Title

Impact of grazing on shaping abundance and composition of active methanotrophs and methane oxidation activity in a grassland soil

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Running Head: Active methanotrophs in a typical steppe

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Abstract

The effect of grazing on the abundance, composition and methane (CH_4) uptake of methanotrophs in grasslands has been well documented in the past few decades, but the dominant communities of active methanotrophs responsible for CH_4 oxidation activity in grazed soils are still poorly understood. In this study, we characterized the metabolically active, aerobic methanotrophs in grasslands with three different levels of grazing (light, medium and heavy) by combining DNA-stable isotope probing (SIP) and quantitative PCR (qPCR) for methane monooxygenase (*pmoA*) gene and 16S rRNA gene-based amplicon sequencing. The CH_4 oxidation potential was as low as 0.51 μmol g dry weight^{-1} day^{-1} in the ungrazed control, while it decreased as grazing intensity increased in grazed fields, ranging from 2.25 μmol g dry weight^{-1} day^{-1} in light grazed fields to 1.59 in heavily grazed fields. Increased CH_4 oxidation activity was paralleled by 2-fold increases in abundance of *pmoA* genes and relative abundance of methanotroph-affiliated 16S rRNA genes in the total microbial community in grazed soils. SIP and sequencing revealed that the genus *Methylobacter* and *Methylosarcina* (type I; *Gammaproteobacteria*) and *Methylocystis* (type II; *Alphaproteobacteria*) were active methanotrophs responsible for CH_4 oxidation in grazed soils. Light and intermediate grazing stimulated the growth and activity of methanotrophs, while heavy grazing decreased abundance and diversity of the active methanotrophs in the typical steppe. Redundancy and correlation analysis further indicated that the variation of bulk density, soil C and N induced by grazing determined the abundance, diversity of active methanotrophs, and methane oxidation activity in the long-term grazed grassland soil.

***Key Words*:** active methanotrophs; *pmoA*; SIP; grazing; grassland.

Introduction

Methane (CH_4) is the second most important anthropogenic greenhouse gas and is about 28 times more efficient at absorbing infrared radiation than CO_2 (Stocker et al. 2013). Methane emissions can be attenuated by aerobic methane-oxidizing bacteria known as methanotrophs. These bacteria have the ability to utilize methane as their source of C and energy. Methanotrophs can act either as a bio-filter in wetland agricultural soils (paddy field) or as a methane sink in well-aerated soils (Bridgham et al. 2013; Fan et al. 2019a, b; Kolb, 2009; Wei et al. 2019). Although methane can also be oxidized anaerobically by microbes using nitrite, nitrate, ferric iron and sulfate as electron acceptors, methanotrophs are ubiquitous in nature and represent the only oxic biogenic sink for the greenhouse gas methane (Fan et al. 2019b; Hanson and Hanson 1996; Shen et al. 2019; Trotsenko and Murrell 2008).

Historically, aerobic methanotrophs have been classified as types I, II and X, based on physiological, biochemical and phenotypical characteristics (Veraart et al. 2015). Nowadays they are preferentially classified based on phylogeny; either belonging to the classes of *Gammaproteobacteria* (referred to as type I or X methanotrophs), *Alphaproteobacteria* (referred to as type II methanotrophs), or to the phyla *Verrucomicrobia* (referred to as type III methanotrophs) and NC10 (Knief 2015; Stein et al. 2012). More specifically, type I methanotrophs belong to the *Methylococcaceae* (type Ia and type Ib) and *Methylothermaceae* (type Ic) families, while type II methanotrophs are divided into type IIa (*Methylocystaceae*) and type IIb (*Beijerinckiaceae*) (Lüke et al. 2011). Converting CH_4 to methanol is the key step in the CH_4 oxidation pathway, which is catalyzed by the enzyme methane monooxygenase (Hanson and Hanson 1996). The *pmoA* gene encodes the β-subunit of the particulate

methane monooxygenase enzyme (pMMO) and is the most commonly used functional marker for identifying methanotrophs in environmental samples (Dumont 2014).

Grasslands are one of the largest terrestrial ecosystems in the world, covering approximately 20-40% of the earth's surface and accounting for up to 40% of the total land area in China (Nan 2005). Grassland soils are one of the largest terrestrial methane sinks with the capacity for methane oxidation (Pachauri et al. 2014). As the most popular management of grasslands, animal grazing may lead to different soil physical conditions (e.g., bulk density and aeration status) and chemical properties (e.g., pH and organic matter content) (Li et al. 2008; Steffens et al. 2008). Soil bulk density and moisture are considered to be critical factors for CH_4 oxidation activity in soil (Serrano-Silva et al. 2014). Animal grazing alters methanotroph composition and methane uptake activities by reducing soil water content and soil aeration through herbage removing and tramping, respectively (Leriche et al. 2001; Zhou et al. 2008). Heavy grazing has been reported to significantly reduce annual CH_4 uptake, while light-to-moderate grazing has been shown to have either a considerable positive impact on CH_4 uptake or to not significantly change CH_4 uptake (Chen et al. 2011; Ma et al. 2018). Previous studies either measured the abundance and/or composition of methanotroph in grassland soils or try to link CH_4 uptake with the abundance of the pmoA gene. However, mere presence of *pmoA* genes under *in situ* soil conditions may not necessarily reflect functional activity of CH_4 oxidation (Nannipieri et al. 2019). To our knowledge there are little studies that links CH_4 oxidation with functional active methanotrophs in grazed grassland soils.

The objective of this study was to obtain information on the presence of aerobic methanotrophs affected by grazing gradients in the field and to identify the active

microorganisms responsible for CH_4 oxidation in a typical steppe grazed soils. We applied quantitative PCR (qPCR) of the *pmoA* gene and Miseq sequencing coupled with DNA based stable isotope probing (SIP) to study the active methanotroph communities from field soils. Based on previous studies, we hypothesized that different levels of grazing would alter the abundance and communities of active methanotrophs and change the methane oxidation activity.

Materials and Methods

Setup of the experiment

Four different stocking rates were established in 2005, with zero, three, nine, and fifteen sheep per two hectares, at the Inner Mongolia Grassland Ecosystem Research Station (IMGERS, 43º37'N, 116°43' E) on the Xilingol steppe of the Xilin River basin. The dominant plant species in the study area is *Leymus chinensis* with some *Stipa grandis* and *Cleistogenes squarrosa*. The four stocking rates were classified as ungrazed control (CK), light grazing (G1), moderate grazing (G2), and heavy grazing (G3). Each year, sheep are in the field from June to September (~95 days), in accordance with the local summer grazing season. The different stocking rate treatments were arranged in a randomized block design with three replicates separated by fences and the plot size for each block was 2 ha. Composite soil samples of each replicate were collected from the upper 10 cm layer from 5 random locations using a 5 cm diameter soil auger in August, 2015, and transported to the laboratory with cold chain. After passed through a 2-mm sieve, soil samples of each replicate were separated into three subsamples for DNA extraction, physicochemical property analysis and SIP incubation experiments, respectively. Soil physicochemical properties were determined according to the protocols of Handbook of Soil Analysis (Pansu and

Gautheyrou 2007). The soil type is dark chestnut (calcic Chernozem according to the ISSS Working Group RB (1998)).

