Point-by-point response to the comments of the referee #1

(Manuscript ID bg-2014-97)

General comments of the referee #1

The authors present an intriguing experiment that demonstrates that urea fertilization combined with a high methane concentration (~10,000 ppm) may inhibit ammonia oxidizers and ammonia oxidation. The authors provide hints that type I MOB were N-limited and outcompeted the obviously the much slower responding AOB when utilizing urea

Used methods (amplicon pyrosequencing, DNA stable isotope probing) are state of the art methods and all experiments were well conducted. The english is largely of good quality.

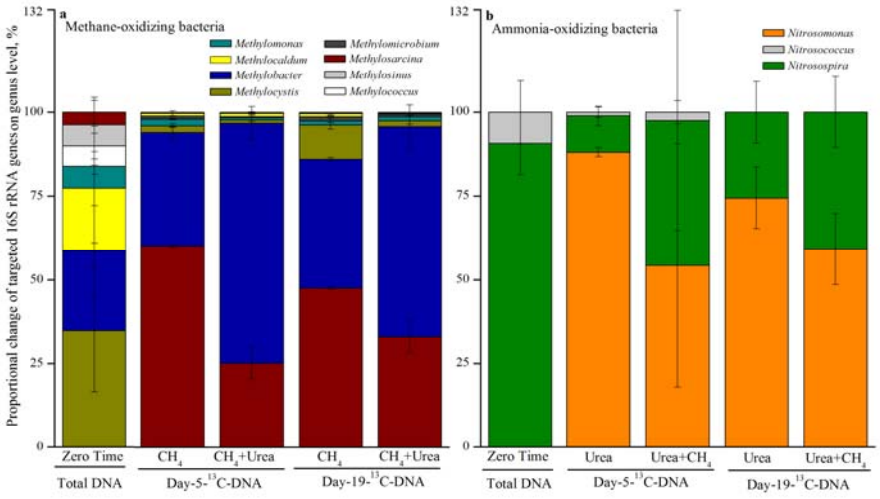
The reviewer has some major concerns

Reply: We are thankful to the referee for the positive comment. The major concerns are addressed in the following, and we are looking forward to further comments for manuscript improvement.

Major Concerns

1. It would be extremely helpful to present in figure 5 not only MOB identities based on the old fashioned classification system (type I or II), but name genera, as the authors do then finally in the discussion section and Fig. 3.

Reply: It has been corrected as follows in the revised Fig. 5



2. Type II methanotrophs did not rapidly respond to added methane or urea. Are the detected organisms known to be diazotrophic, i.e., are

these specialists that respond under N-limited conditions? Please, discuss this issue in the revised manuscript version

Reply: We fully agree with these comments, and believe type II could survive better under N-limited conditions than type I. It was discussed in the revised ms as follows.

^{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. Pls see the revised version from line 535 to line 538 on page 19.

3. Before the experiment soil was pre-incubated. The reviewer did not see any data that documents, which changes in the methanotrophic and ammonia-oxidizing communities occurred during this pre-incubation period. This lack of information make the relevance for the in situ situation less likely. Please, discuss this issue in the revised manuscript

Reply: the new discussion was added in the revised ms as follows..

According to this comment and the related comments of reviewer#2, We have discussed about the issue as follows in the revised version from line 470 to line 487 on page 17~18.

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).

4. Methanol-oxidizers: The authors do not explain how they decided, which of the detected taxa were methanol-utilizers (this is also not documented for ammonia-oxidizers, nitrite oxidizers, and

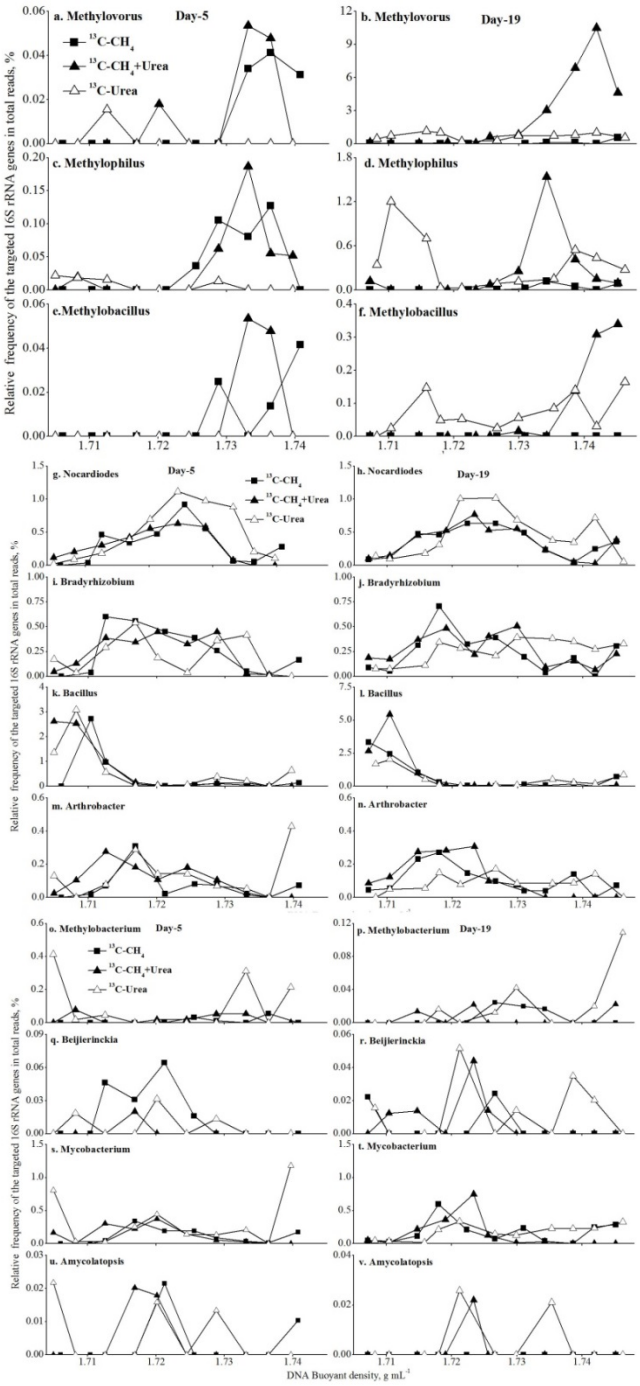
methanotrophs). There a lot of methanol-oxidizers known that occur in soil and were likely overlooked when defining this functional group (for reference Kolb 2009 FEMS Letters, Stacheter & Kolb 2013 Front Mic)

Reply: We greatly appreciate this insightful comment.

(1) Methanol oxidizers: Methanol-oxidizing bacteria utilize methanol as carbon and energy source. The known soil-retrieved methanol-oxidizing bacteria was with high diversity (Kolb, 2009). However, most of them are facultative methylotrophic, indicating the capability to utilize alternative carbon substrate. 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).

(2) In addition, we have analyzed the known methanol-oxidizers mentioned by Kolb 2009. We detected 11 genera of methanol-oxidizing bacteria, while only three genera *Methylovorus*, *Methylophilus*, and *Methylobacillus,* belonging to *Methylophilaceae* were apparently higher in the 'heavy' DNA fractions form labeled microcosms (^{13}C-CH_4 treatment and ^{13}C-CH_4+Urea treatment) than those in the control treatment (^{12}C-CH_4+Urea treatment), indicating that activity of these three methanol-oxidizers in soils after incubation for 5 and 19 days.

Therefore, the methanol-oxidizing bacteria analyzed in our study is *Methylophilaceae*. The revision and correction has been made about methanol-oxidizers in the revised manuscript from line 93 to line 98 on page 4 and from line 323 to line 325 on page 12.



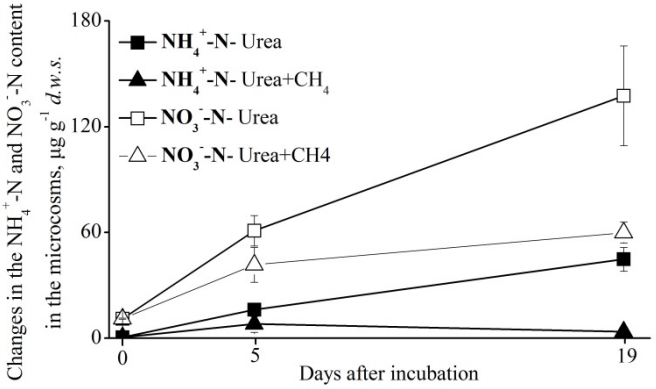
Comment#1-Fig.1 Relative frequency of 16S rRNA gene sequences affiliated with methnol-oxidizers on genus level detected in our study across the buoyant density gradient of DNA fractions from the ^{13}C-labeled and ^{12}C-control microcosms after incubation for 5 and 19 days.

(1) We have stated the taxa of the Methanotrophs from line 82 to line 89 on page 4, AOB from line 106 to line 109 on page 4, AOA from line 109 to line 113 on page 4, and NOB from line 113 to line 117 on page 4 in the revised manuscript.

5. Fig. 1, The reviewer thinks, that it would improve understanding of the complex experiment, when nitrate and ammonia data would be presented as line graphs in a separate figure.

Reply: It has been done as follow as supplementary Fig. S2

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



6. Fig. 5, Is the sequence coverage high enough to allowed for statistical comparison of single datasets? Please, provide coverages and rafaction analyses. Please, correct in the label of the y-axis '...on genus level...'.

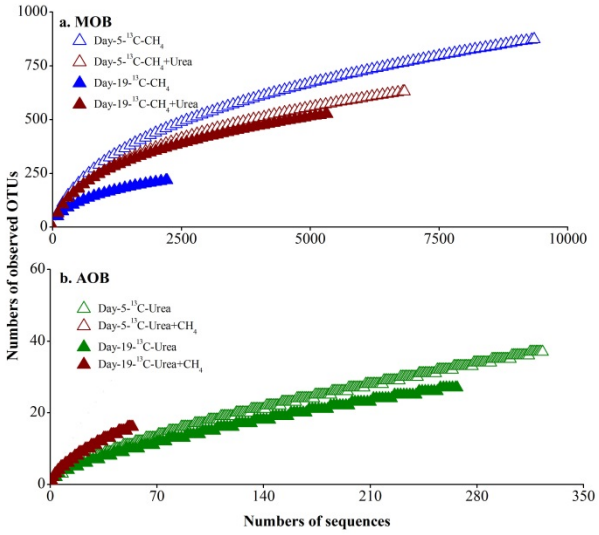
Reply: Yes, the sequencing is deep enough to allow for statistical comparison of single datasets.

(1) The coverage calculated using Good's C(Comment#1-Table1) at sequence identity of 97% of MOB and AOB sequences retrieved from ^{13}C-labeled DNA was 96.2%~96.5% and 83.3%~94.0%, respectively. In addition, rarefractionanalysis (Comment#1-Fig.2) indicated that the OTU numbers of MOB and AOB nearly reached saturation level in our treatments.

(2) We have corrected in the label of the y-axis the '...on genus level...' in the revised version(see Fig. 5).

Comment#1-Table 1. The coverage analysis of 16S rRNA gene sequences affiliated with MOB and AOB in the ^{13}C-labeled DNA from the microcosms after incubation for 5 and 19 days.

Treatment		MOB			AOB		
		sequences	**Coverage**	Observed OTUs	sequences	**Coverage**	Observed OTUs
Day5-^{13}C-DNA	CH_4	9348	**96.2%**	873	---	---	---
	Urea	---	---	---	323	**92.2%**	37
	CH4+Urea	6828	**96.4%**	631	27	**85.1%**	9
Day19-^{13}C-DNA	CH_4	2219	**96.5%**	218	---	---	---
	Urea	---	---	---	267	**94.0%**	27
	CH4+Urea	5325	**96.2%**	526	54	**83.3%**	16



Comment#1-Fig.2 Rare fraction of mentotrophic (a) and ammonia-oxidizng bacterial (b) 16S rRNA gene sequences in the ^{13}C-labeled DNA from the microcosms after incubation for 5 and 19 days.

