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Competitive interactions between methane- and ammonia-oxidizing bacteria modulate carbon and nitrogen cycling in paddy soil

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Abstract

Pure culture studies have demonstrated that methanotrophs and ammonia oxidizers can both carry out the oxidation of methane and ammonia. However, the expected interactions resulting from these similarities are poorly understood, especially in complex, natural environments. Using DNA-based stable isotope probing and pyrosequencing of 16S rRNA and *pmoA* genes, we report on biogeochemical and molecular evidence for growth stimulation of methanotrophic communities by ammonium fertilization, and that methane modulates nitrogen cycling by competitive inhibition of nitrifying communities in a rice paddy soil. Pairwise comparison between microcosms amended with CH_4, CH_4+Urea, and Urea indicated that urea fertilization stimulated methane oxidation activity by 6-fold during a 19 day incubation period, while ammonia oxidation activity was significantly inhibited in the presence of CH_4. Pyrosequencing of the total 16S rRNA genes revealed that urea amendment resulted in rapid growth of *Methylosarcina*-like type Ia MOB, and nitrifying communities appeared to be suppressed by methane. High-throughput sequencing of the ^{13}C-labeled DNA further revealed that methane amendment resulted in clear growth of *Methylosarcina*-related MOB while methane plus urea led to equal increase in *Methylosarcina* and *Methylobacter*-related MOB, indicating the differential growth requirements of representatives of these genera. Strikingly, type Ib MOB did not respond to methane nor to urea. Increase in ^{13}C-assimilation by microorganisms related to methanol oxidizers clearly indicated carbon transfer from methane oxidation to other soil microbes, which was enhanced by urea addition. The active growth of type Ia methanotrops was significantly stimulated by urea amendment, and the pronounced growth of methanol-oxidizing bacteria occurred in CH_4-treated microcosms only upon urea amendment. Methane addition inhibited the growth of *Nitrosospira* and *Nitrosomonas* in urea-amended microcosms, in addition of nitrite-oxidizing bacteria. These results provide comprehensive insights in the interactions between actively growing methanotrophs and ammonia oxidizers in a complex soil ecosystem.

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1 Introduction

The intensive use of nitrogenous fertilizers in rice agriculture is a perquisite to meet the growing demand for food, especially since this crop feeds more than half of world's population (Galloway et al., 2008). The tight coupling between nitrogen fertilization and methane emission from rice paddy ecosystems in combination with the significant contribution of these system to the global methane emission 15 to 45 % of global CH_4 budget (Bodelier, 2011) has evoked numerous studies focusing on this topic. Recent meta-analysis indicate that the increasing rice biomass by nitrogen fertilization may result in the elevated supply of readily available carbon in suport of methanogenesis, stimulating methane emission in paddy fields (Banger et al., 2012). However, opposed to this there is a strong body of evidence demonstrating stimulation of methane oxidation by ammonium-based fertilizers in rice soil, leading to reduced methane flux (Bodelier et al., 2000b). The vast amount of studies following these observations as well as possible underlying mechanisms for nitrogen regulation of methane oxidation in soils and sediments has been reviewed (Bodelier, 2011; Bodelier and Laanbroek, 2004). However, the role of interactions between methanotrophs and ammonia oxidizers and the consequences for interactions between carbon and nitrogen cycling has rarely been investigated in natural complex ecosystems (Bodelier, 2011).

Aerobic methane-oxidizing bacteria (MOB) belong to two phyla: *Proteobacteria* and *Verrucomicrobia* (Bodelier et al., 2009). Whereas proteobacterial MOB are widespread, *Verrucomicrobia* seem to be restricted to extreme environments (Dunfield et al., 2007). Aerobic proteobacterial MOB can be divided into two major groups mainly based on phylogeny being type I (*Gammaproteobacteria*) and type II (*Alphaproteobacteria*). This group assignment used to be supported by differences in biochemical, physiological and morphological properties. However, a number of exceptions exists, and the type I and II classification scheme beyond phylogeny is not fully supported for demarcating ecologically different types of methanotrophs (Stein et al., 2012). Based on congruent 16S rRNA and *pmoA* phylogeny, type I MOB harboring the family *Methylococcaceae*

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can be further divided into type Ia and type Ib. Type II MOB include the family *Methylocystaceae* and *Beijerinckiaceaea* (Stein et al., 2012). The methane monooxygenase (MMO) exist either as a particulate (pMMO) or a soluble (sMMO) form. All known methanotrophs contain pMMO except *Methylocella* and *Methyloferula*, while sMMO is found only in a few species (Hanson and Hanson, 1996; Lipscomb, 1994). Nitrifying bacteria use ammonia monooxygenase (AMO) for oxidation of their primary growth substrate. Though the AMO gene was thought to be unique to ammonia-oxidizing bacteria, the discovery of ammonia-oxidzing archaea (AOA) has suggested important role of archaeal nitrification in the global nitrogen cycle (Lu and Jia, 2013; Venter et al., 2004). However, until now the relative contribution of AOB and AOA to ammonia oxidation in argricultural soil is still unclear (Prosser and Nicol, 2012; Xia et al., 2011).

The key enzymes methane monooxygenase (MMO) in methanotrophs and ammonia monooxygenase in ammonia oxidizers are evolutionarily linked (Holmes et al., 1995), leading to functional similarities enabling both methanotrophs and ammonia oxidizers to oxidize both methane and ammonia (Jones and Morita, 1983; O'Neill and Wilkinson, 1977). Pure culture studies demonstrated that methane can act as a competitive inhibitor for ammonia oxidizers, and ammonia inhibits the growth and activity of methanotrophs (Bedard and Knowles, 1989; Stein et al., 2012). Next to this, both MOB as well as AOB have to deal with toxic intermediates (hydroxylamine in case of MOB and methanol in case of AOB) (Stein et al., 2012). At the microbial community level, however, the growth of methantroph could be nitrogen-limited and nitrogen fertilization might relieve methane oxidizers from nutrient constraint (Bodelier et al., 2000b). At the same time ammonia oxidizers and subsequent nitrification may be inhibited by the methanotrophic N-assimilation. However, the research focus of methane effects on nitrification in natural complex ecosystems is in sharp contrast with the number of studies executed to elucidate effect of nitrogenous fertilizers on methane oxidation. Moreover, the lack of knowledge on this topic is even more evident taking the yet unknown role of AOA in interactions with MOB into account. DNA-based stable isotope probing (DNA-SIP) is generally used to link the metabolisms of ^{13}C-labeled substrates with growing

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microbial communities in the environment. DNA-SIP has been employed to identify the active methanotrophs (Dumont et al., 2011) and ammonia oxidizers in soils (Jia and Conrad, 2009; Lu and Jia, 2013; Xia et al., 2011). The combined use of stable isotope labeling and high throughput pyrosequencing is a powerful combination of approaches that offers great opportunities in elucidating interaction between MOB and AOB/AOA, because both groups can easily and specifically be labeled using $^{13}CH_4$ (Bodelier et al., 2013, 2012) and $^{13}CO_2$ (Jia and Conrad, 2009). However, studies that assessed both functional groups in interaction with each other are missing.

In this study, the oxidation rate and microbial composition in CH_4-amened microcosms and Urea-amended microcosms were compared with microcosms amended with both CH_4 and Urea, respectively. High-throughput pyrosequencing of 16S rRNA gene and functional genes (*pmoA*, bacterial *amoA*) and DNA-SIP were used to determine microbial interactions between methane and ammonia oxidization in a paddy soil.

