The response of methanotrophs to additions of either ammonium, nitrate or urea in alpine swamp meadow soil as revealed by stable isotope probing

Article *in* FEMS Microbiology Ecology · May 2019

DOI: 10.1093/femsec/fiz077

CITATIONS
10

READS
243

6 authors, including:

Dan He
Guangdong Academy of Sciences
39 PUBLICATIONS **579** CITATIONS
SEE PROFILE

Liyan Zhang
Chinese Academy of Sciences
5 PUBLICATIONS **30** CITATIONS
SEE PROFILE

Marc G Dumont
University of Southampton
160 PUBLICATIONS **4,075** CITATIONS
SEE PROFILE

Jin-Sheng He
Peking University
245 PUBLICATIONS **9,759** CITATIONS
SEE PROFILE

FEMS Microbiology Ecology, 95, 2019, fiz077

doi: 10.1093/femsec/fiz077
Advance Access Publication Date: 24 May 2019
Research Article

RESEARCH ARTICLE

The response of methanotrophs to additions of either ammonium, nitrate or urea in alpine swamp meadow soil as revealed by stable isotope probing

Dan He[1,2], Liyan Zhang[1,3], Marc G. Dumont[4], Jin-Sheng He[5,6], Lijuan Ren[7] and Haiyan Chu[1,*,†]

[1]State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, East Beijing Road 71, Nanjing 210008, China, [2]Key Laboratory of Vegetation Restoration and Management of Degraded Ecosystems, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, China, [3]University of Chinese Academy of Sciences, Beijing 100049, China, [4]School of Biological Sciences, University of Southampton, Southampton, SO17 1BJ, UK, [5]Department of Ecology, College of Urban and Environmental Sciences, and Key Laboratory for Earth Surface Processes of the Ministry of Education, Peking University, Beijing 100871, China, [6]State Key Laboratory of Grassland Agro-Ecosystems, and College of Pastoral Agriculture Science and Technology, Lanzhou University, Lanzhou 730000, Gansu, China and [7]Key Laboratory of Tropical Marine Bioresources and Ecology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, China

*Corresponding author: State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, East Beijing Road 71, Nanjing 210008, China. Tel: +862586881356, Fax: +862586881000; E-mail: hychu@issas.ac.cn

One sentence summary: Urea has large effects on the metabolically active methanotroph communities in the swamp meadow soil on the QTP.

Editor: Yong-Guan Zhu

†Haiyan Chu, http://orcid.org/0000-0001-9004-8750

ABSTRACT

Different forms of nitrogen (N) are deposited on the Qinghai-Tibetan plateau (QTP), while their differential effects on soil methanotrophs and their activity remain elusive. We constructed microcosms amended with different N fertilizers (ammonia, nitrate and urea) using the soils sampled from a swamp meadow on the QTP. The responses of active methanotrophs to different forms of nitrogen were determined by stable isotope probing with 5% ^{13}C-methane. At the early stage of incubation, all N fertilizers, especially urea, suppressed methane oxidation compared with the control. The methane oxidation rate increased during the incubation, suggesting an adaptation and stimulation of some methanotrophs to elevated methane. At the onset of the incubation, the type II methanotrophs *Methylocystis* were most abundant, but decreased during the incubation and were replaced by the type Ia methanotrophs *Methylomonas*. Ammonia and urea had similar effects on the methanotroph communities, both characterized by an elevation in the proportion of *Methylobacter* and more diverse methanotroph communities. Nitrate had less effect on the methanotroph community. Our results uncovered the active methanotrophs responding to different nitrogen forms, and suggested that urea-N might have large effects on methanotroph diversity and activity in swamp meadow soils on the QTP.

Received: 18 January 2019; **Accepted:** 23 May 2019

Keywords: methanotrophs; nitrogen deposition; Qinghai-Tibetan Plateau; nitrogen forms; stable Isotope probing

INTRODUCTION

Methane is the second most important greenhouse gas, and makes up 14% of the global greenhouse effect (U.S. Environmental Protection Agency 2006). The concentration of methane in the atmosphere has increased about 2.5 times above the pre-industrial level, from 250 ppb to 1.8 ppm in 2011 (Hartmann *et al.* 2013). The consumption of methane by methanotrophs prevents its release to the atmosphere, and can be a pivotal part for the atmospheric source/sink budget of methane. Methanotrophs typically use methane as their sole source of carbon and energy (Hanson and Hanson 1996). They harbor two different enzymes for the first step of methane oxidation, either a soluble methane monooxygenase (sMMO) or a membrane-bound particulate methane monooxygenase (pMMO). The latter is ubiquitous in all methanotrophs except *Methylocella* and *Methyloferula*, which are methanotrophs often associated with mildly acidic habitats (Dedysh *et al.* 2000; Dedysh *et al.* 2015). Anaerobic methane oxidation is common in deep-sea sediments and other anoxic environments (Boetius *et al.* 2000; Teske, Dhillon and Sogin 2003; Cui *et al.* 2015), whereas aerobic methane oxidation dominates in terrestrial habitats. The aerobic methanotrophs can be classified into two main groups: *Gammaproteobacteria* methanotrophs and *Alphaproteobacteria* methanotrophs. The two groups are often referred to as the conventional type I and type II methanotrophs, respectively, based on phospholipid fatty acid (PLFA) composition, carbon assimilation pathways, intracellular membrane arrangement and phylogeny (Knief 2015). The two groups can exhibit distinct methane affinity and respond differently to environmental change (Nazaries *et al.* 2011).

The Qinghai-Tibetan Plateau (QTP) is the highest plateau in the world. As the 'third pole', it is sensitive to global climate change and can feedback greatly (Kutzbach, Prell and Ruddiman 1993). A large area of the QTP is covered by swamp meadow, which is a hotspot for both methanogenesis and methane oxidation (Zhao *et al.* 2013). The nitrogen deposition on the QTP is 8.7–13.8 kg N ha^{-1} $year^{-1}$ and could be affecting the biogeochemical cycles (Lu and Tian 2007). Studies have examined the composition of methanotroph communities in several wetlands on the QTP (Yun *et al.* 2012; Deng *et al.* 2013), but little is known about how methanotrophs in these soils respond to environmental variables such as nitrogen deposition. It is known from a wide range of reports that nitrogen deposition can induce changes in soil methane oxidation capacity. These can be either positive in N-limited soils (Bodelier and Laanbroek 2004) or, more often, negative in other soils including meadow soils such as those on the QTP (Gulledge *et al.* 2004; Jiang *et al.* 2010). Alongside these observations, the underlying mechanisms are not understood completely. The competing role of ammonia with methane in binding the MMO enzyme site, or the toxic effects of hydroxylamine or nitrite produced during ammonia oxidation by methanotrophs are the reasons often claimed to be the basis of inhibition (Holmes *et al.* 1995; Bodelier and Laanbroek 2004). It is also suggested that the variations in methanotroph community composition can affect the methane oxidation capacity or potential in soil (Gulledge *et al.* 2004; Carini *et al.* 2010). Some studies also revealed that the effect of nitrogen deposition on methane oxidation was dependent on the original methanotroph community. For example, in cold temperate forest soils, the addition of ammonium suppressed methane oxidation only when type II methanotrophs dominated (Mohanty *et al.* 2006). In addition, the effects of different N species (ammonium, nitrate or low-molecular organic-N) on the methanotroph communities were usually different and may depend on the ecosystem type. In paddy soil, the addition of ammonium suppressed type II but stimulated type I methanotrophs, while the addition of nitrate could stimulate both types of methanotrophs (Hu and Lu 2015). In another study of the rice rhizosphere, the addition of ammonium sulfate suppressed type I methanotrophs, while no effect on the methanotroph community was observed after the addition of urea (Shrestha *et al.* 2012).

Stable isotope probing (SIP) has been used in many ecological studies, and is well suited to study the metabolic activities of methanotrophs (McDonald, Radajewski and Murrell 2005). A direct link between methane-uptake activity and methanotroph taxonomy can be established by using SIP and analyzing DNA markers specific for methanotrophs (Radajewski *et al.* 2002; Knief, Lipski and Dunfield 2003; McDonald, Radajewski and Murrell 2005). For example, SIP was used to examine the response of active methanotrophs to urea addition in rice paddy soil, showing a stimulation of type I methanotrophs (Noll, Frenzel and Conrad 2008). Here we used a similar approach to investigate the response of active methanotrophs in the QTP swamp meadow soil to different forms of nitrogen. Given the importance of the QTP in regulating global climate change, it is important to understand how soil methanotrophs influence methane fluxes, and the effects of different N forms. The results delineate the specific effects of added nitrogen on soil methane oxidation, and most importantly, link these effects to specific active methanotrophic taxa. We hypothesized that different chemical forms of added nitrogen would result in different soil methane oxidation potentials, and cause differential effects on the active methanotrophic community.

