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.

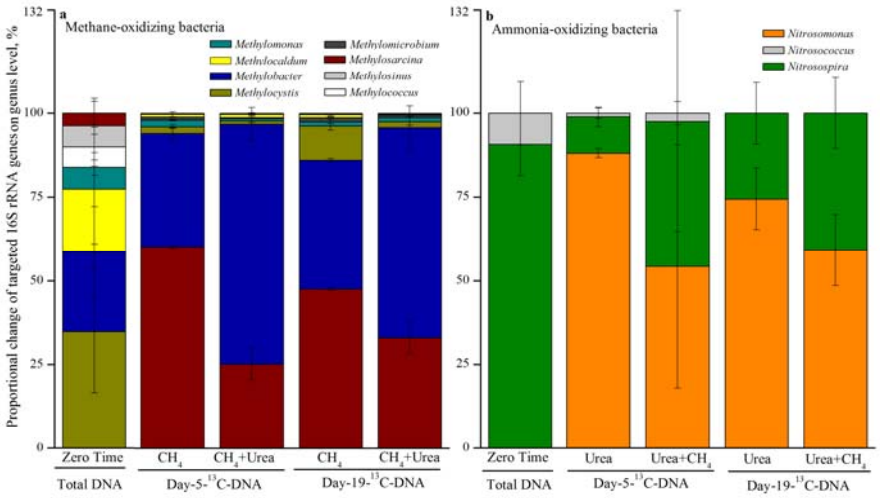
The reviewer has some major concerns

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. 6



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 545 to line 548 on page 20.

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#3, We have discussed about the issue as follows in the revised version from line 477to line 494 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).

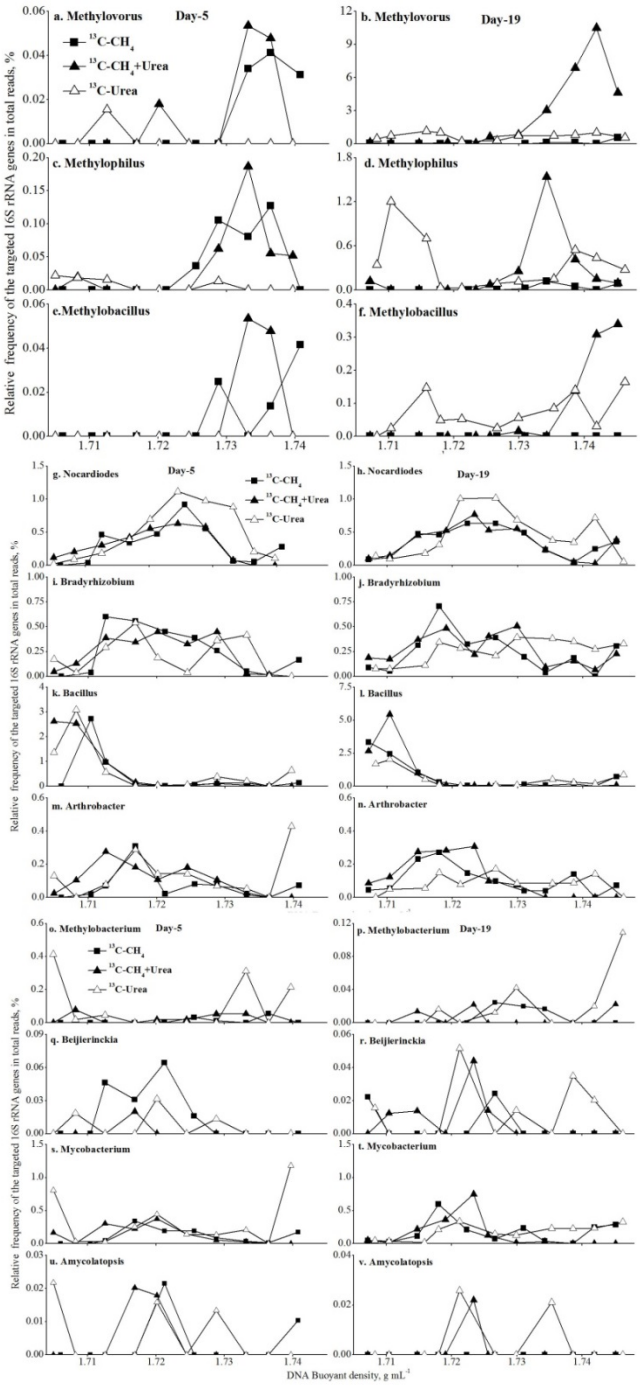
4. Methanol-oxidizers: The authors do not explain how they decided, which of the detected taxa were methanol-utilizers (this is also not documented for ammonia-oxidizers, nitrite oxidizers, and methanotrophs). There a lot of methanol-oxidizers known that occur in soil and were likely overlooked when 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 97 on page 4 and from line 330 to line 332 on page 12.