2 Materials and methods

2.1 Site description and soil sampling

The paddy soil was collected from Yangzhou City (119°42′0″ E, 32°35′5″ N) of Jiangsu province, one of the major regions for rice production in China. The soil was silt clay and classified as Calcaric Glevsols. The field has a history of rice cultivation for more than 50 years. Soil sampling was performed at 0–15 cm depth by steel cores with three replicates. Soil maximum water holding capacity (WHC) was 55 %, and the soil samples were homogenized by passing though a 2 mm meshed sieve. The resulting soil samples were kept at 40 % maximum water holding capacity in fridge until use. Soil characteristics are as follows: 15 g total organic C kg^{-1}, 1.59 g total N kg^{-1}, 1.23 g total P kg^{-1} and pH 7.4 determined with water to soil ratio at 2.5.

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2.2 DNA-SIP microcosms

Four treatments were performed including ^{13}C-CH_4-labeled microcosms (incubated with ^{13}C-CH_4), ^{13}C-Urea-labeled microcosms (incubated with ^{13}C-Urea and ^{13}C-CO_2), ^{13}C-CH_4 + Urea-labeled microcosms (incubated with ^{13}C-CH_4, ^{13}C-Urea and ^{13}C-CO_2) and ^{12}C-CH_4 + Urea control microcosm (incubated with ^{12}C-CH_4, ^{12}C-Urea and ^{12}C-CO_2). The hydrolysis of ^{13}C-labeled urea was employed to generate ammonia and ^{13}C-CO_2 in support of autotrophic nitrifying communities in soil as previously reported (Lu and Jia, 2013). Pairwise comparison among the treatments of ^{13}C-CH_4, ^{13}C-CH_4 + Urea, and ^{13}C-Urea was used to assess the effect of urea fertilization on methane oxidation, and the role of methane on ammonia oxidation. The soil microcosm with ^{12}C-CH_4 + Urea amendment was performed as control treatment for the labeled SIP microcosms.

Microcosms for stable-isotope probing incubations were constructed in triplicate by adding approximately 7.30 g fresh soil (equivalent to 6.0 g dry weight gram soil, i.e., d.w.s.) to 120 mL serum bottles capped with black butyl stoppers for incubation at 28 °C in the dark for 19 days. To increase the labeling efficacy of targeted microorganisms, the pre-incubation of soil at 40 % maximum water-holding capacity (WHC) was performed for 14 days to reduce the amount of soil-respired ^{12}C-CO_2 (Jia and Conrad, 2009; Xia et al., 2011). The ^{13}C-CH_4-labeled microcosms and ^{13}C-CH_4 + Urea-labeled microcosms were injected with $^{13}CH_4$ (99 atom %^{13}C, Sigma-Aldrich Co., St Louis, MO, USA) to reach 9000 ppmv (Table S1). Meanwhile, ^{13}C-Urea fertilization of 100 μg urea – N g^{-1} d.w.s. with 5 % $^{13}CO_2$ (99 atoms %^{13}C, Sigma-Aldrich Co., St Louis, MO, USA) was performed for ^{13}C-Urea-labeled microcosms and for ^{13}C-CH_4 + Urea-labeled microcosms as previously described (Jia and Conrad, 2009). As for ^{13}C-CH_4-labeled microcosms, the distilled water instead of urea was added. SIP control microcosms were established in triplicate by addition of the unlabeled CH_4, urea and CO_2 instead of ^{13}C-substrate. CH_4 and CO_2 concentrations were measured

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every few hours depending on the rate of methane consumption by gas chromatography (Shimadzu GC12-A, Japan) as previously described (Zhu et al., 2010). After more than 90 % of CH_4 was consumed, the headspace was flushed with pressurized synthetic air (20 % O_2, 80 % N_2) for 1 min to maintain oxic conditions before ^{13}C-labeled or unlabeled substrate was renewed, to reach about ~ 10 000 ppmv CH_4 and/or 100 µg urea – N g^{-1} d.w.s. plus 5 % CO_2. Due to strong methane oxidation in microcosms amended with ^{13}C-CH_4 + Urea treatment (Fig. S1), methane addition was regularly repeated, in addition to urea and CO_2 substrates. The scenario of SIP microcosm construction was detailed in supplemental Table S1. The destructive sampling was performed in triplicate after incubation of SIP microcosms for 0, 5 and 19 days. Soil samples were immediately frozen at −20 °C until further use. For SIP microcosm amended with urea, approximately 3 g of fresh soil was removed from each of triplicate microcosms. The rest of the soil was homogenized with 15 mL of 2 M KCl by shaking at 200 rpm for 60 min, and then passed through filter paper for determination of NH_4^+-N and NO_3^--N using a Skalar SAN Plus segmented flow analyzer (Skalar, Inc., Breda, the Netherlands).

2.3 DNA extraction and isopycnic centrifugation

The total DNA from 0.5 g soil (fresh weight) of each microcosm was extracted using the FastDNA spin kit for soil (MP Biomedicals, Cleveland, OH, USA), according to the manufacturer's instruction. Soil DNA quality and quantity were observed by a Nanodrop ND-1000UV-Vis Spectrophotometer (NanoDropTechnoloqies, Wilmington, DE, USA), and soil DNA was stored at −20 °C.

For each treatment, density gradient centrifugation of total DNA was performed to separate the ^{13}C-labeled DNA from ^{12}C-DNA as previously described in detail (Jia and Conrad, 2009). In brief, approximately 2.0 µg DNA was mixed well with CsCl stock solution to achieve an initial CsCl buoyant density of 1.725 g mL^{-1} using gradient buffer (pH 8.0; 100 mM Tris-HCl; 100 mM KCl; 1.0 mM EDTA). The mixture was ultra-centrifuged

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in a 5.1 mL Beckman polyallomer ultracentrifuge tube by using a Vti65.2 vertical rotor (Beckman Coulter, Inc., Palo Alto, CA, USA) at 177 000 g for 44 h at 20 °C. A NE-1000 single syringe pump (New Era Pump Systems, Inc., Farmingdale, NY, USA) with a precisely controlled flow rate of 0.38 mL min^{-1} was used to fractionate DNA by displacing the gradient medium with sterile water from the top. Fourteen or fifteen DNA fractions were obtained with equal volumes of about 340 µL, and a 65 µL aliquot was used for refractive index measurement using an AR200 digital hand-held refractometer (Reichert Inc., Buffalo, NY, USA). The CsCl medium was removed by PEG precipitation (polyethylene glycol 6000), and the DNA pellet was further purified with 70 % ethanol. The fractionated DNA was then dissolved in 30 µL sterile water for downstream analysis.

2.4 Real-time quantitative PCR of fractionated DNA

Real-time quantitative analysis of the *pmoA* gene as a function of buoyant density of DNA gradient fraction was performed to determine the efficacy of ^{13}C incorporation into the genomic DNA of MOB communities by analyzing the distribution patterns of *pmoA* gene copies over the entire density range of SIP gradient on a CFX96 Optical Real-Time Detection System (Bio-Rad, Laboratories Inc., Hercules, CA, USA) as previously described. The labeling of AOB and AOA communities was assessed by real-time quantitative PCR of bacterial and archaeal *amoA* genes, respectively (Lu and Jia, 2013). The amplification efficiencies were 93 ~ 103 % obtained with R^2 values of 99.1 ~ 99.9 %. PCR conditions and primers were described in Supplement Table S2.