Microcosm incubations for SIP and CH_4 oxidation potential were conducted in sterile 120 ml glass serum vials in triplicate, containing 10 g (dry weight) of soils, and sealed with butyl rubber stoppers. Each microcosm was injected with either $^{13}CH_4$ (99 atom % ^{13}C, Sigma-Aldrich, USA) or CH_4 (99.5% pure, as control) to a final concentration of 8% (v/v) in the headspace and incubated at 25°C in dark conditions for 28 days. CH_4 oxidation potential was assessed from the zero-order decrease in CH_4 concentration in the headspace of the serum vials within 12 h, and measured with a gas chromatography (Shimadzu GC12-A, Japan) (Kightley et al. 1995). After more than 90% of the CH_4 was consumed, the headspace was flushed with pressurized synthetic air (20% O_2, 80% N_2) for 1 min to maintain oxic conditions and, after that, the labelled or unlabelled CH_4 was renewed. The atmosphere of the microcosms was renewed three times with pressurized synthetic air and labelled or unlabelled CH_4 during the incubation period. Destructive sampling was performed in triplicate for further soil analysis after incubation of SIP microcosms for 28 days.

Nucleic acid extraction and SIP fractionation

DNA was carefully extracted from 0.5 g of soil using a FastDNA SPIN kit for soil (MP Biomedicals; Solon, OH, USA) according to the manufacturer's instructions. Negative control without soil were not test as this study focus on the active methanotrophs which were labelled with ^{13}C (Vestergaard et al. 2017). The concentrations and quality of DNA were estimated by a Nanodrop® ND-2000 UV-vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and gel electrophoresis.

Density gradient centrifugation was performed on bulk DNA extracted from the

$^{13}CH_4$ and CH_4 treatments as described by Liu et al. (2019a) and Zhang et al. (2019), with a minor modification in scale: 2.5 μg of DNA were mixed with gradient buffer/CsCl solution in a 5.1 mL Beckman ultracentrifuge tube and DNA was fractionated into 14 equal fractions after centrifuged at 177,000 g_{av} for 44 h at 20 °C in a Vti65.2 vertical rotor (Beckman Coulter, Palo Alto, CA, USA) (Jia et al. 2019). The fractionated DNA was purified and dissolved in 30 μL of TE buffer.

Quantitative PCR (qPCR) of the *pmoA* genes

Quantitative *PCR* analysis of the *pmoA* gene in bulk DNA and in each DNA gradient fraction was performed to determine the growth and efficiency of ^{13}C incorporation into the genomic DNA of methanotroph communities, respectively. The primer pair A189f and mb661r (Costello and Lidstrom 1999; Holmes et al. 1995) was used for the qPCR of the *pmoA* gene as described previously (Zheng et al. 2014). qPCR runs were carried out in a LightCycler® 480II (Roche, Germany). Efficiencies of 89–105% were obtained for all gene amplifications, with R^2 values ranging between 0.992 and 0.999.

MiSeq sequencing and phylogenetic analysis

Methanotroph-affiliated 16S rRNA gene was amplified in both $^{13}CH_4$ and CH_4-control microcosms in bulk DNA and in heavy fractions of each gradient with standard PCR conditions and quantified DNA as suggested (Schöler et al. 2017). Methanotroph-affiliated 16S rRNA libraries were constructed and sequenced using an Illumina® MiSeq sequencer (Illumina, San Diego, CA, USA) by Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China) with a universal 515F-907R primer assay as reported previously (Daebeler et al. 2014; Zheng et al. 2014). Raw fastq files were quality-filtered by Trimmomatic and merged by FLASH with the following criteria: (i) The reads were truncated at any site receiving an average quality score <20 over a 50 bp sliding

window. (ii) Sequences with overlap being longer than 10 bp were merged according to their overlap with mismatch no more than 2 bp. (iii) Sequences of each sample were separated according to barcodes (exactly matching) and Primers (allowing 2 nucleotide mismatching), and reads containing ambiguous bases were removed. We obtained a total of 1,809,594 high quality sequences with an average of 37,940 for each sample. Rarefying may bring out some problem, but is still a popular method in the study of microbial ecology (Delgado-Baquerizo et al. 2018). To avoid potential bias caused by sequencing depth, all sequence data were rarefied to 17,454 sequences per sample for the downstream analyses. Operational taxonomic units (OTUs) were clustered with 97% similarity cutoff using UPARSE (version 7.1 http://drive5.com/uparse/) with a novel 'greedy' algorithm that performs chimera filtering and OTU clustering simultaneously. The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the Silva (SSU123) 16S rRNA database using confidence threshold of 97%.

Distribution of the methanotroph communities in situ and in the incubation experiment among different grazing intensities was evaluated by Principal components analysis (PCA) in R using the vegan package. Redundancy analysis (RDA) were also performed to identify the abiotic factors (Bulk density, total C, Olsen P and NO_3^--N) that are most important on shaping active methanotroph communities in the grazed grassland soils. The neighbor-joining tree was constructed by MEGA 7 with 1000-fold bootstrap support (Kumar et al. 2016).

Statistical analysis and sequencing data deposition

Significant differences of CH_4 oxidation potential, *pmoA* and methanotroph-affiliated 16S rRNA genes relative abundance among different treatments were assessed by

One-way analysis of variance (ANOVA) followed by a Tukey's post hoc test. All analyses were conducted by SPSS version 20 (IBM Co., Armonk, NY, USA).

The reads for the 16S rRNA genes of the *in situ* and incubated soil samples were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under accession number PRJNA432864.

Results

Soil physicochemical properties

The general physicochemical characteristics for the soils from different stocking rates (no grazing (CK), light grazing (G1), moderate grazing (G2), and heavy grazing (G3)) are summarized in Table 1. Soil pH was significantly lower in the G2 (6.89) soil compared with other grazed soils (G1: 8.12; G3: 7.69) and the ungrazed control (CK: 7.62). Soil bulk density showed a significant positive relationship with increasing stocking rates ($r=0.96$, $p<0.001$). The contents of total soil C and nutrients, including SOM, Total C, Total N, Olsen P and K, were highest in the G1 soils compared to other samples. Grazing led to significantly less exchangeable NH_4^+-N in the grazed soils of G1, G2 and G3, compared with the ungrazed control.

Abundance and diversity of methanotrophs *in situ*

The abundance of methanotrophs was estimated in soil samples *in situ* by qPCR targeting the *pmoA* gene (Fig.1a). The results showed that *pmoA* gene abundance ranged from $7.74 \times 10^5 \pm 2.13 \times 10^4$ copies g^{-1} soil in G1 soil to $6.59 \times 10^5 \pm 0.95 \times 10^5$ copies g^{-1} soil in G3 soil. The abundance of *pmoA* gene increased with increasing grazing intensities. The light grazing (G1) had a significantly lower abundance of the *pmoA* gene compared with the ungrazed control (CK).