7. Please define in the beginning of the text once the abbreviation 'd.w.s'. It means 'dry weight of soil'?

Reply: Corrected. '*d.w.s.*' means 'dry weight of soil'. We have defined the abbreviation in the revised version line 176 on page 7.

8. Do the authors also consider 16S rRNA phylotypes of the genus *Nitrosococcus* as AOB? Where these AOB detected?

Reply: Yes, we do consider it

(1) The 16S rRNA phylotypes of the genus as AOB based on the the

previous study (Purkhold et al., 2000; Purkhold et al., 2003). Comparative 16S rRNA sequences analyses showed that all the recognized ammonia oxidizers are confined to β and γ-subclass of *Proteobacteria*, and the genus *Nitrosococcus* constitutes a separate branch with the γ-subclass(Purkhold et al., 2000; Purkhold et al., 2003).

(2) It has been reported that *Nitrosococcus* species are restricted to marine environments and salt lakes (Degelmann et al., 2010). However, It has also been reported that organisms similar to the cultivated *N.oceani* strains could be detected from a wide variety of terrestrial environments (Ward and O'Mullan, 2002).

9. Discussion. The authors state that denitrfication took place suggesting a reduced oxygen availability (3908, ln 24-26). a) The authors did not provide any data on this. b) Denitrification can be very active at slightly lowered oxygen levels. The whole issue is pure speculation.

Reply: Thanks! We agree with the comment.

According to this comment and the related comments of reviewers#2, the discussion about N balance and the denitrification activity are rather speculative. To make our discussion precise and focused, we have removed the relevant discussions in the revised ms.

10. Discussion: The authors stated that MOB have a 'memory' for optimal growth conditions.The whole concept sounds arkward. Such a memory might occur somehow on community level or might just be a misinterpretation because the phylogenetic resolution of such studies are too imprecise and the found identical taxa were not identical on phenotypic level. Please, remove it or extend this point with more details.

Reply: We agree with this point and removed the points about 'memory' for optimal growth conditions in the revised version.

Minor comments

1. abstract: The final conclusion (last sentence) is not very concise and convincing. Please, provide a more conclusive statement what can be learned on competition between AOB and MOB in rice field soil. This statement is the take home message.

Reply: It has been rephrased as follows

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.

2. 3895 ln25-27, What do you intend to state here. Please, find a more concise wording.

   Reply: We intend to state that there are methanotrophic groups outside *Proteobacteria*, such as phylum *Verrucomicrobia*. Because this point has been mentioned from line 77 to line 78 on page 3, we removed this sentence to avoid repetition in the revised version.

3. 3896 ln 21, correct '...methanotrophs might...'

   Reply: Corrected

4. 3897 ln9-14,Please provide a more sharpened rational why the study is important.

   Reply: It was rephrased as follows from line 145 to line 150 on page 6 in the revised version.

   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 CH_4, urea and CH_4+urea in a paddy soil using culture-independent techniques.

5. 3898,ln2-12, Why was no additional control with only 12CH4 being used.

   Reply: Methanotrophs are not expected in the control SIP microcosms of both $^{12}CH_4$ and $^{12}CH_4$+urea treatments. Therefore, we do not run $^{12}CH_4$ control

6. 3899-3900, Please, put in references for the used SIP protocol.

   Reply: Reference added including Jia and Conrad (Jia and Conrad, 2009), Xia et al (Xia et al., 2011) and Dumont et al (Dumont et al., 2011).

7. 3901, ln6 correct '...high-quality...'

   Reply: Corrected

8. 3901, ln 25, it does make any sense to cluster *pmoA* sequences at a level of 97% similarity. It has been suggested that an average similarity of 87% is species-indicative. Of course any threshold can be used, but then a rational is mandatory.

Reply: Corrected

The 87% species cutoff value based on *pmoA* gene was shown to correspond to the 3% 16S rRNA gene distance level (Degelmann et al., 2010). We have clustered *pmoA* sequences at a level of 87% similarity in supplementary Fig. S5 and supplementary Fig. S9 in the revised version. The phylogenetic analysis of *pmoA* gene at 87% similarity is similar with that at 97% similarity in our study when we analyze the data based on genus level.

9. 3908, ln9-10 correct 'The ratio of N to CH4 is approximately 0.11 ...'

Reply: Corrected

10. 3908, ln 16 correct '...mineral N,...'

Reply: Corrected.

11. 3911, ln 12, correct '...low methane habitats.'

Reply: Corrected.

12. 3911, ln 15 correct '...in the pmoA gene...'

Reply: Corrected

13. 3911, ln 13-27, Can you exclude that the pmoA primers and 16S rRNA primers did not cover the same diversity of organisms. If not, please, note also this as another technical challenge when comparing 16S rRNA gene with pmoA datasets.

Reply: We agree with the comment that *pmoA* primers and 16S rRNA primers may not cover similar ranges of diversity. We have noted this from line 580 to line 582 on page 21in the revised version.

14. 3913, ln 5 correct '...three species...'

Reply: Corrected

15. 3913, ln 8-10, the reviewer is not convinced that substantial amounts of formaldehyde would be released. Normally formaldehyde is to its largest amount bound to cofactors to keep the cell-internal concentrations as low as possible. This system is highly efficient and works as well at high millimolar CH_4 concentrations. Methanol is a completely different issue since the reaction rate of the MeOH dehydrogenase is usually such low that methanol production at high methane concentrations exceeds its consumption. This process is located in periplasm and thus, substantial amounts of an metabolic intermediate can be released. Please, remove formaldehyde from the statement or provide literature evidence that it might have happened.

Reply: Thanks for the reasoning. The relevant discussion of formaldehyde was

removed in the revised version.

16. 3914, correct '...communities...'

Reply: Corrected

17. 3914, ln 13-15. This is very speculative based on the presented data. The authors did not provide any evidence for oxygen depletion

Reply: We agree with the comments since oxygen concentrations were not measured. The relevant discussion was therefore tuned down, although it seems very likely that oxygen concentrations differed in microcosms with different methane oxidation capacity.

18. 3914, The study did not provide any direct evidence that methanol or any other metabolite was assimilated by other methylotrophs. Thus, the sentence is overstated. Please, down tone it a bit.

Reply: We have rephrased the sentence from line 670 to line 673 on page 24 as follows.

'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.'

19. Fig S3, correct in figure legend '..affiliation...' and NOT '...designation...'

Reply: We are afraid there might have some confusion. It has been rephrased as follows.

The designation of CH_4+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 ^{13}C-CH_4+Urea treatment after incubation for 19 days.

Reference

Bratina, B.J., Brusseau, G.A., and Hanson, R.S. (1992) USE OF 16S RIbosomal-rna analysis to investigate phylogeny of methylotrophic bacteria. *International Journal of Systematic Bacteriology* **42**: 645-648.

Degelmann, D.M., Borken, W., Drake, H.L., and Kolb, S. (2010) Different atmospheric methane-oxidizing communities in European beech and Norway spruce soils. *Appl Environ Microbiol* **76**: 3228-3235.

Dumont, M.G., Pommerenke, B., Casper, P., and Conrad, R. (2011) DNA-, rRNA- and mRNA-based stable isotope probing of aerobic methanotrophs in lake sediment. *Environmental Microbiology* **13**: 1153-1167.

Eller, G., Kruger, M., and Frenzel, P. (2005) Comparing field and microcosm experiments: a case study on methano- and methylo-trophic bacteria in paddy soil. *Fems Microbiology Ecology* **51**: 279-291.

He, R., Wooller, M.J., Pohlman, J.W., Catranis, C., Quensen, J., Tiedje, J.M., and Leigh, M.B. (2012) Identification of functionally active aerobic methanotrophs in sediments from an arctic lake using stable isotope probing. *Environmental Microbiology* **14**: 1403-1419.

Jia, Z., and Conrad, R. (2009) *Bacteria* rather than *Archaea* dominate microbial ammonia

oxidation in an agricultural soil. *Environmental Microbiology* **11**: 1658-1671.
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Murrell, J.C., and Dalton, H. (1983) NITROGEN-FIXATION IN OBLIGATE METHANOTROPHS. *Journal of General Microbiology* **129**: 3481-3486.
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Nouchi, I., Hosono, T., Aoki, K., and Minami, K. (1994) Seasonal-variation in methane flux from rice paddies associated with methane concentration in soil-water, rice biomass and temperature, and its modeling. *Plant and Soil* **161**: 195-208.
Purkhold, U., Wagner, M., Timmermann, G., Pommerening-Roser, A., and Koops, H.P. (2003) 16S rRNA and amoA-based phylogeny of 12 novel betaproteobacterial ammonia-oxidizing isolates: extension of the dataset and proposal of a new lineage within the nitrosomonads. *International Journal of Systematic and Evolutionary Microbiology* **53**: 1485-1494.
Purkhold, U., Pommerening-Roser, A., Juretschko, S., Schmid, M.C., Koops, H.P., and Wagner, M. (2000) Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: Implications for molecular diversity surveys. *Applied and Environmental Microbiology* **66**: 5368-5382.
Ward, B.B., and O'Mullan, G.D. (2002) Worldwide distribution of Nitrosococcus oceani, a marine ammonia-oxidizing gamma-proteobacterium, detected by PCR and sequencing of 16S rRNA and amoA genes. *Applied and Environmental Microbiology* **68**: 4153-4157.
Xia, W., Zhang, C., Zeng, X., Feng, Y., Weng, J., Lin, X. et al. (2011) Autotrophic growth of nitrifying community in an agricultural soil. *Isme Journal* **5**: 1226-1236.

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 communitiesin 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 suppressed 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 partially inhibitedby 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. 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 methanotropswas 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 partially inhibitedthe 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 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 studiesfollowing 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).

Aerobicmethane-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 proteobacterialMOB 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,*Methylomonas, Methylomicrobium, Methylosoma, Methylosphaera and Methylovulum*) and type Ib (including genera *Methylococcus, Methylocaldum, Methylogaea, Methylobius and Methylothermus*). Type II MOB includethe family *Methylocystaceae* (including genera *Methylocystis* and *Methylosinus*)and *Beijerinckiaceaea* (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 CH_4 to methanol, which can be utilized by methanol-oxidzing bacteria as carbon and energy source. The known soil-retrieved methanol-oxidizing bacteria was with high diversity, however, most of them are faculative 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-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).16S rRNA and *amoA* gene analyses of AOB revealed that physiological group are confined to monophyletic groups whithin β- andγ-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, inclusing *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,*

115 *Nitrococcus*, *Nitrospina* and *Nitrospira*, which were assigned to the α-proteobacteria,
116 γ-proteobacteria, δ-proteobacteria and phylum *Nitrospirae*, respectively (Bock and
117 Wagner 2006).

118 The key enzymes methane monooxygenase (MMO) in methanotrophs and ammonia
119 monooxygenase in ammonia oxidizers are evolutionarily linked (Holmes et al., 1995),
120 leading to functional similarities enabling both methanotrophs and ammonia oxidizers
121 to oxidize both methane and ammonia (Jones and Morita, 1983; O'Neill and
122 Wilkinson, 1977). Pure culture studies demonstrated that methane can act as a
123 competitive inhibitor for ammonia oxidizers, and ammonia inhibits the growth and
124 activity of methanotrophs (Bedard and Knowles, 1989; Stein et al., 2012). Next to this,
125 both MOB as well as AOB have to deal with toxic intermediates (hydroxylamine in
126 case of MOB and methanol in case of AOB) (Stein et al., 2012). At the microbial
127 community level, however, the growth of methanotrophsmightbe nitrogen-limited and
128 nitrogen fertilization might relieve methane oxidizers from nutrient constraint
129 (Bodelier et al., 2000b). At the same time ammonia oxidizers and subsequent
130 nitrification may be inhibited by the methanotrophic N-assimilation. However, the
131 research focus of methane effect on nitrificatin in natural complex ecosystems is poor,
132 which is in sharp contrast with a large number of studies executed to elucidate effect
133 of nitrogenous fertilizers on methane oxidation.Moreover, the lack of knowledge on
134 this topic is even more evident taking the yet unknown role of AOA in interactions
135 with MOB into account. DNA-based stable isotope probing (DNA-SIP) is generally
136 used to link the metabolisms of ^{13}C-labeled substrates with growing microbial
137 communities in the environment. DNA-SIP has been employed to identify the active
138 methanotrophs (Dumont et al., 2011) and ammonia oxidizers in soils (Jia and Conrad,
139 2009; Lu and Jia, 2013; Xia et al., 2011). The combined use of stable isotope labeling
140 and high throughput pyrosequencing is a powerful combination of approaches that
141 offers great opportunities in elucidating interaction between MOB and AOB/AOA,
142 because both groups can easily and specifically be labeled using $^{13}CH_4$(Bodelier et al.,
143 2013; Bodelier et al., 2012) and $^{13}CO_2$(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 CH_4, urea and CH_4+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 formore 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%, andthe 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 fridgeuntil 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 ^{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

171 methane oxidation activity and MOB community composition, and the role of
172 methane on ammonia oxidation activity and AOB/AOA community composition. The
173 soil microcosm with ^{12}C-CH_4+Urea amendment was performed as control treatment
174 for the labeled SIP microcosms.