MATERIALS AND METHODS

Soil sampling

On June 28, 2015, we took soil samples in an alpine marsh meadow near the Haibei Alpine Meadow Ecosystem Research Station (37°36′ N, 101°19′ E) of the Chinese Academy of Sciences. The soil type is swamp meadow soil, and the dominant plant species were *Koeleria tibetica* and *Blysmus sinocompressus*. The upper 0–15 cm soils were collected using a 7-cm diameter soil auger. Triplicate soil samples were pooled together aseptically in a clean plastic bag and taken immediately into the laboratory. After removing plant roots, stones and other large debris, basic soil properties were measured three times to get a mean value. Soil moisture was determined gravimetrically by drying at 105°C for 24 h, and the mean value was 47.38% (w/w) (SD, 0.23%); soil pH was determined using a pH meter (E20-FiveEasy™ pH, Mettler Toledo, Germany) in a 1:5 (fresh soil: deionized water, wt/vol) suspension after shaking for 30 min, and the mean value was 6.86 (SD, 0.05).

Incubation experiment

The methane-uptake incubation experiment was done in serum bottles sealed with butyl rubber stoppers. The sampled soil was first partly air-dried to have a soil moisture of 26% (w/w), and then 5 g of this partly air-dried soil was put into the serum bottles. This pre-drying procedure helped with the handling as

the original soil was too viscous. 0.85 ml of N-nutrient solution (final concentration 1.494 μM N g^{-1} dry soil) or pure water was added to the soil to get the identical soil moisture (47%) of the original soil. The added N content was comparable to the content of available nitrogen in the original soil. Four treatments were made by adding NH_4Cl (NH), $NaNO_3$ (NO), $CO(NH_2)_2$ (UR) and sterilized pure water (CK), respectively. Each treatment had three replicates. To confirm that the density of DNA in the SIP gradient was due to ^{13}C enrichment and not to G + C content, we also included a natural abundance methane (hereafter, referred to as ^{12}C-methane) incubation for each treatment (Neufeld *et al.* 2007). Two empty bottles with only methane were used to test the gas tightness of the serum bottles in the experimental system. Thus, there were a total of 26 serum bottles in this incubation experiment. A total of 6.25 ml of pure ^{13}C-methane or ^{12}C-methane was injected into the bottle to get a concentration of 5%, approaching an incubation of 0.0519 mM C g^{-1} dry soil. The bottles were kept in the dark at 20°C. The concentration of methane in the bottle was monitored. When the methane concentration got below 0.5% (i.e. 90% methane consumed), the bottles were reopened and flushed with air. The bottles were resealed with butyl rubber stoppers and 6.25-ml pure methane was added. This was done a total of four times to ensure that sufficient ^{13}C was integrated into the DNA of active methanotrophs for DNA-SIP (Radajewski *et al.* 2002; McDonald, Radajewski and Murrell 2005). After the incubation, all the soils in the bottles were collected and stored at −40°C.

DNA extraction and CsCl ultracentrifugation

Soil DNA was extracted from 0.5 g of soil from each incubation bottle using the FastDNA® SPIN Kit (MP Biomedicals, Santa Ana, CA) according to the manufacturer's protocol. The concentration of DNA was measured using a Nanodrop 1000 instrument (Thermo Scientific, Wilmington, Delaware), and the DNA sample with a 260/230 ratio over 1.7 was stored at −40°C before the downstream experiments.

For each DNA sample, the 'heavy' and 'light' DNA were resolved by isopycnic density gradient centrifugation in CsCl as described previously (Jia and Conrad 2009; Xia *et al.* 2011). Briefly, 2 μg of DNA was mixed with the GB buffer (100 mM Tris-HCl; 100 mM KCl; 1.0 mM EDTA, pH 8.0) and CsCl stock solution (Neufeld *et al.* 2007). The final buoyant density of this mixture is 1.725 g ml^{-1}. The mixture was then added to the centrifuge tube to a volume of 5.1 ml. After sealing, the tubes were centrifuged at a speed of 177 000 *g* at 20°C for 44 hours. After the centrifugation, the DNA solutions in the tubes were fractionated from bottom to top into 15 identical fractions using a peristaltic pump (NE-1000, New Era Pump Systems, Inc., Farmingdale, NY, USA). The peristaltic speed was set at 0.38 ml min^{-1}. Each gradient fraction was collected in a new sterile 2-ml tube. The refractive index of each fraction was measured using an AR200 digital refractometer (Reichert, Inc., Buffalo, NY, USA). The buoyant density (BD) of each fraction (Fig. S1, Supporting Information) was calculated as specified previously (Lueders, Manefield and Friedrich 2004). A total of 550-μl PEG6000 solution (Neufeld *et al.* 2007) was then added into the 2-ml tube containing fractioned DNA solution. The solutions were mixed by inverting the tubes several times and then kept at 25°C for 2 hours. The DNA was pelleted by centrifugation at 15°C and 13 000 *g* for 30 minutes. The supernatant was discarded. A total of 500 μl of 70% (v/v) ethanol was added to the tubes to rinse the DNA, followed by centrifugation at 10 000 *g* for 10 minutes. The supernatant was discarded and the ethanol rinse was repeated a second time. The DNA precipitate was then air-dried for 30 minutes. The DNA was dissolved in 30 μl of sterile water and stored at −40°C.

Representative fractions of the heavy and light DNA

To confirm and choose the representative fractions of heavy and light DNA, we did real-time quantitative PCR (qPCR) for DNA fractions 3 to 13. The fractions 1, 2, 14, and 15 were discarded because they usually contain very little DNA template. The primers A189F (GGNGACTGGGACTTCTGG) and mb661r (CCGGMGCAACGTCYTTACC) were used in the real-time PCR (Costello and Lidstrom 1999). The PCR conditions were 95°C for 3 minutes for pre-heating, 35 cycles of (95°C, 10 s; 55°C, 30 s; 72°C, 30 s), and a final elongation at 72°C for 8 minutes. The reactions were performed in a total volume of 20 μl with 10.0 μL SYBR Premix Ex Taq (Takara, Dalian), 0.5 μM primers and 1 μl of template DNA. The triplex qPCR assays were done on a CFX96 system (Bio-Rad Laboratories, Hercules, CA, USA). The standard curve was obtained by a 10-fold dilution series of plasmids containing the A189F/mb661r amplified *pmoA* gene fragments. The R^2 of all amplification curves of the real-time PCR ranged from 0.992 to 0.996. Based on the results of the real-time PCR (Fig. S2, Supporting Information), we chose fractions 7, 8 and mixed them to represent the heavy DNA fractions, and the fractions 11–12 and mixed them to represent the light DNA fractions.

Amplicon sequencing of the heavy and light DNA

For each heavy and light DNA from both ^{13}C-methane and ^{12}C-methane incubations, 16S rRNA gene amplicon sequencing was performed using primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACVSGGGTATCTAAT-3′) (Caporaso *et al.* 2011). The sequencing was done with MiSeq at Majorbio (Shanghai, China). For the heavy DNA from ^{13}C-methane incubations, the *pmoA* gene amplicon sequencing was done. The primers were chosen as A189F (GGNGACTGGGACTTCTGG) and mb661r (CCGGMGCAACGTCYTTACC) and the sequencing were performed with Roche 454 at Personalbio (Shanghai, China).

Bioinformatics of the sequences

The 16S rRNA gene reads were processed by the software Mothur 1.39.5 (Schloss *et al.* 2009) according to the online protocol (http://www.mothur.org/wiki/MiSeq_SOP). Briefly, the reads were first quality-filtered using the command 'trim.seqs'. Those reads with an average quality score less than 30 were discarded. Reads shorter than 200 bases or with any ambiguous base were removed with the command 'screen.seqs'. The reads were then further denoized with the commands 'pre.cluster' and 'uchime.chimera' sequentially. The high-quality sequences were classified with the command 'classify.seqs' against the RDP database with the method 'wang' and a cutoff of 60. The OTUs (97% similarity) were clustered with the command 'cluster' using the average distance method. The alpha and beta analyses based on the OTU table were done with the package 'vegan' in R.

