General comments of the referee #3

In this study the authors describe community shifts of methane-oxidizing and ammonia oxidizing bacteria in SIP-incubations of paddy soil mesocosms under treatments with either methane or urea or both. Based on the results of methane consumption and nitrate production in the mesocosms and the results of pyrosequencing of 16S rRNA, pmoA and amoA genes, they conclude that addition of methane or methane and urea stimulates Methylosarcina-like MOB or Methylosarcina and Methylobacter-like MOB, respectively, within the methanotroph community. Furthermore, they conclude that urea alone stimulates AOB in general and Nitrosomonas-related AOB within the total AOB community but that AOB are inhibited in the methane- or methane plus urea treated mesocosms. As has often been the case before in similar studies, the authors observed no or only weak growth of AOA in their mesocosms. This manuscript provides interesting data on methanotrophs and ammonia oxidizers in paddy soils and comes up with some interesting conclusions about potential interactions between these two groups.

Reply: We are grateful to the referee for the overall positive comments.

Major comments

1. My major criticism is that it is not clear to what extent these observations are relevant for mechanisms that can be assumed to take place under field conditions. The composition of the methanotrophic community and also their relative fraction within the total microbial community changed substantially under the conditions of the 19-days-incubation experiment. If one assumes that the original community was already adapted to the field conditions, it remains an open question if the observed changes are really representative of mechanisms taking place under field conditions. Here, the authors should provide more information about to what extent the mesocosm incubation was representative of fertilizer applications and methane availability at the sampling site, or to what extent it may reflect responses of microbial communities to fluctuations in environmental conditions.
Reply: We appreciate this comment, which was raised by referee #1 as well. We fully agree that our results could not entirely represent what is occurring under *in situ* conditions. We however hope the results of this study could provide useful insights on complex interactions between methane and ammonia oxidation. For example, our data are consistent with a very recent review paper showing that Type I methanotrophs utilize K-strategy lifestyle, while r-strategy was exploited by type II methanotrophs.

As suggested, the results and discussion were rephrased from line 470 to line 487 on page 17~18 in the revised version as follows:

The pre-incubation was performed to increase the labeling efficiency of targeted microorganisms because the dilution of $^{13}$CO$_2$ by soil-respired $^{12}$CO$_2$ could be decreased significantly as reported previously (Jia and Conrad, 2009; Xia et al., 2011). No apparent changes of ammonia oxidizer communities were observed during a 4-week pre-incubation without ammonium fertilization, significant shift of AOB communities occurred in the ammonium-amended soils (Jia and Conrad, 2009). The nitrogenous fertilization of paddy field in this study is about 250 kg N ha$^{-1}$, which is equivalent to 107 µg N g$^{-1}$ d.w.s., assuming an effective soil depth of 20 cm. In addition, methane concentrations of 900 to 15000 µL L$^{-1}$ were generally detected in paddy soil during rice-growing season (Nouchi et al., 1990; Nouchi et al., 1994). Therefore, the microcosms were incubated with 100 µg urea-N g$^{-1}$ d.w.s. and 10000 µL L$^{-1}$ methane to extrapolate the microbial interactions between methane- and ammonia-oxidation under field conditions. It suggests that microcosms might represent largely what is occurring under *in situ* conditions, although it could not reproduce the physiochemical and biological conditions in field. For instance, it also has been reported that the results of microcosm incubations remained largely consistent with population dynamics of methanotrophic communities in field (Eller et al., 2005).

2. Another critical aspect is that the discussion focuses mostly on biogeochemical processes and interactions while the largest fraction of the results part is dealing with the pyrosequencing derived community data. Consequently, a large part of the biogeochemical conclusions is based on assumed metabolisms derived from the assigned taxonomies. I wonder if this information is sufficient for some of the conclusions regarding biogeochemical interactions.

Reply: We believe that the taxonomic identity of active organisms could be considered with great confidence. Phylogenetic analysis of 16S rRNA genes was based on the RDP and mothur software package. These databases integrated with Silver database of high-quality 16S rRNA genes and are widely used for microbial ecology study. Therefore, we believe this taxonomic information represent the best resolution available by far.
3. Another aspect related to this is that as far as I can see, triplicate samples were pooled for pyrosequencing analysis but the error range of the method itself remains unknown. So here, small changes in community composition over time or between treatments should be interpreted with caution.

Reply: The pooled sample was tested for reliability before pyrosequencing as shown in the following Table. The DNA extracts from triplicate soil samples were analyzed, in addition to the pooled DNA from the triplicate samples. There was no significant difference between the means of the three replicates and the results of the pooled DNA, with respect to the relative abundance (Table 1) and compositions of MOB and AOB communities (Figure 1). Therefore, the pooled DNA was fractionated for molecular analysis.

Table 1  Pyrosequencing summary of total microbial communities in microcosms incubated for 5 days using 515F-907R of total 16S rRNA genes 
(The designation of R1 to R3 represents triplicate microcosm incubation. ‘Average’ represents the mean value of triplicate microcosms. ‘Pool’ represents the pooled DNA of triplicate microcosms.)
Figure 1  The composition of MOB(a) and AOB(b) community based on 16S rRNA gene sequences in the microcosms incubated for 5 days. The designation ‘Average’ represents mean value of triplicate microcosms. The designation of ‘Pool’ represent the pooled DNA of triplicate microcosms.

4. 3896, l. 24-26: "However, the research: : :methan oxidation." It is unclear what this sentence means. Please rephrase.

Reply: Thanks! It has been rephrased from line 130 to line 133 on page 5 as follows.
‘However, the research focus of methane effect on nitrificatin in natural complex ecosystems is poor, which is in sharp contrast with a large number of studies executed to elucidate effect of nitrogenous fertilizers on methane oxidation.’

5.  p. 3898, l. 9: please write "microbial community composition"

Reply: Done.

6.  p. 3898, l. 2-8: The authors state that hydrolysis of 13C-urea was used to generate ammonia and 13C-CO2. Why did they then add extra 13C-CO2? Please explain.

Reply: Thank for the comment!