2.5 Pyrosequencing of 16S rRNA genes at the whole community level

Pyrosequencing of the total 16S rRNA genes was performed in triplicate microcosms (Table S3) and in the fractionated DNA from fraction-3 to 13 of each treatment (Table S4) using the universal primers 515F/907R with primer adaptors, key sequence, and tag sequence as previously described (Lu and Jia, 2013). Tag sequences were

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used to barcode the PCR amplicons, and PCR conditions and primers were described in Supplementary Table S2. PCR reaction mixture of 50 µL was performed and the amplicons were purified and visualized on 1.8 % agarose gels. The purified PCR products were determined by a Nanodrop ND-1000 UV-Vis Spectrophotometer. Pyrosequencing was performed on a Roche 454 GS FLX Titanium sequencer (Roche Diagnostics Corporation, Branford, CT, USA). The read was trimmed to generate high-quantity sequences using mothur software (Schloss et al., 2009). Taxonomic assignment of the high-quantity sequence reads were obtained by RDP Multi Classifier with a confidence threshold of 50 % (Wang et al., 2007). The MOB-like and AOB-like 16S rRNA gene sequences were extracted and clustered into operational taxonomic unit (OTU) at 97 % sequence identify cut-off using mothur software package. One representative sequence of each OTU was then used for phylogenetic analysis.

2.6 Pyrosequencing of *amoA* and *pmoA* genes from total DNA and ^{13}C-labeled DNA

The *pmoA* gene for MOB and bacterial *amoA* gene for AOB were also analyzed using high-throughput pyrosequencing of the total DNA and ^{13}C-labeled DNA in the ^{13}C-labeled microcosms at day 0 and day 19 (Table S5). PCR primer pairs were A189F/mb661r for *pmoA* gene (Costello and Lidstrom, 1999; Holmes et al., 1995), and amoA-1F/amoA-2R for bacterial *amoA* gene (Rotthauwe et al., 1997), respectively (Table S2). The functional genes were amplified using total DNA extract from triplicate microcosms for each treatment. The "heavy" DNA fraction showed the highest relative abundance of AOB and MOB 16S rRNA genes was used as the ^{13}C-DNA for pyrosequencing of functional genes. The preparation of PCR products and pyrosequncing was the same with the 16S rRNA gene as described previously. Raw sequences were trimmed and clustered into operational taxonomic unit (OTU) at 97 % sequence identify cut-off using mothur software (Schloss et al., 2009). Pyrosequencing of *pmoA* genes yielded 36 430 high quality sequence reads with an average length of 482 bp, while 47 303 sequence reads of bacterial *amoA* genes were generated an average length

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of 469 bp (Table S5). One representative sequence was then used from each OTU for phylogenetic analysis.

2.7 Statistical analysis

Effect of urea or CH_4 on measured parameters was tested using one-way analysis of variance analysis (ANOVA). Prior to ANOVA analysis these data were tested for normality (plots of SD vs. means) and for homogeneity of variances (Levene's test). All analyses were performed using SPSS Statistics soft package version 16.0.

2.8 Accession number of nucleotide sequences

The pyrosequencing reads have been deposited at DNA Data Bank of Japan (DDBJ) with accession numbers DRA001245 and DRA001247 for the 16S rRNA genes and functional genes (bacterial *amoA* and *pmoA*), respectively.

3 Results

3.1 Microbial oxidation of methane and ammonia

Methane oxidation activity was assessed by determining the amount of methane consumed in soil microcosms over the incubation course of 19 days, and the strong capacity of methane oxidation was observed in the paddy soil tested (Fig. S1). It is estimated that 4.01 and 32.4 $\mu mol\, CH_4\, g^{-1}$ d.w.s. were oxidized in soil microcosms after incubation with CH_4 for 5 and 19 days, respectively (Fig. 1a). Urea fertilization significantly stimulated methane oxidation activity by 2- and 6-fold at day 5 and 19, respectively (Fig. 1a). Soil nitrification activity was determined as the increase of soil nitrate concentrations during incubation of microcosms for 19 days. Soil nitrate content significantly increased from 11.1 $\mu g\, NO_3^- - N\, g^{-1}$ d.w.s. in urea-amended microcosms at day 0, to 61.0 and 137.6 $\mu g\, NO_3^- - N\, g^{-1}$ d.w.s. at 5 and 19 days, respectively (Fig. 1b). The

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presence of CH_4 in the headspace of urea-amended microcosms significantly inhibited production of soil nitrate at day 19, although statistically significant inhibition was not observed at day 5 (Fig. 1b).

High-throughput fingerprinting of the total microbial communities was performed by pyrosequencing of the total 16S rRNA genes in SIP microcosms over the 19 days incubation period (Table S3). About 346, 000 high-quality sequence reads were obtained with an average length of 377 bp in the V3 ~ V4 region, and methanotrophic 16S rRNA gene comprised only 0.28 % of total microbial communities in paddy soil tested (Fig. 1c). However, methane oxidation led to a remarkable increase of MOB-like 16S rRNA genes up to 27.9 % of the total microbial communities during SIP microcosm incubations (Fig. 1c). Interestingly, methanotrophic proportions appeared to show a decreasing trend with prolonged incubation of microcosms amended only with CH_4 from 14.8 % at day 5 to 7.42 % to day 19. Nonetheless, urea addition resulted in higher abundance of methanotroph-like 16S rRNA gene sequences up to 19.8 % and 27.9 % at day 5 and day 19, respectively, representing 1.3- and 4-fold increase relative to CH_4-amended microcosms (Fig. 1c). The family *Methylophilaceae*, using methanol as sole source of carbon and energy (Devries et al., 1990; He et al., 2012), was the dominant methanol-oxidizing bacteria in our study. Similar trend was observed for 16S rRNA gene sequences affiliated with methanol-oxidizing bacteria (Fig. 1e), the relative abundance of which was 150-fold higher in soil microcosms with CH_4 + Urea treatment (2.76 %) than that in CH_4-amended microcosms (0.02 %) at day 19.

AOB comprised only a tiny fraction of the total microbial communities during a 19 day incubation period (Fig. 1d). The relative abundance increased significantly in urea-amended microcosms from 0.21 % at day 0 to 0.35 % at day 19. The presence of CH_4 significantly inhibited the proportional increase of AOB-like 16S rRNA gene reads leading to a relative frequency down to 0.15 % at day 19 (Fig. 1d). Similar results were observed for soil nitrite-oxidizing bacteria (NOB). For instance, the relative abundance of NOB increased significantly from 0.91 % at day 0 to 1.42 % at day 19 in the urea-amended microcosms, while soil microcosms with Urea + CH_4 displayed a relative

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abundance as low as 0.42 % at day 19 (Fig. 1f). As for AOA, there was no significant change in relative abundances upon urea fertilization during SIP microcosm incubation, although the decreasing trend was observed in the presence of CH_4 (Fig. S2).