PCA showed that over 67% methanotroph variations was explained by the first

two axes, with PC1 and PC2 explaining 48.9% and 17.7% of the total variance, respectively (Fig.1b).

Methane oxidation potential and abundance of methanotrophs

The CH_4 oxidation potential under a headspace of 8% (v/v) CH_4 concentration ranged from 1.59 ± 0.07 to 2.25±0.07 μmol g dry weight^{-1} day^{-1} for the grazed soils (Fig. 2). No significant differences of the CH_4 oxidation potential were observed between $^{13}CH_4$ labelled and CH_4 control microcosms. The CH_4 oxidation potential was as low as 0.51±0.05 μmol g dry weight^{-1} day^{-1} in the ungrazed control, which was significantly lower than the grazed soils. Compared with the light and intermediate grazing, the heavy grazing significantly decreased the CH_4 oxidation potential.

The community size of methane-oxidizing bacteria was not significantly different for the ungrazed control after 28 days of incubation (Fig.3a). The copy number of *pmoA* genes increased significantly from $1.38 \times 10^7 \pm 3.21 \times 10^5$ at day 0, to $3.39 \times 10^7 \pm 0.5 \times 10^6$ in microcosms of the G3 soil after 28 days of incubation.

Significantly higher abundances of the *pmoA* gene were observed in the G1 and G2 soils, representing 12- and 6-fold increases, respectively (Fig. 3a). Similar results were observed from the relative abundance of methanotrophs reads to total 16S rRNA reads by MiSeq amplicon sequencing (Fig. 3b). Methanotrophs reads showed a significant increase after 28 days of incubation in the grazed soils but not in the ungrazed control. Correlation analysis showed strong correlation between the potential CH_4 oxidation rates and *pmoA* quantities ($r=0.92$, $p<0.01$) during incubation, but not with *pmoA* quantities *in situ* ($r=-0.517$, $p=0.085$).

Active methanotrophs in soils

The relative proportion of *pmoA* across CsCl gradients was similar among the three

soils despite the differences in the intensity of grazing (Fig. 4a). The maximum relative proportion of *pmoA* was initially detected in the light fractions (around a buoyant density of 1.723 g mL^{-1}) in the CH_4 microcosms but shifted to the heavy fractions (around a buoyant density of 1.745 g mL^{-1}) in the $^{13}CH_4$ microcosms after the 28-day incubation (Fig. 4a). This indicated that methanotrophs grew by assimilating CH_4 during active methane oxidation. By contrast, no significant shifts in the relative proportion of *pmoA* were observed in the CH_4 and $^{13}CH_4$ incubations of the ungrazed control soil (Fig. 4a). Miseq sequencing of methanotroph-affiliated 16S rRNA gene distributed across the CsCl gradient also indicated the assimilation of CH_4 in the three grazed soils but not in the ungrazed control during the active methane oxidation (Fig. 4b).

Phylogenetic analysis revealed that active methanotrophs in the ^{13}C-DNA from the three grazed soils were most closely affiliated with *Methylobacter* and *Methylosarcina* of type Ia and *Methylocystis* of Type II methanotrophs (Fig. 5; Supplementary fig. 1). *Methylosarcina* was enriched in the heavy fractions in grazed soils G1 and G2 after incubation, while *Methylocystis* was only enriched in G1 after incubations (Fig. 5).

Correlating soil properties with active methanotroph communities

Bulk density, total C, and Olsen P in combination with NO_3^--N explained the highest percentage of the variance of active methanotroph communities (Fig. 6). The soil bulk density and NO_3^--N content were significantly correlated with the first axis (explaining 77.3% of the total variance).

Correlation analysis showed that the soil bulk density was negatively correlated with the increase of *pmoA* gene abundance ($r=-0.767$, $p<0.05$) and methane oxidation

potential (r=-0.782, p<0.05) during the incubation in the grazed grassland soils (Fig.7). The ungrazed control was excluded for the correlation analysis because of no detectable growth of methanotrophs and extremely low CH_4 oxidation potential in the ungrazed control soil (Fig.2 and Fig.3).

Discussion

Effect of grazing on methanotroph community *in situ*

The abundance and community composition of methanotrophic bacteria were evaluated for three field samples all exhibiting various levels of grazing. The abundance of a key gene in the methane oxidation pathway, the *pmoA,* was used as a proxy for the population of methanotrophs in the steppe. Compared with the ungrazed control, the abundance of *pmoA* gene significantly decreased in G1 soil, while increased in G3 soil (Fig.1a). Abell et al. (2009) found that the abundance of the predominant type I methanotrophs was positively affected by long-term cattle grazing in an alpine meadow soil. In contrast, no significant change of methanotroph abundance with grazing was observed in an alpine meadow (Zheng et al. 2012). On the other hand, grazing was reported to impact the composition of the methanotrophic community in a typical grassland, while no impact was apparent in an alpine meadow (Zhou et al. 2008; Zheng et al. 2012). In this study, distribution of methanotrophs among the soils analyzed showing by PCA indicated a significant impact of grazing on the methanotroph communities (Fig.1b). In addition, we observed a significantly lower abundance of methanotrophs in G1 soil, even though the concentrations of SOM and TN were significantly higher than other (Table.1). Some factors like the availability of N, cross-feeding and other C sources, apart from the CH_4 availability, have also been proposed to regulate the population size of

methanotrophs in upland soils (Li et al. 2018; Malghani et al. 2016). In this study, the *pmoA* abundance was strongly correlated with SOM (r=-0.758, p<0.01), TN (r=-0.584, p<0.05) and moisture content (r=-0.611, p<0.05). The low abundance of methanotrophs in G1 soil was mainly ascribed to the competition between methanotrophs, which represent only a small fraction of the total bacterial community, and heterotrophs, which was markedly stimulated by the higher nutrients in G1 soil. These results indicate that long-term grazing not only changes soil properties but also the abundance and composition of functional microbes like methanotrophs *in situ*.

Effect of grazing on CH_4 oxidation potential

The CH_4 oxidation potential measurements are useful for comparing the relative activities of the methanotroph populations within samples from different environmental conditions (Kightley et al. 1995). The potential CH_4 oxidation rates were variable between the grassland samples, ranging from 0.51±0.05 to 2.25±0.07 μmol g^{-1} d^{-1} (Fig. 2). While the potential CH_4 oxidation rates were within ranges seen in wetlands (0.17 to 80 μmol CH_4 g^{-1} d^{-1}) (Graef et al. 2011) and geothermal environments (1.0–141 μmol CH_4 g^{-1} d^{-1}) (Sharp et al. 2014) with similar elevated CH_4 concentrations (>5% v/v), they were higher than those in upland soils (up to 74.64 nmol CH_4 g^{-1} d^{-1}) reported by Knief et al. (2003), and in grassland soils (2.42 to 21.54 nmol CH_4 g^{-1} d^{-1}) reported by Kou et al. (2017) with lower concentrations of CH_4 (<0.1% v/v). The strong correlation (r=0.92, p<0.01) between the potential CH_4 oxidation rates and *pmoA* quantities suggests that there is a constant activity and a consistent abundance of methanotrophs throughout the 28-day incubation period. A similar trend was observed from the relative abundance of methanotroph reads to total 16S