13C-urea was used to prevent the dilution of the label because 13CO2 addition is crucial for the labeling of ammonia oxidizers. As referee point out, 13C-urea
catabolized to ammonia and $^{13}$CO$_2$, the amount of $^{13}$CO$_2$ generated by urea catabolism can be used for labeling of ammonia oxidizers as well. The amount of urea-N we added to the microcosms was 100µg g$^{-1}$ d.w.s., the content of $^{13}$CO$_2$ is about 3.57 µmol g$^{-1}$ d.w.s. assuming all urea is converted to ammonia and $^{13}$CO$_2$. In order to increased the labeling efficiency of targeted microorganisms, we added 50000 ppmv $^{13}$CO$_2$ (44.6 µmol g$^{-1}$ d.w.s.) in the microcosms as previous study used (Jia and Conrad, 2009; Xia et al., 2011).

7. p. 3901, l. 23-24: Please provide more information about denoising and read length of the pyrosequencing reads and provide a reference for "as described previously".

Reply: The information about denosing and read length of the pyrosequencing reads has been provide from line 271 to line 277 on page 10 in the revised version.

‘Raw sequences were imported into mothur software (Schloss et al 2009) for quality check, alignment and phylogenetic tree construction. High quality sequences (e.g. read length longer than 200bp, average quantity score more than 25, without ambiguous base calls) were excluded from further analysis. Pyrosequencing of pmoA gene yield about 36 000 high quality sequence reads with an average length of 482bp, while about 47 000 bacterial amoA gene were generated with an average length of 469bp.’

In addition, the detailed information about the preparation of PCR products was also provided from line 267 to line 271 on page 10 in the revised version as follows.

‘PCR was performed in a 50 µL PCR reaction mixture containing 45µL L$^{-1}$ Platinum PCR SuperMix (Invitrogen, Shanghai, China), a 200 nM final concentration of each primer, and 2 µL template PCR products were gel purified and sent for pyrosequencing on a Roche 454 GS FLX Titanium sequencer (Roche Diagnostics Corporation, Branford, CT, USA).’

8. p. 3904, l.12-13: Do the relative abundances given here refer to the total microbial community or only to the relative fraction within MOB? Please clarify.

Reply: The relative abundance here refers to the total microbial community.

We have rephrased the sentence as follows in the revised version from line 356 to line 357 on page 13.

‘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 methanotrophic 16S rRNA gene sequences in the total 16S rRNA gene sequences from 0.09% at day 0 to 14.4% at day 5.’
9. p. 3905, l. 19: Please write here "16S rRNA gene sequences of methanotrophs"

Reply: Done.

10. p. 3907, l. 18: Please describe "nitrogen effects" in more detail.

Reply: It has been described in a greater detail from line 451 to line 454 on page 17 as follows.

‘The inhibition of mineral nitrogen on methane consumption has been demonstrated from numerous studies, however, ammonium-based fertilization was observed to stimulate methane consumption in rice paddies (Bodelier and Laanbroek, 2004). Mechanistically, there is still poor understanding of nitrogen effects on methane cycling and vice versa.’

11. p. 3912, l. 14-15: Interesting is this link between nitrogen: : :methylotrophs in soil." This statement is too general, please specify

Reply: Specified in the revised ms from line 598 to line 601 on page 22 as follows.

‘Interesting is this link between nitrogen and cross-feeding of methanotrophic metabolites by other microorganism, possibly creating novel niches e.g. more methane-driven carbon substrate, lower-toxic environment for methanotrophs in soil.’

12. p. 3912, l. 28-30: As far as I can see, the statement that a large part of the N applied was assimilated by MOB is just an assumption (see p.3908, l. 11-13). This should be stated more clearly here

Reply: It was clearly rephrased in the revised ms from line 613 to line 615 on page 22.

‘Furthermore, a large part of the applied N disappeared in the presence of CH₄, and presumably assimilated by MOB. This explanation seems plausible for the suppression of methane on ammonia oxidation and the growth of ammonia oxidizers.’

13. p. 3913, l. 28-29: Here, the wording with "former" and "latter" makes the sentence rather complicated. Please rephrase

Reply: It was rephrased in the revised ms from line 641 to line 643 on page 23 as follows:
'The growth of *Nitrosomonas* was stimulated to a much greater extent than that of *Nitrosospira* in urea-amended microcosms, but *Nitrosomonas* appeared to be suppressed more significantly than *Nitrosopira*.'

14. p. 3914, l.17-18: This last sentence remains very general

Reply: It was rephrased as follows.

‘Therefore, we speculated that competition for nitrogen between methane- and ammonia-oxidizers play a dominant role in microbial interactions in our study, which is of help toward predictive understandings of carbon and nitrogen cycle in complex environment.’

Reference


1 Title Page

1. Title:

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

2. Running Title:

Interactions between soil methane and ammonia oxidizers

3. Subject Category:

Microbial Ecology

4. Author Names:

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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 functional genes, we report on biogeochemical and molecular evidence for growth stimulation of methanotrophic communities by ammonium fertilization, and that methanemodulates nitrogen cycling by competitive inhibition of nitrifying communities in a rice paddy soil. Pairwise comparison between microcosms amended with CH₄, CH₄+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 suppressed in the presence of CH₄. 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 partially inhibited by methane. High-throughput sequencing of the ¹³C-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. Increase in ¹³C-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 methanotrophs was significantly stimulated by urea amendment, and the pronounced growth of methanol-oxidizing bacteria occurred in CH₄-treated microcosms only upon urea amendment. Methane addition partially inhibited the growth of Nitrosospira and Nitrosomonas in urea-amended microcosms, in addition of nitrite-oxidizing bacteria. These results suggest that type I methanotrophs could likely outcompete type II methane oxidizers under nitrogen-rich environment and the competitive interactions among methane and ammonia oxidizers are complicated than previously appreciated.