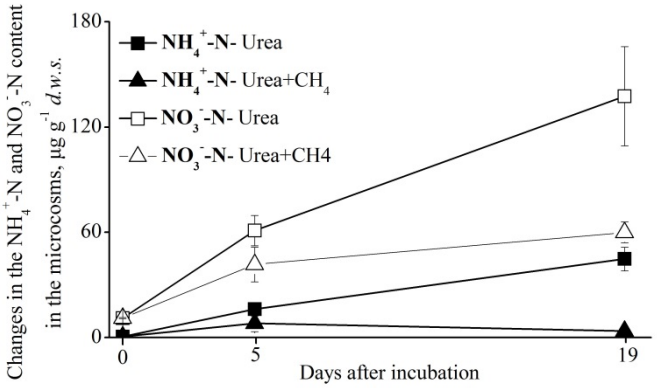
Comment#1-Fig.1 Relative frequency of 16S rRNA gene sequences affiliated with methnol-oxidizers on genus level detected in our study across the buoyant density gradient of DNA fractions from the ^{13}C-labeled and ^{12}C-control microcosms after incubation for 5 and 19 days.

(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 112 to line 116 on page 4~5 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...'.

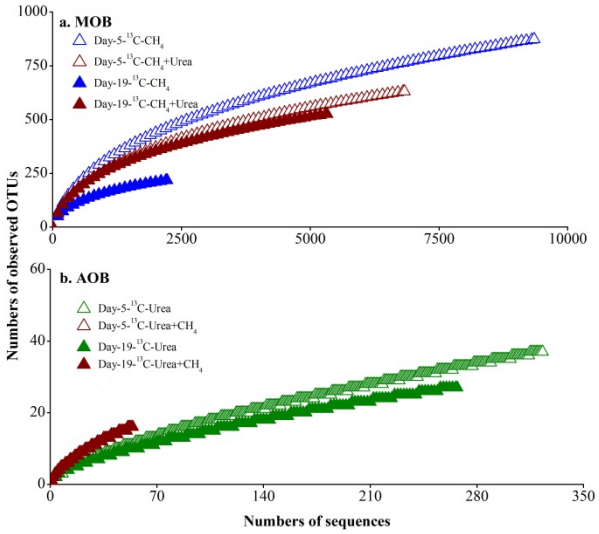
Reply: Yes, the sequencing is deep enough to allow for statistical comparison of single datasets.

(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. 6).

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 Fig. 4b and supplementary Fig. S5b 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 579 to line 581 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 663 to line 666 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.

Kolb, S. (2009) Aerobic methanol-oxidizing Bacteria in soil. *Fems Microbiology Letters* **300**: 1-10.

Murrell, J.C., and Dalton, H. (1983) NITROGEN-FIXATION IN OBLIGATE METHANOTROPHS. *Journal of General Microbiology* **129**: 3481-3486.

Nouchi, I., Mariko, S., and Aoki, K. (1990) Mechanism of methane transport from the rhizosphere to the atmosphere through rice plants. *Plant Physiology* **94**: 59-66.

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.

Point-by-point response to the comments of the referee #2

(Manuscript ID bg-2014-97)

General comments of the referee #2

Zheng et al presented an interesting investigation of "competitive interaction" between MOB and AOB in a paddy soil using molecular ecological approaches. The topic is obviously of intense interest to the environmental microbiology community and this reviewer agrees strongly with the authors that the interaction of methane cycle and nitrogen cycle is very poorly understood. Thus the work is topical and important to the field. I have the following suggestions to improve

Reply: We thank the reviewer for positive comments and a point-to-point reply to the comments was made as follows.

Major Suggestions

11. It was not very clear to me why $^{13}CO_2$ should be used in combination with ^{13}C-urea (page 3898 lines 1-4). obviously urea catabolism to ammonia generate CO_2. It is also unclear to me why ^{13}C-labelled urea (and $^{13}CO_2$) is used in the ^{13}C-methane treatment. it seems to me that the key treatment missing is ^{13}C-methane plus ^{12}C-urea and ^{12}C-methane plus ^{13}C-urea.