175 Microcosms for stable-isotope probing incubations were constructed in triplicate by
176 adding approximately 7.30 g fresh soil (equivalent to 6.0 g dry weight of soil, i.e.,
177 *d.w.s.*) to 120 mL serum bottles capped with black butyl stoppers for incubation at 28°
178 C in the dark for 19 days. To increase the labeling efficacy of targeted microorganisms,
179 the pre-incubation of soil at 40% maximum water-holding capacity (WHC) was
180 performed for 14 days to reduce the amount of soil-respired ^{12}C-CO_2 (Jia and Conrad,
181 2009; Xia et al., 2011). The ^{13}C-CH_4-labeled microcosms and ^{13}C-CH_4+Urea-labeled
182 microcosms were injected with $^{13}CH_4$ (99 atom %^{13}C, Sigma-Aldrich Co., St Louis,
183 MO, USA) to reach 9000 ppmv (Table S1). Meanwhile, ^{13}C-Urea fertilization of 100
184 μg urea-N/g. *d.w.s.* with 5% $^{13}CO_2$ (99 atoms %^{13}C, Sigma-Aldrich Co., St Louis, MO,
185 USA) was performed for ^{13}C-Urea-labeled microcosms and for ^{13}C-CH_4+Urea-labeled
186 microcosms as previously described (Jia and Conrad, 2009). As for ^{13}C-CH_4-labeled
187 microcosms, the distilled water instead of urea was added. SIP control microcosms
188 were established in triplicate by addition of the unlabeled CH_4, urea and CO_2 instead
189 of ^{13}C-substrate. CH_4 and CO_2 concentrations were measured every few hours
190 depending on the rate of methane consumption by gas chromatography (Shimadzu
191 GC12-A, Japan) as previously described (Zhu et al., 2010). After more than 90% of
192 CH_4 was consumed, the headspace was flushed with pressurized synthetic air (20% O_2,
193 80% N_2) for 1 min to maintain oxic conditions before ^{13}C-labeled or unlabeled
194 substrate was renewed, to reach about ~10000 ppmv CH_4 and/or 100 μg urea-N/g.
195 *d.w.s.* plus 5% CO_2. Due to strong methane oxidation in microcosms amended with
196 ^{13}C-CH_4+Urea treatment (Fig. S1), methane addition was regularly repeated, in
197 addition to urea and CO_2substrates. The scenario of SIP microcosm construction was
198 detailed in supplemental Table S1. The destructive sampling was performed in
199 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 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 NH_4^+-N and 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 (NanoDropTechnoloqies ,Wilmington, DE, USA), and soil DNA was stored at -20℃.

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; Xia et al., 2011).. 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 mMTris-HCl; 100 mMKCl; 1.0 mM EDTA). The mixturewas 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 44hours 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/minwas 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 wasthen dissolved in 30μL 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 eachbuoyant 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 andlabeling 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 eachprimer, and 2μL template DNAwas performed and the amplicons were purified and visualized on 1.8% agarosegels. 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-qualitysequence 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-offusing 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 ^{13}C-labeled DNA

The *pmoA* gene for MOBand bacterial *amoA* gene for AOBwere 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 fromtriplicatemicrocosms 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. 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, aligment and phylogenetic tree construction. High quality sequences(e.g. read legth 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 legth 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 wereclustered 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.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 11.1μgNO_3^--N /g *d.w.s* in urea-amended microcosms at day 0, to 61.0 and 137.6 μg NO_3^--N /g *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 theV3~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 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 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 4.44×10^8 copies g^{-1}*d.w.s.* at day 0 to 1.45×10^9copies g^{-1}*d.w.s.* and 1.66×10^9 copies g^{-1}*d.w.s.*in the microcosms incubated with CH_4 for 5 and 19, respectively. Urea addition led to 1.35 and 3.16 times more *pmoA* genes than that in only CH_4-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 CH_4+Urea treatment (2.76%) than that in CH_4-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 CH_4 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 4.08×10^7copies g^{-1}*d.w.s.* at day 0 to 1.06×10^8copies g^{-1}*d.w.s.* at day 19 in the

337 microcosms incubated with urea (Fig. S3b). The increasement also was observed in
338 the urea+CH_4 treatment, however, the presence of CH_4 resulted in 1.33-fold decrease
339 relative to only urae-amended microcosms after incubation for 19 days. This indicated
340 that CH_4 partially inhibited the growth of AOB.Similar results were observed for soil
341 nitrite-oxidizing bacteria (NOB). For instance, the relative abundance of NOB16S
342 rRNA gene sequences in total microbial community increased significantly from 0.91%
343 at day 0 to 1.42% at day 19 in the urea-amended microcosms, while soil microcosms
344 with Urea+CH_4 displayed a relative abundance as low as 0.42% at day 19 (Fig. 1f).As
345 for AOA, there was no significant change in relative abundances upon urea
346 fertilization during SIP microcosm incubation, although the decreasing trend was
347 observed in the presence of CH_4 (Fig. S4).The simialr result was also detected by the
348 real-time PCR of archaeal *amoA* gene (Fig. S3c).

349 ***High-throughput fingerprinting of functional guilds against the total communities***

350 The 16S rRNA genes affiliated with MOB and AOB were selectedfor phylogenetic
351 analysis from the total pyrosequencing reads in soil microcosms, after incubation for
352 5 and 19 days, following the additions of methane and/or urea. Phylogenetic analysis
353 revealed a remarkable shift of MOB community structure based on both 16S
354 rRNAgene (Fig. s5a) and *pmoA* genes (Fig. S5b). Though type II methanotrophs
355 dominate MOB communities in background soil at day 0, the consumption of CH_4 in
356 soil microcosms led to a drastic increase in relative abundance of type Ia
357 methanotrophic 16S rRNA gene sequences in the total 16S rRNA gene sequences
358 from 0.09% at day 0 to 14.4% at day 5 (Fig.2a). Interestingly, type II
359 methanotroph-like 16S rRNA genes stayed at very low proportions in the total
360 microbial communityduring the entire incubation period, whereas significant increase
361 was observed from 0.12% at day 0 to 0.55% at day 19.Urea fertilization further
362 stimulated the relative abundance of type Ia methanotrophs reaching 1.3 and 4 times
363 higher in the CH_4+Urea-amended microcosms than that in the microcosms amended
364 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 IImethanotrophs.Similar results were obtained by pyrosequencing analysis of *pmoA* genes (Fig. S5b). Phylogenetic analysis of*pmoA*genes indicated that type Ia *pmoA* sequenceswere stimulated from 7.4% at day 0 to 69.8% 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 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). CH_4 addition resulted in lower abundance of*Nitrosomonas*-like 16S rRNA genes in the total microbial communitiesat day 5 and day 19, representing2- 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 inhibitedin the presence of CH_4 (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^{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

393 gradient indicated that MOB and AOB were ^{13}C-labeled to different extents. The
394 relative abundance of 16S rRNA gene sequences of methanotrophswas exceptionally
395 high up to 90%of the total 16S rRNA gene sequences in the 'heavy' DNA fractions
396 from the labeled microcosms, suggesting strong labeling of methanotrophic
397 communities in soils after incubation for 5 (Fig.3a) and 19 days (Fig.3b). This was
398 further supported by quantitative analysis of *pmoA* gene copiesreaching the peak in
399 the 'heavy' DNA fractions from the labeled microcosms, while the highest number
400 was observed in the 'light' DNA fractions for the ^{12}C-control treatment (Fig. S7).In
401 addition,the relative abundance of 16S rRNA gene sequences affiliated with
402 methanol-oxidizing bacteria was apparently higher in the 'heavy' DNA fractions from
403 the labeled microcosms (^{13}C-CH_4 and ^{13}C-CH_4+Urea) than those in the control
404 treatments (^{12}C-CH_4+Urea), despite the relatively low proportion of~0.20% at
405 day5(Fig. 3c). The prolonged incubation for 19 days increased the proportion of
406 methanol-oxidizing bacteriasignificantly up to 11.0% of the total 16S rRNAgene
407 sequencesin the ^{13}C-DNA from the labeled soil microcosms amended both with CH_4
408 and Urea, but not in the labeled microcosms that received only CH_4 (Fig. 3d).

409 The 16S rRNA gene sequences of AOB were highly enriched in 'heavy' DNA
410 fractions from the labeled microcosm amended only with urea at day 5 (Fig. 3e) and
411 day 19 (Fig.3f), but not the CH_4+Urea treatment during the 19-day incubation period.
412 For instance, up to 5.73% of total 16S rRNA gene sequences in the 'heavy' DNA
413 fractions could be assigned to AOB for ^{13}C-Urea treatment, while only 0.33% of the
414 total 16S rRNAgene sequences in the ^{13}C-Urea+CH_4 treatments were related to AOB
415 at day 19 (Fig. 3f). Similar results were obtained for nitrite-oxidizing bacteria (Fig.3g
416 and Fig.3h). The relative abundance of NOB in the 'heavy' DNA fractions was
417 significantly higher in microcosms with ^{13}C-urea than ^{13}C-Urea+CH_4 treatment,
418 implying a much greater degree of labeling of NOB cells in ^{13}C-Urea treatments
419 during active nitrification. Furthermore, it is noteworthy that no significant
420 enrichment of archaeal 16S rRNA gene sequences occurred in the 'heavy' DNA
421 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 rRNAsequences 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 MOBin CH_4-amended microcosmsat day 19, whereasall*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 microcosmsat 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_2O 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 oxidizerswill 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 ofMOB. Growth and activity of ammonia oxidizers waspartially 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 approximately0.11 (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 nitrificationonly 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.

509 The relative abundance of both 16S rRNA and *pmoA* genes decreased when
510 incubating with methane only, demonstrating loss of activity and growth potential
511 when N is limiting. A similar result was obtained in microcosms planted with rice
512 (Bodelier et al., 2000a), where MOB even lost their potential for oxidizing methane.
513 However, adding ammonium to these inactive communities led to immediate
514 re-activation of oxidation (Bodelier et al., 2000a), indicating that N-limitation is not
515 only inhibiting growth but also regulated methane consumption enzyme machinery.
516 This inactivation and rapid re-activation of methane oxidation has even been
517 demonstrated on field scale in rice paddies (Dan et al., 2001; Kruger and Frenzel,
518 2003). It has been proposed that nitrogen fixation may deplete reducing equivalents
519 leading to lowering and even cessation of methane oxidation (Bodelier and Laanbroek,
520 2004; Dan et al., 2001). This suggests that under conditions of high methane and low
521 N availability, there is a niche for methanotrophy where they seem to overwhelmingly
522 outcompete nitrifying communities. Nitrifiers can operate in the absence of
523 competition with MOB, which may be inactivated due to energy-depletion as the
524 result of N_2-fixation. Hence, this points to niche differentiation or avoidance strategies
525 of the nitrifiers.