Key Words:
Paddy soil, methane oxidation, ammonia oxidation, microbial interactions, high-throughput pyrosequencing, DNA-SIP
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 support 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. Based on congruent 16S rRNA and pmoA phylogeny, type I MOB harboring the family Methylococcaceae can be further divided into type Ia (including genera Methylosarcina,
Methylobacter, Methylosporococcus, Methylectromonas, Methylosoma, and Methylovulum) and type Ib (including genera Methylococcus, Methylocaldum, Methylogaeae, Methylobius and Methylothermus). Type II MOB include the family Methylocystaceae (including genera Methylocystis and Methylosinus) and Beijerinckiaceae (including genera Methylocella, Methylocapsa and Methyloferula) (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). Methanotrops coverts CH₄ to methanol, which can be utilized by methanol-oxidizing bacteria as carbon and energy source. The known soil-retrieved methanol-oxidizing bacteria was with high diversity, however, most of them are facultative methylotrophic, indicating the capability to utilize alternative carbon substrate (Kolb 2009). The family Methylophilaceae is the known obligate methylotrophs that use methanol as the sole source of carbon and energy (Bratina et al. 1992, He et al. 2012). 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-oxidizing 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). 16S rRNA and amoA gene analyses of AOB revealed that physiological group are confined to monophyletic groups within β- and γ-subclass of Proteobacteria. Nitrosospira and Nitrosomonas form a grouping within β-subclass and Nitrosococcus is affiliated with γ-subclass (Purkhold et al. 2000, Purkhold et al. 2003). Enormous diversity of AOA based on 16S rRNA and amoA gene has been suggested, and four major lineages have been displayed, including Nitrososphaera cluster, Nitrosopumilus cluster, Nitrosotalea cluster, and Nitrosocaldus cluster (Pester et al. 2012, Stahl and de la Torre 2012). The conversion of nitrite into nitrate is caused by nitrite-oxidizing bacteria (NOB). NOB are composed of four genera, including Nitrobacter.
Nitrococcus, Nitrospina and Nitrospira, which were assigned to the α-proteobacteria, γ-proteobacteria, δ-proteobacteria and phylum Nitrospirae, respectively (Bock and Wagner 2006).

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 methanotrophsmight 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 effect on nitrificatin in natural complex ecosystems is poor, which is in sharp contrast with a large 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 13C-labeled substrates with growing 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 13CH4 (Bodelier et al., 2013; Bodelier et al., 2012) and 13CO2 (Jia and Conrad, 2009). However, studies that
assessed both functional groups in interaction with each other are missing.

The interactions between methane- and ammonia-oxidizers are linked to methane-nitrogen cycle in light of climate change. However, the effects of nitrogen on methane oxidation are complicated and contradictory results are often reported. Therefore, the microbial populations and functional dynamics of methane- and ammonia oxidizers were investigated in microcosms incubated with CH₄, urea and CH₄+urea in a paddy soil using culture-independent techniques.

Materials and Methods

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 CalcaricGlevsols. 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 through 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.

DNA-SIP microcosms

Four treatments were performed including ¹³C-CH₄-labeled microcosms (incubated with ¹³C-CH₄), ¹³C-Urea-labeled microcosms (incubated with ¹³C-Urea and ¹³C-CO₂), ¹³C-CH₄+Urea-labeled microcosms (incubated with ¹³C-CH₄, ¹³C-Urea and ¹³C-CO₂) and ¹²C-CH₄+Urea control microcosm (incubated with ¹²C-CH₄, ¹²C-Urea and ¹²C-CO₂). The hydrolysis of ¹³C-labeled urea was employed to generate ammonia and ¹³C-CO₂ in support of autotrophic nitrifying communities in soil as previously reported (Lu and Jia, 2013). Pairwise comparison among the treatments of ¹³C-CH₄, ¹³C-CH₄+Urea, and ¹³C-Urea was used to assess the effect of urea fertilization on
methane oxidation activity and MOB community composition, and the role of methane on ammonia oxidation activity and AOB/AOA community composition. The soil microcosm with $^{12}$C-$\text{CH}_4$+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 of 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-$\text{CH}_4$-labeled microcosms and $^{13}$C-$\text{CH}_4$+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. $d.w.s.$ 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-$\text{CH}_4$+Urea-labeled microcosms as previously described (Jia and Conrad, 2009). As for $^{13}$C-$\text{CH}_4$-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 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 ~10000 ppmv CH$_4$ and/or 100 µg urea-N/g. $d.w.s.$ plus 5% CO$_2$. Due to strong methane oxidation in microcosms amended with $^{13}$C-$\text{CH}_4$+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 -20ºC until further use. For SIP microcosm amended with urea, approximately 3g of fresh soil was removed from each of triplicate microcosms. The rest of the soil was homogenized with 15mL of 2M KCl by shaking at 200 rpm for 60min., and then passed through filter paper for determination of \( \text{NH}_4^+ \)-N and \( \text{NO}_3^- \)-N using a Skalar SAN Plus segmented flow analyzer (Skalar, Inc., Breda, Netherlands).

**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 (NanoDrop Technologies, 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 \(^{13}\text{C}\)-labeled DNA from \(^{12}\text{C}\)-DNA as previously described in detail (Jia and Conrad, 2009; Xia et al., 2011). In brief, approximately 2.0 µg DNA was well mixed 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 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 hours at 20ºC. 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 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.
Real-time quantitative PCR of total and fractionated DNA

Real-time quantitative analysis of the *pmoA* gene in total DNA and in each buoyant density of DNA gradient fraction was performed to determine the growth and efficacy of $^{13}$C incorporation into the genomic DNA of MOB communities on a CFX96 Optical Real-Time Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA), respectively. The growth and 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 primers and PCR conditions were described in Supplementary Table S2. The reactions was performed in a 20 µL mixture containing 10.0 µL SYBR Premix Ex Taq (Takara, Dalian), 0.5µM each primer, and 1µL of DNA template. The amplification efficiencies were 93%–103% obtained with $R^2$ values of 99.1%–99.9%.

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 used to barcode the PCR amplicons, and PCR conditions and primers were described in Supplementary Table S2. 50 µL PCR reaction mixture containing 45µL L⁻¹ Platinum PCR SuperMix (Invitrogen, Shanghai, China), a 200 nM final concentration of each primer, and 2µL template DNA was performed and the amplicons were purified and visualized on 1.8% agarose gels. The purified PCR products were determined by a Nanodrop ND-1000UV-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-quality sequences using mothur software (Schloss et al., 2009). Taxonomic assignment of the high-quality 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 identity cut-off using mothur software package. One representative sequence of each OTU was then used for phylogenetic analysis.

**Pyrosequencing of amoA and pmoA genes from total DNA and 13C-labeled DNA**

The pmoA gene for MOBand bacterial amoA gene for AOB were also analyzed using high-throughput pyrosequencing of the total DNA and 13C-labeled DNA in the 13C-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 13C-DNA for pyrosequencing of functional genes. PCR was performed in a 50 µL PCR reaction mixture containing 45µL L⁻¹ Platinum PCR SuperMix (Invitrogen, Shanghai, China), a 200 nM final concentration of each primer, and 2 µL template PCR products were gel purified and sent for pyrosequencing on a Roche 454 GS FLX Titanium sequencer (Roche Diagnostics Corporation, Branford, CT, USA). Raw sequences were imported into mothur software (Schloss et al 2009) for quality check, alignment and phylogenetic tree construction. High quality sequences (e.g. read length longer than 200bp, average quantity score more than 25, without ambiguous base calls) were excluded from further analysis. Pyrosequencing of pmoA gene yield about 36 000 high quality sequence reads with an average length of 482bp, while about 47 000 bacterial amoA gene were generated with an average length of 469bp (Table S5). pmoA gene sequences and bacterial amoA gene sequences were clustered into operational taxonomic unit at 87% (Degelmann et al 2010) and 97% sequence identity cut-off, respectively. One representative sequence was ten used from each OTU for phylogenetic analysis.