Reply: We appreciate this comment and argue that the current setup of SIP treatments is reasonable as follows.

(1) Why $^{13}CO_2$ should be used in combination with ^{13}C-Urea

^{13}C-urea was used to prevent the dilution of the label because $^{13}CO_2$ addition is crucial for the labeling of ammonia oxidizers. As referee point out, ^{13}C-urea catabolized to ammonia and $^{13}CO_2$, the amount of $^{13}CO_2$ generated by urea catabolism can be used for labeling of ammonia oxidizers as well. The amount of urea-N we added to the microcosms was 100μg g^{-1} *d.w.s.*, the content of $^{13}CO_2$ is about 3.57 μmol g^{-1} *d.w.s.* assuming all urea is converted to ammonia and $^{13}CO_2$. In order to increased the labeling efficiency of targeted microorganisms, we added 50000 ppmv $^{13}CO_2$ (44.6 μmol g^{-1} *d.w.s.*) in the microcosms as previous study used (Jia and Conrad, 2009; Xia et al., 2011).

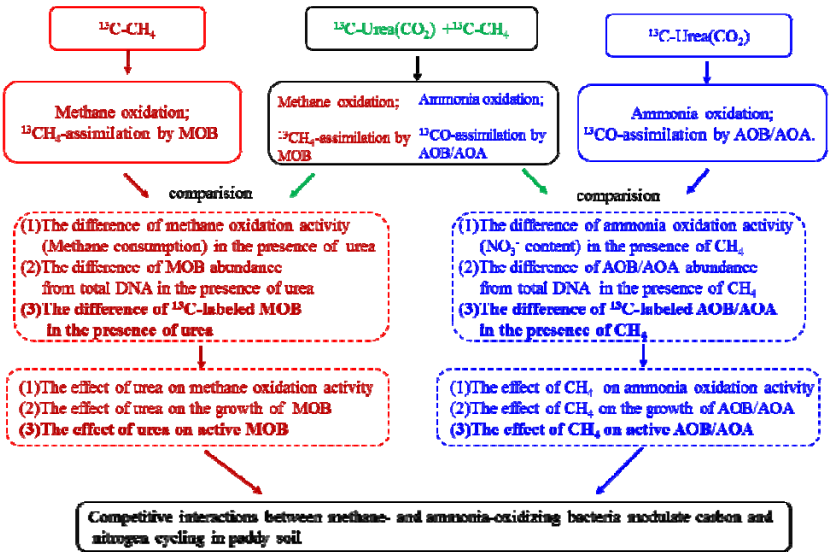
(2) Why not ^{13}C-methane plus ^{12}C-urea and ^{12}C-methane plus ^{13}C-urea

The reason we used ^{13}C-labelled urea (and $^{13}CO_2$) plus ^{13}C-labelled CH_4 as follows. In $^{13}CH_4$ incubated microcosms, methanotrophs can oxidize ^{13}C-methane and assimilated ^{13}C to synthesize ^{13}C-DNA. In the ^{13}C-urea (CO_2) incubated microcosms, autotrophic ammonia oxidizing organisms use ^{13}C-CO_2 as carbon source to synthesize ^{13}C-DNA. In the ^{13}C-urea (CO_2) plus $^{13}CH_4$ incubated microcosms, ammonia and methane oxidation may proceed in parallel, so methanotrophs and ammonia organisms can be both ^{13}C-labeled in the microcosms. Pairwise comparison between ^{13}C-CH_4 and

^{13}C-CH_4+^{13}C-Urea can be used to assess the effect of urea on the methane oxidation activity and the active MOB based on the ^{13}C-labeled DNA. To this end, the treatment ^{13}C-methane plus ^{12}C-urea is largely equivalent to ^{13}C-CH_4+^{13}C-Urea because the labeled MOB were concerned

Comparison between ^{13}C-urea (CO_2) and ^{13}C-CH_4+^{13}C-Urea can be used to assess the effect of urea on the methane oxidation activity and the active AOA/AOB based on the ^{13}C-labeled DNA. In this comparison, the treatment ^{12}C-methane plus ^{13}C-urea can serve the same purpose as ^{13}C-CH_4+^{13}C-Urea.

The detailed setup can be seen as follows.