526 It is obvious that only a subset of the MOB profit substantially from the combined
527 addition of methane and urea-N. Although type II MOB increase in relative
528 abundance of 16S rRNA gene sequences in toal microbial community with the
529 addition of methane they do not profit from the addition of urea, but are also not
530 affected by it. Addition of ammonium to rice soil has been demonstrated to inhibit
531 type II MOB (Mohanty et al., 2006). This is obviously not the case in our study where
532 the rapid growth of type Ia MOB keeps ammonium N-low. The growth of type II
533 MOB is apparently independent of the N-availability suggesting that they can rely on
534 N_2-fixation only.^{13}C-labeled methanotrophic 16S rRNA gene sequences are closely
535 affiliated with *Methylocystis parvus* OBBP, which possesses nitrogenase and capable
536 of nitrogen fixing (Murrell and Dalton 1983). This suggested that these *Methylocystis*
537 *parvus*-like type II may respond under N-limited conditions in our study. Next to this,

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

548 The strong stimulation of type Ia MOB upon methane application alone and in
549 combination with urea-N application has been observed frequently in rice soils but
550 also in other environments, reflecting their competitive life-strategy as reviewed and
551 synthesized (Ho et al., 2013). The most responsive MOB species in high methane
552 habitats seem to be *Methylobacter* species (Krause et al., 2012). Our experiments
553 show that *Methylosarcina* species are clearly the most responsive without addition of
554 urea. This is in contrast with the niche differentiation observed at high spatial
555 resolution in rice soil microcosms (Reim et al., 2012). The presence of
556 *Methylosarcina* related MOB in the surface layer of thin layer microcosms and not in
557 the methane-oxygen interface, implying that *Methylosarcina* thrives under
558 low-methane ('oligotrophic') conditions, in contrast to *Methylobacter* which
559 dominates the zone of high methane flux. However, remarkably, in our experiments
560 *Methylosarcina* clearly is dominant at high methane supply but is replaced partly by
561 *Methylobacter* when urea-N is added. This might be attributed to competition for
562 methane, nitrogen, or even oxygen. A similar result was observed in SIP analyses of
563 lake sediment microcosms using a metagenomic approach (Beck et al., 2013)**.** Hence,
564 we speculate that observations by Reim et al (Reim et al., 2012) may also be
565 explained by weak competitive abilities of *Methylosarcina* instead of being restricted
566 to lowmethane habitats.

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). The variation of *pmoA* copy numbers may occur among different MOB. The two *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 *Methylocloccus capsulatus* Bath (Stolyar et al., 1999). However, this assessment may misestimate the *pmoA* copies in other MOB which is not identified until now.Furthermore, another possible explanation for incongruence may be that*pmoA* primers and 16S rRNA primers may not completely cover simiar ranges of diversity, as reported previously(Costello and Lidstrom 1999).

Interestingly, we found significant increase of putative methanol-oxidizing bacteria related to *Undibacterium* (Fig. S10) which are affiliated the family *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 *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

596 removal of methanol by the methylotrophs is beneficial to methanotrophs given the
597 toxic nature of the compound. However, this would be subject of further
598 study.Interesting is this link between nitrogen and cross-feeding of methanotrophic
599 metabolites by other microorganism, possibly creating novel niches e.g. more
600 methane-driven carbon substrate, lower-toxic environment for methanotrophs in soil.

601 Our results revealed that the presence of CH_4 in microcosmspartially inhibited the
602 nitrification activity in the paddy soil tested. Physiologically, the enzymatic similarity
603 of ammonia-oxidizers and MOB may result in ammonia oxidation by MOB (Bodelier
604 and Frenzel, 1999), leading to reduced availability of ammonia for ammonia oxidizers.
605 However, previous studies showed that MOB had lower affinity for ammonia than for
606 CH_4(Banger et al., 2012; Bedard and Knowles, 1989; Yang et al., 2011). Moreover, it
607 has been proposed that ammonia oxidation by MOB occurred only when the ratio of
608 ammonia to CH_4 is higher than 30 in soils (Banger et al., 2012; Bodelier and
609 Laanbroek, 2004; Yang et al., 2011). The molecular ratio of ammonia to $CH_4$was
610 about 0.11in our study, thus the suppression of ammonia oxidizers growth and activity
611 in the presence of CH_4 may not be explained by ammonia oxidation by MOB.
612 Furthermore, a large part of the applied N disappeared in the presence of CH_4, and
613 presumably assimilated by MOB. This explanation seems plausible for the
614 suppression of methane on ammonia oxidation and the growth of ammonia oxidizers.
615 It is interesting to note that up to 4.8% of the ^{13}C labeled sequences in the
616 urea-amended microcosm were phylogenetically closely related to *Pseudomonas*
617 *fluorescens, Pseudomonas syringae* and *Pseudomonas aeruginosa* (Fig. S10b). These
618 three speciesuse nitrite as nitrogen source and catalyze denitrification (Betlach and
619 Tiedje, 1981; Modolo et al., 2005; Rinaldo et al., 2007). In the meantime, it remains
620 elusive about the toxic effect of intermediates substance during methane oxidation on
621 nitrifying communities. For example, methanol may inhibit the growth of AOA and
622 AOB communities, and we detected no archaeal *amoA* genes and 16S rRNA genes.
623 The possibility of heterotrophic AOA lifestyle could also not be excluded (Ingalls et
624 al., 2006; Stahl and de la Torre, 2012).

625 The genus *Nitrosospira* was the dominant AOB in the native soil, being consistent
626 with general observations that *Nitrosospira* are ubiquitous in upland soils as important
627 members of nitrifying population (Hastings et al., 1997; Stephen et al., 1996).In our
628 study, the apparent growth of *Nitrosospira* was observed in the microcosms amended
629 with urea-N, and the cluster 3 was the dominant active *Nitrosospira* group. It has been
630 reported that *Nitrosospira* cluster 3 was the dominant AOB group in a number of
631 neutral soil receiving nitrogen fertilization (Bruns et al., 1999; Mendum et al., 1999).
632 Intriguingly, methane addition suppressed the growth of *Nitrosospira*, and AOB
633 within the cluster 3 appeared to be inhibited to a greater extent than those of cluster 4.
634 It has been proposed that the reduced ammonia supply may select for the cluster 4
635 populations (Kowalchuk and Stephen, 2001). In the presence of methane, the growth
636 of methanotrophs were significantly stimulated and methanotrophic N assimilation
637 could have likely led to the depletion of ammonium in support of nitrification activity.
638 It was noteworthy thatnone of 16S rRNA and *amoA* genes were affiliated with
639 *Nitrosomonas* in the native soil at day zero. The growth of *Nitrosomonas* was
640 stimulated to a much greater extent than that of *Nitrosospira* in urea-amended
641 microcosms, but *Nitrosomonas* appeared to be suppressed more significantly than
642 *Nitrosopira*.This might be explained by the fact that hat *Nitrosomonas* are markedly
643 responsive to ammonia input (Hastings et al., 1997). Similar to methanotrophic
644 communities, the proportion of *Nitrosospira*in AOB community detected by 16S
645 rRNA gene sequences was lower than that detected by *amoA* gene. It could be in part
646 attributed to the variation of *amoA* copy numbers among different AOB.For instance,
647 the species *N.briensis* and *N.europaea* have two copies of *amoA* genes and *N.tenuis*
648 contained three identical *amoA* genes (Norton et al., 1996; Sayavedra-Soto et al.,
649 1998).

650 The abilities to catalyze the hydrolysis of urea to yield ammonia can be observed in a
651 wide range of microorganisms possessing urease activity (Mobley and Hausinger
652 1989). Some methanotrophs have been identified with the ability of urea hydrolysis
653 (Boden et al 2011, Khmelenina et al 2013), however, the ^{13}C-labeled active
654 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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936 Atmos. Environ., 44, 304-311, 2010.

Table1. Changes in pH,moisture content, NH_4^+-N and NO_3^--Ncontentin soil microcosms over the course of 19 days of incubation

Treatments	pH[a]	Moisture (%)[b]	NH_4^+-N(μg/g *d.w.s*) [c]	NO_3^--N(μg/g *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.

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 lettersabove the columns indicate a significant difference (P<0.05)using analysis of variance.

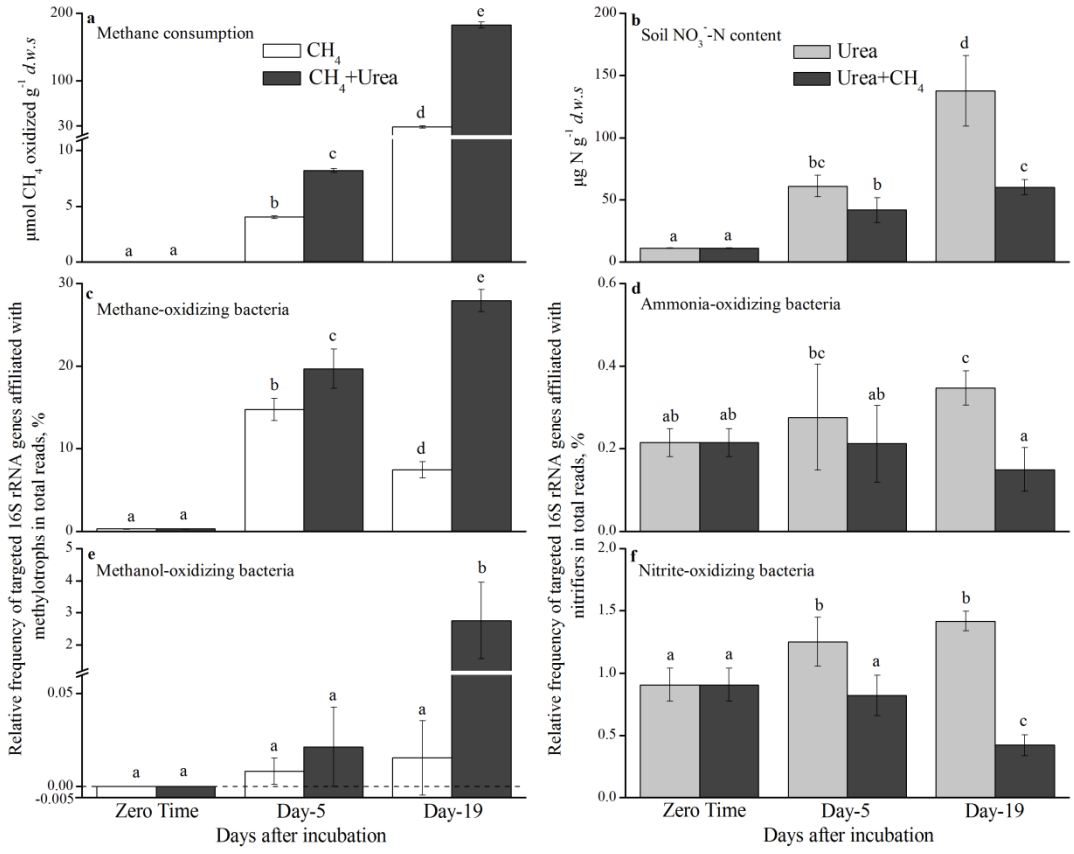


Figure 2. Change in relative abundance of methane-oxidizing bacteria (a) and ammonia-oxidizing bacteria (b) in soil microcosms incubated for 5 and 19 days. The relative abundance of type Ia, type IImethanotrophs, *Nitrosospira*and*Nitrosomonas*are expressed as the targeted 16S rRNAgene to total 16S rRNA genereads 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.