The *pmoA* gene reads were first processed using the software mothur 1.39.5. The reads with an average quality score less than 25 or a length less than 350 bases were discarded. Chimeras were found with the command 'chimera.uchime' using the 'self' or the '*pmoA* gene database' (Dumont *et al.* 2014) as reference. Reads found to be chimeric with either of the two methods were discarded. The non-chimeric *pmoA* gene reads were then checked for frameshift errors using the 'FrameBot' tool (Wang

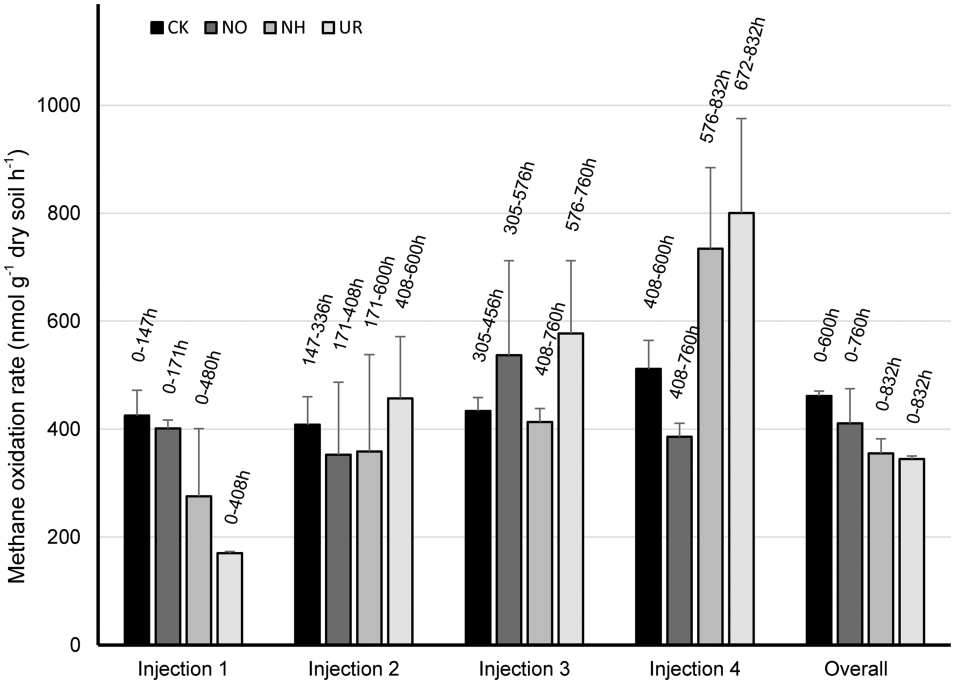

Figure 1. The methane oxidation rates in the serum bottles during the incubation. The mean values and their standard deviations of methane oxidation rates for each treatment are shown. The time above the bar is the maximum of the time lag for the specific injection taking all three replicates together. CK, no N added control; NO, nitrate-N added treatment; NH, ammonia-N added treatment; UR, urea-N added treatment.

et al. 2013). The clean reads were classified against a mothur-formatted database of *pmoA* genes (Dumont *et al*. 2014). OTUs of methanotrophs were clustered using the command 'cluster' in mothur. Those sequences with a similarity value of 86% were clustered together (Wen, Yang and Liebner 2016). Representative sequences of OTUs were combined with *pmoA* sequences from known methanotroph genera. Their translated amino acid sequences were used to construct a phylogenetic tree using the software 'MEGA7' software with the 'Neighbor-Joining' method (Kumar, Stecher and Tamura 2016).The optimal tree with a sum of branch lengths equal to 3.09 was chosen. The branch lengths in the tree are proportional to the evolutionary distances, which were computed using the Poisson correction method (Zuckerkandl and Pauling 1965) and correspond to the number of amino acid substitutions per site. The analysis involved 60 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 117 positions for each sequence in the final dataset. In addition, the clean reads were blast-aligned against the *pmoA* gene database. The blast scores were then transferred to the software MEGAN4 (Huson *et al*. 2011) and all reads classified with the 'LCA' method.

Statistical analyses

The differences in the relative percentages of taxonomic groups among treatments were tested using ANOVA. The pairwise comparisons were done by Tukey HSD tests where necessary. The grouping effects of incubation systems or heavy-light fractions on the phyla compositions were tested with the 'adonis' function in the 'vegan' package in R 3.3.2 (R Core Team 2016). LEfSe analyses were done to identify the OTUs specific to the heavy fractions, the light fractions, or the N-form treatments with the command 'LEfSe' implemented in Mothur 1.39.5 (Schloss *et al*. 2009).

DNA Accession numbers

The *pmoA* gene and 16S rRNA gene sequences obtained in this study were stored in the Bioproject archive in NCBI (https://www.ncbi.nlm.nih.gov/bioproject/) under the accession numbers PRJNA503078 and PRJNA503100, respectively.

RESULTS

Methane oxidation potentials in incubation systems

The soil methane oxidation potentials were calculated from the methane concentrations in serum bottles (Fig. 1; Fig. S3, Supporting Information). The oxidation potentials were determined as the average values in the respective injections. In general, the soil in the CK treatment had the highest methane oxidation potential, followed by NO, NH and UR. This difference was most pronounced when calculated from the first injection. The mean oxidation potential was 425.1 nmol g^{-1} dry soil h^{-1}, 401.5 nmol g^{-1} dry soil h^{-1}, 275.5 nmol g^{-1} dry soil h^{-1} and 170.1 nmol g^{-1} dry soil h^{-1} for CK, NO, NH and UR treatments, respectively (Fig. 1; Fig. S3, Supporting Information). In general, the methane oxidation rates increased with elapsed incubation time for all the treatments. The methane oxidation potentials calculated from the latter injections (especially the 3rd and 4th injections for NH and UR treatments) had increased about 2–3 fold (Fig. 1). Due to the relatively low methane uptake rates at the early stage, the 3rd or 4th injections in the NH and UR treatments corresponded to nearly 600 hours after the start of the incubation, which might have induced methanotroph populations with high rates of methane oxidation. Because of the earlier termination of incubations for CK and NO treatments, we could not directly compare the methane oxidation rates in the four treatments at the same period.

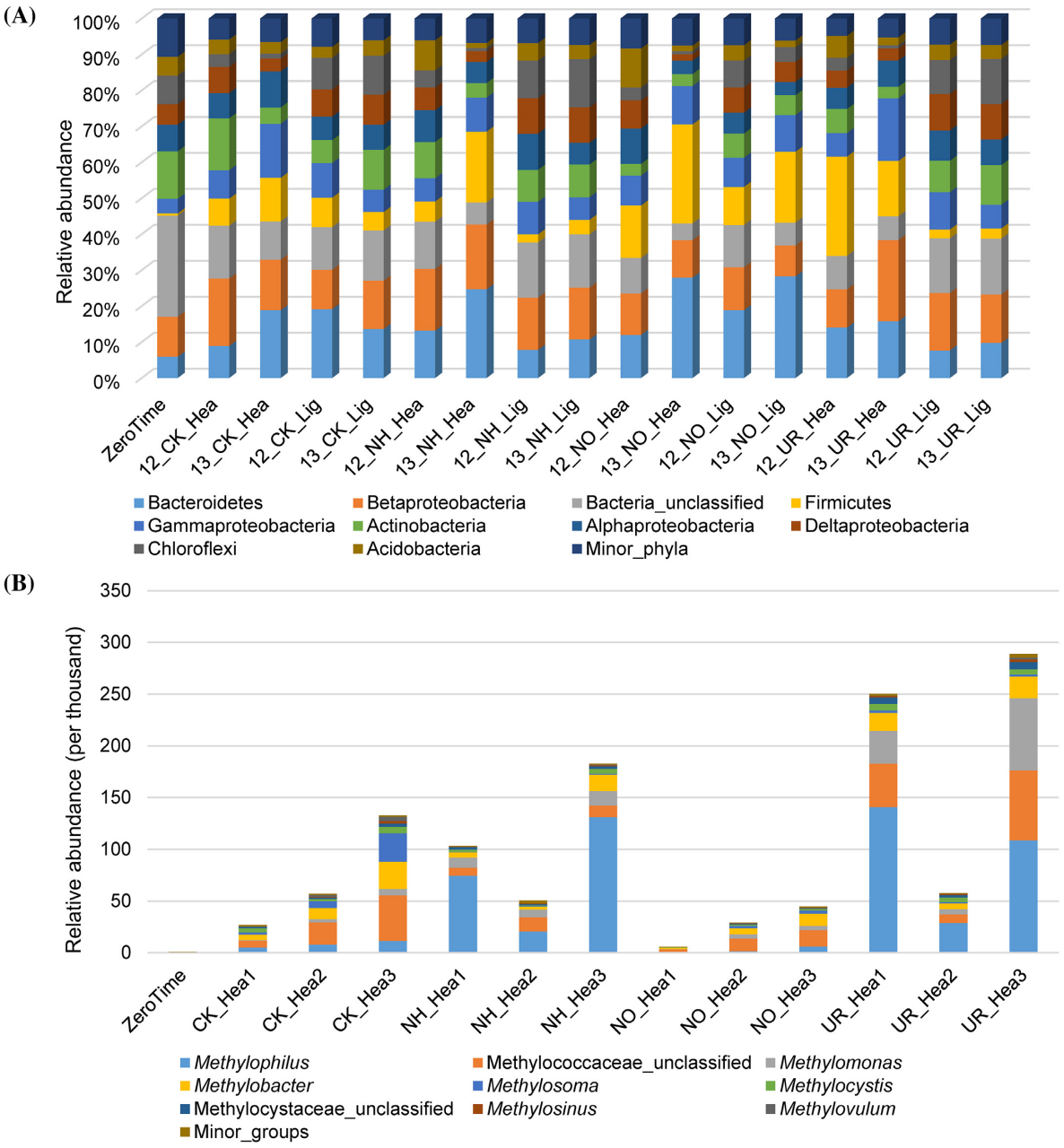

Figure 2. The results of 16S rRNA gene analyses of the DNA fractions. (a), Relative abundances (%) of main phyla/subphyla in heavy (Hea) and light (Lig) DNA fractions in both $^{13}CH_4$ (13.) and $^{12}CH_4$ (12.) incubation systems. (b), Relative abundances (per thousand) of the genera with a prefix of 'methylo' in heavy DNA fractions. The meanings of abbreviations of treatments (CK, NO, NH, UR) can be found in the caption of Fig. 1. ZeroTime means before the incubation start.