rRNA gene reads by MiSeq amplicon sequencing (Fig. 3b), further proving the activity of methanotrophs during incubation. However, the CH_4 oxidation potential was as low as 0.51±0.05 μmol g^{-1} dry weight day^{-1} in the ungrazed control. In agreeing with this, no growth of methanotrophs was detected in the ungrazed control using qPCR of *pmoA* genes and relative abundance of methanotroph reads to total 16S rRNA gene reads by MiSeq amplicon sequencing (Fig. 3). Also, methanotrophs were not labelled by ^{13}C-CH_4 confirming our expectation of low CH_4 oxidation activity in the site and negligible growth of methanotrophs (Fig. 4). It is well known that N fertilizers inhibit CH_4 oxidation by ammonia, which competes with CH_4 for the methane monooxygenases in methanotrophs (Bédard and Knowles 1989). Even though the affinity of MMO for CH_4 is 600–1300-fold higher than its affinity for ammonia, high concentrations of ammonium (40 mg NH_4^+-N kg^{-1}) are known to substantially inhibit CH_4 oxidation (Alam and Jia 2012). Grazing exclusion has been reported to increasing plant biomass, root biomass, root exudate and available soil N (Wang et al. 2018). The concentrations of exchangeable NH_4^+-N were significantly higher in the ungrazed control than the grazed soils, which could partly explain the low CH_4 oxidation in the ungrazed control soil. Ho et al. (2019) suggest that 'high-affinity' methanotrophs predominate CH_4 oxidation in native upland soils, while canonical methanotrophs predominate in the anthropogenic-impacted upland soils. Another possible explanation for the low CH_4 oxidation potential in the ungrazed control could be due to the methanotrophs in this site predominantly comprised of the putative 'high-affinity' methanotrophs. Moreover, no correlation (r=-0.517, p=0.085) between the potential CH_4 oxidation rates and *pmoA* abundance *in situ* was observed, further proved that the mere presence of *pmoA* genes under *in situ* soil conditions may not

necessarily reflect functional activity of CH_4 oxidation (Nannipieri et al. 2019).

It is widely accepted that heavy grazing would cause a decline of CH_4 oxidation rate (Chen et al. 2011). Previous studies have indicated that an increase in stocking rate induced a reduction in CH_4 uptake (Holst et al. 2008; Wang et al. 2012). Simulating the effects of grazing management with the PaSim model, Soussana et al. (2004) have suggested that a decline in the greenhouse gas sink activity of managed steppes occurs with increased stocking intensity. In this study, heavy grazing significantly decreased the potential CH_4 oxidation rate in G3 soil compared to light and intermediate grazing in G1 and G2 soils (Fig.2). Heavy grazing could significantly increase soil bulk density and directly affect the air permeability incurred from sheep trampling (Table.1) (Ball et al. 2012; Pan et al. 2018a). Furthermore, heavy grazing would decrease aboveground plant and litter biomass and consequently increase water stress, potentially inhibiting the activities of methanotrophs indirectly (Chen et al. 2011; Cui et al. 2018; Liu et al. 2007). Heavy grazing has been previously reported to inhibit the growth of ammonia-oxidizing bacteria, reducing the nitrification activity in grazed grassland soils (Pan et al. 2018b). In contrast to these negative impacts on plants, soil properties, and microbes by heavy grazing, light grazing could lead to a greater diversity of plant species, and the dense fibrous rooting systems of plants would benefit soil organic matter formation and soil C sequestration (Reeder and Schuman 2002). N returned in animal excreta, and/or modification of N uptake and C exudation by frequently defoliated plants could also promote soil fertility and enhance microbial activities (Le Roux et al. 2003; Luo et al. 2018; Pan et al. 2018a; Zhou et al. 2010; Zhu et al. 2018). This study showed that grazing alters soil functional traits with light and intermediate grazing stimulating the growth and activity of methanotrophs, while

heavy grazing significantly decreased the abundance of methanotrophs and the methane oxidation potential.

Active methanotrophs in grazed soils

Overall, the methanotroph communities present before and after incubation were similar (Supplementary fig. 1). Communities of the active ^{13}C-labelled methanotrophs were more diverse in the light and intermediate grazed soils than in the heavily grazed soil (Fig.5). The active methanotroph community included members of *Methylobacter* and *Methylosarcina* (type I methanotrophs), and *Methylocystis* (type II methanotrophs) (Supplementary fig. 2). Most of the active methanotrophs (>90%) were closely related to the type I methanotroph, *Methylobacter luteus* (Fig. 5), a species originally isolated from a sewage (Bowman et al. 1993; Romanovskaia et al. 1978). *Methylobacter*-related type I methanotrophs have been reported to be responsible for the majority of methane oxidation in a long-term grazing site in Austria and also in six grazed grassland soils across New Zealand (Abell et al. 2009; Di et al. 2010). The mean annual temperature of 0.3°C and maximum monthly mean temperatures of 19°C in the studied field favoured the growth of *Methylobacter*-related type I methanotrophs, as *Methylobacter* species have been reported to prefer cold environments such as the active layer of Arctic permafrost (Liebner et al. 2009), high Arctic wetlands (Graef et al. 2011), lake sediments (He et al. 2012) and plateau wetlands (Deng and Dumont 2016) and rice fields from cold regions (Sultana et al. 2019). No active methanotrophs in this study were affiliated with the proposed atmospheric methane-oxidizing lineages USCα and USCγ (Knief et al. 2003). Moreover, *Methylosarcina* of type I methanotrophs, which have shown a transient ability to oxidize methane at atmospheric levels and also possible support 'high-affinity'

methane oxidation activity in paddy soil (Cai et al. 2016), were detected during CH_4 oxidation in G1 and G2 soils. Recently, canonical methanotrophs have been suggested to predominate CH_4 oxidation as high-affinity methane-oxidizers in anthropogenically-impacted upland soils (Ho et al. 2019). Furthermore, *Methylocystis* of type II methanotrophs were only detected in the G1 soil. The significantly higher content of SOM in the G1 soil might partly explain the result, as some *Methylocystis* species are known to oxidize and grow on acetate and ethanol in addition to methane (Belova et al. 2011; Im et al. 2011). Another reason for it should be the significantly higher Olsen P in the G1 soil, because the abundance of type II methanotrophs are positively related to phosphorus and adopt a competitor-ruderal life-style (Ho et al. 2013). It thus suggests that the diverse active methanotrophs and higher abundance of *pmoA* genes in the light and intermediate grazed soils enabled the significantly higher CH_4 oxidation potential. These results indicate that light and intermediate grazing stimulate the growth and activity of diverse methanotrophs, while heavy grazing significantly decreases the abundance and diversity of active methanotrophs in this typical steppe.