**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 versus means) and for homogeneity of variances (Levene’s test). All analyses were performed using SPSS Statistics soft package version 16.0.

**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.

**Results**

**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 µmol CH$_4$ g$^{-1}$ d.w. 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 µg NO$_3$−N g$^{-1}$ d.w.s in urea-amended microcosms at day 0, to 61.0 and 137.6 µg NO$_3$−N g$^{-1}$ d.w.s. at 5 and 19 days, respectively (Fig. 1b, Fig. S2). The 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, Fig. S2).

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. 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 CH4 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 CH4-amended microcosms (Fig. 1c). The population size of MOB community determined by real-time PCR of pmoA genes (Fig. S3a) showed the similar result with 16S rRNA pyrosequencing analysis. The copy number of pmoA genes increased significantly from $4.44 \times 10^8$ copies g$^{-1}$ d.w.s. at day 0 to $1.45 \times 10^9$ copies g$^{-1}$ d.w.s. and $1.66 \times 10^9$ copies g$^{-1}$ d.w.s. in the microcosms incubated with CH4 for 5 and 19, respectively. Urea addition led to 1.35 and 3.16 times more pmoA genes than that in only CH4-incubated microcosms at day 5 and day 19, respectively. The family Methylophilaceae, using methanol as sole source of carbon and energy (Devries et al., 1990; He et al., 2012), was methanol-oxidizing bacteria analyzed 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 CH4+Urea treatment (2.76%) than that in CH4-amended microcosms (0.02%) at day 19.

AOB16S rRNA gene sequences 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 CH4 significantly suppressed the proportional increase of AOB-like 16S rRNA gene reads leading to a relative frequency down to 0.15% at day 19 (Fig. 1d). The copies of bacterial amoA gene detected by real-time PCR increased from $4.08 \times 10^7$ copies g$^{-1}$ d.w.s. at day 0 to $1.06 \times 10^8$ copies g$^{-1}$ d.w.s. at day 19 in the
microcosms incubated with urea (Fig. S3b). The increase also was observed in the urea+CH₄ treatment, however, the presence of CH₄ resulted in 1.33-fold decrease relative to only urea-amended microcosms after incubation for 19 days. This indicated that CH₄ partially inhibited the growth of AOB. Similar results were observed for soil nitrite-oxidizing bacteria (NOB). For instance, the relative abundance of NOB16S rRNA gene sequences in total microbial community increased significantly from 0.91% at day 0 to 1.42% at day 19 in the urea-amended microcosms, while soil microcosms with Urea+CH₄ displayed a relative 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 CH₄ (Fig. S4). The similar result was also detected by the real-time PCR of archaeal amoA gene (Fig. S3c).

**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. S5a) and pmoA genes (Fig. S5b). Though type II methanotrophs dominate MOB communities in background soil at day 0, the consumption of CH₄ in soil microcosms led to a drastic increase in relative abundance of type Ia methanotrophic 16S rRNA gene sequences in the total 16S rRNA gene sequences from 0.09% at day 0 to 14.4% at day 5 (Fig. 2a). Interestingly, type II methanotroph-like 16S rRNA genes stayed at very low proportions in the total microbial community 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 CH₄+Urea-amended microcosms than that in the microcosms amended only with CH₄ 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. S5b). Phylogenetic analysis of pmoA genes indicated that type Ia pmoA sequences were stimulated from 7.4% at day 0 to 69.8% of total methanotrophic communities after incubation with CH4 for 19 days. Urea addition further stimulated the proportion of type Ia methanotroph pmoA gene sequences to a greater extent up to 84.7%.

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. S6a). However, the relative abundance of *Nitrosomonas*-like 16S rRNA genes rose to 0.04% and 0.06% of the total microbial communities in urea-amended microcosms after incubation for 5 and 19 days, respectively (Fig. 2b). CH4 addition resulted in lower abundance of *Nitrosomonas*-like 16S rRNA genes in the total microbial communities 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 partially inhibited in the presence of CH4 (Fig. 2b). These results were further verified by phylogenetic analysis of the amoA pyrosequencing reads (Fig. S6b). 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.

**Stable isotope probing of active methanotrophs and ammonia oxidizers**

The incorporation of 13C-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 16S rRNA gene sequences of methanotrophs 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. S7).
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 proportion 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 Fig. 3h). 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. S8).
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 in the $^{13}$C-DNA (Fig. S9a). For instance, 85.0% 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 day 5 and day 19, respectively. Pyrosequencing of *amoA* genes in the $^{13}$C-DNA lend further support for the suppression of *Nitrosomonas*-like AOB since it decreased from 21% to 2% of active AOB communities upon by CH$_4$ addition (Fig. S9b).

**Discussion**

The interaction between methane and nitrogen has been identified as one of the major gaps in carbon-nitrogen cycle interactions (Gardenas et al., 2011; Gärdenäs et al., 2011). There are many possible feedbacks to climate change through effects on
methane and N$_2$O emissions and eutrophication of soils and sediments as a consequence of interactions between methane- and ammonia oxidizers. The inhibition of mineral nitrogen on methane consumption has been demonstrated from numerous studies, however, ammonium-based fertilization was observed to stimulate methane consumption in rice paddies (Bodelier and Laanbroek, 2004). 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 molecular 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.