DNA stable-isotope probing

The *pmoA* gene abundance in each CsCl gradient fraction was quantified (Fig. S2, Supporting Information). Fractions 1, 2, 14 and 15 usually contained very little DNA, so were excluded from the downstream analysis. We also performed real-time quantitative PCR of *pmoA* genes from all fractions to confirm that methanotrophs were labelled. The highest gene copy number came from fractions 7–8 for most ^{13}C-methane incubation samples (range from 4.95×10^5 to 8.35×10^5 copies μl^{-1}), and from fractions 11–12 for all ^{12}C-methane incubation samples (ranging from 1.05×10^5 to 2.32×10^5 copies μl^{-1}) (Fig. S2, Supporting Information). These results confirm that we had successful separation of DNA as a function of ^{13}C content.

16S rRNA gene analysis

We compared the 16S rRNA gene analysis results in both ^{13}C-methane and ^{12}C-methane incubations. There were significant differences in the phyla compositions between the heavy and light fractions for the ^{13}C-methane (adonis, $P < 0.001$), but not for the ^{12}C-methane control incubation (Fig. S4, Table S1, Supporting Information). Generally, *Firmicutes* were more abundant in heavy fractions, and *Chloroflexi* were more abundant in light fractions. There were also significant differences in phylum compositions between the heavy fractions from the ^{13}C-methane and ^{12}C-methane incubations (adonis, $P < 0.01$) (Fig. 2; Fig. S4, Table S1, Supporting). These results proved that the separation of heavy and light DNA fractions from the ^{13}C-methane

incubation were primarily the result of ^{13}C-methane incorporation and not G+C content of the DNA. In the latter analyses we focused on the heavy fractions from the ^{13}C-methane systems. The LEfSe analysis showed that the 12 OTUs specific to heavy fractions mainly came from Gammaproteobacteria (five OTUs), Alphaprotebacteria (three OTUs), Betaproteobacteria (two OTUs) and Firmicutes (one OTU). Most of these OTUs are from known methylotrophs, such as *Methylophilus*, Methylococcaceae and *Methylocystis* (Table S2, Supporting Information). Alphaproteobacteria and Betaproteobacteria had different relative abundances among the four treatments (ANOVA, $P < 0.05$). Specifically, for Alphaproteobacteria, CK had the highest values (mean, 9.90%) versus NO with the lowest value (3.70%); for Betaproteobacteria, UR had the highest values (mean, 22.35%) versus NO with the lowest value (10.45%) (Tukey HSD tests, $P < 0.05$). The LEfSe analysis of the heavy fractions indicated that the UR and CK treatments had the largest number of specific OTUs (five and six OTUs, respectively). OTUs from *Methylophilus*, *Methylocystis* and Methylococcaceae were the main OTUs specific to the UR treatment. OTUs from Bacteroides, Acidobacteria and Clostridiales were specific to the NO treatment. Only one OTU from *Porphyromonas* (Bacteroidetes) was specific to the NH treatment (Table S3, Supporting Information). In addition, we specially extracted the genera with the prefix of 'methy' from the taxonomy information. These genera represent bacteria known for their function in metabolism of methane or methylated-compounds. The relative abundance of genus *Methylophilus* was particularly high in UR (mean, 9.28%) and NH (7.54%) treatments. Unclassified Methylocystaceae were significantly more abundant in UR treatment (5.40%) compared with the other three treatments (Tukey HSD tests, $P < 0.1$).

Analysis of *pmoA* genes

We used two methods to classify the *pmoA* gene sequences, the naïve Bayes method implemented in mothur software and the Megan LCA methods based on blast results (Fig. 3, Table 1). The two methods showed very similar results for the classifications. Before incubation, *Methylocystis* was dominant (relative abundance, >70%), but decreased sharply after incubation in CK, NO (final abundances <3%), and relatively mildly for NH and UR (final abundances >15%). In contrast, *Methylomonas* increased sharply after incubation for all treatments, but especially for CK, NO and UR (relative abundances >40%). The RPC_1 and FWs clades also showed high relative abundance at the start of the incubation, but decreased in all treatments after incubation. The UR and NH treatments had relatively higher diversity of methanotrophs; for example, the clades *Methylosinus*, RPC_1, JRC_3 and LP20 had proportions >1% in UR treatment; and the clades LP20 and aquifer_cluster had proportions >1% in NH treatment. The mean *pmoA* gene OTU number was 22.0 (SD, 5.1) in the UR treatment, 23.3 (SD, 3.3) in the NH treatment, 5.7 (SD, 1.0) in the CK treatment and 7.7 (SD, 0.9) in the NO treatment. The OTU number in the sample at time zero was 29.

Phylogenetic analysis of *pmoA* gene derived amino acid sequences

The representative sequences for the top 25 OTUs (making up >95% of sequences) were used to construct a neighbor-joining phylogenetic tree (Fig. 4). The tree was built based on the translated amino acids of the *pmoA* gene sequences. Most of the OTUs were affiliated with the type I methanotrophs. OTU1 (8842 sequences) is related to *Methylomonas*. These sequences were highest in CK and NO treatments, and were not detected at time zero. OTU2 (3796 sequences) is related to *Methylobacter*, which distributed relatively evenly in the four treatments, but were scarce at time zero. OTU3 (2998 sequences) belongs to the taxon *Methylocystis*, which made up the most part of time zero sequences, a moderate amount from the NH and UR treatments, and a tiny fraction of the CK and NO treatments. There were five OTUs (OTU7, OTU10, OTU22, OTU23 and OTU24) only occurring in the UR and/or NH treatments; OTU22 has relatively distant relationships with known methanotrophs and might represent unknown methanotrophs in the QTP soils that can be enriched by the addition of ammonia. Three OTUs (OTU5, OTU11 and OTU17) from type Ib methanotrophs occurred in samples from time zero, UR and/or NH treatments.

DISCUSSION

The inhibitory effect of nitrate-N on methane oxidation was weaker than ammonia-N and urea-N (Fig. 1; Fig. S3, Supporting Information), consistent with previous studies where nitrate-N had no or less effect than ammonia-N on the uptake of methane in forest, grassland or paddy soils (Crill *et al.* 1994; Hütsch, Webster and Powlson 1994; Zhang, Wang and He 2012). The reason may lie in the competitive inhibition of ammonia on the methane monooxygenase enzyme (Le Mer and Roger 2001), or general inhibitory effects of Cl^- (King and Schnell 1998), which is the counter ion of NH_4^+ in the NH treatment. In alpine meadow or temperate forest, where the nitrogen was limited in soil, the added nitrate could stimulate the uptake of methane (Jang *et al.* 2011; Fang *et al.* 2014). The effect of added nitrogen on methane uptake can also vary depending on the concentrations of methane and the soil type (King and Schnell 1994). Nitrate inversely inhibited methane uptake in a temperate forest soil when the methane concentration was higher than 300 ppmv (Jang *et al.* 2011). Other studies have shown that ammonia had no or promoting effect on methane consumption in high concentrations of methane (>1000 ppmv) (Mohanty *et al.* 2006), and the inhibitory effects of ammonia only occurred with high amounts of fertilizers (e.g. >5 μM N g^{-1} dry soil) (Alam and Jia 2012). In contrast, the inhibition of low-concentration methane (e.g. atmospheric methane) uptake could be incurred by relatively small amounts of fertilizer (Mosier *et al.* 1991; Adamsen and King 1993). In our incubation system, the concentration of methane was high (5% methane), and the addition of nitrogen (~1.5 μM N g^{-1} dry soil) for all three forms (ammonia, nitrate and urea) inhibited methane uptake, especially at the early stage of incubations (e.g. before 408 hours).