Methanotroph activity

Animal grazing alters soil water and energy balance by reducing vegetation, increasing soil compaction or reducing soil aeration by trampling and also soil chemical properties (e.g., pH and organic matter content), which would subsequently induce variation of microbial communities and activity (Li et al. 2019; Liu et al. 2019b; Lu et al. 2019; Pan et al. 2018c; Saggar et al. 2004; Steffens et al. 2008; Yu et al. 2018). Numerous studies have estimated the impact of grazing on either methanotroph communities or methanotroph activity (CH_4 uptake), even though the abundance and

composition of methanotrophs may not necessarily reflect their activity (Abell et al. 2009; Savian et al. 2014; Van den Pol-van Dasselaar et al. 1999; Zheng et al. 2012). In this study, DNA-SIP was used to link identity and function of methanotrophs in grazed grassland soils. RDA showed that soil bulk density and NO_3^--N were significantly correlated with the distribution of active methanotrophs in grazed grassland soils (Fig. 6). Methane diffusion, which is determined by the soil bulk density and moisture, is considered the limiting factor for CH_4 oxidation in soil (Serrano-Silva et al. 2014; Walkiewicz et al. 2018). The single quantification of a target genes may not necessarily reflect their functional activity (Nannipieri et al. 2019). In this study, no correlation (r=-0.517, p=0.085) between the potential CH_4 oxidation rates and *pmoA* abundance *in situ* was observed. We linked the potential CH_4 oxidation rates with the increase of *pmoA* gene abundance during the incubation. The significant correlation between the soil bulk density and the increases of *pmoA* gene abundance ($r=-0.767$, $p<0.05$), and also methane oxidation potential ($r=-0.782$, $p<0.05$) during the incubation in the grazed grassland soils further indicated the important impact of bulk density on the methanotroph communities and functional activity (Supplementary fig. 3). These results indicate that grazing induced variation of bulk density, soil C and N altering the abundance and communities of active methanotrophs and subsequently changes the CH_4 oxidation activity.

SIP studies often require *in vitro* incubations and only partially reflect conditions *in situ.* This method may distort the relative abundance of organisms active in a particular process (Chen and Murrell 2010; McDonald et al. 2005). Fairly high CH_4 concentrations, which probably do not reflect on *in situ* methane levels (typically atmospheric methane levels), were selected in our incubations in order to ensure the

labelling of active microbial communities during the CH_4 oxidation. The often occurs cross-feeding effect for SIP experiments isn't problem in this study because methanotrophs prefer to utilize CH_4 as their source of C and energy even with exist of microbial metabolites or microbial residues from labelled methanotrophs (Bao et al. 2019). No significant changes in the methanotroph communities before and after incubation were found in this study, which indicates that SIP results might largely reflect the functional process of methane oxidation under field conditions (Supplementary fig. 1). The lack of labelled methanotrophs in the ungrazed control could mainly be due to little microbial growth during incubation, as DNA-SIP relies on cell proliferation. The lowest CH_4 oxidation potential rate observed in the ungrazed control further supports the negligible activity of methanotrophs, even though the activity might have resulted from the activation of dormant microbial populations rather than by their growth (Ho et al. 2015). It is worth mentioning that Phospholipid Fatty Acid Analysis (PLFA)-based SIP could ensure labelling methanotrophs at low methane concentrations, however, which would enable the detection of microbial groups but not on microbial genera or species as DNA-SIP (Ho et al. 2019).

Conclusions

Overall, this study showed that high abundance and diversity of methanotroph communities under *in situ* soil conditions may not necessarily reflect high functional activity of CH_4 oxidation. Light and intermediate grazing stimulated the growth and activity of active methanotrophs, while heavy grazing significantly decreased the abundance and diversity of active methanotrophs. Phylogenetic analysis of the ^{13}C-enriched DNA fractions from the DNA-SIP microcosms revealed that the active methanotrophs were dominated by the genus *Methylobacter* of type I. This study also

showed that soil Physicochemical properties, bulk density, soil C and N are key factors determining the abundance and composition of active methanotrophs and subsequently the CH_4 oxidation activity in the long-term grazed grassland soil.

Acknowledgements

We thank the Inner Mongolia Grassland Ecosystem Research Station, Institute of Botany, the Chinese Academy of Sciences for assistance of soil sampling. This work was financially supported by the Natural Science Foundation of China (41671249 and 41721001), Fundamental Research Funds for the Central Universities (2019QNA6011) and National Key Basic Research Program of China (2014CB138801). Yong Li extends his thanks to the Pao Yu-Kong and Pao Zhao-Long Scholarships for financial support.

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718 Biol Biochem 116: 369–377.

Figure Legends

Fig. 1. Abundance of *pmoA* gene (a) revealed by quantitative PCR and principal components analysis (PCA) of methanotroph- affiliated 16S rRNA reads (b) by MiSeq amplicon sequencing in grazed grassland soil *in situ*. Bars represent standard error of triplicate samples. The different letters above the columns indicate a significant difference ($P<0.05$) based on the analysis of variance with Tukey's post hoc test.

Fig. 2. CH_4 oxidation potential in different grazing soils with a CH_4 concentration of 8% (v/v) in air. Bars represent standard error of triplicate samples. The different letters above the columns indicate a significant difference ($P<0.05$) based on the analysis of variance with Tukey's post hoc test.

Fig. 3. Abundances of *pmoA* gene by quantitative PCR (a) and relative abundance of methanotrophic reads to total 16S rRNA genes reads by MiSeq amplicon sequencing (b) in soil microcosms over an incubation period of 28 days. The error bars represent the standard errors of triplicate microcosms. The different letters above the columns indicate a significant difference ($P<0.05$) based on the analysis of variance with Tukey's post hoc test.

Fig. 4. Quantitative distribution of the *pmoA* gene based on qPCR across the entire buoyant density gradient of the DNA fractions (a) and percentage distribution of methanotroph-affiliated 16S rRNA reads by MiSeq amplicon sequencing for the heavy DNA (fractionas 4-7) (b) from soil microcosms incubated with $^{12}CH_4$ or $^{13}CH_4$ for 28 days. The normalized data are the ratios of the gene copy number in each DNA gradient to the maximum quantities from each treatment.

Fig. 5. Proportional changes of methanotrophic phylotypes in SIP microcosms over an incubation period of 28 days. HF indicated the methanotrophs from heavy fraction of DNA at 28 days.

Fig. 6. Redundancy analysis (RDA) between soil physicochemical properties (Bulk density, total C, Olsen P and NO_3^--N) and active methanotrophs revealed by MiSeq amplicon sequencing of labelled DNA during the incubation in the grazed grassland soils.