3.2 High-throughput fingerprinting of functional guilds against the total communities

The 16S rRNA genes affiliated with MOB and AOB were selected for phylogenetic analysis from the total pyrosequencing reads in soil microcosms, after incubation for 5 and 19 days, following the additions of methane and/or urea. Phylogenetic analysis revealed a remarkable shift of MOB community structure based on both 16S rRNA gene (Fig. S3a) and *pmoA* genes (Fig. S3b). Though type II methanotrophs dominate MOB communities in background soil at day 0, the consumption of CH_4 in soil microcosms led to a drastic increase in relative abundance of type Ia methanotrophs from 0.09 % at day 0 to 14.4 % at day 5 (Fig. 2a). Interestingly, type II methanotroph-like organisms stayed at very low proportions during the entire incubation period, whereas significant increase was observed from 0.12 % at day 0 to 0.55 % at day 19. Urea fertilization further stimulated the relative abundance of type Ia methanotrophs reaching 1.3 and 4 times higher in the CH_4 + Urea-amended microcosms than that in the microcosms amended only with CH_4 at day 5 and day 19, respectively. However, urea nitrogen appeared to have no effect on the relative abundance of type II methanotrophs. Similar results were obtained by pyrosequencing analysis of *pmoA* genes (Fig. S3b). Phylogenetic analysis of *pmoA* genes indicated that type Ia *pmoA* sequences were stimulated from 9 % at day 0 to 75 % of total methanotrophic communities after incubation with CH_4 for 19 days. Urea addition further stimulated the proportion of type Ia methanotroph *pmoA* gene sequences to a greater extent up to 85 %.

AOB communities were exclusively dominated by *Nitrosospira*-like 16S rRNA gene sequences at day-0, and none of 16S rRNA gene sequences could be assigned to *Nitrosomonas* (Fig. S4a). However, the relative abundance of *Nitrosomonas*-like 16S rRNA genes rose to 0.04 % and 0.06 % of the total microbial communities in urea-

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amended microcosms after incubation for 5 and 19 days, respectively (Fig. 2b). CH_4 addition resulted in lower abundance of *Nitrosomonas*-like 16S rRNA genes at day 5 and day 19, representing 2- and 3-fold decrease relative to that in urea-amended microcosms (Fig. 2b). The relative abundance of *Nitrosospira*-like AOB was stimulated by urea fertilization, but inhibited in the presence of CH_4 (Fig. 2b). These results were further verified by phylogenetic analysis of the *amoA* pyrosequencing reads (Fig. S4b). For instance, none of *amoA* gene sequences was affiliated with *Nitrosomonas* in background soil at day 0, whereas 7 % of *amoA* gene sequences were affiliated with *Nitrosomonas* at day 19 in the urea-amended microcosms.

3.3 Stable isotope probing of active methanotrophs and ammonia oxidizers

The incorporation of ^{13}C-label into nucleic acid of active microbial communities in complex soil was analyzed by isopycnic centrifugation of total DNA extracted from SIP microcosms. The fractionated DNA over the entire density range of a given gradient was further assessed by pyrosequencing of the total 16S rRNA gene. About 418 000 high-quality reads were generated with an average length of 356 bp in the V3 ~ V4 region of the 16S rRNA gene (Table S4). Pyrosequencing the relative abundance of microbial guilds as a function of the buoyant density of the DNA gradient indicated that MOB and AOB were ^{13}C-labeled to different extents. The relative abundance of methanotrophic 16S rRNA sequences was exceptionally high up to 90 % of the total 16S rRNA gene sequences in the "heavy" DNA fractions from the labeled microcosms, suggesting strong labeling of methanotrophic communities in soils after incubation for 5 (Fig. 3a) and 19 days (Fig. 3b). This was further supported by quantitative analysis of *pmoA* gene copies reaching the peak in the "heavy" DNA fractions from the labeled microcosms, while the highest number was observed in the "light" DNA fractions for the ^{12}C-control treatment (Fig. S5). In addition, the relative abundance of 16S rRNA gene sequences affiliated with methanol-oxidizing bacteria was apparently higher in the "heavy" DNA fractions from the labeled microcosms (^{13}C-CH_4 and ^{13}C-CH_4 + Urea) than those in the control treatments (^{12}C-CH_4 + Urea), despite the relatively low pro-

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portion of ~ 0.20 % at day 5 (Fig. 3c). The prolonged incubation for 19 days increased the proportion of methanol-oxidizing bacteria significantly up to 11.0 % of the total 16S rRNA gene sequences in the ^{13}C-DNA from the labeled soil microcosms amended both with CH_4 and Urea, but not in the labeled microcosms that received only CH_4 (Fig. 3d).

The 16S rRNA gene sequences of AOB were highly enriched in "heavy" DNA fractions from the labeled microcosm amended only with urea at day 5 (Fig. 3e) and day 19 (Fig. 3f), but not the CH_4 + Urea treatment during the 19 day incubation period. For instance, up to 5.73 % of total 16S rRNA gene sequences in the "heavy" DNA fractions could be assigned to AOB for ^{13}C-Urea treatment, while only 0.33 % of the total 16S rRNA gene sequences in the ^{13}C-Urea+CH_4 treatments were related to AOB at day 19 (Fig. 3f). Similar results were obtained for nitrite-oxidizing bacteria (Fig. 3g and h). The relative abundance of NOB in the "heavy" DNA fractions was significantly higher in microcosms with ^{13}C-urea than ^{13}C-Urea + CH_4 treatment, implying a much greater degree of labeling of NOB cells in ^{13}C-Urea treatments during active nitrification. Furthermore, it is noteworthy that no significant enrichment of archaeal 16S rRNA gene sequences occurred in the "heavy" DNA fractions from the labeled microcosms (Fig. S6).

Phylogenetic analysis of the ^{13}C-labeled 16S rRNA genes demonstrated that active MOB were affiliated with Type Ia (*Methylobacter*- and *Methylosarcina*-like) and *Methylocystis*-related type II methanotrophs, while type Ib methanotrophic sequences were not detected during active methane oxidation (Fig. 4a). Active ammonia oxidizers were phylogenetically assigned to distinctly different phylotypes including the *Nitrosospira* clusters and the *Nitrosomonas communis* lineage on the basis of ^{13}C-16S rRNA gene analysis (Fig. 4b). DNA-SIP demonstrated remarkable community shifts of methanotrophs and ammonia oxidizers during the 19 day incubation period (Fig. 5). Type Ia-like MOB accounted for 89 % of the ^{13}C-labeled methanotrophic 16S rRNA sequences in CH_4-amended microcosms at day 19, while up to 98 % of the active methanotrophs could be assigned to Type Ia MOB in soil microcosms amended with both CH_4 and urea (Fig. 5a). This was further supported by pyrosequencing analysis of *pmoA* genes

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in the ^{13}C- DNA (Fig. S7a). For instance, 86 % of *pmoA* genes were affiliated to type Ia MOB in CH_4-amended microcosms at day 19, whereas all *pmoA* sequences were detected exclusively as type Ia MOB in the microcosms amended with both CH_4 and urea. As for ammonia oxidizers, the relative abundance of *Nitrosomonas*-like 16S rRNA genes was as high as 88.2 % of the ^{13}C-labeled AOB communities in microcosms after incubation with urea for 5 days (Fig. 5b). However, the presence of CH_4 resulted in lower proportions of *Nitrosomonas*-like 16S rRNA genes, represented by 1.6 and 1.3 times lower than that in urea-amended microcosms at day 5 and day 19, respectively. Pyrosequencing of *amoA* genes in the ^{13}C-DNA lend further support for the inhibition of *Nitrosomonas*-like AOB since it decreased from 21 % to 2 % of active AOB communities upon by CH_4 addition (Fig. S7b).

4 Discussion

The interaction between methane and nitrogen has been identified as one of the major gaps in carbon-nitrogen cycle interactions (Gärdenäs et al., 2011). There are many possible feedbacks to climate change through effects on methane and N_2O emissions and eutrophication of soils and sediments as a consequence of interactions between methane- and ammonia oxidizers. Mechanistically, there is still a poor understanding of nitrogen effects on methane cycling and vice versa. Elucidation of these mechanisms is of utmost importance to obtain comprehensive understanding of the nature of the effects of e.g. climate change on the release of major greenhouse gases from various ecosystems.

