifier community (Le Roux et al. 2008). Moreover, the different grazing intensities have conspicuous impact on the structure of methanotroph community in the Inner Mongolia steppe, China (Zhou et al. 2008). Abell et al. (2009) demonstrated that predominant type II methanotrophs in an alpine meadow soil in Austria were not influenced by grazing, while the CH_4 oxidation and the abundance of type I methanotrophs increased due to grazing. However, knowledge of the grazing effect on the community structure and activity of methanotrophs in alpine meadow soil of the Tibetan Plateau is less available.

Alpine meadow is the main vegetation type on the Tibetan Plateau, which covers an area of approximately 2.5 million km^2 in China. Evidence shows that the Tibetan Plateau is experiencing climatic warming (Thompson et al. 2000). Moreover, this region is predicted to experience "much greater than average" increases in surface temperatures in the future (Houghton et al. 2001). Concurrent with climate changes, there are profound changes to pastoral land-use dynamics on the plateau that are resulting in increased grazing pressure on the alpine meadow (Zhou et al. 2005). Due to its unique geographical location, the ecosystems on the Tibetan Plateau are therefore thought to be fragile and sensitive to climate change and human activities (Wu et al. 2010). Some ecological studies concerning climate change have been carried out, such as soil properties and carbon dynamics (Wang et al. 2007; Luo et al. 2009, 2010), microclimate (Klein et al. 2005), and CO_2, CH_4, and N_2O fluxes (Zhao et al. 2006; Cao et al. 2008; Wang et al. 2009; Hu et al. 2010). However, methanotrophs, which have an important ecological function in attenuating the greenhouse effect partly caused by CH_4, have not been extensively studied.

The aims of this study were to determine (1) the response of methanotrophic abundance, community composition, and diversity to the controlled warming and grazing based on the analyses of the field soil using real-time PCR and *pmoA* gene sequences, and (2) whether CH_4 oxidation potential of the soils was influenced by warming and grazing in alpine meadow on the Tibetan Plateau.

Materials and methods

Study site

This study was carried out in an alpine meadow of the Haibei Alpine Meadow Ecosystem Research Station (HAMERS) of Chinese Academy of Sciences (37°37′ N, 101°12′ E) in Qinghai Province, the northeast corner of Tibetan Plateau. This site has a typical plateau continental climate with a mean annual temperature of −2°C, a mean annual precipitation of 500 mm, and a mean altitude of 3,200 m asl (Zhao and Zhou 1999). The soil developed is Mat-Gryic Cambisol, corresponding to Gelic Cambisol.

A controlled warming–grazing experiment system has been established to investigate the effect of simulated warming and grazing on the alpine meadow ecosystem in HAMERS since 2006 (Luo et al. 2010). A two-factorial

design (warming and animal grazing) was used with four replicates of each of four treatments, i.e., no warming with no grazing (NWNG), no warming with grazing (NWG), warming with no grazing (WNG), and warming with grazing (WG). In total, 16 plots of 3-m diameter were used in a complete randomized block distribution in the field (Fig. S1).

The infrared heating system, herein called a free-air temperature enhancement, was described previously by Kimball et al. (2008). The setpoint differences between heated and corresponding reference plots were 1.2°C during daytime and 1.7°C at night in summer, which falls within limits of predicted temperature increases for this century (1.5–5°C, Houghton et al. 2001). The canopy temperatures were measured with infrared thermometers every second, and the heaters were modulated at 1-s intervals. Fifteen minute averages were output by the dataloggers. During winter from October to April, because some infrared thermometers were not working, the power outputs of the heaters were manually set at 1,500 W per plot (Luo et al. 2010, i.e., approximate 211 $W m^{-2}$).

For grazing, initially, one adult Tibetan sheep was fenced in the grazing plots on the morning of 15 August 2006 for approximately 2 h. The canopy height was about 8–9 and 4–5 cm before and after grazing, respectively. The stocking rate roughly corresponded to a moderate stocking rate in the region. Similarly, two adult Tibetan sheep were fenced for approximate 1 h in the grazing plots in the mornings of 12 July, 3 August, and 12 September in 2007, and 8 July and 20 August in 2008 (Hu et al. 2010).

Soil sampling and soil physicochemical analysis

Five cores (2 cm in diameter) were collected randomly from the top 10 cm of grassland soil in each replicate plot in early August 2009 and then were mixed as a representative sample. The soil samples were independently packed in ice upon collection and transported to the laboratory. The soil samples were sieved (<2 mm) to remove fine roots and large organic debris and stored at −80°C for DNA extraction and at 4°C for analyzing potential CH_4 oxidation and soil basic characteristics.