The pre-incubation was performed to increase the labeling efficiency of targeted microorganisms because the dilution of $^{13}$CO$_2$ by soil-respired $^{12}$CO$_2$ could be decreased significantly as reported previously (Jia and Conrad 2009, Xia et al 2011). No apparent changes of ammonia oxidizer communities were observed during a 4-week pre-incubation without ammonium fertilization, significant shift of AOB communities occurred in the ammonium-amended soils (Jia and Conrad 2009). The nitrogenous fertilization of paddy field in this study is about 250 kg N ha$^{-1}$, which is equivalent to 107 µg N g$^{-1}$d.w.s, assuming an effective soil depth of 20 cm. In addition, methane concentrations of 900 to 15000 µL L$^{-1}$ were generally detected in paddy soil during rice-growing season (Nouchi et al 1990, Nouchi et al 1994).
Therefore, the microcosms were incubated with 100 µg urea-N g\(^{-1}\) d.w.s. and 10000 µL L\(^{-1}\) methane to extrapolate the microbial interactions between methane- and ammonia-oxidation under field conditions. It suggests that microcosms might represent largely what is occurring under *in situ* conditions, although it could not reproduce the physiochemical and biological conditions in field. For instance, it also has been reported that the results of microcosm incubations remained largely consistent with population dynamics of methanotrophic communities in field (Eller et al. 2005).

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 partially inhibited in the presence of CH\(_4\). It is obvious that competitive inhibition of the methanemonooxygenase did not occur in our microcosms. The ratio of N-CH\(_4\) is approximately 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 fate of unaccount remaining nitrogen (11%) need further experiment to investigate.

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). 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 of 16S rRNA gene sequences in total microbial community 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 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. $^{13}$C-labeled methanotrophic 16S rRNA gene sequences are closely affiliated with *Methylocystis parvus* OBBP, which possesses nitrogenase and capable of nitrogen fixing (Murrell and Dalton 1983). This suggested that these *Methylocystis parvus*-like type II may respond under N-limited conditions in our study. 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 CO₂ (Yang et al., 2013) as their main C-source for assimilation (Matsen et al., 2013). Labelled CO₂ 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).

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 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 (Reim et al., 2012) may also be explained by weak competitive abilities of Methylosarcina instead of being restricted to low-methane habitats.
A comparison of 16S rRNA gene and \textit{pmoA} gene sequences revealed that \textit{Methylobacter} was detected in a higher proportion in the MOB-16S rRNA gene phylogenetic tree than in the \textit{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). The variation of \textit{pmoA} copy numbers may occur among different MOB. The two \textit{pmoA} copies was assumed to exist in methanotrophs (Gilbert et al., 2000; Kolb et al., 2003), which is only the average copies that has been identified in some strains of methanotrophs, such as \textit{Methylocloccus capsulatus} Bath (Stolyar et al., 1999). However, this assessment may misestimate the \textit{pmoA} copies in other MOB which is not identified until now. Furthermore, another possible explanation for incongruence may be that \textit{pmoA} primers and 16S rRNA primers may not completely cover similar ranges of diversity, as reported previously (Costello and Lidstrom 1999).

Interestingly, we found significant increase of putative methanol-oxidizing bacteria related to \textit{Undibacterium} (Fig. S10) which are affiliated the family \textit{Methylophilaceae}(Fig. S10a), 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 \textit{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 cross-feeding of methanotrophic metabolites by other microorganism, possibly creating novel niches e.g. more methane-driven carbon substrate, lower-toxic environment for methanotrophs in soil.

Our results revealed that the presence of CH$_4$ in microcosms partially 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 applied N disappeared in the presence of CH$_4$, and presumably assimilated by MOB. This explanation seems plausible for the suppression of methane on ammonia oxidation and the growth of ammonia oxidizers. 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. S10b). These three species 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 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 *Nitrosomonas* appeared to be suppressed more significantly than *Nitrosospira*. This might be explained by the fact that *Nitrosomonas* are markedly responsive to ammonia input (Hastings et al., 1997). Similar to methanotrophic communities, the proportion of *Nitrosospiira*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).

The abilities to catalyze the hydrolysis of urea to yield ammonia can be observed in a wide range of microorganisms possessing urease activity (Mobley and Hausinger 1989). Some methanotrophs have been identified with the ability of urea hydrolysis (Boden et al 2011, Khmelenina et al 2013), however, the $^{13}$C-labeled active methanotrophs on the basis of 16S rRNA gene (Fig.4a) and *pmoA* gene (Fig.S9a).
were phylogenetically distinctly different with these known ureolytic methanotrophs. However, the $^{13}$C-labeled AOB showed high sequence similarity with ureolytic 
*Nitrosomonas nitrosa* and *Nitrosomonas oligotrophs*. This indicates the potential of hydrolyzing urea in these active ammonia-oxidizing bacteria. It was estimated that 30%–50% of ammonia could be released from hydrolysis of urea by AOB in batch culture (Pommerening-Roser and Koops 2005). This suggests that ammonia oxidizers may have to compete for the ammonia released into environment with other ammonia-utilizing microorganisms such as methanotrophs, intensifying the competition for nitrogen between AOB and MOB. It is noteworthy that there was no report about the ureolysis of AOA in non-acid soils.

Taken together, the results of this study demonstrate the stimulation of methane consumption and growth of MOB by urea and the subsequent suppression of nitrifier growth and activity. 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. In addition, our results revealed the cross-feeding of methane-derived carbon in the soil system upon urea fertilization, indicating urea might play an important role in carbon cycle through the microbial food web processing carbon from methane oxidation in paddy soil. Assimilation of N possibly might provide mechanistic mechanisms for inhibition of ammonia oxidizers by methane addition. Therefore, we speculated that competition for nitrogen between methane- and ammonia-oxidizers play a dominant role in microbial interactions in our study, which is of help toward predictive understandings of carbon and nitrogen cycle in complex environment.

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Table1. Changes in pH, moisture content, NH$_4^+$-N and NO$_3^-$-N content in soil microcosms over the course of 19 days of incubation

<table>
<thead>
<tr>
<th>Treatments</th>
<th>pH$^a$</th>
<th>Moisture (%)$^b$</th>
<th>NH$_4^+$-N (µg/g d.w.s)$^c$</th>
<th>NO$_3^-$-N (µg/g d.w.s)$^c$</th>
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<td>Zero Time</td>
<td>7.39±0.04</td>
<td>19.4±0.42</td>
<td>0.51±0.10</td>
<td>11.1±0.31</td>
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<td>7.53±0.01</td>
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<td>Day-5-Urea</td>
<td>7.35±0.06</td>
<td>25.5±0.51</td>
<td>16.1±3.81</td>
<td>61.0±8.62</td>
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<td>Day-5-CH$_4$+Urea</td>
<td>7.37±0.12</td>
<td>24.8±1.31</td>
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<tr>
<td>Day-19-Urea</td>
<td>7.27±0.30</td>
<td>30.5±1.85</td>
<td>44.8±6.69</td>
<td>137.6±28.3</td>
</tr>
<tr>
<td>Day-19-CH$_4$+Urea</td>
<td>6.85±0.09</td>
<td>28.6±2.03</td>
<td>3.66±1.56</td>
<td>59.9±6.01</td>
</tr>
</tbody>
</table>