As for the interactions between active methanotroph and AOA/AOB, we fully agree that ^{13}C-methane plus ^{12}Curea and ^{12}C-methane plus ^{13}C-urea are reasonable, and the lack of these treatments would have not significant impact on our results if the key treatment of ^{13}C-CH_4+^{13}C-Urea were included.

12. I am not sure I agree with the authors with regarding to the use of "inhibition" of AOB activity (e.g. figure 1b, 1d) in microcosms where methane is added (e.g. see discussion section Page 3903. What is very likely (also suggested by the authors) is that in the present of both urea and methane, MOB cell numbers are increased. In fact, this should be quantified e.g. by qPCR. the sequencing data only show relative abundance of AOB/MOB in the total microbial community. The relative abundance ofAOB was indeed low in the treatment without methane, however, it is very likely that AOB cell numbers still increased in those treatments. Therefore strictly speaking, AOB activity was in fact enhanced in those treatments (by urea of course). Again the AOB cell numbers should be quantified (eg. by QPCR). Therefore, my point is that the use of "inhibition" of AOB by methane is in fact misleading since this implies that methane direct inhibits AOB activity (which is very difficult to perceive). The authors should make it absolutely clear that it

is the relative numbers ofAOB-to-MOB they refer to, but not the absolute cell counts, therefore either cell numbers need to be presented to justify the use of "inhibition" or rewording is required.

Reply: We thank the referee for this comment and fully agree that methane does not directly inhibit AOB activity. It is scientifically more sound to say that methane inhibited the growth of AOB cells.

We have quantified the methanotrophic *pmoA* gene, bacterial and archaeal *amoA* genes by qPCR (supplementary Fig. S3 in the revised ms). The results were presented from line 324 to line 330on page 12(MOB), from line 341 to line 346 on page 13 (AOB), from line 353 to line 354 on page 13(AOA) in the revised version.

The data did show the increased cell numbers of AOB after incubation with urea in the presence of CH_4 as the reviewer suggested. However, the AOB cell numbers in the microcosms incubated with urea-treatment was significantly higher than those in Urea+CH_4-treatment. This indicated that the growth of AOB was partially inhibited by CH_4. The suppression of AOB growth in the presence of CH_4 was supported not only by the relative numbers of AOB from the 16S rRNA gene pyrosequencing analysis but also by the absolute cell count from Q-PCR.

Also, we have clarified both relative abundance and absolute cell count of MOB, AOB and AOA in our result to avoid the misleading in the revised version.

13. In page 3908 when the authors discussed mass balance of N. in general, I felt that many assumptions were made and this section reads rather speculative. for example, it is assumed that methane-carbon is assimilated with N at 4:1 ration. it is assumed that MOB oxidise 70% methane in order to assimilate 30% methane-carbon into biomass.with these assumptions it is calculated that 11% of N from urea is denitrified. Whilst these assumptions are perceivable, it does not justify the fact that no efforts were made to quantify the denitrification activity and subsequent gas products (N2O, NO2 etc). My overall impression of the discussion is that it is lengthy and not focused. In my opinion,the authors do not need to discuss every aspect of the results, and discussions such as mentioned above, as it stands, is too speculative. Further experiments should be carried out to investigate the unaccounted N in the system

Reply: We agree with the comments, and the mass balance of N was only briefly discussed from line 509 to line 515 on page 18~19 in the revised ms

14. An obvious missing discussion point is the investigation of the genetic potential of urea catabolism to ammonia in/with AOB and MOB. there are two well known systems for urea degradation to ammonia though either urease or urea decaboxylase/allophanate hydrolase. Do sequenced MOB have the genetic potential in urea degradation? How about AOB? How about AOA? was it simply because AOA cannot

release ammonia from urea? These data are readily available and should be discussed with respect to the competition between ammonia oxidizers and methane oxidizers.

Reply:Thanks for the comment!

We analyzed the ^{13}C-labeled MOB 16S rRNA gene(Fig.4a) and *pmoA* gene (Fig. 4b) and ^{13}C-labeledAOB 16S rRNA gene(Fig.5a) and bacterial *amoA* gene (Fig.5b) to detec the genetic potential of urea catabolism to ammonia. It revealed that MOB may not hydrolyze urea, but AOB were closely clustered with ureolytic AOB in our study. There are not reported about ureolytic AOA by far in neutral soil. We have added the discussion of urea catabolism to ammonia in AOB and MOB from line 644 to line 658 on page 23~24 in the revised version as follows.