Soil moisture was manually measured through a tube in the ground down to 40-cm depth using a frequency domain reflectometer (FDR; model Diviner-2000, Sentek Pty Ltd., Stepney, Australia). The soil moisture was expressed as a volume percentage. Soil pH was determined with a soil to water ratio of 2:5 (*W*/*V*). Soil total nitrogen (TN) was determined using the Kjeldahl method (Bremner 1996). Soil ammonium (NH_4–N) was extracted with 2 M KCl and measured by a continuous flow analyser (SAN++, Skalar, Holand). Soil microbial biomass carbon was measured using the fumigation extraction method (Wu et al. 1990). The selected physicochemical characteristics of the soil samples are shown in Table 1.

DNA extraction and concentration regulation

Soil DNAwas extracted using the FastDNA® Spin Kit for Soil according to the manufacturer's instructions. The quantity and quality of the extracted DNA were analyzed by electrophoresis on a 1.0% agarose gel and by spectroscopic analysis (NanoDrop Technologies). The DNAs were diluted by 10-, 100-, and 1,000-fold to test the possibly inhibitory effects of humic substances via PCR method. Consequently, the 10-fold diluted DNAs (10–20 ng μL^{-1}) were found to be the optimal concentration as template.

Real-time PCR

Methanotrophic abundance in the soils of the four treatments was analyzed based on the *pmoA* gene copy numbers using real-time PCR, which was performed on an iCycler iQ5 thermocycler (Bio-Rad). Specific primer pairs A189 (GGNGACTGGGACTTCTGG, Holmes et al. 1999) and mb661 (CCGGMGCAACGTCYTTACC, Costello and Lidstrom 1999) were used to amplify the methanotrophic *pmoA* gene fragments. Amplification was performed by using SYBR® Premix Ex Taq™ as described by the

Table 1 Physicochemical characteristics of soils in the four treatments (mean±SD, n=4)

Treatment	Moisture (%)	pH (H_2O)	TN (%)	NH_4–N (mg kg^{-1})	MBC (mg kg^{-1})
NWNG	35.4±6.7a	7.59±0.24a	0.51±0.15a	28.2±2.1a	1415±727a
NWG	35.6±6.2a	7.61±0.20a	0.50±0.15a	26.5±3.4a	1311±630a
WNG	26.3±2.7b	7.60±0.13a	0.51±0.13a	23.9±4.1a	1491±630a
WG	28.1±5.4b	7.57±0.22a	0.55±0.17a	25.1±1.2a	1577±717a

Values within the same column followed by the different letters indicate significant difference at $P<0.05$ level

NWNG no warming with no grazing, *NWG* no warming with grazing, *WNG* warming with no grazing, *WG* warming with grazing, *TN* total nitrogen, *MBC* microbial biomass carbon

suppliers (TaKaRa). Real-time PCR assay was performed with protocols of Zheng et al. (2010). The 25-μL reaction mixture consisted of 12.5 μL of 2× SYBR® Premix, 1 μL of bovine serum albumin (25 mg mL^{-1}), 0.5 μL of each primer (10 μmol L^{-1}), and 1 μL of 10-fold diluted extracted DNA as template. The PCR was programmed as follows: initial denaturation for 2 min at 95°C, followed by 40 cycles of 1 min at 94°C, 1 min at 56°C, and plate read at 83°C. For confirming specificity of the PCR product, a melting curve analysis was performed by measuring fluorescence continuously as the temperature increased from 55°C to 95°C after the above three temperature steps. The external standard curve for real-time PCR was set up by amplifying *pmoA* gene fragments from *Methylocystis parvus* (NCIMB 11129) as described by Zheng et al. (2010). All assays were performed at least in triplicate. Data analysis was carried out with iCycler software (version 1.0.1384.0 CR).

Construction of clone libraries

The primer pairs A189/mb661 were used to amplify *pmoA* gene fragments. PCR amplification was performed in 50 μL of mixtures including 5 μL of 10× PCR buffer (plus Mg^{2+}), 10 nmol of deoxyribonucleoside triphosphate, 20 pmol of each primer, 2.5 U of *Taq* DNA polymerase (TaKaRa), and approximately 10 ng of template DNA. The PCR programs were as follows: an initial 4 min at 94°C followed by a touchdown procedure (94°C for 1 min, annealing for 1 min at temperatures decreasing from 60°C to 52°C during the first 16 cycles, and ending with an extension step at 72°C for 1 min). Then, additional 20 cycles (94°C for 1 min, 52°C for 1 min, and 72°C for 1 min) and a final extension of 10 min at 72°C were performed.