$^a$ pH was determined using a ratio of H$_2$O 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.
**Figure 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.
Figure 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.
Figure 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.
**Figure 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 of Urea denotes incubation with $^{13}$C-Urea plus $^{13}$C-CO$_2$. CH$_4$-HF-OTU-1-(1068)-72.3% indicates that OTU-1 contained 1068 reads with sequence identity of >97%, accounting for 72.3% 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.
Figure 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.
Supplemental Material for

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

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This file includes:

Supplemental Table S1 to S6
Supplemental Figure S1 to S10
Supplemental Reference
Table S1. The scenario of SIP microcosm construction over the course of 19 days of incubation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day-0 - 18:00 pm†</th>
<th>Day-5 - 8:00 ‡</th>
<th>Day-11 - 20:00 pm ‡</th>
<th>Day-12 - 10:00 am †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>CH4 added (ppmv)*</td>
<td>Urea added (µg N/g d.w.s.)*</td>
<td>CO2 added (ppmv)*</td>
<td></td>
</tr>
<tr>
<td>$^{13}$C-CH$_4$</td>
<td>9460</td>
<td>---</td>
<td>9229</td>
<td>9541</td>
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<tr>
<td>$^{13}$C-Urea</td>
<td>---</td>
<td>---</td>
<td>7770</td>
<td>9552</td>
</tr>
<tr>
<td>$^{13}$C-CH$_4$+Urea</td>
<td>9222</td>
<td>---</td>
<td>8020</td>
<td>9164</td>
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<tr>
<td>$^{12}$C-CH$_4$+Urea</td>
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<td>---</td>
<td>6755</td>
<td>9113</td>
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<td>---</td>
<td>---</td>
<td>6201</td>
<td>9229</td>
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<tr>
<td>$^{13}$C-CH$_4$+Urea</td>
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<td>9766</td>
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<td>---</td>
<td>9113</td>
<td>---</td>
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<tr>
<td>$^{13}$C-CH$_4$</td>
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<td>---</td>
<td>---</td>
<td>18947</td>
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<td>---</td>
<td>11724</td>
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<td>11762</td>
<td>12491</td>
</tr>
<tr>
<td>$^{12}$C-CH$_4$+Urea</td>
<td>---</td>
<td>---</td>
<td>8678</td>
<td>10431</td>
</tr>
<tr>
<td>$^{13}$C-Urea</td>
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<td>11225</td>
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<tr>
<td>$^{13}$C-CH$_4$+Urea</td>
<td>---</td>
<td>---</td>
<td>17923</td>
<td>18598</td>
</tr>
<tr>
<td>$^{12}$C-CH$_4$+Urea</td>
<td>---</td>
<td>---</td>
<td>18190</td>
<td>17706</td>
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<tr>
<td>$^{13}$C-Urea</td>
<td>---</td>
<td>---</td>
<td>8792</td>
<td>10788</td>
</tr>
<tr>
<td>$^{13}$C-CH$_4$+Urea</td>
<td>---</td>
<td>---</td>
<td>17533</td>
<td>15901</td>
</tr>
<tr>
<td>$^{12}$C-CH$_4$+Urea</td>
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<td>---</td>
<td>10286</td>
<td>10430</td>
</tr>
<tr>
<td>$^{13}$C-Urea</td>
<td>---</td>
<td>---</td>
<td>11928</td>
<td>12638</td>
</tr>
</tbody>
</table>

The destructive sampling performed and the remaining microcosms were flushed with pressurized synthetic air (20% O$_2$, 80% N$_2$).

* The amount of substrate added to microcosms. The $^{13}$C and $^{12}$C-substrates were used for labeled and control microcosms, respectively.
† The timing of substrate added to microcosms, and the numbers in brackets indicate the time of day.
‡ The date of SIP microcosms were flushed with pressurized synthetic air (20% O$_2$, 80% N$_2$), and subsequently amended with fresh substrate.
--- No substrate added
## Table S2. Primers and PCR conditions used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>primer sequence(5'-3')</th>
<th>Targeted gene</th>
<th>Thermal Profile</th>
<th>Molecular analysis</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>515F</td>
<td>CCAGCMGCCGCGGG</td>
<td>16S rRNA gene</td>
<td>95℃,3.0min;30×(95℃,30s; 55℃, 30s;72℃, 45s);72℃,10min</td>
<td>Pyrosequencing</td>
<td>(Xia et al., 2011)</td>
</tr>
<tr>
<td>907R</td>
<td>CCGTCAATTCTMTTRAGTTT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A189F</td>
<td>GGN GAC TGG GAC TTC TGG</td>
<td>pmoA gene</td>
<td>95℃,3.0min;40×(95℃,10s; 55℃, 30s;72℃, 30s; 80℃ 5s; with plate read); melt curve 65℃ to 95℃, incremental 0.5℃, 0:05+plate read</td>
<td>Real-time PCR</td>
<td>(Costello and Lidstrom, 1999; Holmes et al., 1995)</td>
</tr>
<tr>
<td>mb661r</td>
<td>CCG GMG CAA CGT CYT TAC C</td>
<td></td>
<td>95℃,3.0min;30×(95℃,30s; 55℃, 30s;72℃, 45s);72℃,10min</td>
<td>Pyrosequencing</td>
<td></td>
</tr>
<tr>
<td>amoA-1F</td>
<td>GGGGTTTTCTACTGGTTG</td>
<td>bacterial amoA gene</td>
<td>95℃,3.0min;40×(95℃,10s; 55℃, 30s;72℃, 30s; with plate read); melt curve 65℃ to 95℃, incremental 0.5℃, 0:05+plate read</td>
<td>Real-time PCR</td>
<td>(Rotthauwe et al., 1997)</td>
</tr>
<tr>
<td>amoA-2R</td>
<td>CCCCTCGGGAAAGCCTTCTTTC</td>
<td></td>
<td>95℃,3.0min;30×(95℃,30s; 55℃, 30s;72℃, 45s);72℃,10min</td>
<td>Pyrosequencing</td>
<td></td>
</tr>
<tr>
<td>Arch-amoAF</td>
<td>STAATGGTCTGGCTTAGACG</td>
<td>Archaeal amoA gene</td>
<td>95℃,10.0min;40×(95℃,30s; 55℃, 45s;72℃, 30s;82℃ 15s with plate read); melt curve 65℃ to 95℃, incremental 1.0℃, 0:05+plate read</td>
<td>Real-time PCR</td>
<td>(Francis et al., 2005)</td>
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<tr>
<td>Arch-amoAR</td>
<td>GCGGCCATCCATCTGTATGT</td>
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Table S3. Pyrosequencing summary of the total microbial communities in SIP microcosms using the universal primers 515F-907R of the total 16S rRNA genes