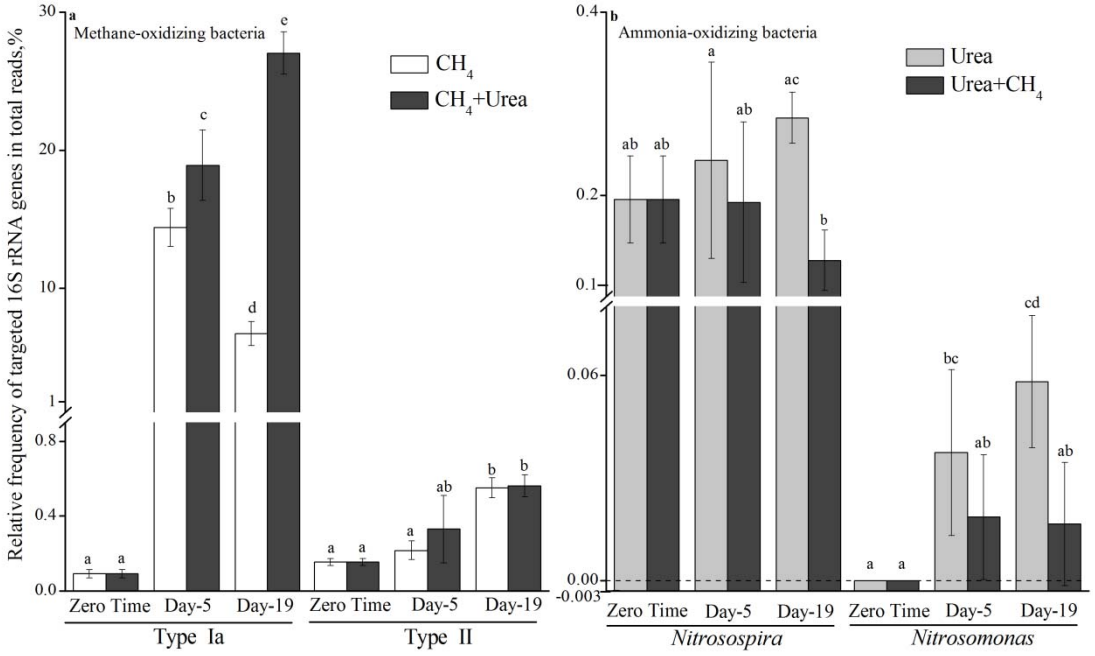


Figure 3. Relative frequency of the 16S rRNA gene sequences affiliated with methane-oxidizing bacteria (a, b), methanol-oxidizing bacteria (c, d), ammonia-oxidizing bacteria (e, f) and nitrite-oxidizing bacteria (g, h) across the buoyant density gradient of DNA fractions from the ^{13}C-labeled and ^{12}C-control 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.

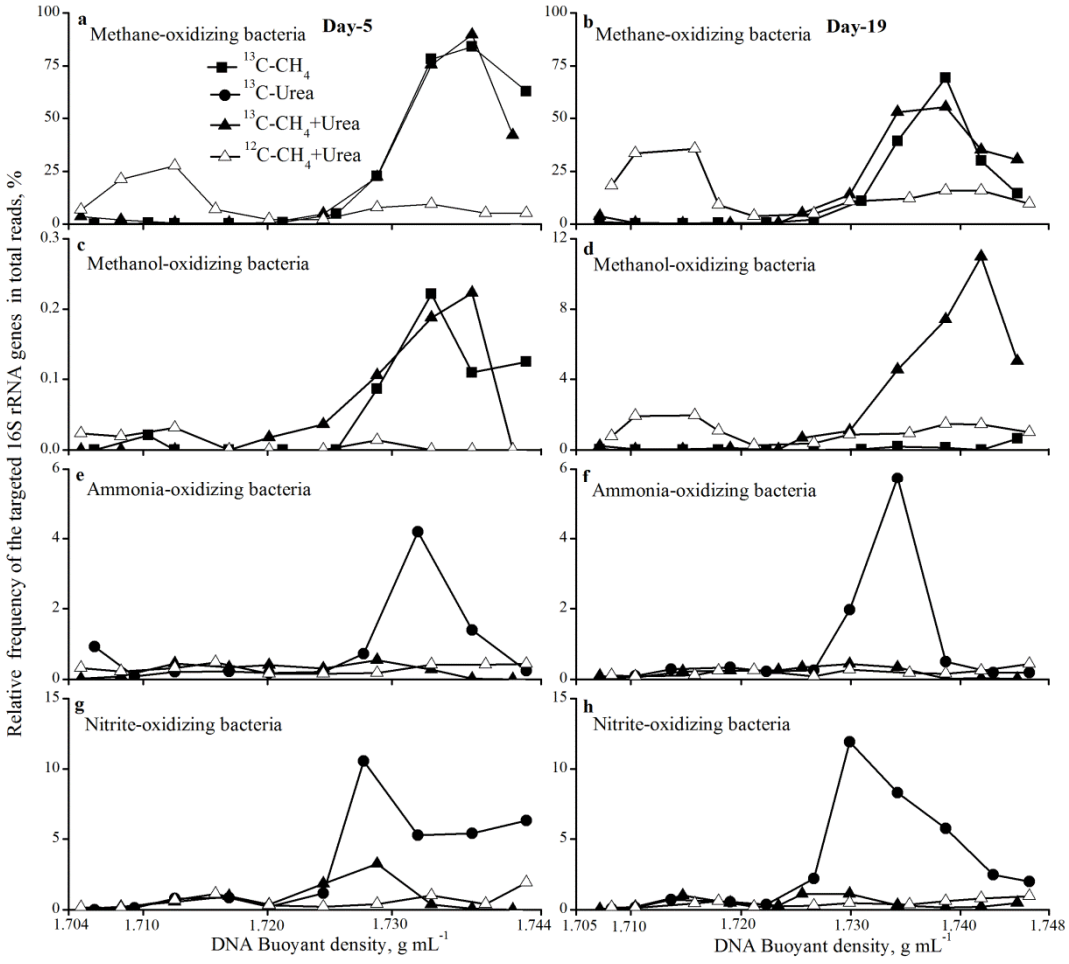


Figure 4. Phylogenetic tree of the ^{13}C-labeled 16S rRNA genes affiliated with methane-oxidizing bacteria (a) and ammonia-oxidizing bacteria (b) from the labeled microcosm after incubation for 19 days. The designationsCH_4 representsoil microcosms incubated with ^{13}C-CH_4, and the designation of 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 genesin 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.

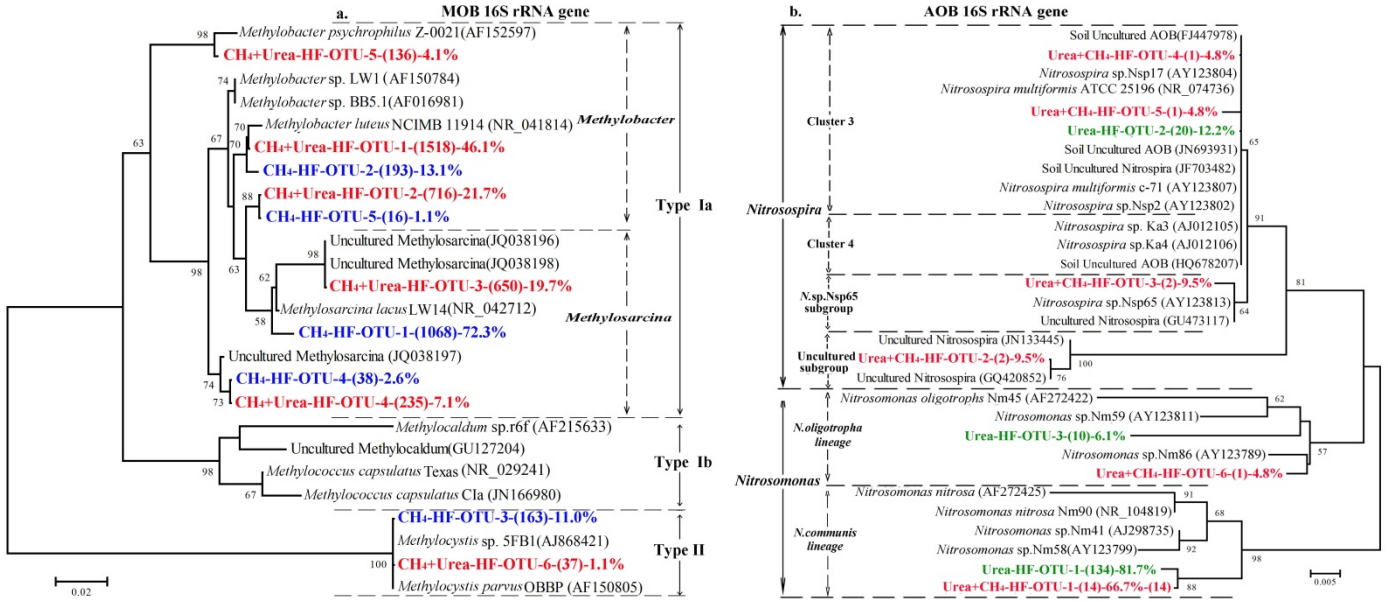
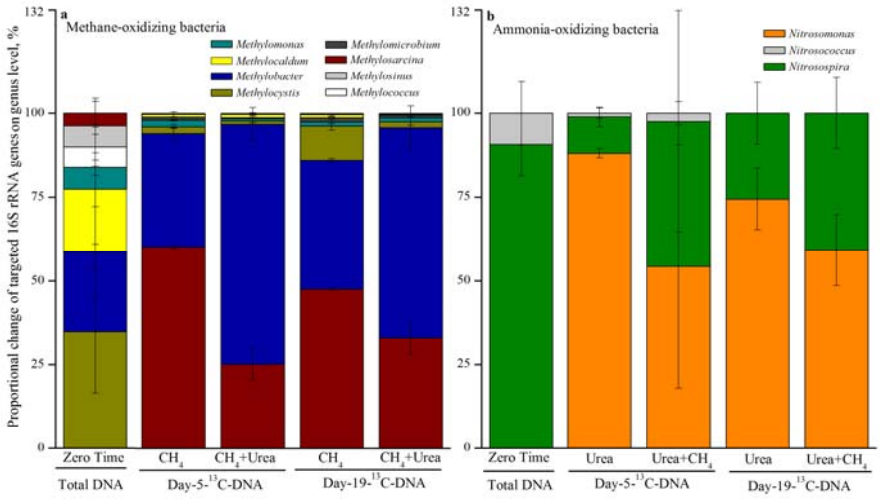


Figure 5. Percent changes of bacterial phylotypes affiliated with methane-oxidizing bacteria (a) and ammonia-oxidizing bacteria (b) in the ^{13}C-DNA fractions from the labeled microcosm after incubation for 5 and 19 days. The designation CH_4+Urea represents soil microcosms incubated with ^{13}C-CH_4 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

Treatment	^{13}C-CH_4	^{13}C-Urea	^{13}C-CH_4+Urea	^{12}C-CH_4+Urea	^{13}C-CH_4	^{13}C-Urea	^{13}C-CH_4+Urea	^{12}C-CH_4+Urea	^{13}C-CH_4	^{13}C-Urea	^{13}C-CH_4+Urea	^{12}C-CH_4+Urea
	CH_4 added (ppmv)*				Ureaadded (μg N/g *d.w.s.*)*				CO_2added (ppmv)*			
Day-0 -18:00pm†	9460	---	9322	9035	---	100	100	100	---	50000	50000	50000
Day-5- 8:00‡	The destructive sampling performed and the remaining microcosms were flushed with pressurized synthetic air (20% O_2, 80% N_2)											
Day-5-18:00 pm	6114	---	7770	6821	---	100	100	100	---	50000	50000	50000
Day-7-18:00 pm	7946	---	8020	6362	---	---	---	---	---	---	---	---
Day-8-20:00 pm	8355	---	8018	8482	---	---	---	---	---	---	---	---
Day-9-18:00 pm	---	---	6755	7067	---	---	---	---	---	---	---	---
Day-10-10:00am	---	---	6201	6718	---	---	---	---	---	---	---	---
Day-10-18:00 pm	---	---	9766	9552	---	---	---	---	---	---	---	---
Day-11-10:00am	---	---	9113	9164	---	---	---	---	---	---	---	---
Day-11-20:00 pm	---	---	9229	9541	---	---	---	---	---	---	---	---
Day-12-10:00am‡	The microcosms were flushed with pressurized synthetic air (20% O_2, 80% N_2).											
Day-12-21:00pm	18947	---	16081	15720	---	100	100	100	---	50000	50000	50000
Day-14-11:00am	---	---	11724	14355	---	---	---	---	---	---	---	---
Day-14-21:00pm	---	---	11762	12491	---	---	---	---	---	---	---	---
Day-15-11:00pm	---	---	8678	10431	---	---	---	---	---	---	---	---
Day-15-21:00pm	---	---	9591	11225	---	---	---	---	---	---	---	---
Day-16-11:00pm	---	---	17923	18598	---	---	---	---	---	---	---	---
Day-16-21:00pm	---	---	18190	17706	---	---	---	---	---	---	---	---
Day-17-11:00am	---	---	8792	10788	---	---	---	---	---	---	---	---
Day-17-21:00pm	---	---	17533	15901	---	---	---	---	---	---	---	---
Day-18-11:00am	---	---	10286	10430	---	---	---	---	---	---	---	---
Day-18-21:00pm	---	---	11928	12638	---	---	---	---	---	---	---	---
Day-19-11:00am ‖	The destructive sampling performed.											