The PCR products were purified using Agarose Gel DNA Purification Kit (Ver.2.0, TaKaRa) and were ligated into the *p*GEM-T Easy Vector (Promega) for blue and white clone screening. For each library, 120 white colonies were picked and propagated in fresh Luria–Bertani media. Vector primers T7 and SP6 were used to reamplify these clones. The amplicons that contain the correct size of *pmoA* gene fragment were sequenced. Evaluation of the clone libraries was performed by a rarefaction analysis using software aRarefactWin (version 1.3, http://www.uga.edu/strata/software/Software.html). The results obtained from rarefaction analysis of each clone library were used to construct cumulative curves using Microsoft® Office Excel 2007.

Sequence analyses

The obtained *pmoA* gene sequences from the 16 libraries were manually proofread, corrected, and edited to start and end with the primers of A189 and mb661 (doing reverse complement transform if necessary) using the MEGA software version 4.0 (Tamura et al. 2007). The sequences with correct inserts were then aligned and in silico translated. Amino acid sequences of *pmoA* gene were analyzed with the programs DOTUR (Schloss and Handelsman 2005). The number of operational taxonomic units (OTUs) achieved by DOTUR was calculated for a 7% distance, which is an estimated cutoff value for methanotrophic species-level OTUs (Degelmann et al. 2010). The coverage (*C*) for each clone library was calculated using equation $C=[1-(n_1/N)]\times 100$, in which n_1 is the number of OTU containing only one *pmoA* gene sequence, and *N* is the number of corrected sequences analyzed (Singleton et al. 2001). One representative sequence of each OTUs was used for similar sequence search in the GenBank database using the BLAST program, and the most similar reference sequences were downloaded from the database. Phylogenetic analysis based on PmoA (amino acid) sequences was performed using MEGA (version 4.0), and a neighbor-joining tree of all the representative OTU sequences and reference sequences was constructed using *p*-distance with 1,000 replicates to produce bootstrap values (1,000 replicates). Representative *pmoA* nucleotide sequences obtained in this study have been deposited in the EMBL nucleotide sequence databases with the accession numbers FR734224–FR734287.

Methane oxidation

A soil incubation experiment was carried out according to the modified method described by Mohanty et al. (2007). Ten grams of fresh soil from each samples was transferred to culture serum bottles (250 mL). The moistures of the tested soils in bottles were kept unchanged from field samplings. No soil added into the bottle was served as control to detect the gas tightness of incubation system. The bottles were sealed with thick butyl rubber stoppers and aluminum caps. All bottles were supplemented with CH_4 at an initial concentration of approximate 120 ppmv, which is a moderate CH_4 concentration according to other incubation studies (Wang and Ineson 2003; Menyailo et al. 2008). The starting concentration of head-space CH_4 in each bottle was determined by gas chromatography. Bottles were incubated at 25°C in dark for 2 weeks, and the change in the CH_4 concentration in the head-space was analyzed at seventh and 14th day of incubation, respectively.

The concentration of CH_4 in the headspace was determined by removing a 1-mL gas sample from the bottle and was measured using a gas chromatography (Agilent 6820, Agilent Technologies) equipped with a flame ionization detector. Nitrogen was the carrier gas (30 mL min^{-1}) and the injector, oven, and detector temperatures were 100°C, 80°C, and 180°C, respectively. The flame gases including H_2 and compressed air were set at 20 and 30 mL min^{-1}, respectively.

The gas volume withdrawn from the bottles was replaced by N_2 at each sampling.

Data analyses

Shannon diversity index (H) of OTUs was calculated using the following formula: $H = -\sum_{i=1}^{S} p_i \ln p_i = -\sum_{i=1}^{S} (N_i/N) \ln(N_i/N)$, where N_i is the abundance (number of clones) of the ith OTU, N is the total abundance of all OTUs in one sample, and S is the number of OTUs. The similarity of methanotroph community compositions among the four treatments were calculated using the Sorensen similarity index (C_s). $C_s=2j/(a+b)$, where j is the number of OTUs share in both A and B treatments, and a and b are the number of OTUs in treatment A and B, respectively (Magurran 1988). The relative abundance was calculated as the number of clones of a certain OTU divided by the total number of clones. The dominant OTUs were defined when their relative abundances were more than 5%. Fisher's test of the OTU distribution data was performed in the R statistical software (version 2.9.2, R Development Core Team 2009). Canonical correspondence analyses (CCA) of methanotroph community structure were performed using CANOCO for Windows 4.5 software (ter Braak and Šmilauer 2002). A Monte Carlo permutation test based on 499 random permutations was used to analyze the relationship among the methanotroph community, treatments, and soil properties.

The statistical analyses were carried out using SPSS software (SPSS for windows, version 13.0). Two-way analysis of variance was used to determine significant differences in soil physicochemical characteristics, Shannon diversity index, and relative abundance of methanotrophic OTUs, *pmoA* gene copy numbers, and potential methane-oxidation rates among the four treatments at $P<0.05$ level.