In general, the methane uptake rates increased over the course of the incubations for all treatments (Fig. 1, the latter injections). The methane oxidation rate was often observed as a function of incubation time, which were low initially with an extended lag, then increased to a higher level until the nutrients or methane were depleted (Mor *et al.* 2006). Similar patterns were observed for soils (Kightley, Nedwell and Cooper 1995; Hilger, Cranford and Barlaz 2000) and composts (Wilshusen, Hettiaratchi and Stein 2004). The stimulation of methane oxidation under elevated concentrations of methane has been observed for pure cultures (King and Schnell 1994; Dunfield and Conrad 2000) and in soil (Bender and Conrad 1992; Cai *et al.* 2016). In a field study, the long term (8 year) exposure to high methane concentrations from a landfill resulted in a relatively high methane oxidation rate (437.5 nmol g^{-1} soil h^{-1}) (Tate, Walcroft and Pratt

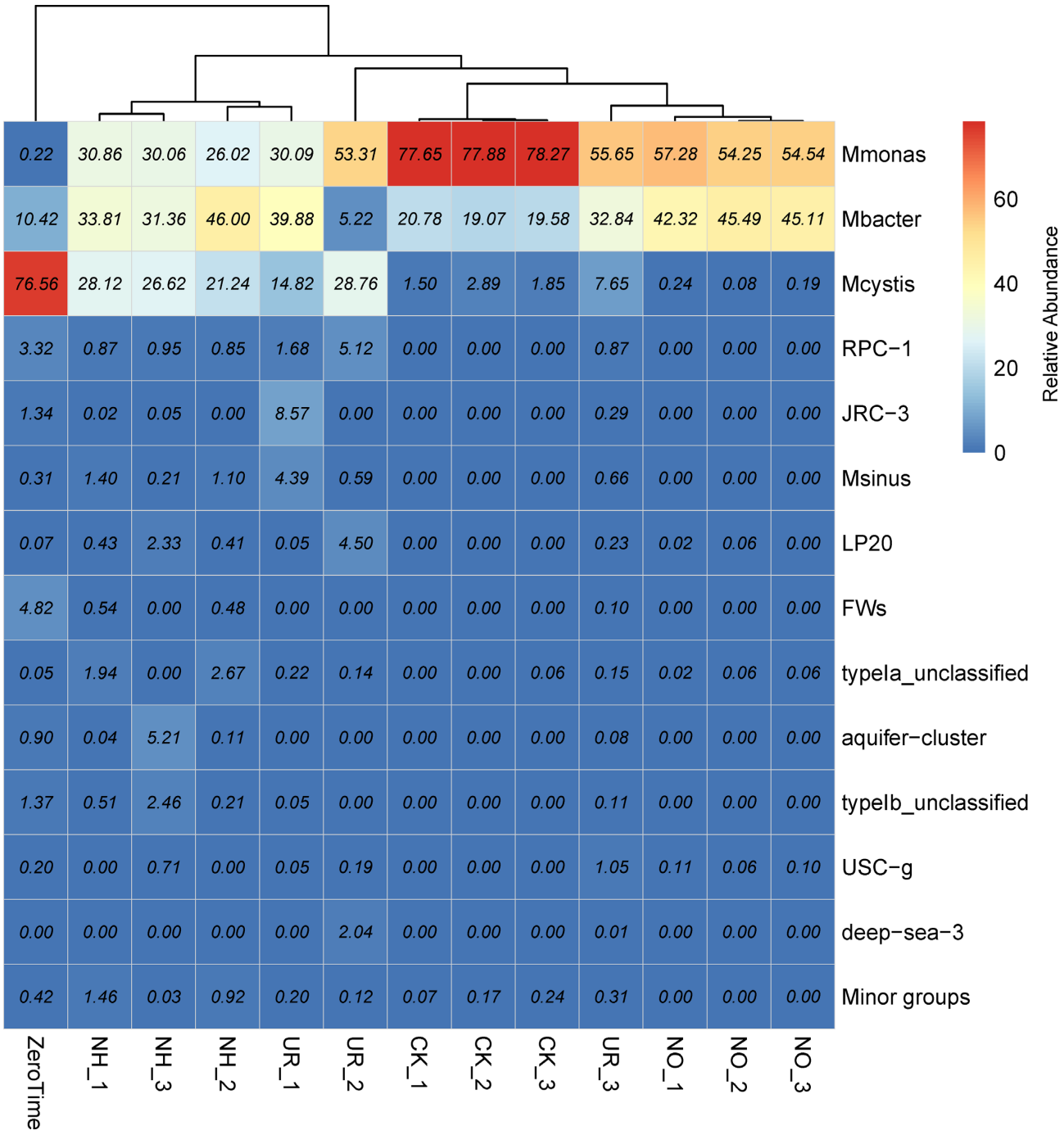

Figure 3. Heatmap of the main methanotroph taxa classified by mothur. The relative abundances (%) are indicated in the cells. The meanings of abbreviations of treatments (CK, NO, NH, UR) can be found in the caption of Fig. 1. ZeroTime means before the incubation start.

2012). In addition, the negative effects of ammonia in our experiment might have ceased after the utilization or transformation of ammonia during the incubation. It was also reported that ammonia could promote methane oxidation at high concentration in soils originating from landfill cover (De Visscher and Cleemput 2003).

Urea can be readily transformed to ammonia by the urease enzyme. Urease activity in meadow soil on the QTP was reported to be 10–20 μg NH_3-N g^{-1} dry soil 24 h^{-1} (Suo *et al.* 2012; Li and He 2014); accordingly, all the added urea in the incubation system (44.9 μg urea-N g^{-1} dry soil) might have been converted to ammonia in about 2–4 days (corresponding to the first methane injection, Fig. S3, Supporting Information) in the incubation system (44.9 μg urea-N g^{-1} dry soil). The transformation from urea to ammonia also partly explain why the two treatments UR and NH had similar effects on the methanotroph communities. However, there might still exist some mechanism by which urea itself directly or indirectly influences the methane uptake in the incubation, because the UR treatment had the strongest inhibitory effect on the methane uptake after the first methane addition (Fig. 1). The initial changes in the community immediately after the addition of urea might have provided clues to the

Table 1. The relative abundance (%) of taxa classified by Megan using the LCA method based on the 'blast' results in each treatment. Mean values are shown with the SD values followed in parenthesis. CK, no N added control; NO, nitrate-N added treatment; NH, ammonia-N added treatment; UR, urea-N added treatment; ZeroTime means before the incubation start. The character 'M' in the first column is the abbreviation of 'Methylo'.

	ZeroTime	CK	NH	NO	UR
Mbacter	10.18	19.72 (0.81)	39.06 (9.32)	44.26 (1.71)	25.78 (18.01)
Mmonas	0.21	77.52 (0.61)	28.89 (2.55)	55.32 (1.66)	45.92 (14.29)
Msarcina	0.16	0	0.22 (0.21)	0	0.08 (0.12)
LP20	0.07	0	1.05 (1.09)	0.03 (0.03)	1.59 (2.51)
Aquifer cluster	0.88	0	1.77 (2.94)	0	0.03 (0.05)
RPC_1	4.39	0	1.88 (1.31)	0	2.57 (2.21)
JRC_3	1.3	0	0.02 (0.03)	0	2.89 (4.76)
FWs	4.67	0	0.34 (0.30)	0	0.03 (0.06)
USCγ	0.19	0	0.24 (0.41)	0.09 (0.03)	0.43 (0.54)
USCα	0	0	0.01 (0.02)	0	0.04 (0.06)
Mcystis	74.74	2.07 (0.72)	25.26 (3.59)	0.17 (0.08)	16.91 (10.70)
Msinus	0.3	0	0.9 (0.62)	0	1.87 (2.10)
Minor groups	0.21	0.16 (0.09)	0.04 (0.07)	0	0.75 (1.16)
no hits	2.69	0.52 (0.39)	0.31 (0.46)	0.13 (0.09)	1.09 (0.84)

mechanism by which urea suppressed methane oxidation at the early stages, but we only analyzed the community at the beginning and end of the experiment.