Table 1 Physicochemical properties of the grassland soils used in this study

Soil properties	CK	G1	G2	G3
pH	7.62±0.61a	8.12±0.02a	6.89±0.21b	7.69±0.18a
Bulk density (g cm^{-3})	1.25±0.01d	1.28±0.01c	1.35±0.01b	1.37±0.01a
Moisture content (%)	20.09±0.91a	21.72±1.26a	17.78±0.77b	16.90±0.99b
SOM (g kg^{-1})	20.44±1.83b	32.16±1.68a	22.95±2.20b	22.66±1.67b
TN (g kg^{-1})	0.18±0.02ab	0.23±0.03a	0.17±0.02b	0.16±0.02b
TC (g kg^{-1})	1.94±0.20b	2.43±0.32a	1.69±0.25b	1.70±0.20b
Olsen P (mg kg^{-1})	2.22±0.19b	2.70±0.22a	2.22±0.13b	2.03±0.19b
K (mg kg^{-1})	321.3±35.52c	652.1±36.65a	488.9±53.26b	342.9±43.48c
NO_3^- -N (mg kg^{-1})	7.92±1.39a	9.45±2.17a	5.39±1.50a	7.12±2.69a
Exchangeable NH_4^+-N (mg kg^{-1})	1.87±0.49a	1.03±0.38b	1.10±0.56b	1.12±0.43b

Note: SOM: Soil organic matter; TC: Total C; TN: Total N; K: Available K. Different letters within the same row denote significant differences ($P < 0.05$) among soils using ANOVA with Tukey's post hoc test.

Figure

Fig.1

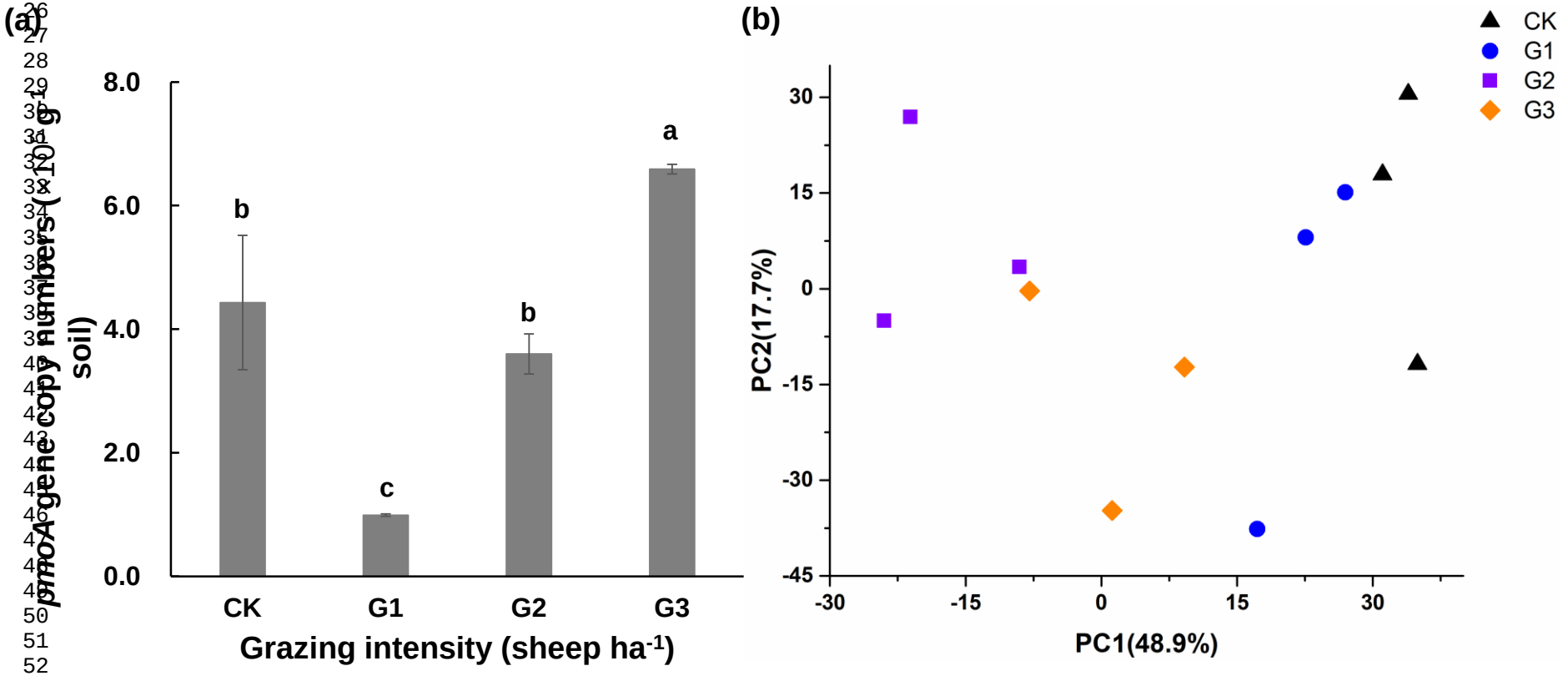

(a)
pmoA gene copy numbers (×10) g soil)
8.0
6.0
4.0
2.0
0.0
b
c
b
a
CK
G1
G2
G3
Grazing intensity (sheep ha-1)
(b)
CK
G1
G2
G3
PC2(17.7%)
30
15
0
-15
-30
-45
-30
-15
0
15
30
PC1(48.9%)

Fig.2

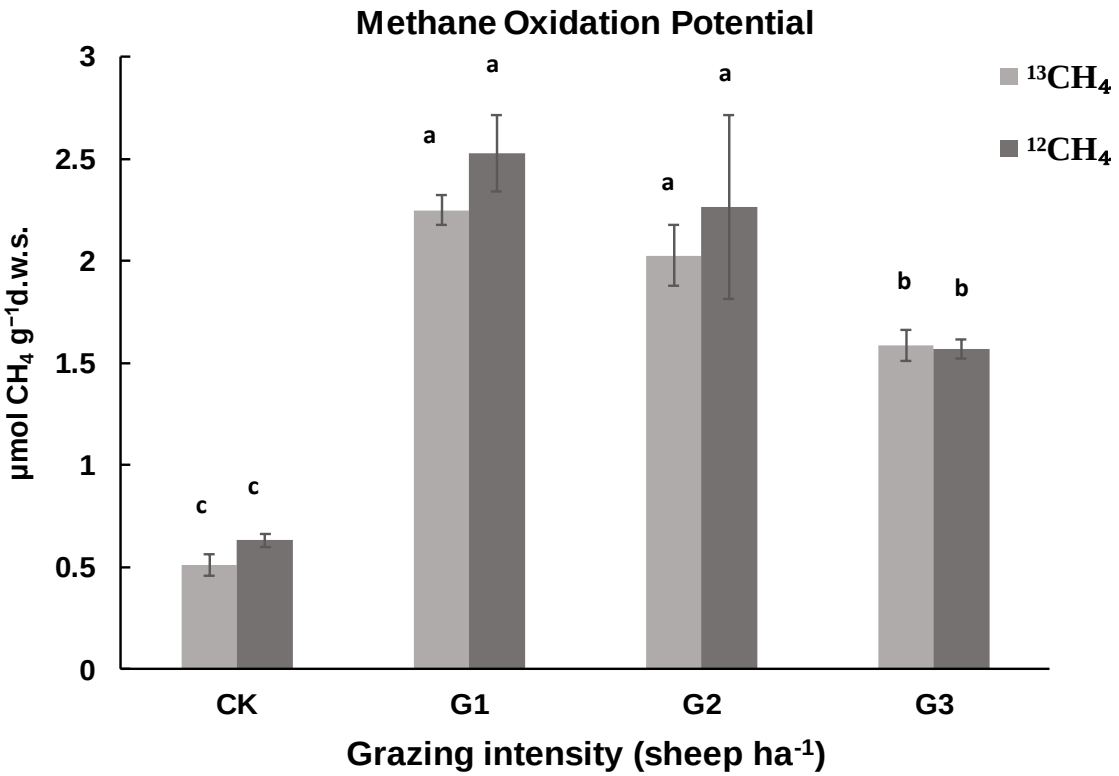

Methane Oxidation Potential
$^{13}CH_4$
$^{12}CH_4$
μmol CH_4 g^{-1}d.w.s.
3
2.5
2
1.5
1
0.5
0
a
a
a
a
b
b
c
c
CK
G1
G2
G3
Grazing intensity (sheep ha^{-1})