The abilities to catalyze the hydrolysis of urea to yield ammonia can be observed in a wide range of microorganisms possessing urease activity (Mobley and Hausinger, 1989). Some methanotrophs have been identified with the ability of urea hydrolysis (Boden et al., 2011; Khmelenina et al., 2013), however, the ^{13}C-labeled active methanotrophs on the basis of 16S rRNA gene (Fig.4a) and *pmoA* gene (Fig.4b) 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* (Fig. 5a). 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.

Reference

Boden, R., Cunliffe, M., Scanlan, J., Moussard, H., Kits, K.D., Klotz, M.G. et al. (2011) Complete Genome Sequence of the Aerobic Marine Methanotroph Methylomonas methanica MC09. *Journal of Bacteriology* **193**: 7001-7002.

Jia, Z., and Conrad, R. (2009) *Bacteria* rather than *Archaea* dominate microbial ammonia oxidation in an agricultural soil. *Environmental Microbiology* **11**: 1658-1671.

Khmelenina, V.N., Beck, D.A.C., Munk, C., Davenport, K., Daligault, H., Erkkila, T. et al. (2013) Draft Genome Sequence of Methylomicrobium buryatense Strain 5G, a Haloalkaline-Tolerant Methanotrophic Bacterium. *Genome announcements* **1**.

Mobley, H., and Hausinger, R. (1989) Microbial ureases: significance, regulation, and molecular characterization. *Microbiological Reviews* **53**: 85-108.

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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.

Point-by-point response to the comments of the referee #3

(Manuscript ID bg-2014-97)

General comments of the referee #3

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

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

Major comments

15. My major criticism is that it is not clear to what extent these observations are relevant for mechanisms that can be assumed to take place under field conditions. The composition of the methanotrophic community and also their relative fraction within the total microbial community changed substantially under the conditions of the 19-days-incubation experiment. If one assumes that the original community was already adapted to the field conditions, it remains an open question if the observed changes are really representative of mechanisms taking place under field conditions. Here, the authors should provide more information about to what extent the mesocosm incubation was representative of fertilizer applications and methane availability at the sampling site, or to what extent it may reflect responses of microbial communities to fluctuations in environmental conditions

Reply: We appreciate this comment, which was raised by referee #1 as well. We fully agree that our results could not entirely represent what is occurring under *in situ* conditions. We however hope the results of this study could provide useful insights on complex interactions between methane and ammonia oxidation. For example, our data are consistent with a very recent review paper showing that Type I methanotrophs utilize K-strategy lifestyle, while r-strategy was exploited by type II methanotrophs.

As suggested, the results and discussion were rephrased from line 477to line 494 on page 17~18 in the revised version as follows

The pre-incubation was performed to increase the labeling efficiency of targeted microorganisms because the dilution of $^{13}CO_2$ by soil-respired $^{12}CO_2$ could be decreased significantly as reported previously (Jia and Conrad, 2009; Xia et al., 2011). No apparent changes of ammonia oxidizer communities were observed during a 4-week pre-incubation without ammonium fertilization, significant shift of AOB communities occurred in the ammonium-amended soils (Jia and Conrad, 2009). The nitrogenous fertilization of paddy field in this study is about 250 kg N ha^{-1}, which is equivalent to 107 μg N g^{-1}*d.w.s*, assuming an effective soil depth of 20 cm. In addition, methane concentrations of 900 to 15000 μL L^{-1} were generally detected in paddy soil during rice-growing season (Nouchi et al., 1990; Nouchi et al., 1994). Therefore, the microcosms were incubated with 100 μg urea-N g^{-1}*d.w.s.* and 10000 μL L^{-1} methane to extrapolate the microbial interactions between methane- and ammonia-oxidation under field conditions. It suggests that microcosms might represent largely what is occurring under *in situ* conditions, although it could not reproduce the physiochemical and biological conditions in field. For instance, it also has been reported that the results of microcosm incubations remained largely consistent with population dynamics of methanotrophic communities in field (Eller et al., 2005).

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

Reply: We believe that the taxonomic identity of active organisms could be considered with great confidence. Phylogenetic analysis of 16S rRNA genes was based on the RDP and mothur software package. These databases integrated with Silver database of high-quality 16S rRNA genes and are widely used for microbial ecology study. Therefore, we believe this taxonomic information represent the best resolution available by far.

