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**Manuscript title:** The response of methanotrophs to additions of different nitrogen resources in alpine swamp meadow soil as revealed by stable isotope probing

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**Abstract**

Different forms of nitrogen (N) are deposited on the Qinghai-Tibetan plateau (QTP), while their effects on the activity of methanotrophs in soil 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% 13C-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 profound effects on methanotroph diversity and activity in swamp meadow soils on the QTP.

**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. EPA 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 et al., 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 oten 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 et al., 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 et al., 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 (Deng et al., 2013; Yun et al., 2012), 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 et al., 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 et al., 2005). A direct link between methane-uptake activity and methanotroph taxonomy can be established by using SIP and analyzing DNA markers specific for methanotrophs (Knief et al., 2003; McDonald et al., 2005; Radajewski et al., 2002). 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 et al., 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-FiveEasyTM 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 NH4Cl (NH), NaNO3 (NO), CO(NH2)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 13C enrichment and not to G + C content, we also included a natural abundance methane (hereafter referred to as 12C-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. 6.25 ml of pure 13C-methane or 12C-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 re-sealed with butyl rubber stoppers and 6.25-ml pure methane was added. This was done a total of four times to ensure that sufficient 13C was integrated into the DNA of active methanotrophs for DNA-SIP (Radajewski et al., 2002; McDonald et al., 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 177000 *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 (Fig. S1) was measured using an AR200 digital refractometer (Reichert, Inc., Buffalo, NY, USA). The buoyant density (BD) of each fraction was calculated as specified previously (Lueders et al., 2004). 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 13000 *g* for 30 minutes. The supernatant was discarded. 500 μl of 70 % (v/v) ethanol was added to the tubes to rinse the DNA, followed by centrifugation at 10000 *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 min. 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, 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 0.992-0.996. Based on the results of the real-time PCR (Fig. S2), 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 13C-methane and 12C-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 13C-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 denoised 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 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 et al., 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 et al., 2016). All positions containing gaps and missing data were removed, leaving a total of 117 positions in the final dataset. In addition, all positions containing gaps and missing data were removed, leaving 60 amino acid residues per sequence. The optimal tree with a sum of branch lengths equal to 3.09332905 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. 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). 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). 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.

**DNA stable-isotope probing**

The *pmoA* gene abundance in each CsCl gradient fraction was quantified (Fig. S2). 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 13C-methane incubation samples (range from 4.95×105 to 8.35×105 copies μl-1), and from fractions 11-12 for all 12C-methane incubation samples (ranging from 1.05x105 to 2.32x105 copies μl-1) (Fig. S2). These results confirm that we had successful separation of DNA as a function of 13C content.

**16S rRNA gene analysis**

We compared the 16S rRNA gene analysis results in both 13C-methane and 12C-methane incubations. There were significant differences in the phyla compositions between the heavy and light fractions for the 13C-methane(adonis, *P* < 0.001), but not for the 12C-methanecontrol incubation (Fig. S4, Table S1). 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 13C-methaneand 12C-methaneincubations (adonis, *P* < 0.01) (Fig. 2, Fig. S4, Table S1). These results proved that the separation of heavy and light DNA fractions from the 13C-methane incubation were primarily the result of 13C-methane incorporation and not G+C content of the DNA. In the latter analyses we focused on the heavy fractions from the 13C-methanesystems. 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). *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). 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), 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 et al., 1994; Zhang et al., 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 counterion of NH4+ 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 (Fang, et al., 2014; Jang, et al., 2011). 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 effect on methanotrophs when grown with high concentrations of methane (1-10%), and the inhibitory effects of ammonia often occurred with high amounts of fertilizers (e.g., > 10 mM ammonia g-1 dry soil) (Whittenbury et al., 1970; Harrits and Hanson, 1980; Yoshinari, 1985). 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 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 (Mor et al., 2005). The methane oxidation rate tended to be low initially with an extended lag, followed by an increase in uptake and a final decline. Similar patterns were observed for soils (Kightley et al., 1995; Hilger et al., 2000; De Visscher and Van Cleemput, 2003) and composts (Wilshusen et al., 2004). The methane concentration in our incubations typically remained above 0.5%, and we may not have incubated long enough to observe a decline in uptake rate. 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 longterm (eight 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 et al., 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 Van 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 NH3-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) could have been converted to ammonia in about 2-4 days (corresponding to the first methane injection, Fig. S3) in the incubation system (44.9 μg urea-N g-1 dry soil). The transformation from urea to ammoniamight 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 methaneuptake 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 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 (Deng et al., 2013; Yun et al., 2012), 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 et al., 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 13C, as has been used in many DNA-SIP studies (McDonald et al., 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 et al., 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 et al., 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* and stimulate the methane oxidation rate (Zhang 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).