* 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

Primer name	primer sequence(5'-3')	Targeted gene	Thermal Profile	Molecular analysis	Reference
515F	CCAGCMGCCGCGG	16S rRNA gene	95℃,3.0min;30×(95℃,30s; 55℃, 30s;72℃, 45s);72℃,10min	Pyrosequencing	(Xia et al., 2011)
907R	CCGTCAATTCMTTTRAGTTT				
A189F	GGN GAC TGG GAC TTC TGG	*pmoA* gene	95℃,3.0min;40×(95℃,10s; 55℃, 30s;72℃, 30s; 80℃ 5s; with plate read); melt curve 65℃ to 95℃, incremental0.5℃, 0:05+plate read	Real-time PCR	(Costello and Lidstrom, 1999; Holmes et al., 1995)
mb661r	CCG GMG CAA CGT CYT TAC C		95℃,3.0min;30×(95℃,30s; 55℃, 30s;72℃, 45s);72℃,10min	Pyrosequencing	
amoA-1F	GGGGTTTCTACTGGTGGT	bacterial *amoA* gene	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	Real-time PCR	(Rotthauwe et al., 1997)
amoA-2R	CCCCTCGGGAAAGCCTTCTTC		95℃,3.0min;30×(95℃,30s; 55℃, 30s;72℃, 45s);72℃,10min	Pyrosequncing	
Arch-amoAF	STAATGGTCTGGCTTAGACG	Archaeal*amoA* gene	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	Real-time PCR	(Francis et al., 2005)
Arch-amoAR	GCGGCCATCCATCTGTATGT				

Table S3. Pyrosequencing summary of the total microbial communities in SIP microcosms using the universal primers 515F-907R of the total 16S rRNA genes

Treatment*		Pyrosequencing reads number†				
		High-quality read number	Methane oxidizing bacteria	Methanol oxidizing bacteria	Ammonia oxidizing bacteria	Nitrite oxidizing bacteria
Zero time	Zero Time-R1	9519	28 (0.29%)	---	22 (0.23%)	81 (0.85%)
	Zero Time-R2	9110	26 (0.29%)	---	16 (0.18%)	74 (0.81%)
	Zero Time-R3	9369	24 (0.26%)	---	22 (0.23%)	99 (1.06%)
Day-5	^{13}C-CH_4-R1	7758	1252 (16.1%)	1 (0.01%)	12 (0.15%)	64 (0.82%)
	^{13}C-CH_4-R2	8630	1273 (13.5%)	1 (0.01%)	16 (0.19%)	55 (0.64%)
	^{13}C-CH_4-R3	8829	1192 (13.5%)	---	18 (0.20%)	50 (0.57%)
	^{13}C-Urea-R1	7803	31 (0.40%)	---	10 (0.13%)	80 (1.03%)
	^{13}C-Urea-R2	7807	13 (0.17%)	2 (0.03%)	26 (0.33%)	108 (1.38%)
	^{13}C-Urea-R3	6541	17 (0.26%)	1 (0.02%)	24 (0.37%)	88 (1.35%)
	^{13}C-CH_4+Urea-R1	7431	1637 (22.0%)	---	15 (0.20%)	60 (0.81%)
	^{13}C-CH_4+Urea-R2	8372	1633 (19.5%)	1 (0.01%)	30 (0.36%)	88 (1.05%)
	^{13}C-CH_4+Urea-R3	7568	1559 (20.6%)	4 (0.05%)	22 (0.29%)	47 (0.62%)
	^{12}C-CH_4+Urea-R1	6995	1109 (15.9%)	---	9 (0.13%)	67 (0.96%)
	^{12}C-CH_4+Urea-R2	8083	1782 (22.1%)	3 (0.04%)	12 (0.15%)	55 (0.68%)
	^{12}C-CH_4+Urea-R3	7809	1433 (18.4%)	2 (0.03%)	11 (0.14%)	63 (0.81%)
Day-19	^{13}C-CH_4-R1	10104	640 (6.33%)	---	18 (0.18%)	67 (0.66%)
	^{13}C-CH_4-R2	41172	3330 (8.09%)	18 (0.04%)	86 (0.21%)	274 (0.67%)
	^{13}C-CH_4-R3	41230	3235 (7.85%)	1 (0.00%)	104 (0.25%)	305 (0.74%)
	^{13}C-Urea-R1	8294	23 (0.28%)	---	26 (0.31%)	121 (1.46%)
	^{13}C-Urea-R2	31675	110 (0.35%)	2 (0.01%)	125 (0.39%)	465 (1.47)
	13C-Urea-R3	44313	129 (0.29%)	1 (0.00%)	149 (0.34%)	587 (1.32%)
	^{13}C-CH_4+Urea-R1	10370	2961 (28.6%)	465 (4.48%)	12 (0.12%)	34 (0.33%)
	^{13}C-CH_4+Urea-R2	7309	1963 (26.9%)	238 (3.26%)	7 (0.10%)	33 (0.45%)
	^{13}C-CH_4+Urea-R3	6494	1955 (30.1%)	231 (3.56%)	15 (0.23%)	21 (0.32%)
	^{12}C-CH_4+Urea-R1	9485	2672 (28.2%)	163 (1.72%)	13 (0.14%)	51 (0.54%)
	^{12}C-CH_4+Urea-R2	7695	2129 (27.7%)	121 (1.57%)	9 (0.12%)	33 (0.43%)
	^{12}C-CH_4+Urea-R3	6663	1750 (26.3%)	132 (1.98%)	13 (0.20%)	30 (0.45%)
	Average	12831				
	Total reads	346428				

*: 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

DNA gradient fraction*	High-quality reads number							
	Day-5				Day-19			
	^{13}C-CH_4	^{13}C-Urea	^{13}C-CH_4+Urea	^{12}C-CH_4+Urea	^{13}C-CH_4	^{13}C-Urea	^{13}C-CH_4+Urea	^{12}C-CH_4+Urea
Fraction-13	39	107	4240	4593	4488	1384	5859	6410
Fraction-12	5134	4677	3861	5387	7446	3318	8093	8171
Fraction-11	4318	4658	3615	6492	6046	6916	7277	5441
Fraction-10	3227	4531	4941	5556	3682	6224	2472	6090
Fraction-9	4647	4710	5583	6323	6149	6102	4542	3867
Fraction-8	6195	5239	5534	4925	4108	6825	7147	8140
Fraction-7	8080	6620	3796	7488	5079	5527	6684	7118
Fraction-6	5889	7060	3736	1925	2570	3087	6374	4740
Fraction-5	7270	3306	6278	481	2134	5643	5804	5725
Fraction-4	9622	427	195	931	402	4526	7471	9873
Fraction-3	---	---	---	---	5568	5644	4424	1835
Average	5442	4134	4178	4410	4334	5018	6013	6128
Subtotal	54421	41335	41779	44101	47672	55196	66147	67410
Total	418061							

*: 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.

Organisms*	Replicate	Day-0	Day-19					
			^{13}C-CH_4		^{13}C-Urea		^{13}C-CH_4+Urea	
		Total DNA†	Total DNA†	^{13}C-DNA‡	^{13}C-DNA	^{13}C-DNA	Total DNA†	^{13}C-DNA
pmoA genes of MOB	R1	4295	8244		---		1106	
	R2	3616	5297	384	---	---	5074	7159
	R3	---	5878		---		6303	
amoA genes of AOB	R1	5484	---		7572		2728	
	R2	472	---	---	10656	472	3262	1115
	R3	6261	---		4832		4449	

* 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

Treatment [a]	μmol CH_4-C/microcosm[b]			μg urea-N/microcosms		
	CH_4consumed	CO_2 produced	CO_2 assimilated by methanotrophs	Assimilation of urea-N by methanotrophs [c]	Nitrate produced from urea-N by ammoniaoxidizers	Urea-N recovery
^{13}C-CH_4+Urea-R1	1111	730.9	380.5 (34.2%)	1332 (74.0%)	364.2 (20.2%)	94.2%
^{13}C-CH_4+Urea-R2	1081	688.8	392.4 (36.3%)	1373 (76.3%)	339.6 (18.9%)	95.2%
^{13}C-CH_4+Urea-R3	1030	746.3	283.9 (27.6%)	994 (55.9%)	372.4 (20.69%)	76.6%
Average	1074±41.1	722.0±29.8	352.3±59.5 (32.7%±4.54%)	1234±208.4 (68.7%±11.2%)	358.7±17.1 (19.9%±0.93%)	88.7±10.5%

[a.] The designation R1 to R3 represents incubation of triplicate microcosms.

[b.] The amount of CH_4consumed was calculated as the net difference in CH_4 concentration between day 0 and day 19. The amount of CO_2 produced was estimated in a similar way. Assuming that all CH_4 consumed were converted to CO_2, the amount of CO_2 assimilated by methanotrophs could be calculated as the net difference between the consumed CH_4 and the produced CO_2 at day 19 as previously described (Whalen et al., 1990).

[c.] For every mole of assimilated carbon 0.25 moles of nitrogen have to be taken up(Bodelier and Laanbroek, 2004Bodelier 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 microcosns 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.

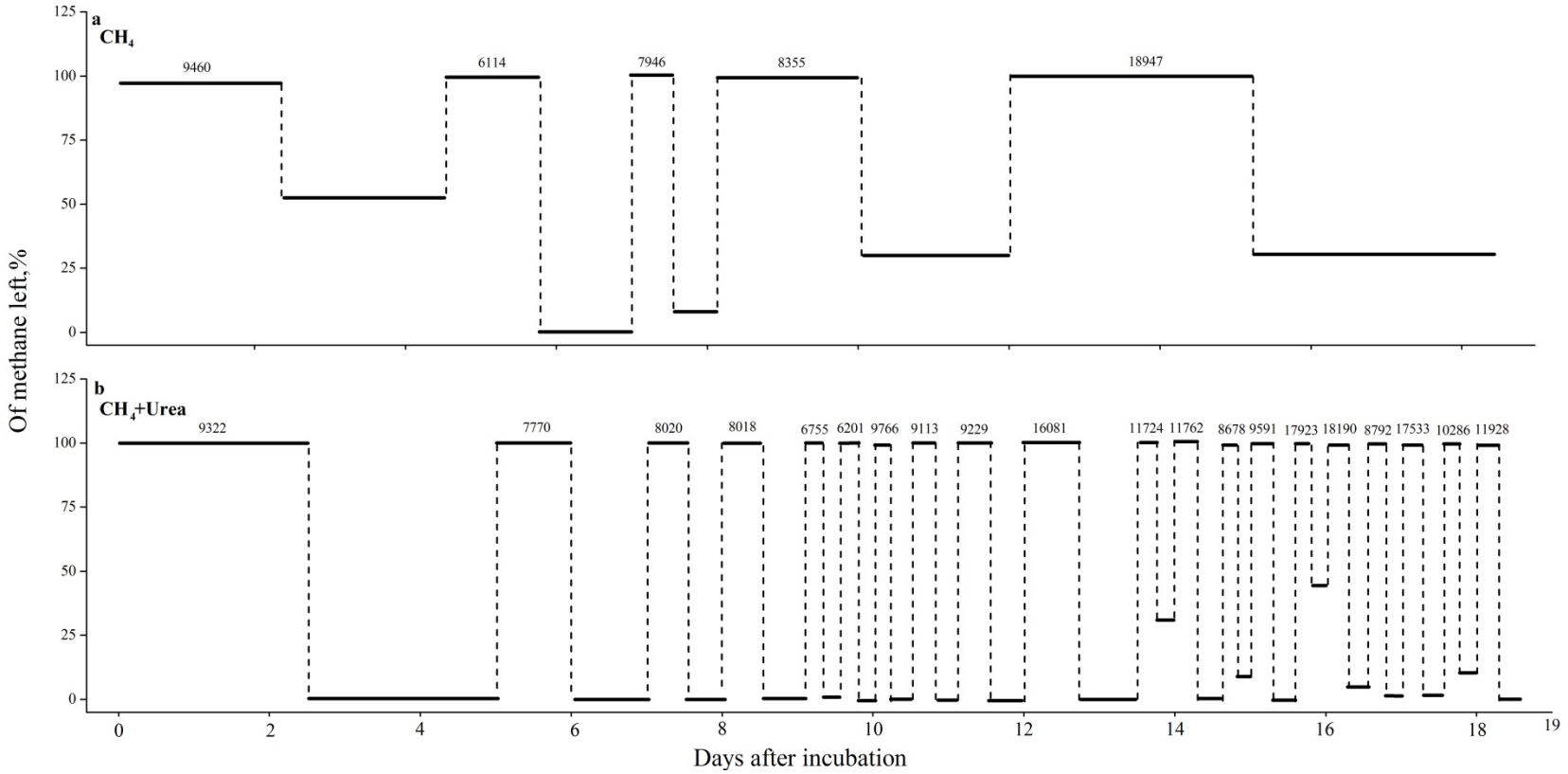


Figure S2. Changes in NH_4^+-N and NO_3^--N content in soil microcosms incubated with urea with or without CH_4over the course of 19 days of incubation