17. Another aspect related to this is that as far as I can see, triplicate samples were pooled for pyrosequencing analysis but the error range of the method itself remains unknown. So here, small changes in community composition over time or between treatments should be interpreted with caution

Reply: The pooled sample was tested for reliability before pyrosquencing as shown in the following Table. The DNA extracts from triplicate soil samples were analyzed, in addition to the pooled DNA from the triplicate samples. There was no significant difference between the means of the three replicates and the results of the pooled DNA, with respect to the relative abundance

(Table 1) and compositions of MOB and AOB communities (Figure 1). Therefore, the pooled DNA was fractionated for molecular analysis.

Table 1 Pyrosequencing summary of total microbial communities in microcosms incubated for 5 days using 515F-907R of total 16S rRNA genes

(The designation of R1 to R3 represents triplicate microcosm incubation. 'Average' represents the mean value of triplicate microcosms. 'Pool' represents the pooled DNA of triplicate microcosms.)

Treatment		Relative abundance of targeted 16S rRNA gene in total reads,%				
		High-quality read number	Methane-oxidizing bacteria	Methanol oxidizing bacteria	Ammoniaoxidizing bacteria	Nitriteoxidizing bacteria
Day-5 -^{13}C-CH_4	R1	7758	16.1%	0.01%	0.15%	0.82%
	R2	8630	13.5%	0.01%	0.19%	0.64%
	R3	8829	13.5%	---	0.20%	0.57%
	Average		**14.4%**	**0.01%**	**0.18%**	**0.68%**
	Pool	**8694**	**13.3%**	**0.02%**	**0.16%**	**0.70%**
Day-5- ^{13}C-Urea-	R1	7803	0.40%	---	0.13%	1.03%
	R2	7807	0.17%	0.03%	0.33%	1.38%
	R3	6541	0.26%	0.02%	0.37%	1.35%
	Average		**0.28%**	**0.03%**	**0.28%**	**1.25%**
	Pool	**9058**	**0.33%**	**0.01%**	**0.31%**	**1.37%**
Day-5- ^{13}C-CH_4+Urea	R1	7431	22.0%	---	0.20%	0.81%
	R2	8372	19.5%	0.01%	0.36%	1.05%
	R3	7568	20.6%	0.05%	0.29%	0.62%
	Average		**20.7%**	**0.03%**	**0.28%**	**0.83%**
	Pool	**9040**	**18.48%**	**0.03%**	**0.17%**	**0.91%**
Day-5- ^{12}C-CH_4+Urea	R1	6995	15.9%	---	0.13%	0.96%
	R2	8083	22.1%	0.04%	0.15%	0.68%
	R3	7809	18.4%	0.03%	0.14%	0.81%
	Average		**18.8%**	**0.04%**	**0.14%**	**0.82%**
	Pool	**10279**	**18.74%**	**0.02%**	**0.23%**	**1.04%**

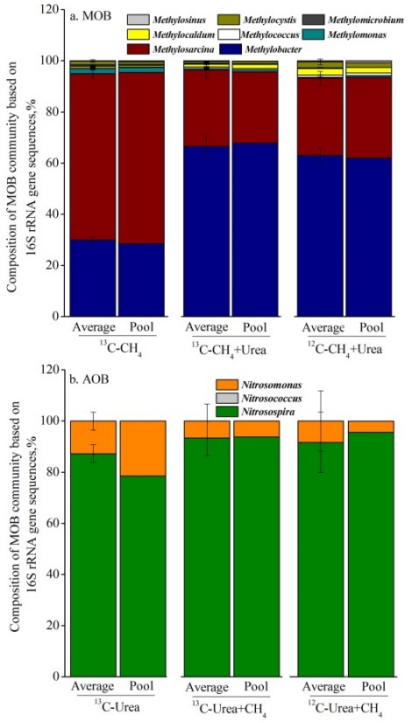

Figure 1 The composition of MOB(a) and AOB(b) community based on 16S rRNA gene sequences in the microcosms incubated for 5 days. The designation of 'Average' represents mean value of triplicate microcosms. The designation of 'Pool' represent the pooled DNA of triplicate microcosms.

18. Moreover, in the description and discussion of the pyrosequencing data, the authors should generally point out clearly that these data provide relative abundances of sequence reads but not necessarily relative abundances of microorganims within the total community.

Reply: Thanks for the comment! It has been pointed out from line 313 to line 314 on page 12 in the revised version as follows.
'Pyrosequencing data provided the information about relative abundance of targeted microbial 16S rRNA gene sequences in the total microbial communities.'

