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Methane and Ammonia Oxidations Interact in Paddy Soils

M Saiful Alam[1,2], Weiwei Xia[1,2] and Zhongjun Jia[1*]
[1]*State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, Nanjing, 210008, Jiangsu Province, P.R. China*
[2]*University of Chinese Academy of Sciences, Beijing, 100049, China*
*For correspondence: jia@issas.ac.cn

Abstract

The interactions of methane and ammonia oxidations in a paddy soil by methane-oxidizing bacteria (MOB) and ammonia-oxidizing bacteria (AOB) and archaea (AOA) were investigated through microcosm incubation study. Addition of $(NH_4)_2SO_4$ stimulated the activity of methane oxidation, while Na_2SO_4 amendment resulted in no apparent changes in methane oxidation kinetics. The inhibition of methane oxidation was observed in soil microcosms amended with Na_2CO_3 or phosphate buffer (PB buffer). After incubation for 28 days, the $(NH_4)_2SO_4$-amended microcosms showed the highest abundance of MOB, AOB and AOA, whereas lowest abundance for MOB and AOB were found in PB buffer-amended soil microcosms but Na_2CO_3 showed the lowest value for AOA abundance. Microbial ammonia oxidation in the soil microcosm was stimulated by methane addition, although it was not matched by changes in the abundances of AOA, AOB and MOB. The results of this study indicated that methane and ammonia oxidizers interacts with each other and might play important roles in regulating carbon and nitrogen turnovers in paddy soil.

Keywords: Methane oxidation; Nitrification; Interaction; Gene copy number; Paddy soil

Introduction

Methane (CH_4) is considered as one of the important greenhouse gases (GHG) and is responsible for various physical and chemical processes in the atmosphere, and so far it has contributed to an estimated 18-20% (Knittel and Boetius, 2009; Zhuang *et al*., 2009) of postindustrial global warming. It is estimated that approximately 70% of CH_4 appeared into the environment is originated from different human activities predominantly by agricultural management, disposal of waste material, and burning of the biomass from different sources (Houghton *et al*., 2001).

Methane-oxidizing bacteria (MOB) are capable of assimilating CH_4 as their exclusive carbon and energy source and thus perform vital role to reduce the global CH_4 load. Numerous studies have indicated that nitrogenous compounds are closely associated with methane oxidation kinetics (Bodelier, 2011). It is generally accepted that methane oxidation is inhibited by nitrogenous substrates, as was reported for agricultural soil (Sitaula *et al*., 2000), forest soil (King and Schnell, 1994a) and sediments (Van der Nat *et al*., 1997). The inhibition was often explained by the close evolutionary relations between the *amoA* and *pmoA* genes encoding the key enzymes responsible for CH_4 and ammonia oxidation (Holmes *et al*., 1995). It has indeed been demonstrated that MOB and ammonia-oxidizing bacteria (AOB) can switch substrates (Dunfield and Knowles, 1995). Conversely, many other studies have illustrated that methane oxidation was stimulated upon fertilization in paddy soil (Bodelier *et al*., 2000a, b; Krüger *et al*., 2002; Krüger and Frenzel, 2003; Mohanty *et al*., 2006), and forest soil (Börjesson and Nohrstedt, 2000). The observed stimulation might be due to the relief of N-source limitation or a direct stimulation of CH_4 oxidation by NH_4^+-N by an as yet unidentified mechanism (Bodelier *et al*., 2000a). However, it was also demonstrated that methane oxidation was unaffected by ammonium based N-fertilization (Dunfield and Knowles, 1995; Dunfield *et al*., 1995; Delgado and Mosier, 1996; Dan *et al*., 2001). Recently, a schematic representation showing the influence of nitrogen compounds on CH_4 oxidation activity for wetland and upland ecosystems has been projected (Bodelier, 2011). The effect of nitrogenous substrates on methane oxidation has been the most investigated but no consistent patterns could be generalized, and hence the interactions between the nitrogen and methane cycle are far more complicated than previously appreciated (Bodelier, 2011).