We used three methods (Bayes classifier, Megan LCA and a phylogenetic tree of amino acid sequences) to classify *pmoA* sequences detected in this study. All methods showed very similar results. The main taxa found in this study, *Methylocystis*, *Methylobacter*, *Methylomonas*, *Methylosinus*, RPC and LP20 were also detected in wetlands on the QTP in earlier studies (Yun *et al.* 2012; Deng *et al.* 2013), and to the best of our knowledge represent the principle methanotrophs responsible for methane uptake in wetland or swamp meadow soils on the QTP. The type II methanotrophs *Methylocystis* were most abundant at time zero. In another study, *Methylocystis* were also found to be most abundant in soils in a natural wetland on the QTP (Deng *et al.* 2013). The dominance of type II methanotrophs (i.e. *Methylocystis*) at low methane concentrations could be a selective advantage in natural wetlands where growth is periodically restricted by the fluctuations in the methane supply (Baani and Liesack 2008). Indeed, in the natural habitat in our studied site, where there is periodic flooding and drying of soil in the swamp meadow, may result in the fluctuations of methane from low to high concentrations. The type I methanotrophs (*Methylobacter*, *Methylomonas*) increased during the incubations with 5% methane in the headspace, while the dominance of *Methylocystis* decreased for all treatments (especially in CK and NO) (Fig. 3). This high concentration of methane was added to ensure sufficient labelling of bacterial DNA by ^{13}C, as has been used in many DNA-SIP studies (McDonald, Radajewski and Murrell 2005). Methane concentration has its own effect on methanotroph community composition. Studies have reported that the type II methanotrophs (e.g. *Methylocystis*) are less competitive than type I methanotrophs at high concentrations of methane (Knief and Dunfield 2005; Knief *et al.* 2006). It is possible that the methane concentration in our SIP study might have masked, at least partially, the effects of different N treatments. Nonetheless, there are cases where type I methanotrophs did not dominate the type II methanotrophs at high methane concentrations (Henckel, Roslev and Conrad 2000; Macalady *et al.* 2010). Studies also reported that added N fertilizer favors the competitiveness of type I methanotrophs, even at high methane concentration (Bodelier *et al.* 2000; Noll, Frenzel and Conrad 2008). According to our results, there appear to be specific effects on different genera of methanotrophs within the same family. For example, nitrate favored *Methylobacter* over *Methylomonas*, which are both type I methanotrophs in the family Methylococcaceae. Ammonia and urea might also increase the competitiveness of *Methylocystis*, which lost their dominance in the CK and NO treatments (Fig. 3).

The effects of urea-N and ammonia-N on the methanotroph communities were similar, while nitrate-N had less effect. In the control, where the soil methane oxidation rate was highest, there was a particularly high abundance of *Methylomonas*, agreeing with a previous study where *Methylomonas* correlated with high methane oxidation activity (Shrestha *et al.* 2010). In comparison with the control, we found that in addition to *Methylomonas*, the added N also favored *Methylobacter*, which had a high relative abundance in all N treatments. In landfill biocover and paddy soil, ammonia or urea additions were also found to cause an increase in the abundances of *Methylobacter*, which corresponds to the high level of methane oxidation rate (Zheng *et al.* 2014; Wei *et al.* 2016). This was also observed in the UR and NH treatments towards the end of the incubation. Our results also indicated that nitrate might be preferable to ammonia and urea for the selection of *Methylobacter* (Fig. 3). In addition, the effect of ammonia or urea could lessen the decrease in relative abundance in *Methylocystis* typically observed under high methane, since in these two treatments, the relative abundance of *Methylocystis* remained at relatively high levels (Fig. 3, Table 1).

The inhibition of methanotrophs by ammonia has been reported for specific species and strains (King and Schnell 1994; Nyerges and Stein 2009). With respect to the N nutrition of methanotroph communities, ammonia might be preferable to nitrate in the swamp meadow soil since it supported a more diverse methanotroph community (Fig. 4, Table 1). The taxa USCγ and USCα, associated with atmospheric methane uptake and seldom detected in wetlands on the QTP (Deng *et al.* 2013), were mainly detected after the UR and NH treatments. The nutrition of methanotrophs with ammonia was discussed previously, concluding that at high levels of methane it acts more for nutrition and less as an inhibitor for some methanotrophs (Stein and Klotz 2011).

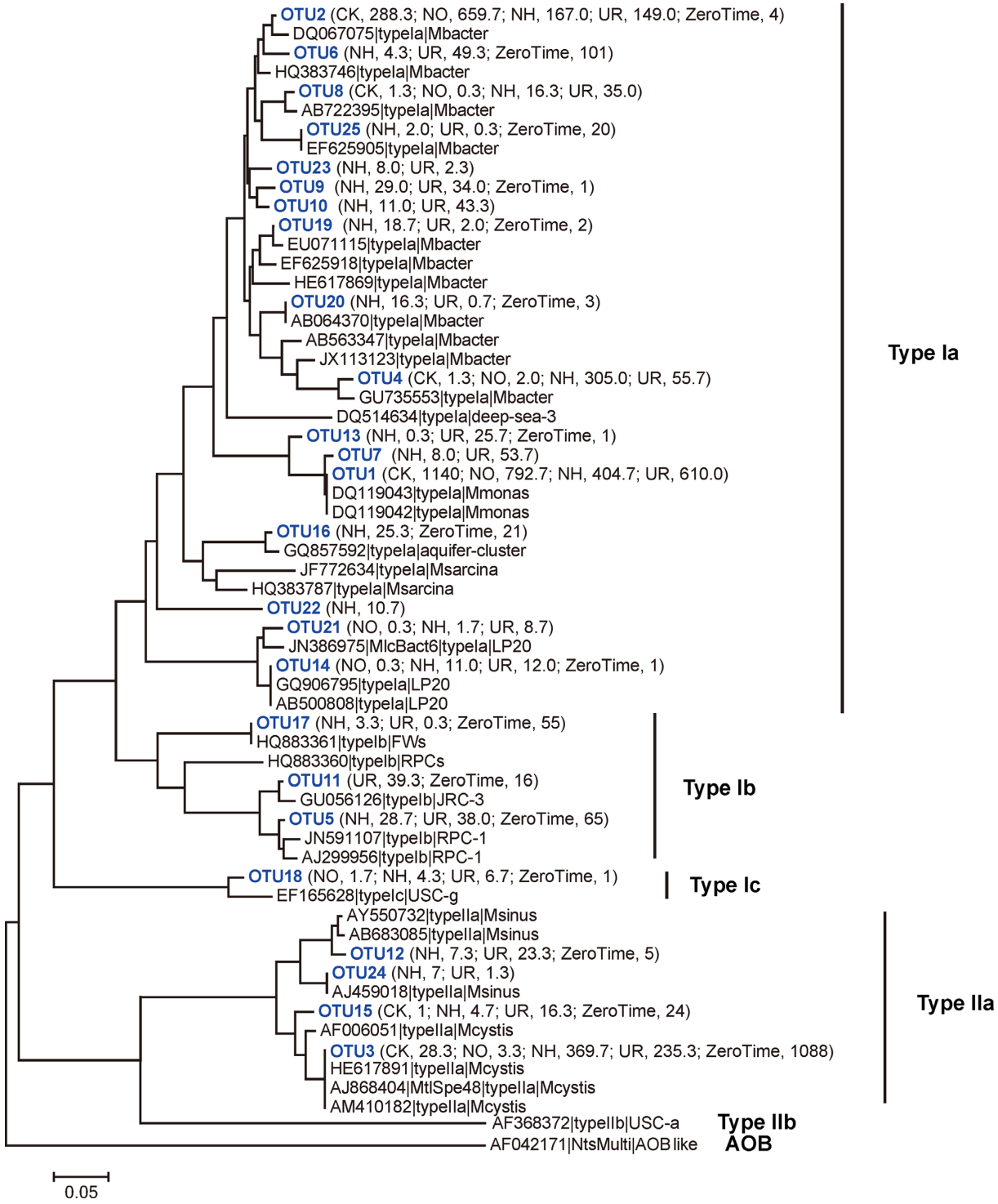

Figure 4. Molecular phylogenetic analysis by Neighbor-Joining method of the amino acid sequences translated from the representative *pmoA* gene sequences of the top 25 OTUs. For each OTU, the average number of sequences (n = 3) in each treatment was shown in parenthesis. The OTUs occurring only in the UR and/or NH treatments were underlined. The meanings of abbreviations of treatments (CK, NO, NH, UR) can be found in the caption of Fig. 1. ZeroTime means before the incubation start.

The 16S rRNA gene analysis identified non-methanotrophs in the heavy DNA fractions, such as Firmicutes and Betaproteobacteria, which might incorporate the ^{13}C into their DNA by cross-feeding carbon from methanotrophs. Cross-feeding has often been reported in SIP studies and can reflect the labelled carbon transferring within the microbial food chain. In another SIP study, Betaproteobacteria were also detected from heavy DNA fractions (Hutchens *et al.* 2004). Bacteria from Betaproteobacteria and Firmicutes were found to be able to metabolize one-carbon compounds, thus prone to the assimilation of metabolites such as methanol or formaldehyde synthesized by methanotrophs (Beck *et al.* 2013; Krause *et al.* 2017). The methylotrophs, including methanotrophs, usually make up less than 1% of the microbial community in natural environments, but were found

elevated to proportions of 3%–25% in total bacterial communities after incubation. This elevation was also found in another incubation study (Zheng *et al*. 2014). Because of the dietary links, the interactions between methylotrophs might be ubiquitous and strong. These interactions could also vary in different treatments. For example, in the UR and NH treatments, the proportion of methylotrophs were elevated to a higher extent than in CK and NO treatments (Fig. 2). The OTUs affiliated in *Methylophilus* and Methylococcaceae were abundant and specific to the UR treatment, while OTUs from the non-methylotrophs, such as Bacteroides and Acidobacteria, were specific to the NO treatment (Fig. 2; Table S3, Supporting Information). Different N-fertilizers could have different effects on the interactions of methanotrophs with other heterotrophs, and thus have varying influences on the methanotrophic activity of the whole system (Ho *et al*. 2014).