Due to the enzymatic similarity of methane and ammonia monooxygenase, methane and ammonia-oxidizers can oxidize methane as well as ammonia (Bodelier and Frenzel, 1999; Oneill and Wilkinson, 1977; Stein et al., 2012). However, methane oxidizers do not gain energy out of the oxidation of ammonia while ammonia oxidizers do not grow on methane (Stein et al., 2012). Moreover, mineral nitrogen is essential for biomass formation, especially for those methanotrophs lacking the ability to fix molec-

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ular nitrogen (Semrau et al., 2010). The latter indicates that next to direct enzymatic effects, interactions at the level of competition for N will play an important role in this matter, especially in high methane environments where ammonia oxidizers will face enzymatic as well as competitive stress, with respect to which sparse information is available.

In our study, it is demonstrated that urea fertilization significantly stimulated methane oxidation activity and growth of MOB. Growth and activity of ammonia oxidizers was suppressed in the presence of CH_4. It is obvious that competitive inhibition of the methane monooxygenase did not occur in our microcosms. The ratio of N-CH_4 is approx.0.11 (assuming all urea is converted to ammonium). In other studies ratios of up to 200 (Bodelier et al., 2000b) did not lead to inhibition. Hence, it is safe to conclude that the ammonium formed out of urea or the subsequently produced nitrate acted as nitrogen source for biomass generation of MOB. The decreased NH_4^+-N concentrations corresponded with the increased NO_3^--N concentrations via nitrification only in the microcosms without methane amendment. Addition of methane to microcosms led to lower recovery of mineral N (Table 1) despite the equal addition of urea (Table S1), suggesting that part of consumed ammonia was not oxidized to nitrate via nitrification or part of the nitrate disappeared. We deduce that the consumed ammonia, which was not involved in ammonia oxidation, may be assimilated as a nitrogen nutrient for cell growth of MOB. Assuming that for oxidation of every mol CH_4-C, 0.25 mol N has to be assimilated by MOB (Bodelier and Laanbroek, 2004), the amount of N-assimilated can be calculated using a 70 : 30 ratio of respiration of CH_4 vs. assimilation. This calculation shows that of the total amount of urea added 69 % was assimilated by MOB, while 20 % was nitrified (Table S6). The unaccounted remaining N (11 %) is probably denitrified which may also be an indication that oxygen concentration in the soil microcosms was substantially lowered. Hence, next to the fact that ammonia oxidizers may be suppressed by ammonium assimilation by MOB, they may also suffer from oxygen limitation. Moreover, the remaining nitrate produced may also have been the result of ammonia oxidation by MOB, which has shown to be up to 84 % of the total nitrifica-

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tion in the rhizosphere of rice (Bodelier and Frenzel, 1999; Bodelier et al., 2000a). It appears that assimilation combined with methanotrophic nitrification may account for the whole consumption of the added urea strongly indicating that MOB are far better competitors for N than AOB or AOA.

Our results even demonstrate the dependency of the MOB on sufficient N-availability. The relative abundance of both 16S rRNA and *pmoA* genes decreased when incubating with methane only, demonstrating loss of activity and growth potential when N is limiting. A similar result was obtained in microcosms planted with rice (Bodelier et al., 2000a), where MOB even lost their potential for oxidizing methane. However, adding ammonium to these inactive communities led to immediate re-activation of oxidation (Bodelier et al., 2000a), indicating that N-limitation is not only inhibiting growth but also regulated methane consumption enzyme machinery. This inactivation and rapid re-activation of methane oxidation has even been demonstrated on field scale in rice paddies (Dan et al., 2001; Kruger and Frenzel, 2003). It has been proposed that nitrogen fixation may deplete reducing equivalents leading to lowering and even cessation of methane oxidation (Bodelier and Laanbroek, 2004; Dan et al., 2001). The available inorganic nitrogen source was indeed almost depleted after incubation in CH_4-amended microcosms, decreasing from 11.6 $\mu g\,g^{-1}$ d.w.s. at day 0, to 1.37 and 1.19 $\mu g\,g^{-1}$ d.w.s. at day 5 and day 19, respectively (Table 1). This suggests that under conditions of high methane and low N availability, there is a niche for methanotrophy where they seem to overwhelmingly outcompete nitrifying communities. Nitrifiers can operate in the absence of competition with MOB, which may be inactivated due to energy-depletion as the result of N_2-fixation. Hence, this points to niche differentiation or avoidance strategies of the nitrifiers.

It is obvious that only a subset of the MOB profit substantially from the combined addition of methane and urea-N. Although type II MOB increase in relative abundance with the addition of methane they do not profit from the addition of urea, but are also not affected by it. Addition of ammonium to rice soil has been demonstrated to inhibit type II MOB (Mohanty et al., 2006). This is obviously not the case in our study where

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the rapid growth of type Ia MOB keeps ammonium N-low. The growth of type II MOB is apparently independent of the N-availability suggesting that they can rely on N_2-fixation only. Next to this, the presence of highly active type I MOB did not prevent the growth of type II. However, significant growth of type II MOB only occurs after 19 days of incubation suggesting that either lower growth rates as compared to type I or dependency of type II MOB on the activity of type I. The former is indeed the case as was demonstrated in wetland soil microcosms (Steenbergh et al., 2010) while the latter maybe the result from the fact that type II MOB may use CO_2 (Yang et al., 2013) as their main C-source for assimilation (Matsen et al., 2013). Labelled CO_2 in the microcosms can only be formed by methane oxidation carried by type Ia in the early stages of the experiment. Another explanation may be succession of MOB, with type II MOB increasing in number when type I MOB are getting limited by N (Krause et al., 2010).

It is striking that type Ib MOB do not increase in numbers nor do they show up in the SIP analyses. This is in sharp contrast with other observations, where *Methylocaldum*-related MOB consisted the dominant part of the ^{13}C-CH_4 incorporation MOB community in rice soil microcosms (Noll et al., 2008). One explanation may be the discrepancy between the offered conditions in the microcosms and the rice field where the soils were obtained from. It has been shown that response of MOB to methane availability in rice soils of different ages depends on historical contingencies in these fields (Ho et al., 2011). Type Ib MOB from 2000 years old responded more vigorously to methane addition than those from young rice fields. Hence, the MOB have been proposed to have "memory" for optimal environmental conditions, which in our case may not have matched with the incubation conditions offered.

The strong stimulation of type Ia MOB upon methane application alone and in combination with urea-N application has been observed frequently in rice soils but also in other environments, reflecting their competitive life-strategy as reviewed and synthesized (Ho et al., 2013). The most responsive MOB species in high methane habitats seem to be *Methylobacter* species (Krause et al., 2012). Our experiments show that *Methylosarcina* species are clearly the most responsive without addition of urea. This

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is in contrast with the niche differentiation observed at high spatial resolution in rice soil microcosms (Reim et al., 2012). The presence of *Methylosarcina* related MOB in the surface layer of thin layer microcosms and not in the methane–oxygen interface, implying that *Methylosarcina* thrives under low-methane ("oligotrophic") conditions, in contrast to *Methylobacter* which dominates the zone of high methane flux. However, remarkably, in our experiments *Methylosarcina* clearly is dominant at high methane supply but is replaced partly by *Methylobacter* when urea-N is added. This might be attributed to competition for methane, nitrogen, or even oxygen. A similar result was observed in SIP analyses of lake sediment microcosms using a metagenomic approach (Beck et al., 2013). Hence, we speculate that observations by Reim et al. (2012) may also be explained by weak competitive abilities of *Methylosarcina* instead of being restricted to low methane niches.