The particulate methane monooxygenase (*pmoA*) genes of MOB and the ammonia monooxygenase (*amoA*) genes of AOB are evolutionarily related to each other (Holmes *et al*., 1995). A number of similarities between CH_4 and ammonia oxidizers could promote interactions that can extensively shape the carbon and nitrogen turnovers in soil. Compared to nitrogenous effects on methane oxidation, the impact of methane on ammonia oxidizers remains poorly understood, and both stimulation and inhibition are apparently being involved (O'Neill and Wilkinson, 1977) as was observed for the kinetics changes of methane oxidation

in response to ammonium availability. No consistent interaction patterns were observed in the complex environment, although both inhibition (Megraw and Knowles, 1987; Roy and Knowles, 1994) and stimulation (Bodelier and Frenzel, 1999) of nitrification activity by MOB were often demonstrated.

Culture-dependent techniques have significantly advanced our understandings about the effect of ammonium on microbial methane oxidation activity, whereas the effect of methane on ammonia oxidation is rarely tested. However, it remains poorly understood about the putative interactions between ammonia and methane oxidations in soil ecosystem. Considering the similarities between methane and ammonia oxidizers, we expect that ammonia and methane oxidizers could promote interactions with each other in paddy soils. Therefore, microcosm incubations were performed to investigate the influence of ammonium on microbial CH_4 oxidation activity, as well as to investigate the influence of CH_4 on microbial ammonia oxidation simultaneously in a single soil microcosm.

Materials and Methods

Collection of Soil Samples

Soil samples were collected from the field trials established with free-air CO_2 enrichment (FACE) system located at Jiangdu (32°35'N, 119°42' E), Jiangsu, China. The soil was described as Shajiang-Aquic Cambosols according to CRGCST (2001). The experimental site was established with a rice–wheat rotation system. To conduct the microcosm study, soil samples from 0 to 15 cm depth were collected from the ambient plots of the wheat cultivation field. Soil samples were randomly taken from triplicate plots of the ambient treatment. Soil samples were immediately taken to the laboratory and kept at −20°C for molecular analysis and 4°C for soil physicochemical analyses.

Incubation Study

Five gram of soil was placed into a 120 mL crimp top serum vial for microcosm construction. Using different substrates, a total of 5 treatments were then generated including (1) H_2O (50 mL) +CH_4, (2) $(NH_4)_2SO_4$ (final conc. 1.0 mM) + CH_4, (3) Na_2SO_4 (final conc. 1.0 mM) + CH_4, (4) PB (0.1 M K phosphate buffer-PB buffer 50 mL) + CH_4 and (5) Na_2CO_3 (1 mL of 5% Na_2CO_3) + CH_4. All treatments were conducted in triplicate microcosms. The experimental design was established to generate conclusive evidence of methane and ammonia oxidation kinetics linked inhibitors by pairwise comparison. For instance, comparison between $(NH_4)_2SO_4$ and Na_2SO_4 amended treatments would illustrate the effects of ammonium rather than sulfate anion on methane oxidation activity. Similarly, comparison between Na_2SO_4 and Na_2CO_3 could provide convincing evidence for the effects of carbonate rather than sodium. After generating the treatments, rubber stoppers were used to seal the serum vials and then methane was injected into the headspaces to get the targeted methane concentrations of ~ 6,000 part per million. The incubation of soil microcosms were performed at 28°C in darkness with shaking at 200 rpm for 28 days. After consumption of > 95% CH_4, flushing of the vials with fresh air was carried out to remove the CO_2 and to maintain the soil slurries under aerobic condition. Methane concentration was measured on a daily basis or every other day. Gas samples (one milliliter) were analyzed by a gas chromatograph as described previously (Liu *et al*., 2011). Soil slurries were collected at 0, 14 and 28 days during the incubation study. The vials were strongly shaken, and 10 milliliter of the soil slurries were transferred and then centrifuged for 5 min at 10,000 rpm to collect the soil pellets. The collected soil pellets were then kept at -20°C for DNA extraction.