In summary, we used SIP technology to link the activity of methanotrophs with their phylogeny and the methane uptake rate in soils retrieved from a swamp meadow on the QTP. We found that additions of all nitrogen forms (ammonia-N, urea-N and nitrate-N) suppressed methane oxidation, with the strongest effect in the urea treatment at the onset of incubation. Urea-N and ammonia-N had similar effects on methane uptake and shaping the methanotroph communities, which both resulted in methanotroph communities with higher diversity (including type Ia, type 1b, and also type II methanotrophs, with relative abundances over 1%). Nitrate-N had less effect on methane uptake than urea-N and ammonia-N, and favored the type Ia *Methylomonas* and *Methylobacter* genera. There were also differential effects of N forms on the cross-feeding or interactions of other methylotrophs and non-methylotrophs. Our results suggested that urea-N in particular might have profound effects on methanotroph communities and activities in swamp meadow soils on the QTP.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

ACKNOWLEDGEMENTS

We thank Ms Hongfei Wang, Dr. Chao Wang, Dr. Cheng Han and Dr. Junji Yuan for their helps in sampling and laboratory work. This study was funded by the National Program on Key Basic Research Project (973 Program, 2014CB954002), the Strategic Priority Research Program (XDB15010101) of Chinese Academy of Sciences, the Basic work of science and technology of Ministry of Science and Technology of China (2015FY110100) and the China Postdoctoral Science Foundation (2015M571834). .

Conflicts of interest. None declared.

REFERENCES

Adamsen APS, King GM. Methane consumption in temperate and subarctic forest soils: rates, vertical zonation, and response to water and nitrogen. *Appl Environ Microbiol* 1993;**59**:485–90.

Alam MS, Jia Z. Inhibition of methane oxidation by nitrogenous fertilizers in a paddy soil. *Front Microbiol* 2012;**3**:00246.

Baani M, Liesack W. Two isozymes of particulate methane monooxygenase with different methane oxidation kinetics are found in *Methylocystis* sp. strain SC2. *Proc Natl Acad Sci USA* 2008;**105**:10203–08.

Beck DAC, Kalyuzhnaya MG, Malfatti S *et al*. A metagenomic insight into freshwater methane–utilizing communities and evidence for cooperation between the *Methylococcaceae* and the *Methylophilaceae*. *Peer J* 2013;**1**:e23.

Bender M, Conrad R. Kinetics of CH_4 oxidation in oxic soils exposed to ambient air or high CH_4 mixing ratios. *FEMS Microbiol Ecol* 1992;**101**:261–70.

Bodelier PLE, Laanbroek HJM. Nitrogen as a regulatory factor of methane oxidation in soils and sediments. *FEMS Microbiol Ecol* 2004;**47**:265–77.

Bodelier PLE, Roslev P, Henckel T *et al*. Stimulation by ammonium–based fertilizers of methane oxidation in soil around rice roots. *Nature* 2000;**403**:421–24.

Boetius A, Ravenschlag K, Schubert CJ *et al*. A marine microbial consortium apparently mediating anaerobic oxidationof methane. *Nature* 2000;**407**:623–26.

Cai Y, Yan Z, Bodelier PLE *et al*. Conventional methanotrophs are responsible for atmospheric methane oxidation in paddy soils. *Nat Commun* 2016;**7**:11728.

Caporaso JG, Lauber CL, Walters WA *et al*. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci USA* 2011;**108**:4516–22.

Carini S, Bano N, Lecleir G *et al*. Aerobic methane oxidation and methanotroph community composition during seasonal stratification in Mono Lake, California (USA). *Environ Microbiol* 2010;**7**:1127–38.

Costello AM, Lidstrom ME. Molecular characterization of functional and phylogenetic genes from natural populations of methanotrophs in lake sediments. *Appl Environ Microbiol* 1999;**65**:5066–74.

Crill PM, Martikainen PJ, Nyka″nen H *et al*. Temperature and N fertilization effects on methane oxidation in a drained peatland soil. *Soil Biol Biochem* 1994;**26**:1331–39.

Cui M, Ma A, Qi H *et al*. Anaerobic oxidation of methane: an "active" microbial process. *MicrobiologyOpen* 2015;**4**:1–11.

De Visscher A, Cleemput OV. Induction of enhanced CH_4 oxidation in soils: NH_4^+ inhibition patterns. *Soil Biol Biochem* 2003;**35**:907–13.

Dedysh SN, Liesack W, Khmelenina VN *et al*. *Methylocella palustris* gen. nov., sp. nov., a new methane-oxidizing acidophilic bacterium from peat bogs, representing a novel subtype of serine-pathway methanotrophs. *Int J Syst Evol Microbiol* 2000;**50**:955–69.

Dedysh SN, Naumoff DG, Vorobev AV *et al*. Draft genome sequence of *Methyloferula stellata* AR4, an obligate methanotroph possessing only a soluble methane monooxygenase. *Genome Announc* 2015;**3**:e01555–14.

Deng Y, Cui X, Lüke C *et al*. Aerobic methanotroph diversity in Riganqiao peatlands on the Qinghai-Tibetan plateau. *Environ Microbiol Rep* 2013;**5**:566–74.

Dumont MG, Lüke C, Deng Y *et al*. Classification of *pmoA* amplicon pyrosequences using BLAST and the lowest common ancestor method in MEGAN. *Front Microbiol* 2014;**5**:77–79.

Dunfield PF, Conrad R. Starvation alters the apparent half-saturation constant for methane in the type II methanotroph *methylocystis* strain LR1. *Appl Environ Microbiol* 2000;**66**:4136–38.

Fang H, Cheng S, Yu G *et al*. Low-level nitrogen deposition significantly inhibits methane uptake from an alpine meadow soil on the Qinghai–Tibetan plateau. *Geoderma* 2014;**213**:444–52.

Gulledge J, Hrywna Y, Cavanaugh C *et al*. Effects of long-term nitrogen fertilization on the uptake kinetics of atmospheric methane in temperate forest soils. *FEMS Microbiol Ecol* 2004;**49**:389–400.

Hütsch BW, Webster CP, Powlson DS. Methane oxidation in soil as affected by land use, soil pH and N fertilization. *Soil Biol Biochem* 1994;**26**:1613–22.

Hanson RS, Hanson TE. Methanotrophic bacteria. *Microbiology Reviews* 1996;**60**:439–71.

Hartmann DL, Klein Tank AMG, Rusticucci M *et al*. Observations: atmosphere and surface. In: Stocker TF, Qin D, Plattner GK *et al*.(ed). *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge, United Kingdom: Cambridge University Press, 2013, 159–254.

Henckel T, Roslev P, Conrad R. Effects of O_2 and CH_4 on presence and activity of the indigenous methanotrophic community in rice field soil. *Environ Microbiol* 2000;**2**:666–79.

Hilger HA, Cranford DF, Barlaz MA. Methane oxidation and microbial exopolymer production in landfill cover soil. *Soil Biol Biochem* 2000;**32**:457–67.

Ho A, Roy KD, Thas O *et al*. The more, the merrier: heterotroph richness stimulates methanotrophic activity. *ISME J* 2014;**8**:1945–48.

Holmes AJ, Costello A, Lidstrom ME *et al*. Evidence that participate methane monooxygenase and ammonia monooxygenase may be evolutionarily related. *FEMS Microbiol Lett* 1995;**132**:203–08.

Hu A, Lu Y. The differential effects of ammonium and nitrate on methanotrophs in rice field soil. *Soil Biol Biochem* 2015;**85**:31–38.

Huson DH, Mitra S, Weber N *et al*. Integrative analysis of environmental sequences using MEGAN4. *Genome Res* 2011;**21**:1552–60.

Hutchens E, Radajewski S, Dumont MG *et al*. Analysis of methanotrophic bacteria in Movile Cave by stable isotope probing. *Environ Microbiol* 2004;**6**:111–20.

Jang I, Lee S, Zoh KD *et al*. Methane concentrations and methanotrophic community structure influence the response of soil methane oxidation to nitrogen content in a temperate forest. *Soil Biol Biochem* 2011;**43**:620–7.