Fig.3

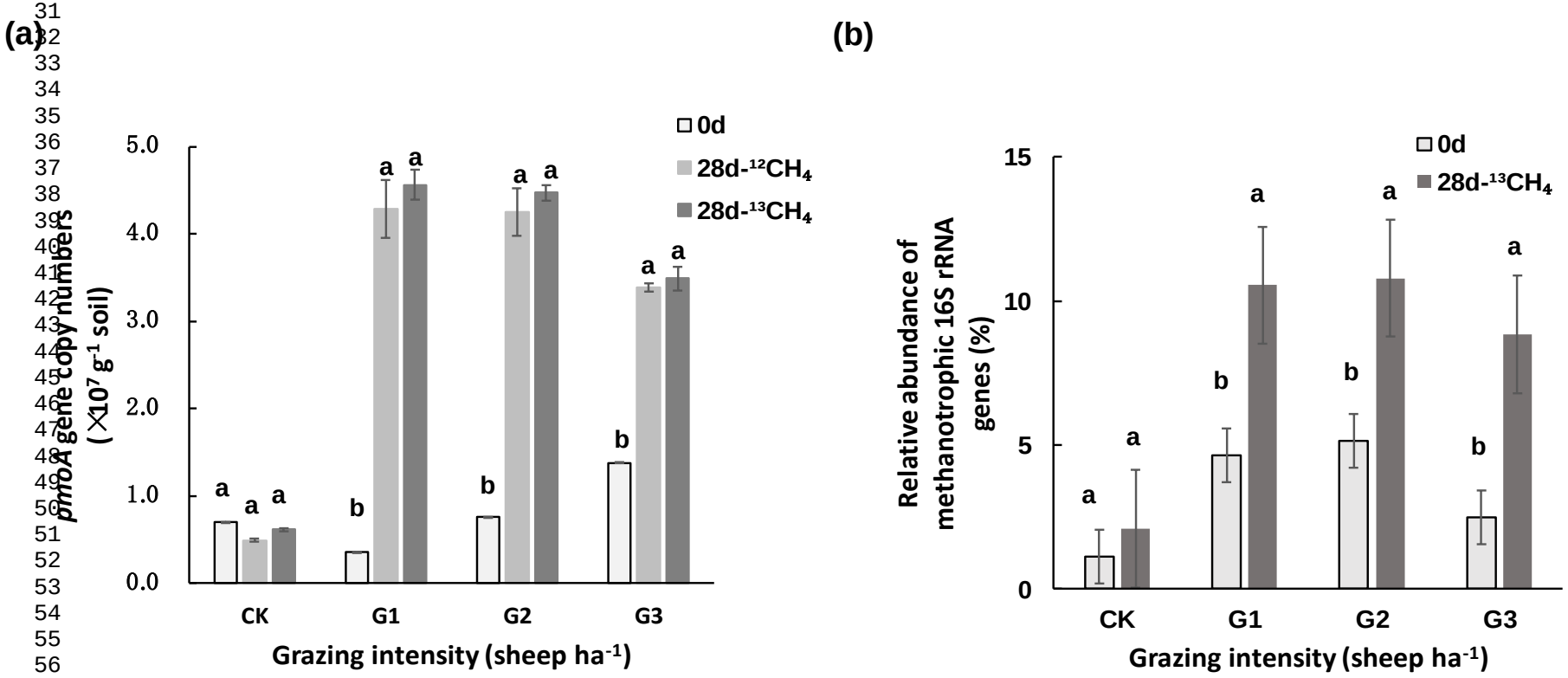

(a)
pmoA gene copy numbers (×10⁷ g⁻¹ soil)
0d
28d-¹²CH₄
28d-¹³CH₄
5.0
4.0
3.0
2.0
1.0
0.0
a a a
b a a
b a a
b a a
CK
G1
G2
G3
Grazing intensity (sheep ha⁻¹)
(b)
Relative abundance of methanotrophic 16S rRNA genes (%)
0d
28d-¹³CH₄
15
10
5
0
a a
b a
b a
b a
CK
G1
G2
G3
Grazing intensity (sheep ha⁻¹)