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Pyrosequencing reads number†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High-quality read number</td>
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<tr>
<td>Zero Time-R1</td>
<td>9519</td>
</tr>
<tr>
<td>Zero Time-R2</td>
<td>9110</td>
</tr>
<tr>
<td>Zero Time-R3</td>
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<tr>
<td>13C-CH₄-R1</td>
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<tr>
<td>13C-CH₄-R2</td>
<td>8630</td>
</tr>
<tr>
<td>13C-CH₄-R3</td>
<td>8829</td>
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<tr>
<td>13C-Urea-R1</td>
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<td>13C-Urea-R3</td>
<td>6541</td>
</tr>
<tr>
<td>13C-CH₄+Urea-R1</td>
<td>7431</td>
</tr>
<tr>
<td>13C-CH₄+Urea-R2</td>
<td>8372</td>
</tr>
<tr>
<td>13C-CH₄+Urea-R3</td>
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<td>7809</td>
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<tr>
<td>12C-CH₄-R1</td>
<td>10104</td>
</tr>
<tr>
<td>12C-CH₄-R2</td>
<td>41172</td>
</tr>
<tr>
<td>12C-CH₄-R3</td>
<td>41230</td>
</tr>
<tr>
<td>13C-Urea-R1</td>
<td>8294</td>
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<td>13C-Urea-R2</td>
<td>31675</td>
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<td>13C-Urea-R3</td>
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<tr>
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<td>7309</td>
</tr>
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<td>12C-Urea-R3</td>
<td>6494</td>
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<tr>
<td>12C-Urea-R1</td>
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<td>12C-Urea-R3</td>
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<tr>
<td>Average</td>
<td>12831</td>
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<tr>
<td>Total reads</td>
<td>346428</td>
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</table>

*: The designation of R1 to R3 represents triplicate microcosm incubations.
†: The value in parentheses represents the percentage of the targeted 16S rRNA phylotype reads to total 16S rRNA gene sequence reads in each microcosm.
--- Not detected
Table S4. Pyrosequencing summary of the total microbial communities in the fractionated DNA by isopycnic centrifugation of total DNA extracted from SIP microcosms using the universal primers 515F-907R of the total 16S rRNA genes.

<table>
<thead>
<tr>
<th>DNA gradient fraction*</th>
<th>Day-5</th>
<th>Day-19</th>
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<tbody>
<tr>
<td></td>
<td>$^{13}$C-CH$_4$</td>
<td>$^{13}$C-Urea</td>
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<tr>
<td>Fraction-13</td>
<td>39</td>
<td>107</td>
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<tr>
<td>Fraction-12</td>
<td>5134</td>
<td>4677</td>
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<tr>
<td>Fraction-11</td>
<td>4318</td>
<td>4658</td>
</tr>
<tr>
<td>Fraction-10</td>
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<td>Fraction-9</td>
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<td>4710</td>
</tr>
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<td>Fraction-8</td>
<td>6195</td>
<td>5239</td>
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<tr>
<td>Fraction-7</td>
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<td>Fraction-5</td>
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<tr>
<td>Fraction-4</td>
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<td>427</td>
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<tr>
<td>Fraction-3</td>
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<td>---</td>
</tr>
<tr>
<td>Average</td>
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<td>4134</td>
</tr>
<tr>
<td>Subtotal</td>
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</tr>
<tr>
<td>Total</td>
<td>418061</td>
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</tbody>
</table>

*: indicates DNA gradient fractions with different buoyant densities, and the smaller the number, the heavier the fractionated DNA.
--- Not determined.
Table S5. Pyrosequencing summary of *pmoA* and *amoA* genes in the total DNA extract from SIP microcosms and in the $^{13}$C-DNA fractions after isopycnic centrifugation of total DNA using primer pairs A189F-mb661r and amoA1F-2R, respectively.

<table>
<thead>
<tr>
<th>Organisms*</th>
<th>Replicate</th>
<th>Day-0</th>
<th>Day-19</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$^{13}$C-CH$_4$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total DNA†</td>
<td>Total DNA†</td>
</tr>
<tr>
<td><em>pmoA</em> genes of MOB</td>
<td>R1</td>
<td>4295</td>
<td>8244</td>
</tr>
<tr>
<td></td>
<td>R2</td>
<td>3616</td>
<td>5297</td>
</tr>
<tr>
<td></td>
<td>R3</td>
<td>---</td>
<td>5878</td>
</tr>
<tr>
<td><em>amoA</em> genes of AOB</td>
<td>R1</td>
<td>5484</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>R2</td>
<td>472</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>R3</td>
<td>6261</td>
<td>---</td>
</tr>
</tbody>
</table>

* MOB and AOB represent methane-oxidizing bacteria and ammonia-oxidizing bacteria, respectively.
† indicates that pyrosequencing was performed on the total DNA extract from the $^{13}$C-labeled microcosms.
‡ indicates that pyrosequencing was performed on the $^{13}$C-DNA fraction after ultracentrifugation of total DNA extract.
---Not determined
Table S6. The estimated budget of carbon and nitrogen assimilation by methanotrophs and ammonia oxidizers in microcosms at day 19

<table>
<thead>
<tr>
<th>Treatment</th>
<th>µmol CH₄-C/microcosm ¹</th>
<th>µg urea-N/microcosms</th>
<th>CO₂ produced</th>
<th>CO₂ assimilated by methanotrophs</th>
<th>Assimilation of urea-N by methanotrophs ²</th>
<th>Nitrate produced from urea-N by ammonia oxidizers</th>
<th>Urea-N recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>¹³C-CH₄+Urea-R1</td>
<td>1111</td>
<td>730.9</td>
<td>380.5 (34.2%)</td>
<td>1332 (74.0%)</td>
<td>364.2 (20.2%)</td>
<td>94.2%</td>
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</tr>
<tr>
<td>¹³C-CH₄+Urea-R2</td>
<td>1081</td>
<td>688.8</td>
<td>392.4 (36.3%)</td>
<td>1373 (76.3%)</td>
<td>339.6 (18.9%)</td>
<td>95.2%</td>
<td></td>
</tr>
<tr>
<td>¹³C-CH₄+Urea-R3</td>
<td>1030</td>
<td>746.3</td>
<td>283.9 (27.6%)</td>
<td>994 (55.9%)</td>
<td>372.4 (20.69%)</td>
<td>76.6%</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>1074±41.1</td>
<td>722.0±29.8</td>
<td>352.3±59.5</td>
<td>1234±208.4</td>
<td>358.7±17.1</td>
<td>88.7±10.5%</td>
<td></td>
</tr>
</tbody>
</table>

¹The designation R1 to R3 represents incubation of triplicate microcosms.