Jia ZJ, Conrad R. Bacteria rather than archaea dominate microbial ammonia oxidation in an agricultural soil. *Environ Microbiol* 2009;**11**:1658–71.

Jiang C, Yu G, Fang H *et al*. Short-term effect of increasing nitrogen deposition on CO_2, CH_4 and N_2O fluxes in an alpine meadow on the Qinghai-Tibetan plateau, China. *Atmos Environ* 2010;**44**:2920–26.

King GM, Schnell S. Ammonium and nitrite inhibition of methane oxidation by *Methylobacter albus* BG8 and *Methylosinus trichosporium* OB3b at low methane concentrations. *Appl Environ Microbiol* 1994;**60**:3508–13.

King GM, Schnell S. Effects of ammonium and non-ammonium salt additions on methane oxidation by *Methylosinus trichosporium* OB3b and Maine forest soils. *Appl Environ Microbiol* 1998;**64**:253–57.

Kightley D, Nedwell DB, Cooper M. Capacity for methane oxidation in landfill cover soils measured in laboratory scale microcosms. *Appl Environ Microbiol* 1995;**61**:592–601.

Knief C. Diversity and habitat preferences of cultivated and uncultivated aerobic methanotrophic bacteria evaluated based on *pmoA* as molecular marker. *Front Microbiol* 2015;**6**:01346.

Knief C, Dunfield PF. Response and adaptation of different methanotrophic bacteria to low methane mixing ratios. *Environ Microbiol* 2005;**7**:1307–17.

Knief C, Kolb S, Bodelier PL *et al*. The active methanotrophic community in hydromorphic soils changes in response to changing methane concentration. *Environ Microbiol* 2006;**8**:321–33.

Knief C, Lipski A, Dunfield PF. Diversity and activity of methanotrophic bacteria in different upland soils. *Appl Environ Microbiol* 2003;**69**:6703–14.

Krause SMB, Johnson T, Samadhi Karunaratne Y *et al*. Lanthanide-dependent cross-feeding of methane-derived carbon is linked by microbial community interactions. *Proc Natl Acad Sci USA* 2017;**114**:358–63.

Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol* 2016;**33**:1870–74.

Kutzbach JE, Prell WL, Ruddiman WF. Sensitivity of Eurasian climate to surface uplift of the Tibetan Plateau. *J Geol* 1993;**101**:177–90.

Le Mer J, Roger P. Production, oxidation, emission and consumption of methane by soils: a review. *Eur J Soil Biol* 2001;**37**:25–50.

Li EY, He GY. Tibetan plateau alpine meadow soil properties and enzyme activity in response to fertilization gradients in different seasons. *Guihaia* 2014;**34**:467–72.

Lu CQ, Tian HQ. Spatial and temporal patterns of nitrogen deposition in China: synthesis of observational data. *J Geophys Res Atmos* 2007;**112**:D22S05.

Lueders T, Manefield M, Friedrich MW. Enhanced sensitivity of DNA- and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. *Environ Microbiol* 2004;**6**:73–78.

Macalady JL, Mcmillan AMS, Dickens AF *et al*. Population dynamics of type I and II methanotrophic bacteria in rice soils. *Environ Microbiol* 2010;**4**:148–57.

McDonald IR, Radajewski S, Murrell JC. Stable isotope probing of nucleic acids in methanotrophs and methylotrophs: a review. *Org Geochem* 2005;**36**:779–87.

Mohanty SR, Bodelier PL, Floris V *et al*. Differential effects of nitrogenous fertilizers on methane-consuming microbes in rice field and forest soils. *Appl Environ Microbiol* 2006;**72**:1346–54.

Mor S, Visscher AD, Ravindra K *et al*. Induction of enhanced methane oxidation in compost: temperature and moisture response. *Waste Manag* 2006;**26**:381–8.

Mosier A, Schimel D, Valentine D *et al*. Methane and nitrous oxide fluxes in native, fertilized and cultivated grasslands. *Nature* 1991;**350**:330–32.

Nazaries L, Tate KR, Ross DJ *et al*. Response of methanotrophic communities to afforestation and reforestation in New Zealand. *ISME J* 2011;**5**:1832–36.

Neufeld JD, Vohra J, Dumont MG *et al*. DNA stable-isotope probing. *Nat Protoc* 2007;**2**:860–66.

Nyerges G, Stein LY. Ammonia cometabolism and product inhibition vary considerably among species of methanotrophic bacteria. *FEMS Microbiol Lett* 2009;**297**:131–36.

Noll M, Frenzel P, Conrad R. Selective stimulation of type I methanotrophs in a rice paddy soil by urea fertilization revealed by RNA-based stable isotope probing. *FEMS Microbiol Ecol* 2008;**65**:125–32.

R Core Team. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria, 2016. https://www.R–project.org(16 January 2019, date last accessed).

Radajewski S, Webster G, Reay DS *et al*. Identification of active methylotroph populations in an acidic forest soil by stable-isotope probing. *Microbiology* 2002;**148**:2331–42.

Schloss PD, Westcott SL, Ryabin T *et al*. Introducing mothur: open–source, platform–independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* 2009;**75**:7537–41.

Shrestha M, Shrestha PM, Frenzel P *et al*. Effect of nitrogen fertilization on methane oxidation, abundance, community structure, and gene expression of methanotrophs in the rice rhizosphere. *ISME J* 2010;**4**:1545–56.

Shrestha PM, Kammann C, Lenhart K *et al*. Linking activity, composition and seasonal dynamics of atmospheric methane oxidizers in a meadow soil. *ISME J* 2012;**6**:1115–26.

Stein LY, Klotz MG. Nitrifying and denitrifying pathways of methanotrophic bacteria. *Biochem Soc Trans* 2011;**39**:1826–31.

Suo NJ, Tan YR, Zhu WX *et al*. A study on soil enzyme activity in four different grasslands of the eastern Tibetan Plateau. *Cao Ye Xue Bao* 2012;**21**:10–15.

Tate KR, Walcroft AS, Pratt C. Varying atmospheric methane concentrations affect soil methane oxidation rates and methanotroph populations in pasture, an adjacent pine forest, and a landfill. *Soil Biol Biochem* 2012;**52**:75–81.

Teske A, Dhillon A, Sogin ML. Genomic markers of ancient anaerobic microbial pathways: sulfate reduction, methanogenesis, and methane oxidation. *Biol Bull* 2003;**204**:186–91.

U.S. Environmental Protection Agency (EPA). *Global Anthropogenic Emissions of Non-CO Greenhouse Gases 1990–2020 (June 2006)*. https ://www.epa.gov/sites/production/files/2016-05/documents/globalanthroemissionsreport.pdf(16 January 2019, date last accessed).

Wang Q, Quensen JF, III, Fish JA *et al*. Ecological patterns of *nifH* genes in four terrestrial climatic zones explored with targeted metagenomics using FrameBot, a new informatics tool. *mBio* 2013;**4**:e00592–13.

Wei M, Qiu Q, Qian Y *et al*. Methane oxidation and response of *Methylobacter/Methylosarcina* methanotrophs in flooded rice soil amended with urea. *Appl Soil Ecol* 2016;**101**: 174–84.

Wen X, Yang S, Liebner S. Evaluation and update of cutoff values for methanotrophic *pmoA* gene sequences. *Arch Microbiol* 2016;**198**:629–36.

Wilshusen JH, Hettiaratchi JPA, Stein VB. Long-term behavior of passively aerated compost methanotrophic biofilter columns. *Waste Manag* 2004;**24**:643–53.

Xia W, Zhang C, Zeng X *et al*. Autotrophic growth of nitrifying community in an agricultural soil. *ISME J* 2011;**5**: 1226–36.

Yun J, Zhuang G, Ma A *et al*. Community structure, abundance, and activity of methanotrophs in the Zoige wetland of the Tibetan Plateau. *Microb Ecol* 2012;**63**: 835–43.

Zhang Z, Wang G, He J. Short-term effect of fertilization on CO_2, CH_4 and N_2O fluxes in alpine meadow on the Tibetan Plateau. *American Geophysical Union, Fall Meeting*, 2012. Abstract id:ED23B–0769.

Zhao YH, Zhao L, Yue GY *et al*. A study of methane oxidation rates in soil samples from swamp meadow on the Tibetan Plateau. *J Glaciol Geocryol* 2013;**35**:1126–32.

Zheng Y, Huang R, Wang BZ *et al*. Competitive interactions between methane- and ammonia-oxidizing bacteria modulate carbon and nitrogen cycling in paddy soil. *Biogeosciences* 2014;**11**:3893–926.

Zuckerkandl E, Pauling L. Evolutionary divergence and convergence in proteins. In: Bryson V, Vogel HJ (ed.). *Evolving Genes and Proteins*. New York, USA.: Academic Press, 1965, 97–166.