19. The authors should state more precisely the objectives of their work and present a clear hypothesis what kind of interactions they expected to find. Moreover, the discussion is lengthy in some parts and should be shortened. Repetition of results should be avoided.

Reply: Thanks for the comments! A number of changes have been made to keep the results more concise and focused. For example we have deleted the discussions about type Ib (Page 3910, line 13-23), denitrification (page 3908, line 26-29; page 3909, line 1-4), *Undibacterium* (page 3911, line29-30; page 3912, line 1-3) and *Pseudomonas* (page 3913, line 2-5) in the revised manuscript. In addition, repetition of results has been avoided.

Specific comments

1. 3896, l. 24-26: "However, the research: : :methan oxidation.“ It is unclear what this sentence means. Please rephrase.

   Reply: Thanks! It has been rephrased from line 129 to line 132 on page 5 as follows.
   ‘However, the research focus of methane effect on nitrification in natural complex ecosystems is poor, which is in sharp contrast with a large number of studies executed to elucidate effect of nitrogenous fertilizers on methane oxidation.’

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

   Reply: Done.

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

   Reply: Thank for the comment!

   ^{13}C-urea was used to prevent the dilution of the label because $^{13}CO_2$ addition is crucial for the labeling of ammonia oxidizers. As referee point out, ^{13}C-urea catabolized to ammonia and $^{13}CO_2$, the amount of $^{13}CO_2$ generated by urea catabolism can be used for labeling of ammonia oxidizers as well. The amount of urea-N we added to the microcosms was 100μg g^{-1} *d.w.s.*, the content of $^{13}CO_2$ is about 3.57 μmol g^{-1} *d.w.s.* assuming all urea is converted to ammonia and $^{13}CO_2$. In order to increase the labeling efficiency of targeted microorganisms, we added 50000 ppmv $^{13}CO_2$ (44.6 μmol g^{-1} *d.w.s.*) in the microcosms as previous study used (Jia and Conrad, 2009; Xia et al., 2011).

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

   Reply: The information about denosing and read length of the pyrosequencing reads has been provide from line 272 to line 278 on page 10 in the revised version.
   ‘Raw sequences were imported into mothur software (Schloss et al 2009) for quality check, alignment and phylogenetic tree construction. High quality sequences (read length longer than 200bp, average quality score more than 25, without ambiguous base calls) were extracted for further analysis. Pyrosequencing of *pmoA* gene yield about 36 000 high quality sequence reads with an average length of 482bp, while about 47 000 bacterial *amoA* gene were generated with an average length of 469bp.’
   In addition, the detailed information about the preparation of PCR products was also provided from line 268 to line 272 on page 10 in the revised version

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

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

Reply: The relative abundance here refers to the total microbial community. We have rephrased the sentence as follows in the revised version from line 362 to line 364 on page 13

'Though type II methanotrophs dominate MOB communities in background soil at day 0, the consumption of CH_4 in soil microcosms led to a drastic increase in relative abundance of type Ia methanotrophic 16S rRNA gene sequences in the total 16S rRNA gene sequences from 0.09% at day 0 to 14.4% at day 5.'

6. p. 3905,l. 19: Please write here "16S rRNA gene sequences of methanotrophs

Reply: Done.

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

Reply: It has been described in a greater detail from line 458 to line 461 on page 17 as follows.

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

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

Reply: Specified in the revised ms from line 596 to line 599 on page 22 as follows.
'Interesting is this link between nitrogen and cross-feeding of methanotrophic metabolites by other microorganism, possibly creating novel niches e.g. more methane-driven carbon substrate, lower-toxic environment for methylotrophs

in soil.'

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

Reply: It was clearly rephrased in the revised ms from line 611 to line 613 on page 22.

'Furthermore, a large part of the applied N disappeared in the presence of CH_4, and presumably assimilated by MOB. This explanation seems plausible for the suppression of methane on ammonia oxidation and the growth of ammonia oxidizers.'

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

Reply: It was rephrased in the revised ms from line 633 to line 636 on page 23 as follows:
'The growth of *Nitrosomonas* was stimulated to a much greater extent than that of *Nitrosospira* in urea-amended microcosms, but *Nitrosomonas* appeared to be suppressed more significantly than *Nitrosospira*.'

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

Reply: It was rephrased as follows.
'Therefore, we speculated that competition for nitrogen between methane- and ammonia-oxidizers play a dominant role in microbial interactions in our study, which is of help toward predictive understandings of carbon and nitrogen cycle in complex environment.'