²The amount of CH₄ consumed was calculated as the net difference in CH₄ concentration between day 0 and day 19. The amount of CO₂ produced was estimated in a similar way. Assuming that all CH₄ consumed were converted to CO₂, the amount of CO₂ assimilated by methanotrophs could be calculated as the net difference between the consumed CH₄ and the produced CO₂ at day 19 as previously described (Whalen et al., 1990).

³For every mole of assimilated carbon 0.25 moles of nitrogen have to be taken up (Bodelier and Laanbroek, 2004).
**Figure S1.** Methane consumption in soil microcosms over an incubation period of 19 days. Methane consumption is expressed as the percentage of the methane concentrations left in the headspace of the microcosms relative to the initial methane concentration in the microcosms in the absence (a) and presence (b) of urea nitrogen. The numbers above the columns denote the initial concentration (ppmv) immediately after the methane additions.
Figure S2. Changes in NH$_4^+$-N and NO$_3^-$-N content in soil microcosms incubated with urea with or without CH$_4$ over the course of 19 days of incubation.

Figure S3. Quantitative distribution of pmoA gene copy numbers (a), amoA gene copy numbers of Bacteria (b) and Archaea (c) in total DNA from microcosms after incubation for 5 and 19 days. The error bars represent standard deviations of the triplicate microcosms. The different letters above the columns indicate a significant difference (P<0.05) using analysis of variance.
Figure S4. The effect of methane on ammonia-oxidizing archaea (AOA) in soil microcosms incubated for 19 days. The relative frequency is expressed as the percentage of the targeted reads to the total 16S rRNA gene sequences reads in soil sample. The error bars represent standard deviation of the triplicate microcosms, while for the soil microcosms of CH₄+Urea treatment 6 replicates were used including both ¹²C-control and ¹³C-labeled treatments. The different letters above the columns indicate a significant difference ($P<0.05$) using analysis of variance.
Figure S5. Phylogenetic tree showing the relationship of methane-oxidizing bacterial 16S rRNA gene (a) and pmoA gene (b) sequences in soil microcosms to those deposited in the GenBank. Pyrosequencing reads of methanotrophic 16S rRNA genes and pmoA genes were used from triplicate microcosms at day 0 and day 19, and representative sequences were chosen for analysis. The designation of CH4+Urea-OTU-1-38%-(616) indicates that OTU-1 containing 616 sequences with identity of >97% comprised 38% of methanotrophic 16S rRNA gene sequences in 13C-CH4+Urea treatment after incubation for 19 days. CH4-OTU-1-69.8%-(3844) indicates that OTU-1 containing 616 sequences with identity of >87% comprised 69.8% of pmoA gene sequences in 13C-CH4 treatment after incubation for 19 days. One representative sequence was extracted using mothur software package for tree construction. The scale bar represents nucleotide acid substitution percentage.
Figure S6. Phylogenetic tree showing the relationship of ammonia-oxidizing bacterial 16S rRNA gene (a) and amoA gene (b) sequences in soil microcosms to those deposited in the GenBank. Pyrosequencing reads of AOB 16S rRNA genes and amoA genes were used from triplicate microcosms at day 0 and day 19. As for 16S rRNA genes, all AOB sequence reads were retrieved for analysis using mother software package, and only representative amoA gene reads were included for clarity to construct phylogenetic tree. The designation of Urea+CH4-OTU-1-52%-(2326) indicates that OTU-1 containing 2326 sequences with identity of >97% comprised 52% of ammonia-oxidizing bacterial amoA gene sequences in 13C-Urea+CH4 treatment after incubation for 19 days, and one representative sequence was extracted using mothur software package for tree construction. The scale bar represents nucleotide acid substitution percentage.
Figure S7. Quantitative distribution of *pmoA* gene copy numbers (a,b), *amoA* gene copy numbers of *Bacteria* (c,d) and *Archaea* (e,f) across the entire buoyant density gradient of the fractionated DNA from SIP microcosms after incubation for 5 and 19 days. The normalized data are the ratio of gene copy number in each DNA gradient fraction to the maximum quantities for each treatment. The error bars represent standard deviations of the duplicate microcosms.
Figure S8. Relative frequency of the archaeal 16S rRNA gene sequences reads in DNA gradient fractions with a buoyant density gradient isolated from SIP microcosms after incubation for 5 and 19 days. The frequency is expressed as the percentage of the targeted archaeal reads to the total 16S rRNA gene sequences reads in each DNA gradient fraction.
**Figure S9.** Phylogenetic tree of *pmoA* genes for methane-oxidizing bacteria (a) and *amoA* genes for ammonia-oxidizing bacteria (b) in the 13C-DNA ‘heavy’ fraction from the labeled microcosm after incubation for 19 days. The designation of CH4-HF-OTU-1-(308)-83.9% indicates that OTU-1 containing 308 reads with sequence identity of >87% comprised 83.9% of *pmoA* gene sequences retrieved from the ‘HF’ fraction in microcosms amended with 13C-CH4 for incubation after 19 days, and one representative sequence was extracted using mothur software package for tree construction. The scale bar represents nucleotide acid substitution percentage.
Figure S10. Phylogenetic tree showing the relationship of the high-throughput sequence reads of *Undibacterium* (a) and *Pseudomonas* (b) in the $^{13}$C-labeled ‘heavy’ DNA fractions (HF) to those deposited in the GenBank. The designation of of CH$_4$+Urea-HF -OTU-1-13‰-(172) indicates that OTU-1 contains 172 sequences associated with *Undibacterium* comprising 13‰ of 16S rRNA gene sequence reads in the ‘heavy’ DNA fractions with identity of >97%, and one representative sequence was extracted using mothur software package for tree construction. The scale bar represents nucleotide acid substitution percentage.
Reference