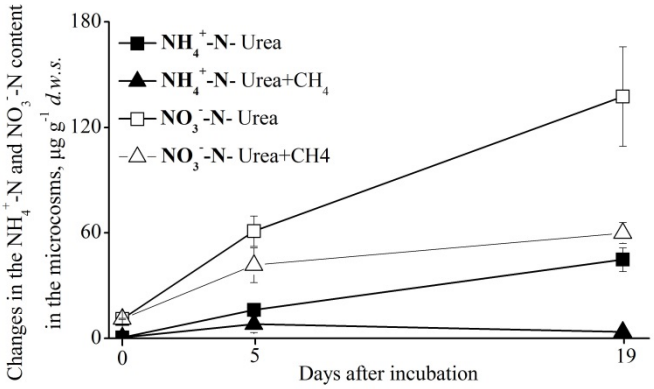


Figure S3. Quantitative distribution of pmoA gene copy numbers (a), amoA gene copy numbers of Bacteria (b) and Archaea (c) in total DNA from microcosms after incubation for 5 and 19 days. The error bars represent standard deviations of the triplicate microcosms. The different letters above the columns indicate a significant difference ($P<0.05$) using analysis of variance.

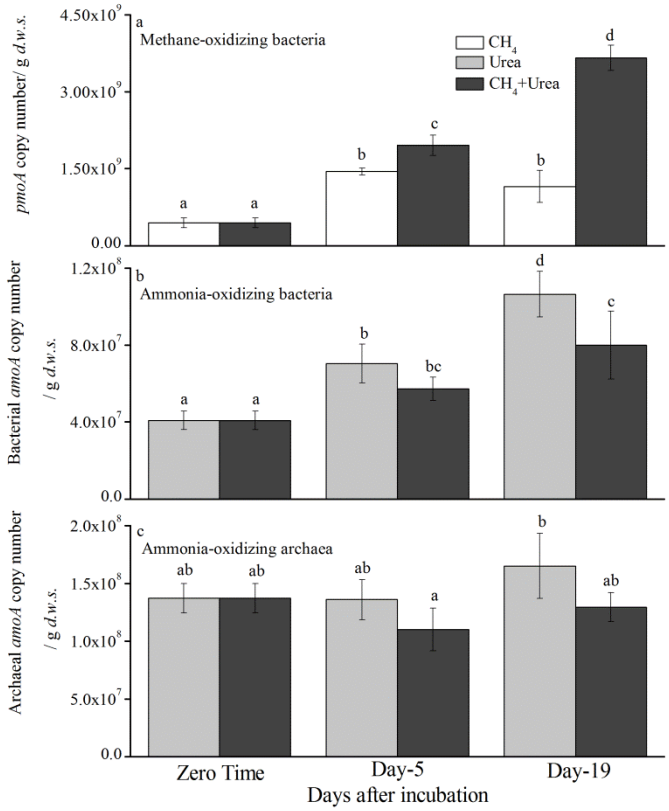


Figure S4. The effect of methane on ammonia-oxidizing archaea (AOA) in soil microcosms incubated for 19 days. The relative frequency is expressed as the percentage of the targeted reads to the total 16S rRNA gene sequences reads in soil sample.The error bars represent standard deviation of the triplicate microcosms, while for the soil microcosms of 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.

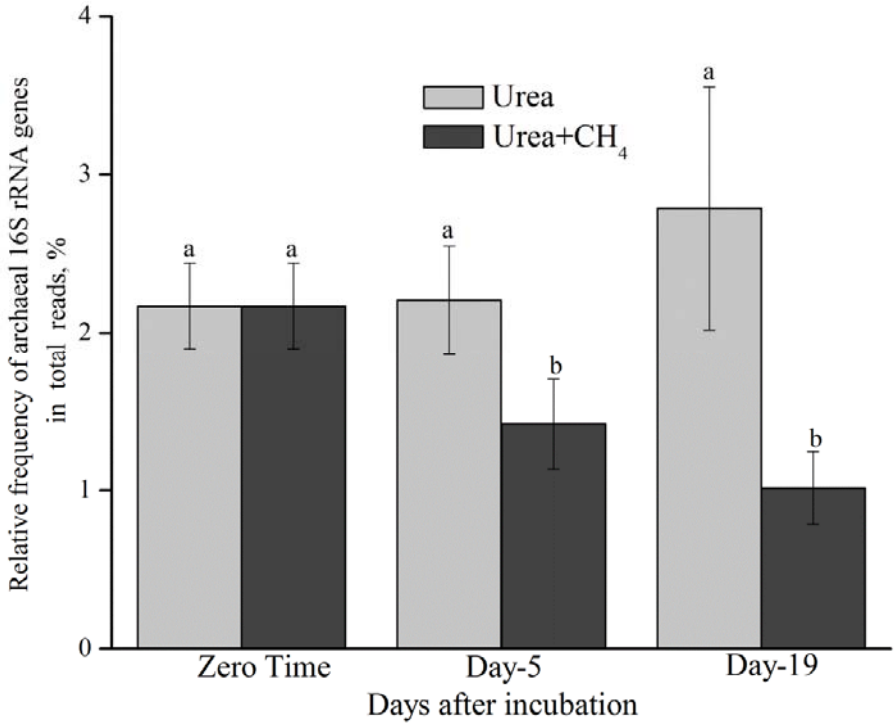


Figure S5. Phylogenetic tree showing the relationship of methane-oxidizing bacterial 16S rRNA gene (a) and *pmoA*gene(b) sequences in soil microcosms to those deposited in the GenBank. Pyrosequencing reads of methanotrophic 16S rRNA genes and *pmoA* genes were used from triplicate microcosms at day 0 and day 19, and representative sequences were chosen for analysis. The designation of CH_4+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 ^{13}C-CH_4+Urea treatment after incubation for 19 days.CH_4-OTU-1-69.8%-(3844)indicates that OTU-1 containing 616 sequences with identity of >87% comprised 69.8% of *pmoA* gene sequences in ^{13}C-CH_4 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.

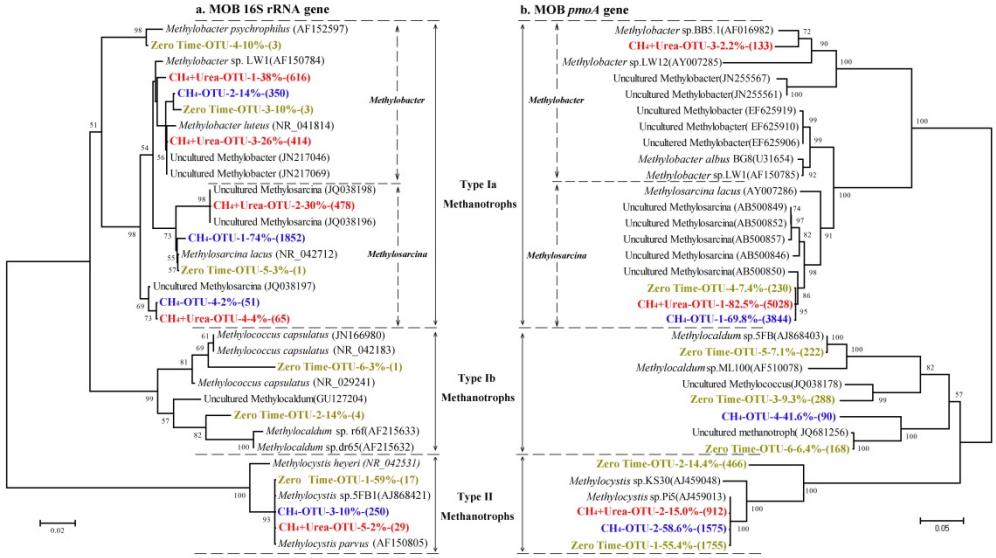


Figure S6. Phylogenetic tree showing the relationship of ammonia-oxidizing bacterial 16S rRNA gene (a) and *amoA*gene(b) sequences in soil microcosms to those deposited in the GenBank. Pyrosequencing reads of AOB 16S rRNA genes and *amoA* genes were used from triplicate microcosms at day 0 and day 19. 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+CH_4-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 ^{13}C-Urea+CH_4 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.

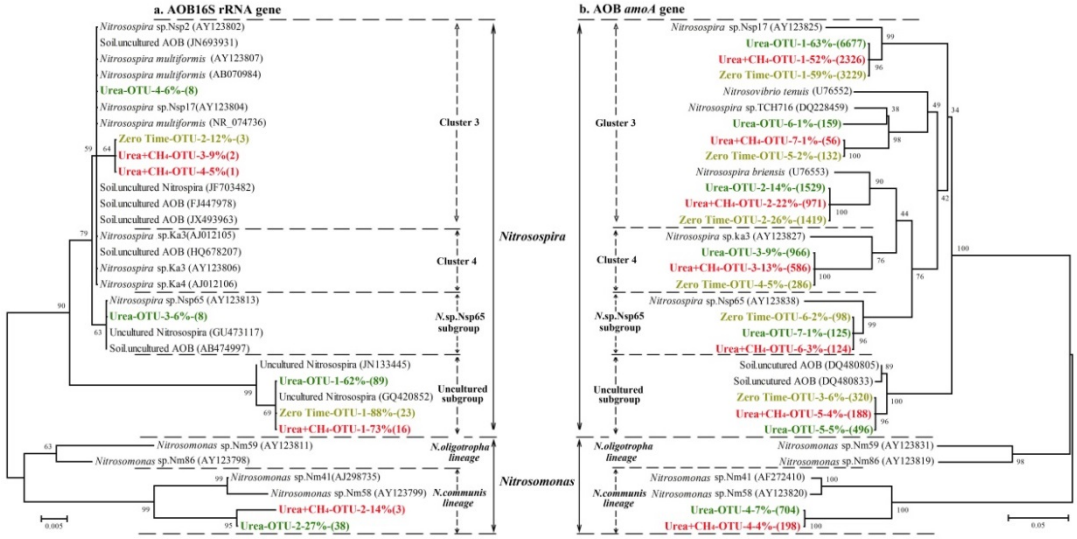


Figure S7. Quantitative distribution of *pmoA* gene copy numbers (a,b), *amoA* gene copy numbers of *Bacteria* (c,d) and *Archaea* (e,f) across the entire buoyant density gradient of the fractionated DNA from SIP microcosms after incubation for 5 and 19 days. The normalized data are the ratio of gene copy number in each DNA gradient fraction to the maximum quantities for each treatment. The error bars represent standard deviations of the duplicate microcosms.