Fig.4

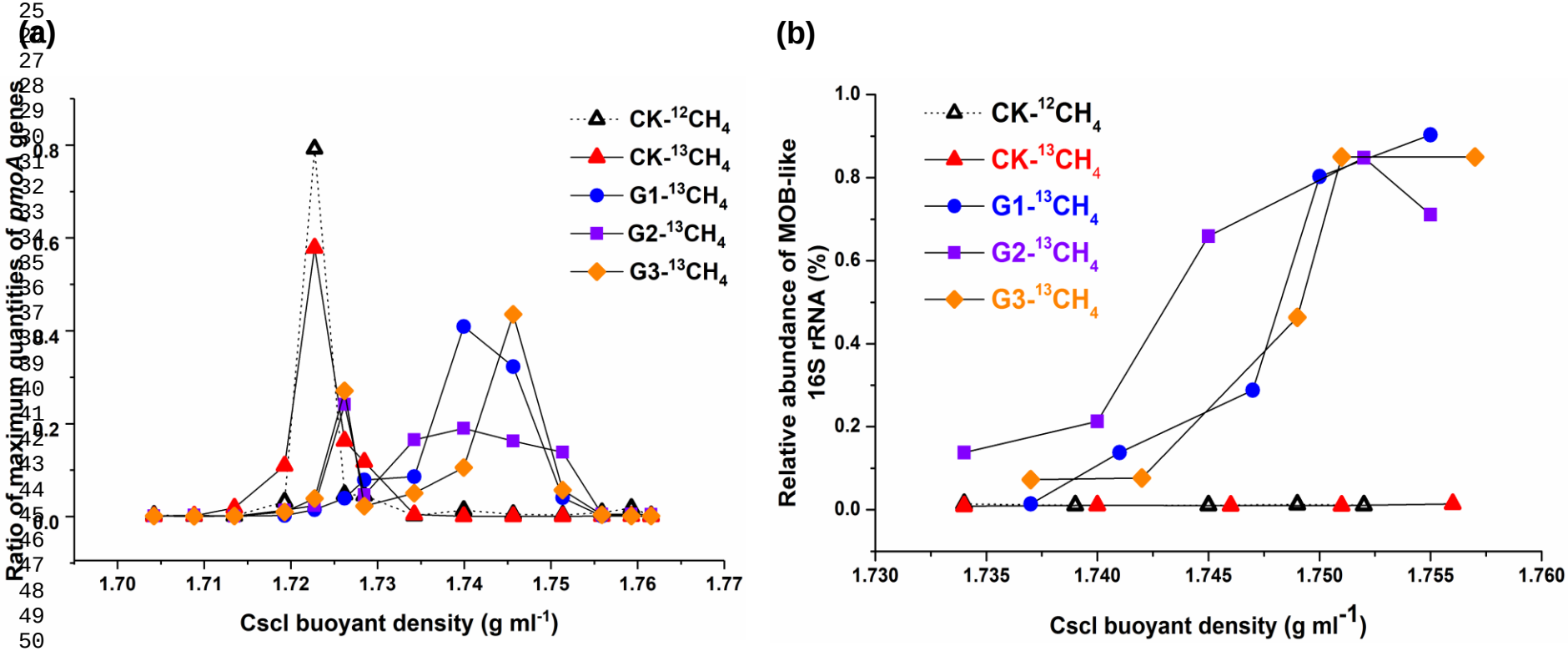

(a)
Ratio of maximum quantities of pmoA genes
0.0
0.2
0.4
0.6
0.8
1.70
1.71
1.72
1.73
1.74
1.75
1.76
1.77
CsCl buoyant density (g ml-1)
CK-12CH4
CK-13CH4
G1-13CH4
G2-13CH4
G3-13CH4
(b)
Relative abundance of MOB-like 16S rRNA (%)
0.0
0.2
0.4
0.6
0.8
1.0
1.730
1.735
1.740
1.745
1.750
1.755
1.760
CsCl buoyant density (g ml-1)
CK-12CH4
CK-13CH4
G1-13CH4
G2-13CH4
G3-13CH4

Fig.5

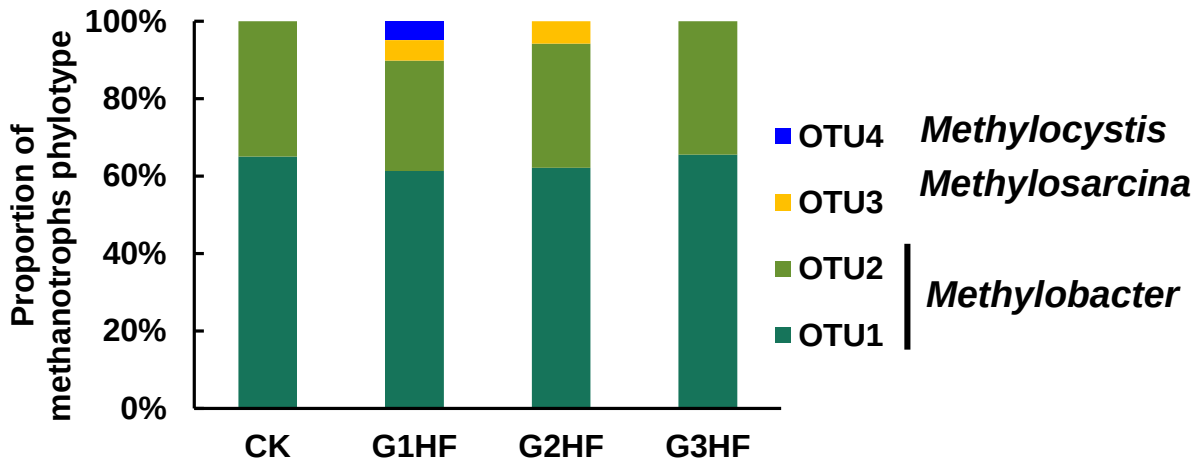
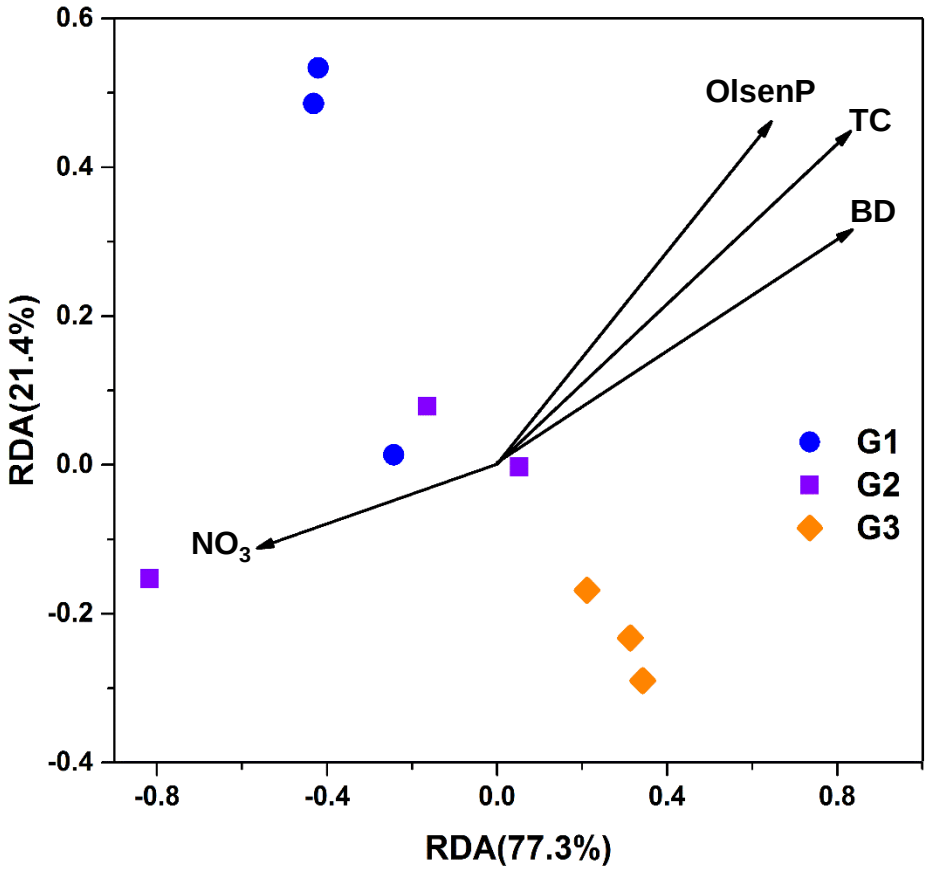

Proportion of methanotrophs phylotype
100%
80%
60%
40%
20%
0%
CK
G1HF
G2HF
G3HF
OTU4
OTU3
OTU2
OTU1
Methylocystis
Methylosarcina
Methylobacter

Fig.6


0.6
0.4
0.2
0.0
-0.2
-0.4
-0.8
-0.4
0.0
0.4
0.8
RDA(21.4%)
RDA(77.3%)
OlsenP
TC
BD
NO3
G1
G2
G3