Results

Methanotrophic abundance

In the current study, real-time PCR was used to quantify the methanotrophic *pmoA* gene copies in 16 soil samples collected from an alpine meadow under four treatments of NWNG, NWG, WNG, and WG. There was high abundance of methanotrophs (*pmoA* gene copies up to $1.2–3.4\times10^8$ per gram of dry weight soil) in this alpine meadow ecosystem. The abundances of methanotrophs were significantly higher in WG (3.4×10^8 *pmoA* gene copies per gram of dry weight soil) and WNG (3.1×10^8 *pmoA* gene copies per gram of dry weight soil) than in two no warming treatments NWNG (1.6×10^8 *pmoA* gene copies per gram of dry weight soil) and NWG (1.2×10^8 *pmoA* gene copies per gram of dry weight soil), whereas grazing did not significantly affect the abundance regardless of warming (Fig. 1). There was no significant interaction between warming and grazing on abundance of methanotrophs ($F_{1,\ 12}=1.345$, $P=0.276$). Correlation analyses indicated that the abundance of methanotrophs were significantly negatively correlated with the soil moisture ($r=-0.900$, $P<0.01$) and with NH_4–N content in soils ($r=-0.661$, $P<0.05$), respectively (Table 2).

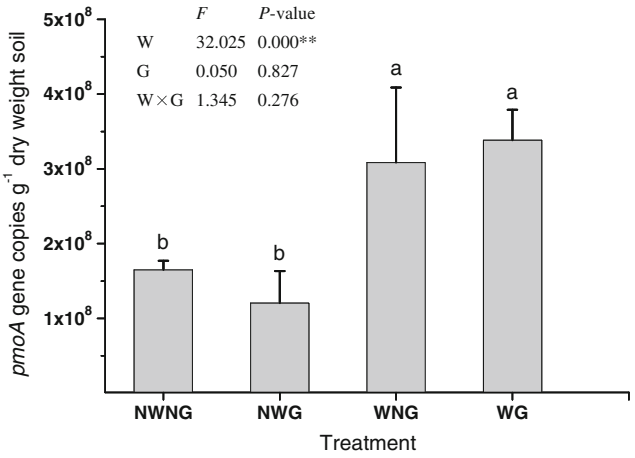


Fig. 1 The number of *pmoA* gene copies of methanotrophs in the soils of four treatments (mean+SD, $n=4$). The *different letters above the bars* indicate significant difference at $P<0.05$ level. *NWNG* no warming with no grazing, *NWG* no warming with grazing, *WNG* warming with no grazing, *WG* warming with grazing

Phylogenetic analysis of the *pmoA* gene

A total of 1,439 *pmoA* gene sequences with the average length of 507.4 bp were obtained, which was consistent with the theoretical insert length of 508 bp. These sequences were grouped into 64 OTUs based on 93% threshold of PmoA (amino acids) sequence identity. The 64 representative OTU sequences, 26 reference sequences, and one PmoA-related sequence of *Verrucomicrobiae* bacterium V4 as an outgroup downloaded from GenBank were included in the neighbor-joining (NJ) analysis. A NJ

Table 2 Correlation analyses among the methanotrophic abundance and soil physicochemical characteristics

	Kendall's correlation coefficient				
	Moisture	pH	TN[a]	NH_4–N	MBC[a]
Abundance	−0.900**	−0.004	0.342	−0.661*	0.178
Activity	−0.557	0.246	0.051	−0.381	−0.122

[a] The abbreviations are the same as Table 1

*$P<0.05$; **$P<0.01$

phylogenetic tree was obtained in the analysis (Fig. 2). Within the tree, 63 OTUs formed a clade with 22 reference sequences of type I methanotrophs. Within the clade, the 63 OTUs clustered together with 12 references belonging to upland soil cluster γ (USC-γ) methane-oxidizing group with a 100% bootstrap support. OTU-64 formed a clade with four references of type II methanotrophs with a 93% bootstrap support and further clustered together with two *Methylocystis* species with a 95% bootstrap support. OTU-64 was therefore identified as *Methylocystis* sp.

Methanotroph community composition and diversity

In the 64 OTUs, 45, 48, 49, and 50 OTUs were recovered from NWNG, NWG, WNG, and WG treatments, respectively (Table S1). The Shannon diversity indices were not significantly different among the four treatments (Table S1). The Sorensen similarity indices of OTU community composition among the four treatments from high to low were between NWG and WG (86%), between NWG and WNG (85%), between WNG and WG (83%), between NWNG and NWG (82%), between NWNG and WG (80%), and between NWNG and WNG (77%). Within the 64 OTUs, 31 were found in the four treatments, 15 in the three treatments, five in two treatments, and 13 in one treatment. Moreover, OTU-05, OTU-17, OTU-19, OTU-22, OTU-39, and OTU-40 were dominant in all the four treatments (Table S1). According to the Fisher's test, the relative abundances of OTU-12 (P=0.028), OTU-39 (P=0.005), and OTU-41 (P=0.044) were significantly different among the four treatments. CCA of the clone library data showed that no treatment clustered separately from other treatments (Fig. S2) and indicated that no clearly consistent character was given by each treatment. Moreover, no significant correlations were found between methanotroph community composition and treatments and between community composition and the selected soil environmental variables deduced from the Monte Carlo permutation tests in CCA (P>0.05, Table S2).