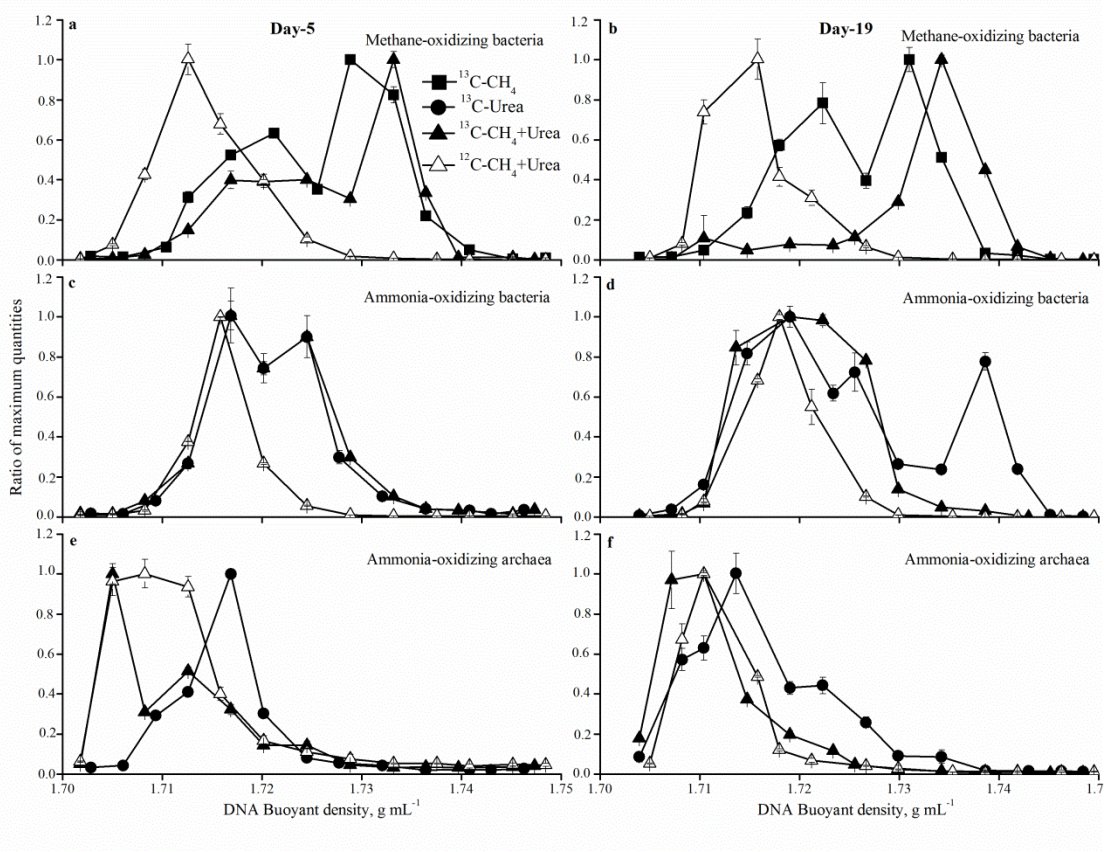


Figure S8. Relative frequency of the archaeal 16S rRNA gene sequences reads in DNA gradient fractions with a buoyant density gradient isolated from SIP microcosms after incubation for 5 and 19 days. The frequency is expressed as the percentage of the targeted archaealreads to the total 16S rRNA gene sequences reads in each DNA gradient fraction.

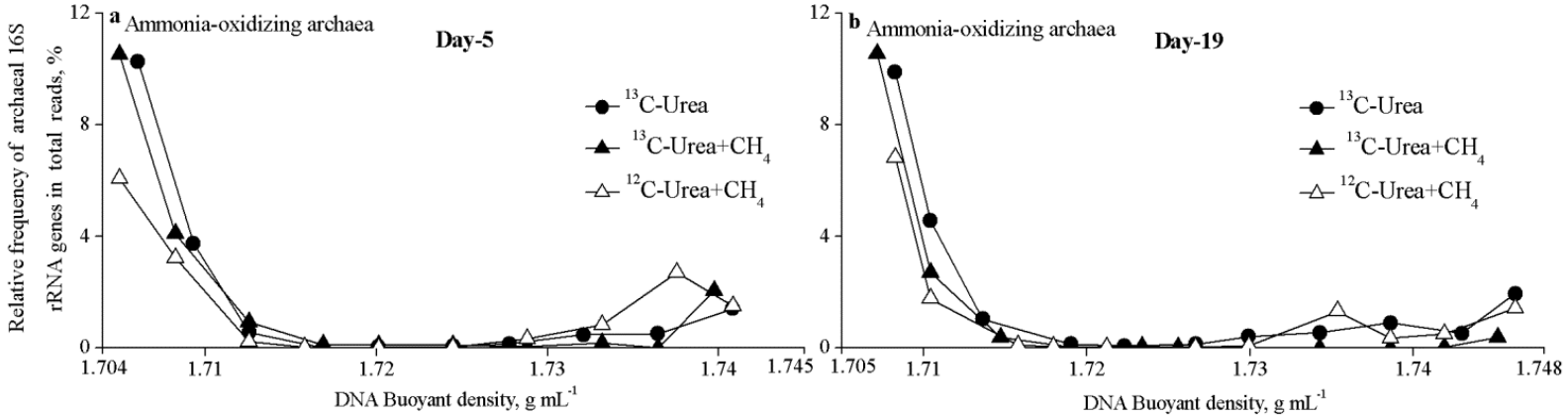


Figure S9. Phylogenetic tree of *pmoA* genes for methane-oxidizing bacteria (a) and *amoA* genes for ammonia-oxidizing bacteria (b) in the ^{13}C-DNA 'heavy' fraction from the labeled microcosm after incubation for 19 days. The designation of CH_4-HF-OTU-1-(308)-83.9% indicates that OTU-1 containing 308reads with sequence identity of >87% comprised 83.9% of *pmoA* gene sequences retrieved from the 'HF' fraction in microcosms amended with ^{13}C-CH_4 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.

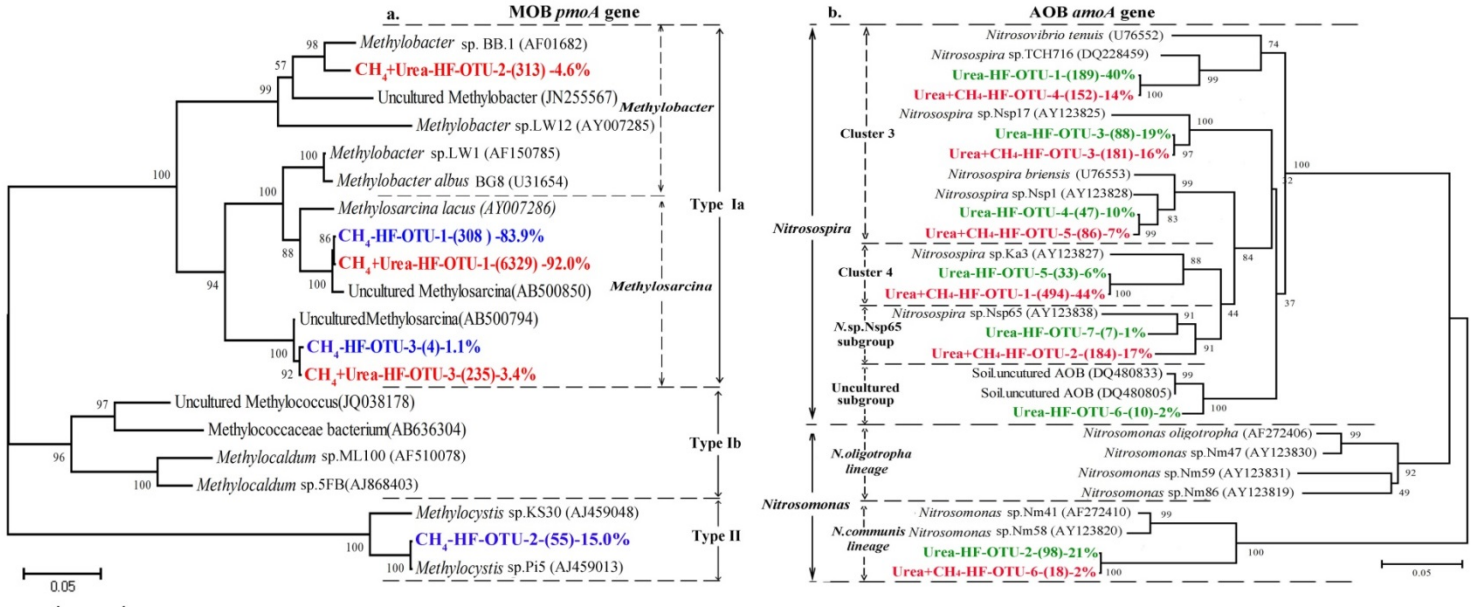
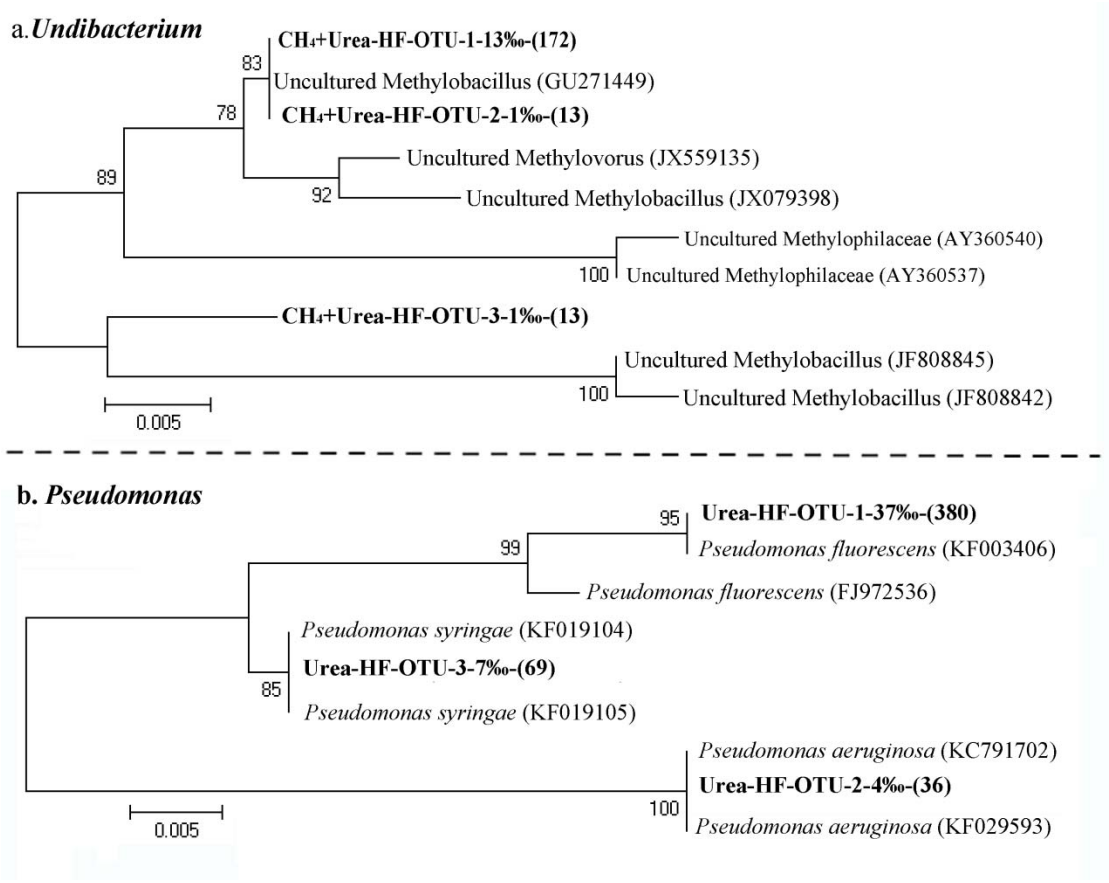


Figure S10. Phylogenetic tree showing the relationship of the high-throughput sequence reads of *Undibacterium* (a) and *Pseudomonas* (b) in the ^{13}C-labeled 'heavy' DNA fractions (HF) to those deposited in the GenBank. The designation of of CH_4+Urea-HF -OTU-1-13‰-(172) indicates that OTU-1 contains 172 sequences associated with *Undibacterium* comprising 13‰ of 16S rRNA gene sequence readsin 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.



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