A comparison of 16S rRNA gene and *pmoA* gene sequences revealed that *Methylobacter* was detected in a higher proportion in the MOB-16S rRNA gene phylogenetic tree than in the*pmoA* gene phylogenetic tree. It may be explained by that the 16S rRNA gene copies varied in the different genus of MOB community. It has been reported that the 16S rRNA gene copies ranged from 1 to 15 in the bacterial and archaeal genomes (Lee et al., 2009). Moreover, the number of 16S rRNA in the closely related species is not entirely consistent (Fogel et al., 1999; Lee et al., 2009). Thus we supposed that the *Methylobacter* may contain more 16S rRNA gene copies than other MOB. In addition, it has been assumed that two *pmoA* copies exist in methanotrophs (Gilbert et al., 2000; Kolb et al., 2003), which is only the average 16S rRNA gene copies that has been identified in some strains of methanotrophs, such as *Methylocloccus capsulatus* Bath (Stolyar et al., 1999). However, this assessment may misestimate the *pmoA* copies in other MOB which is not identified until now. The *pmoA* copies may vary in different genus of MOB, and *Methylobacter* may have less *pmoA* gene copies than other MOB, which led to its lower proportion in the MOB community.

Interestingly, we found significant increase of putative methanol-oxidizing bacteria related to *Undibacterium* (Fig. S8) which are affiliated the family *Methylophilaceae*

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(Fig. S8a), a family of microbes known to utilize methanol as sole carbon and energy source. The occurrence 16S rRNA of these sequences in the "heavy" DNA fractions indicates that these *Undibacterium*-like organisms assimilated methane derived carbon. Cross feeding of methylotrophs by methanotrophs releasing methanol has been demonstrated before (Antony et al., 2010; Beck et al., 2013; He et al., 2012; Noll et al., 2008). The direct mechanism for this cross feeding and what compound actually is exchanged have not been elucidated yet. We can add another component to this body of unsolved mechanisms which is the strong stimulation of methylotrophs upon urea fertilization, thereby linking the nitrogen and the carbon cycle. It is very likely that the enhanced methane consumption and growth of methanotrophs leads to higher availability of methanol. However, we can not exclude that urea has stimulatory effect on the methylotrophs directly. We also speculate that the active removal of methanol by the methylotrophs is beneficial to methanotrophs given the toxic nature of the compound. However, this would be subject of further study. Interesting is this link between nitrogen and the consequences for the methane-derived soil food web, possibly creating novel niches for methylotrophs in soil.

Our results revealed that the presence of CH_4 in microcosms inhibited the nitrification activity in the paddy soil tested. Physiologically, the enzymatic similarity of ammonia-oxidizers and MOB may result in ammonia oxidation by MOB (Bodelier and Frenzel, 1999), leading to reduced availability of ammonia for ammonia oxidizers. However, previous studies showed that MOB had lower affinity for ammonia than for CH_4 (Banger et al., 2012; Bedard and Knowles, 1989; Yang et al., 2011). Moreover, it has been proposed that ammonia oxidation by MOB occurred only when the ratio of ammonia to CH_4 is higher than 30 in soils (Banger et al., 2012; Bodelier and Laanbroek, 2004; Yang et al., 2011). The molecular ratio of ammonia to CH_4 was about 0.11 in our study, thus the suppression of ammonia oxidizers growth and activity in the presence of CH_4 may not be explained by ammonia oxidation by MOB. Furthermore, a large part of the N-applied was assimilated by MOB which is therefore the most likely explanation for the suppression of methane on ammonia oxidation rate and the growth of ammonia

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oxidizers. However, oxygen consumption by MOB might also have resulted in suppression of aerobic AOB growth. It is interesting to note that up to 4.8 % of the ^{13}C labeled sequences in the urea-amended microcosm were phylogenetically closely related to *Pseudomonas fluorescens*, *Pseudomonas syringae* and *Pseudomonas aeruginosa* (Fig. S8b). These three genera use nitrite as nitrogen source and catalyze denitrification (Betlach and Tiedje, 1981; Modolo et al., 2005; Rinaldo et al., 2007). In the meantime, it remains elusive about the toxic effect of intermediates substance during methane oxidation on nitrifying communities. For example, methanol and/or formaldehyde may inhibit the growth of AOA and AOB communities, and we detected no archaeal *amoA* genes and 16S rRNA genes. The possibility of heterotrophic AOA lifestyle could also not be excluded (Ingalls et al., 2006; Stahl and de la Torre, 2012).

The genus *Nitrosospira* was the dominant AOB in the native soil, being consistent with general observations that *Nitrosospira* are ubiquitous in upland soils as important members of nitrifying population (Hastings et al., 1997; Stephen et al., 1996). In our study, the apparent growth of *Nitrosospira* was observed in the microcosms amended with urea-N, and the cluster 3 was the dominant active *Nitrosospira* group. It has been reported that *Nitrosospira* cluster 3 was the dominant AOB group in a number of neutral soil receiving nitrogen fertilization (Bruns et al., 1999; Mendum et al., 1999). Intriguingly, methane addition suppressed the growth of *Nitrosospira*, and AOB within the cluster 3 appeared to be inhibited to a greater extent than those of cluster 4. It has been proposed that the reduced ammonia supply may select for the cluster 4 populations (Kowalchuk and Stephen, 2001). In the presence of methane, the growth of methanotrophs were significantly stimulated and methanotrophic N assimilation could have likely led to the depletion of ammonium in support of nitrification activity. It was noteworthy that none of 16S rRNA and *amoA* genes were affiliated with *Nitrosomonas* in the native soil at day zero. The growth of *Nitrosomonas* was stimulated to a much greater extent than that of *Nitrosospira* in urea-amended microcosms, but the former was suppressed more significantly than the latter. This might be explained by the fact that hat *Nitrosomonas* are markedly responsive to ammonia input (Hastings

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et al., 1997). Similar to methanotrophic communiiets, the proportion of *Nitrosospira* in AOB community detected by 16S rRNA gene sequences was lower than that detected by *amoA* gene. It could be in part attributed to the variation of *amoA* copy numbers among different AOB. For instance, the species *N. briensis* and *N. europaea* have two copies of *amoA* genes and *N. tenuis* contained three identical *amoA* genes (Norton et al., 1996; Sayavedra-Soto et al., 1998).

Taken together, the results of this study demonstrate the stimulation of methane consumption and growth of MOB by urea and the subsequent repression of nitrifier growth and activity. High-throughput pyrosequencing of 16S rRNA gene and functional gene (*pmoA* and *amoA*) in combination with DNA-SIP elucidated the microbial interactions between methane and ammonia oxidation. Only a sub-set of the MOB profited from the urea addition, with *Methylobacter* species responding the most vigorous, showing that urea addition gives rise to niche differentiation in MOB communities. Assimilation of N possibly in combination with oxygen consumption might provide mechanistic mechanisms for inhibition of ammonia oxidizers by methane addition. Our results provide strong evidence for the cross-feeding of methane-derived carbon in the soil system upon urea fertilization, pointing out another as yet unknown link between the nitrogen and the carbon cycle.