The clone libraries constructed from the four treatments were evaluated by rarefaction analysis (Fig. 3). Almost all of 16 rarefaction curves within four treatments tended to reach the saturation platform, and the mean coverage values of clone libraries were 89%, 86%, 90%, and 88% for NWNG, NWG, WNG, and WG treatments, respectively, indicating that approximate 100 clone sequences used in each library construction could well cover the diversity of the methanotrophs.

Methane consumption and methanotrophic activity

The CH_4 consumption of the soils from the four treatments showed a clear reduction in head-space CH_4 concentration in 2 weeks of incubation (Fig. 4). Compared to the NWNG treatment, CH_4 was much more sharply oxidized in the treatments of NWG, WNG, and WG, in which more than 58% of CH_4 were consumed after 1 week and 88% after 2 weeks of incubation, respectively. Significantly higher CH_4 was oxidized in the treatments of NWG, WNG, and WG (88–94%) than that in NWNG (20%) treatments within the whole incubation period (P<0.01, Fig. 4). An interactive effect was detected between warming and grazing ($F_{1,\ 12}$=33.801, P=0.000) on the methanotrophic activity (Fig. S3). No significant correlation was observed between the methanotrophic activity and soil physicochemical characteristics (Table 2).

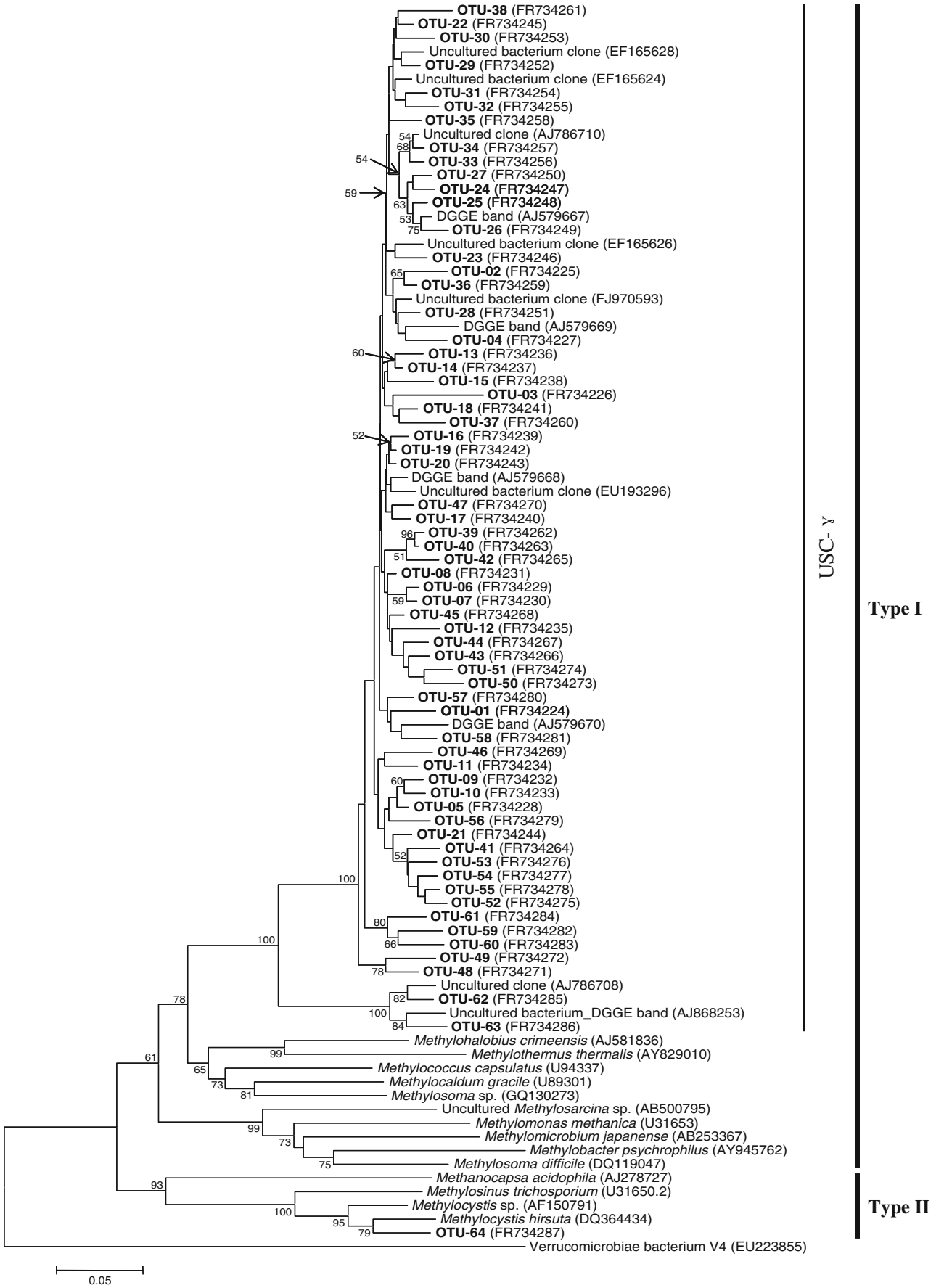
Fig. 2 A neighbor-joining tree constructed based on the methanotrophic ▶ PmoA (amino acid) sequences. Bootstrap values were calculated on the basis of 1,000 data resamplings, and more than 50% are shown. *Bold OTUs* (01–64) were obtained in our study. Accession numbers are given in *parentheses*. The *Verrucomicrobiae* bacterium V4 was used as an outgroup. The *scale bar* represents 5% sequence divergence