The 16S rRNA gene analysis identified non-methanotrophs in the heavy DNA fractions, such as *Firmicutes* and *Betaproteobacteria*, which might incorporate the 13C 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). Different N-fertilizers could have different effects on the interactions of methanotrophs with other heterotrophs, and thus have varying impacts 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 species 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.

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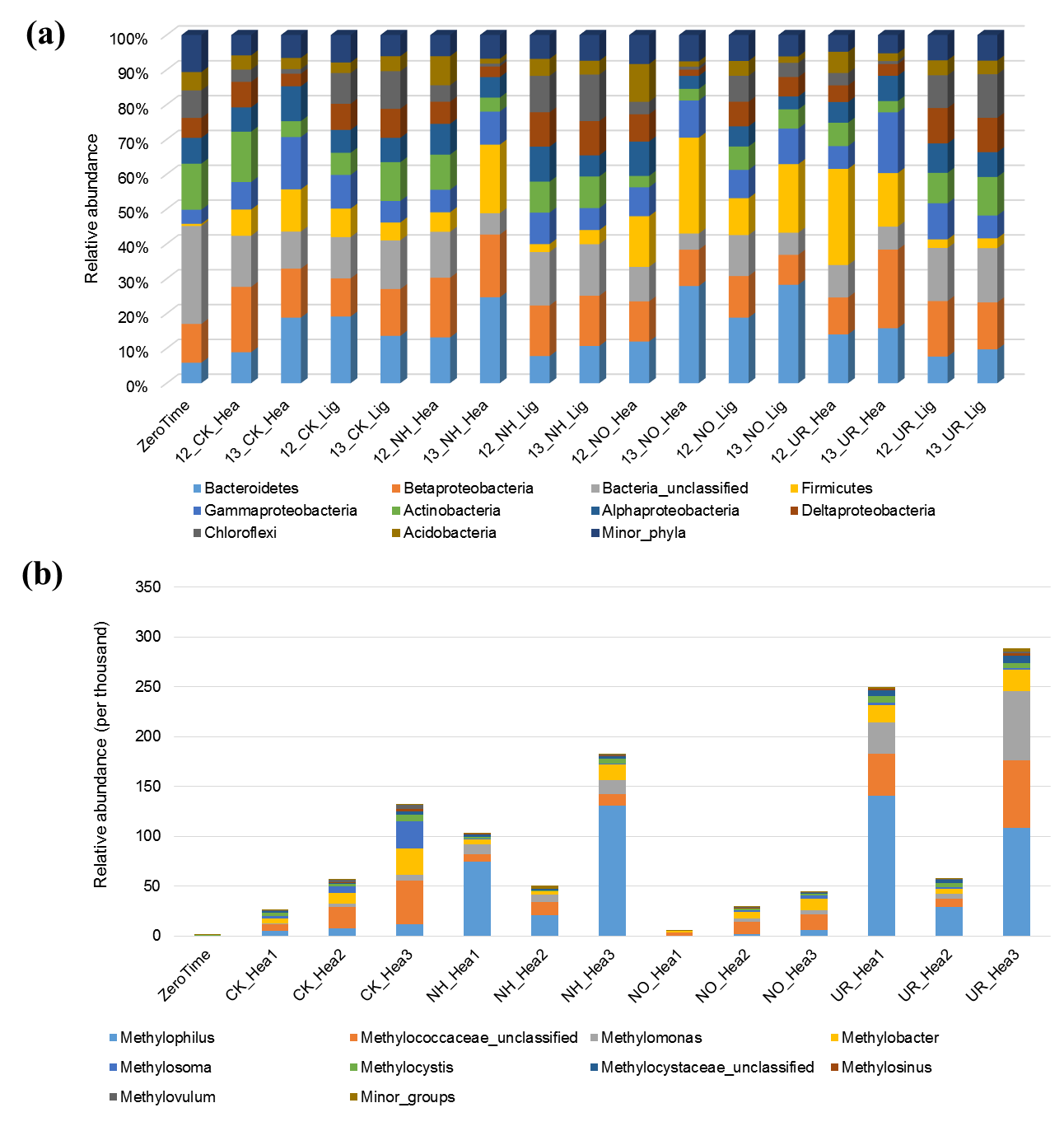
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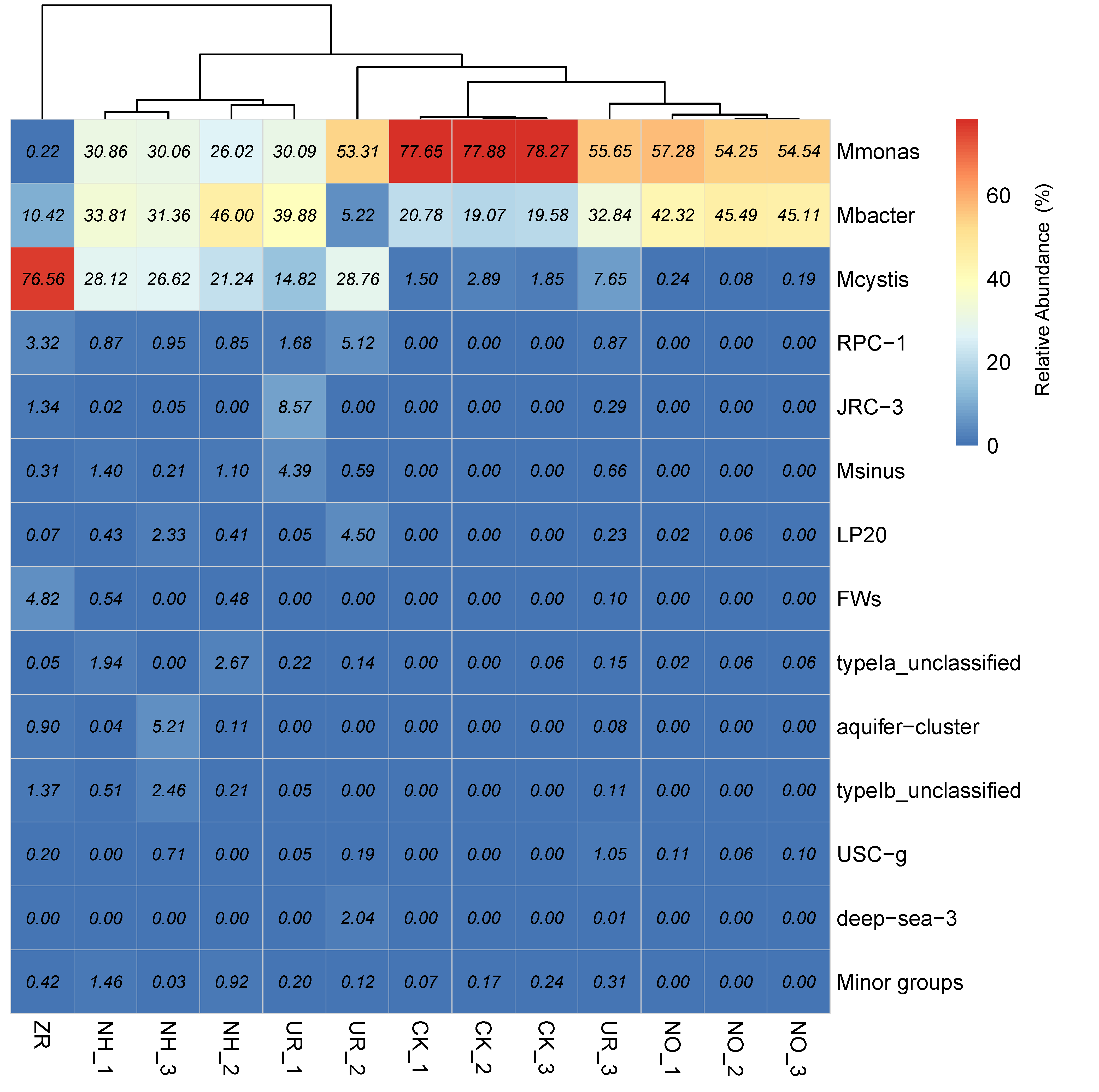
**Fig. 1**



**Fig. 2**



**Fig. 3**



**Fig. 4**