Supplementary material related to this article is available online at http://www.biogeosciences-discuss.net/11/3893/2014/bgd-11-3893-2014-supplement.pdf.

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Table 1. Changes in pH, moisture content, NH_4^+-N and NO_3^--N content in soil microcosms over the course of 19 days of incubation.

Treatments	pH[a]	Moisture (%)[b]	NH_4^+-N ($\mu g\,g^{-1}$ d.w.s.)[c]	NO_3^--N ($\mu g\,g^{-1}$ d.w.s.)[c]
Zero Time	7.39 ± 0.04	19.4 ± 0.42	0.51 ± 0.10	11.1 ± 0.31
Day-5-CH_4	7.53 ± 0.01	26.1 ± 0.16	0.47 ± 0.33	0.90 ± 0.35
Day-5-Urea	7.35 ± 0.06	25.5 ± 0.51	16.1 ± 3.81	61.0 ± 8.62
Day-5-CH_4 + Urea	7.37 ± 0.12	24.8 ± 1.31	8.01 ± 4.66	41.6 ± 9.87
Day-19-CH_4	7.54 ± 0.03	28.3 ± 1.89	0.78 ± 0.12	0.41 ± 0.49
Day-19-Urea	7.27 ± 0.30	30.5 ± 1.85	44.8 ± 6.69	137.6 ± 28.3
Day-19-CH_4 + Urea	6.85 ± 0.09	28.6 ± 2.03	3.66 ± 1.56	59.9 ± 6.01

[a] pH was determined using a ratio of H_2O to soil as 2.5 (v/w). The mean ± standard deviation of triplicate microcosms was given for each treatment.
[b] The mean ± standard deviation of triplicate microcosms was given for each treatment.
[c] The mean ± standard deviation of triplicate microcosms was given for each treatment, while for the CH_4 + Urea treatment 6 replicates were used including both ^{12}C-control and ^{13}C-labeled treatments.

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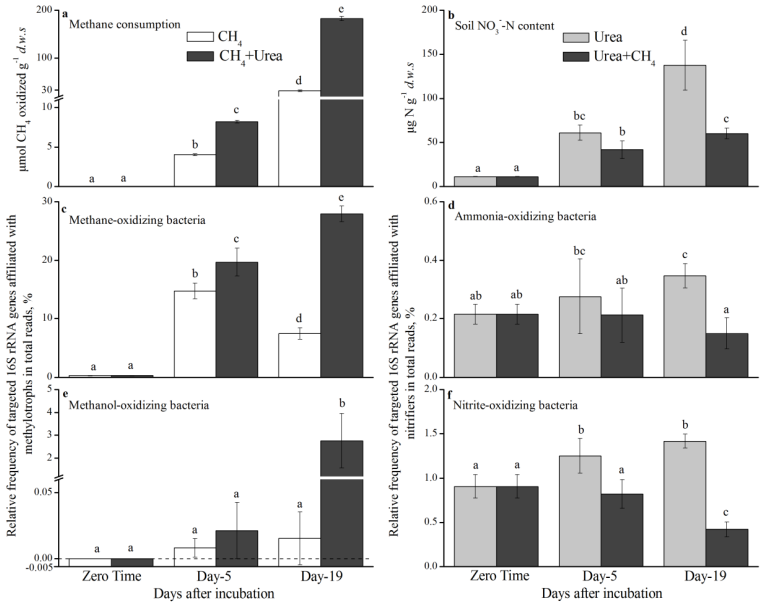

Fig. 1. Interactions between microbial methane and ammonia oxidation in a paddy soil. The left panel shows urea effect on methane oxidation activity **(a)**, methane-oxidizing bacteria **(c)** and methanol-oxidizing bacteria **(e)**. The right panel refers to methane effect on ammonia oxidation activity **(b)**, ammonia-oxidizing bacteria **(d)** and nitrite-oxidizing bacteria **(f)** in soil microcosms after incubation for 5 and 19 days. The amount of methane consumed was used to assess methane oxidation activity and soil nitrate production was used to evaluate ammonia oxidation activity. The total microbial communities were pyrosequenced using universal primers of the 16S rRNA gene. The relative frequency is expressed as the percentage of the targeted 16S rRNA genes to the total 16S rRNA reads for each soil sample. The error bars represent standard deviations of the triplicate microcosms, while for the CH_4 + Urea treatment 6 replicates were used including both ^{12}C-control and ^{13}C-labeled treatments. The different letters above the columns indicate a significant difference ($P < 0.05$) using analysis of variance.

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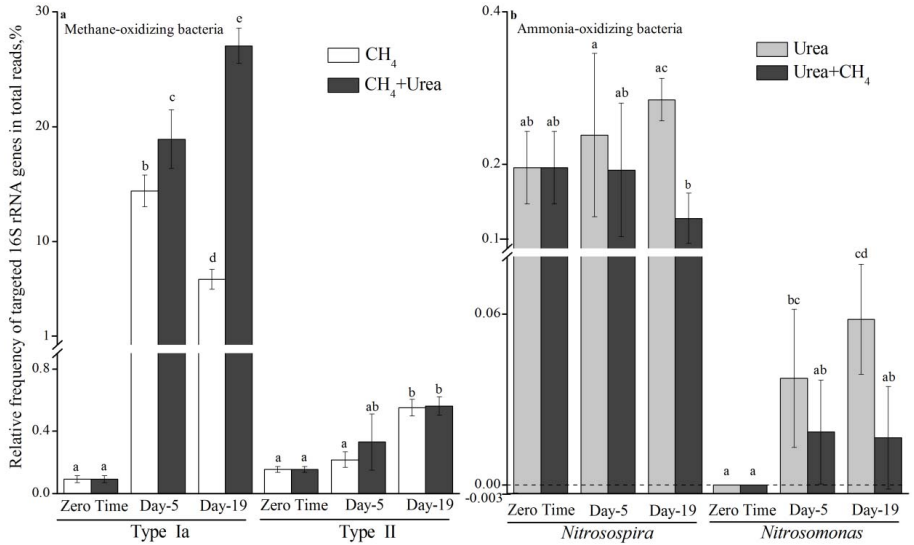

Fig. 2. Change in relative abundance of methane-oxidizing bacteria **(a)** and ammonia-oxidizing bacteria **(b)** in soil microcosms incubated for 5 and 19 days. The relative abundance of type Ia, type II methanotrophs, *Nitrosospira* and *Nitrosomonas* are expressed as the targeted 16S rRNA gene to total 16S rRNA gene reads in soil microcosms incubated with CH_4, urea and CH_4 + Urea. The error bars represent standard deviation of the triplicate microcosms, while for the CH_4 + Urea treatment 6 replicates were used including both ^{12}C-control and ^{13}C-labeled treatments. The different letters above the columns indicate a significant difference ($P < 0.05$) using analysis of variance.