Discussion

Effect of warming and grazing on methanotrophic abundance and activity

Surprisingly, high abundance of methanotrophs was found in alpine meadow soil on the Tibetan Plateau in this study. Warming significantly increased the methanotrophic abundance, regardless of grazing treatment in this study. In addition, warming significantly enhanced methane oxidation under no grazing condition. The mainly direct effects of climate change on soil microorganisms are likely to be caused by changes in temperature and moisture (Singh et al. 2010). Here, lower soil moistures were observed in the warming treatments, regardless of grazing treatments, and a significantly negative correlation was found between the methanotrophic abundance and soil moisture, which was consistent with the previous report in a California upland grassland soil (Horz et al. 2005). Both temperature and soil moisture were regarded as crucial factors in controlling the abundance and activity of methanotrophs (King 1997; Börjesson et al. 2004; Einola et al. 2007; Urmann et al. 2009). Particularly, the water content is an important factor in regulating the substrate (CH_4 and O_2) transport in soil (Urmann et al. 2009). Hence, probably warming promoted CH_4/O_2 diffusion due to decrease soil water content and enlarged the abundance of methanotrophs and thus increased their activities. However, it is unclear why warming did not strengthen the activity under grazing in this study.

Our results indicated that the sheep grazing had little effect on methanotrophic abundance. However, we observed that grazing significantly increased CH_4 oxidation potential under ambient temperature (no warming). Abell et al. (2009) also found that the abundance of the predominant type II methanotrophs was not significantly affected by cattle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OTU-22 (FR734245)
OTU-30 (FR734253)
Uncultured bacterium clone (EF165628)
OTU-29 (FR734252)
Uncultured bacterium clone (EF165624)
OTU-31 (FR734254)
OTU-32 (FR734255)
OTU-35 (FR734258)
Uncultured clone (AJ786710)
OTU-34 (FR734257)
OTU-33 (FR734256)
OTU-27 (FR734250)
OTU-24 (FR734247)
OTU-25 (FR734248)
DGGE band (AJ579667)
OTU-26 (FR734249)
Uncultured bacterium clone (EF165626)
OTU-23 (FR734246)
OTU-02 (FR734225)
OTU-36 (FR734259)
Uncultured bacterium clone (FJ970593)
OTU-28 (FR734251)
DGGE band (AJ579669)
OTU-04 (FR734227)
OTU-13 (FR734236)
OTU-14 (FR734237)
OTU-15 (FR734238)
OTU-03 (FR734226)
OTU-18 (FR734241)
OTU-37 (FR734260)
OTU-16 (FR734239)
OTU-19 (FR734242)
OTU-20 (FR734243)
DGGE band (AJ579668)
Uncultured bacterium clone (EU193296)
OTU-47 (FR734270)
OTU-17 (FR734240)
OTU-39 (FR734262)
OTU-40 (FR734263)
OTU-42 (FR734265)
OTU-08 (FR734231)
OTU-06 (FR734229)
OTU-07 (FR734230)
OTU-45 (FR734268)
OTU-12 (FR734235)
OTU-44 (FR734267)
OTU-43 (FR734266)
OTU-51 (FR734274)
OTU-50 (FR734273)
OTU-57 (FR734280)
OTU-01 (FR734224)
DGGE band (AJ579670)
OTU-58 (FR734281)
OTU-46 (FR734269)
OTU-11 (FR734234)
OTU-09 (FR734232)
OTU-10 (FR734233)
OTU-05 (FR734228)
OTU-56 (FR734279)
OTU-21 (FR734244)
OTU-41 (FR734264)
OTU-53 (FR734276)
OTU-54 (FR734277)
OTU-55 (FR734278)
OTU-52 (FR734275)
OTU-61 (FR734284)
OTU-59 (FR734282)
OTU-60 (FR734283)
OTU-49 (FR734272)
OTU-48 (FR734271)
Uncultured clone (AJ786708)
OTU-62 (FR734285)
Uncultured bacterium_DGGE band (AJ868253)
OTU-63 (FR734286)
Methylohalobius crimeensis (AJ581836)
Methylothermus thermalis (AY829010)
Methylococcus capsulatus (U94337)
Methylocaldum gracile (U89301)
Methylosoma sp. (GQ130273)
Uncultured Methylosarcina sp. (AB500795)
Methylomonas methanica (U31653)
Methylomicrobium japanense (AB253367)
Methylobacter psychrophilus (AY945762)
Methylosoma difficile (DQ119047)
Methanocapsa acidophila (AJ278727)
Methylosinus trichosporium (U31650.2)
Methylocystis sp. (AF150791)
Methylocystis hirsuta (DQ364434)
OTU-64 (FR734287)
Verrucomicrobiae bacterium V4 (EU223855)
0.05
USC- γ
Type I
Type II