Reference

Bodelier, P.L.E., and Laanbroek, H.J. (2004) Nitrogen as a regulatory factor of methane oxidation in soils and sediments. *Fems Microbiology Ecology* **47**: 265-277.

Boden, R., Cunliffe, M., Scanlan, J., Moussard, H., Kits, K.D., Klotz, M.G. et al. (2011) Complete Genome Sequence of the Aerobic Marine Methanotroph Methylomonas methanica MC09. *Journal of Bacteriology* **193**: 7001-7002.

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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.
Pommerening-Roser, A., and Koops, H.P. (2005) Environmental pH as an important factor for the distribution of urease positive ammonia-oxidizing bacteria. *Microbiological Research* **160**: 27-35.
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Point-by-point response to the comments of the referee #4

(Manuscript ID bg-2014-97)

General comments of the referee #4

In their study, Zheng and co-workers investigated the competition between methane and ammonia oxidizers in paddy soil microcosms. Soil slurries were incubated with 13-C labelled CH4, 13-C urea, and 13-C-CH4 and 13-C-urea. Soils were incubated for 5 and 19 days. Methane oxidation and nitrification rates were determined and the microbial community was analyzed by qPCR and amplicon sequencing targeting the 16SrRNA and the marker genes pmoA/amoA. The authors could show a strong stimulation of methane oxidation by urea addition and on the other side, a decrease of nitrification rates by methane addition. Within the methane oxidizing community, type Ia methanotrophs were highly enriched under the tested conditions and also labelled. Within the ammonia oxidizers, Nitrosospira was most abundant; however, Nitrosomonas dominated the labelled fraction. Ammonia oxidizing archaea do not seem to play a role in this system. Furthermore, the authors describe the labelling of 16S rRNA genes affiliated to known methanol degraders, indicating the close food web between methanotrophs and methylotrophs that feed on methanol. This is an interesting topic and the authors used an appropriate experimental approach to address this question.

Reply: We thank the referee for the overall positive comments.

Major comments

1. Nevertheless, the documentation of results and discussion is in my opinion not always concise and the manuscript contains too many figures and tables. This experiment contains a large dataset and not every aspect has to be discussed.

   Reply: We agree with this comment.

   A number of changes have been made to keep the results more concise and focused. For example we have deleted the discussions about type Ib (Page 3910, line 13-23), denitrification (page 3908, line 26-29; page 3909, line 1-4), *Undibacterium* (page 3911, line28-29; page 3912, line 1-3) and *Pseudomonas* (page 3913, line 2-5) in the revised manuscript.

2. However, what is missing in my opinion is the overall result of the 16S rRNA pyrosequencing (Archaea and bacteria). Of course it has not to be discussed in detail, but it should be shown to follow the authors'

argumentations and the selection of specific subsets.

Reply: The overall result of the 16S rRNA pyrosequncing has been shown in the Supplemental Table S3 in the revised version. The result revealed that bacteria were overwhelmingly dominant in total microorganism community.

We have described these overall results in the revised manuscript from line 309 to line 312 on page 12 as follows.

'About 346, 000 high-quality sequence reads were obtained with an average length of 377 bp in the V3~V4 region, while about 337,000 sequences was affiliated with bacteria. Relative abundance of bacterial 16S rRNA genes in total microorganism ranged from 95.3% to 98.8% in these microcosms, which was much higher than that of archaeal 16S rRNA genes.'

Specific comments:

1. In my opinion, not all phylogenetic trees have to be shown.Information on abundance and labelling could be combined in single trees

   Reply: Corrected. We agree with this comment. Phylogenetic trees of the ^{13}C-labeled MOB 16S rRNA genes and *pmoA* genes have been presented in a single Figure 4, while ^{13}C-labeled AOB 16S rRNA gene and *amoA* gene in Figure 5 in the revised version

2. Figure S8 is not important here.

   Reply: Removed.

3. Side 3911 Lines 13-27: There are already genomes of methanotrophs published. The authors should check this. As far as I remember, sequenced type Ia methanotrophs have a single copy of the rRNA operon as well as the pmoA (excluding the very different isoenzyme)

   Reply: We agree with this comment which was raised by referee #1 as well. The incongruence has been discussed briefly as follows from line 576 to line 581 on page 21.

   '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. The incongruence might result from the bias associated with the different coverage of *pmoA* and 16S rRNA gene primers as reported previously (Costello and Lidstrom 1999).'