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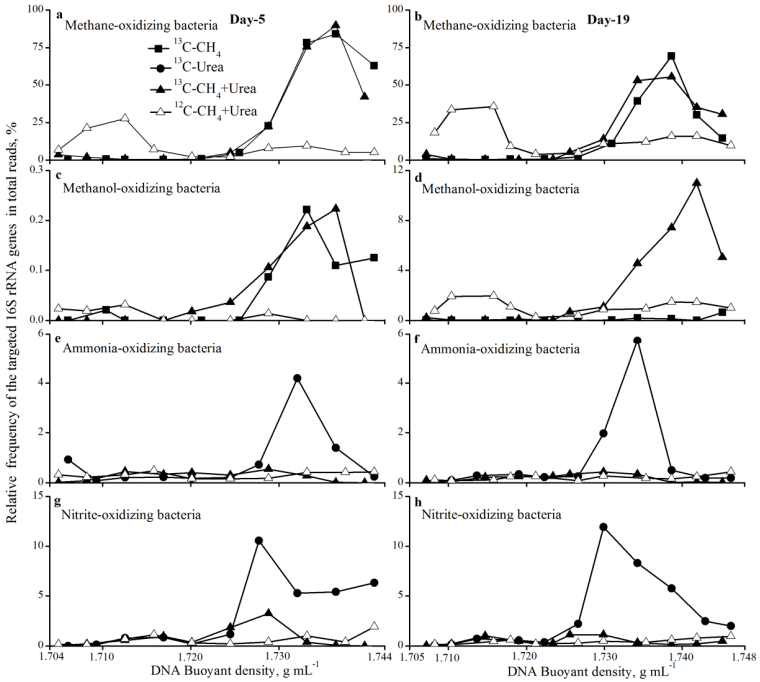

Fig. 3. Relative frequency of the 16S rRNA gene sequences affiliated with methane-oxidizing bacteria **(a, b)**, methanol-oxidizing bacteria **(c, d)**, ammonia-oxidizing bacteria **(e, f)** and nitrite-oxidizing bacteria **(g, h)** across the buoyant density gradient of DNA fractions from the ^{13}C-labeled and ^{12}C-control microcosms after incubation for 5 and 19 days. ^{13}C-CH$_4$ refers to microcosm incubation with ^{13}CH$_4$ for labeling of methane-metabolizing communities, and ^{13}C-Urea represents incubation with ^{13}C-Urea plus ^{13}CO$_2$ for labeling of nitrifying communities. The relative frequency is expressed as the percentage of the targeted 16S rRNA genes to total 16S rRNA reads in each DNA gradient fraction.

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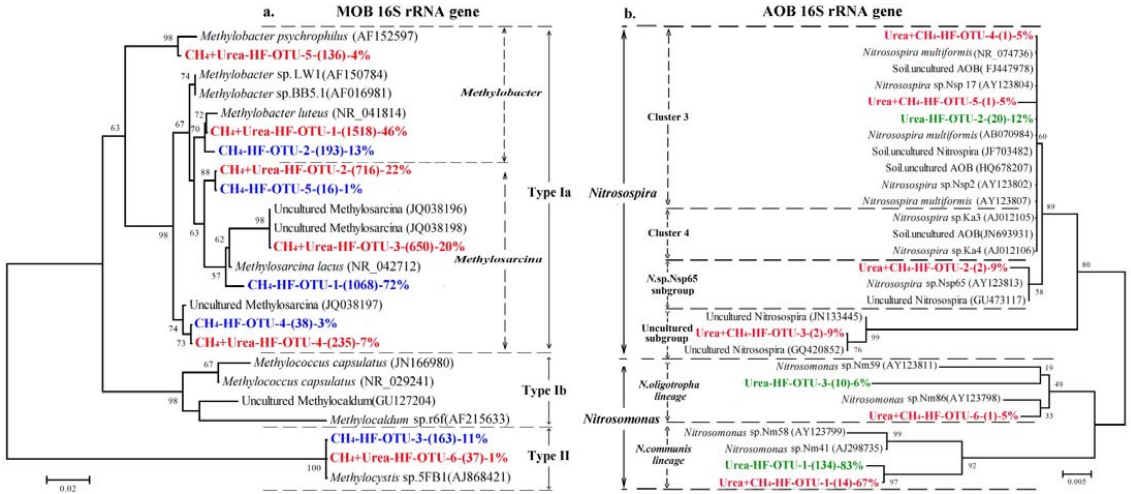

Fig. 4. Phylogenetic tree of the ^{13}C-labeled 16S rRNA genes affiliated with methane-oxidizing bacteria **(a)** and ammonia-oxidizing bacteria **(b)** from the labeled microcosm after incubation for 19 days. The designations CH_4 represent soil microcosms incubated with ^{13}C-CH_4, and the designation Urea denotes incubation with ^{13}C-Urea plus ^{13}C-CO_2. CH_4-HF-OTU-1-(1068)-72 % indicates that OTU-1 contained 1068 reads with sequence identity of > 97 %, accounting for 72 % of the total methanotroph-like 16S rRNA genes in the "heavy DNA fraction" from the labeled microcosms. One representative sequence was extracted using mothur software package for tree construction. The scale bar represents nucleotide acid substitution percentage.

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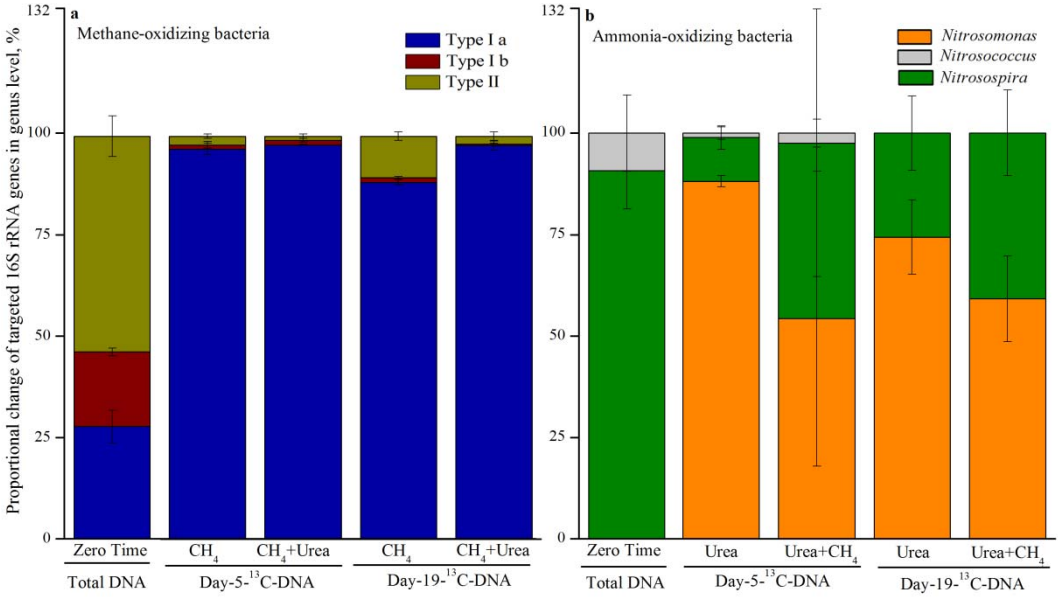

Fig. 5. Percent changes of bacterial phylotypes affiliated with methane-oxidizing bacteria **(a)** and ammonia-oxidizing bacteria **(b)** in the ^{13}C-DNA fractions from the labeled microcosm after incubation for 5 and 19 days. The designation CH_4 + Urea represents soil microcosms incubated with ^{13}C-CH_4 and ^{13}C-Urea plus ^{13}C-CO_2, and the designation Day-5-^{13}C-DNA denotes the ^{13}C-labeled methanotrophic communities in the "heavy" DNA fractions after isopycnic centrifugation of the total DNA extracted from microcosms after incubation with the labeled substrates for 5 days. The percentage of different phylotypes is expressed as the targeted 16S rRNA gene reads to the total 16S rRNA gene reads affiliated with methane-oxidizing bacteria and ammonia-oxidizing bacteria in duplicate.

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