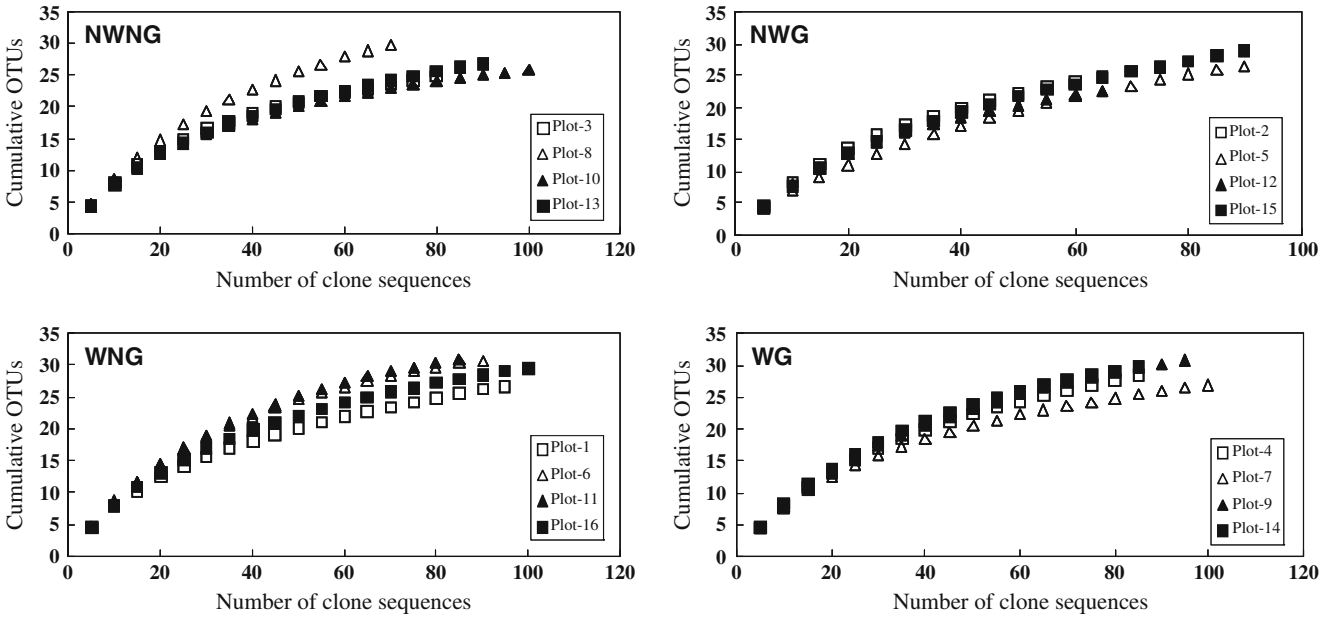

Fig. 3 Rarefaction analysis of the *pmoA* gene sequences obtained from four treatments using software aRarefactWin Version 1.3. There were four plots as replicates in each treatment. The abbreviations are the same as Fig. 1

grazing in any season, whereas grazing apparently enhanced the CH_4 oxidation in an alpine meadow soil in Europe. In our study, although the grazing intensity was about moderate, the aboveground biomass (canopy height), and thus the growth of plant, was significantly changed by grazing (Luo et al. 2010). Indeed, methane oxidation activity was influenced by the growth of plant (Wang et al. 2008). Given that no significant difference in soil properties was found between grazing and no-grazing treatments, thus plant biomass alteration resulted from grazing may be important factor influencing methanotrophic activity. However, in case of warming, weak effects of grazing on methanotrophic activity were observed here. A possible explanation could be that the effect of grazing was overwhelmed by warming.

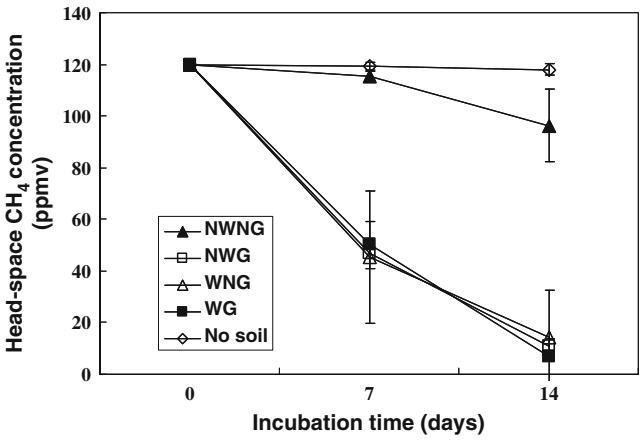

Fig. 4 Changes in head-space CH_4 concentration during a 2-week incubation of soil samples collected from the four treatments. The abbreviations are the same as Fig. 1

Effect of warming and grazing on methanotroph community composition

There was no remarkable shift in methanotroph community composition and diversity among the four treatments in our study. No correlation was found between in situ temperatures (0–18°C) and the methanotroph distribution (ratio of type I to type II methanotrophs; Liebner and Wagner 2007). Urmann et al. (2009) found that methanotrophs remain the same community composition at a wide temperature range (from 4°C to 20°C) using a laboratory-column system. It is generally thought that the growth of methanotrophs is limited by gas diffusion rather than by temperature, particularly under field condition, while temperature was expected to out-compete substrate limitation in well aerated soils such as landfill cover soils (Börjesson et al. 2004). Therefore, the weak temperature effect on methanotroph community composition in this study is attributed to the fact that CH_4/O_2 flux in soil might be limited by diffusion rather than by temperature.

For grazing, similarly, Abell et al. (2009) found that the composition of the predominant type II methanotrophs was not significantly affected by cattle grazing in any season in an alpine meadow soil (1,340 m asl) of Austria. However, Zhou et al. (2008) demonstrated that sheep grazing had an apparent impact on the methanotroph community structure in a semi-arid temperate steppe (1,180–1,250 m asl, annual mean temperature of 2°C, and annual precipitation of 350 mm) of Inner Mongolia steppe in China. The possible reason is that our study site is situated at much higher elevation (>3,200 m asl), with a lower annual mean temperature (−2°C) and a higher annual precipitation

(500 mm) on the Tibetan Plateau than those in Inner Mongolia steppe. Therefore, the differences in the geographic characteristics may cause these conflicting results. Another possible explanation for this discrepancy could be that a moderate grazing was employed in our study site, but heavy-intensity grazing treatment in Inner Mongolia steppe (Zhou et al. 2008). Therefore, probably grazing intensity was another important factor in shaping the methanotroph community.

In our study, the major OTUs (98.4%) belonged to type I methanotrophs (USC-γ). USC-γ methanotrophs were considered to be usually detected in soils with pH more than 6.0 (McDonald et al. 2008), such as pH 7.57–7.61 in this study. In addition, methanotroph community was commonly dominated by type I in cold environment such as in permafrost soils of Siberia (a mean annual temperature of −11.9°C in Liebner and Wagner 2007; a mean annual temperature of −14.7°C in Knoblauch et al. 2008), in Canadian high Arctic soil (a mean annual temperature of −16°C in Martineau et al. 2010), and in our study site (a mean annual temperature of −2°C). Therefore, type I methanotrophs as dominant member may contribute mostly to CH_4 oxidation in the alpine meadow on the Tibetan Plateau.

In summary, we here reported that there was much high abundance of methanotrophs, and type I was the dominant methanotrophs, which may play a key role in the CH_4 biogeochemical cycles in the alpine meadow ecosystem on the Tibetan Plateau. The fact of warming and moderate sheep grazing enhancing the CH_4 oxidation implies that Tibetan Plateau may remove more CH_4 under future climate conditions. Further investigation should be carried out to determine what extent of warming and grazing could help to increase CH_4 consumption and potentially mitigate global greenhouse effect on the Tibetan Plateau.

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