Microcosm incubation was further performed to investigate the influence of CH_4 on nitrification activity including two treatments: (1) microcosms without CH_4 containing 5 g soil and $(NH_4)_2SO_4$ (final conc. 1.0 mM); (2) microcosms with CH_4 containing 5 g soil and $(NH_4)_2SO_4$ (final conc. 1.0 mM). Both of the treatments were performed with 3 triplicate microcosms. The slurry was brought up to 50 mL using sterile distilled water, and the initial concentration of CH_4 was established as ~ 6,000 part per million. In addition 1.0 mL of 5% Na_2CO_3 was added to both treatments in order to eliminate the carbon source constraints for autotrophic growth of ammonia oxidizers. Soil slurries were collected at 0 and 7 days of incubation. Soil pellets were collected and stored for DNA extraction as described above. For inorganic nitrogen analysis (NO_3^-, NO_2^- and NH_4^+), supernatants were collected and kept at -20°C.

DNA Extraction from Soil and Real-time qPCR Assays

About 0.5 g of soil pellet was used to extract soil DNA as previously described by Griffiths *et al*. (2000) with slight modifications. DNA extractions were carried out for three times from each soil sample to obtain the highest amount of nucleic acids. The quantity and purity of the extracted soil DNA were determined using a Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, U.S.A.). To obtain the population sizes of *amoA* and *pmoA* genes, Real-time quantitative PCR (qPCR) was carried out using an optical designed CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.). Abundance of the *amoA* genes of archaea and bacteria were calculated with primer pairs Arch-amoAF/Arch-amoAR (Francis *et al*., 2005), amoA-1F/amoA-2R (Rotthauwe *et al*., 1997) respectively, whereas primer pairs A189f/ mb661r were used for MOB (Costello and Lidstrom, 1999). PCR reaction was carried out in a 20 µL volume including 10.0 µL SYBR Premix Ex Taq (TaKaRa Biotechnology Co. Ltd., Dalian, China), 2 µL soil

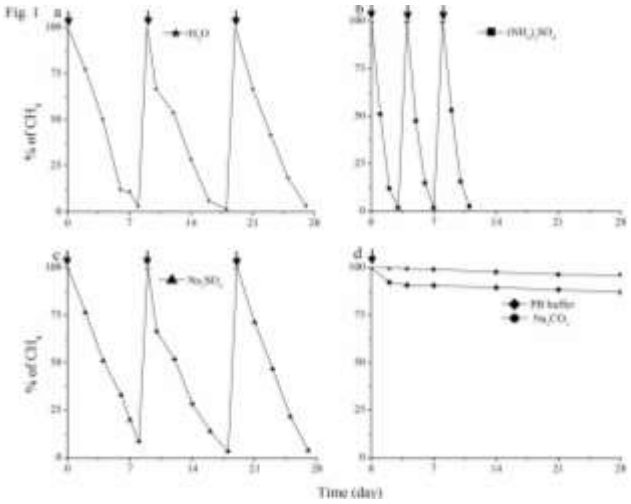

Fig. 1: Methane consumption kinetics during microcosm incubations with water (a), $(NH_4)_2SO_4$ (b), Na_2SO_4 (c) and Phosphate buffer and Na_2CO_3 (d). The arrows indicate the repeated addition of CH_4, i.e., initial concentration of ~6,000 ppm methane in the headspace was re-established after consumption of > 95% CH_4. Methane oxidation activity was determined in triplicate, and the data presented are mean values of the triplicate microcosms

DNA and 0.25 µM from each primer. The thermal conditions of the PCR reactions were similar as previously reported for *pmoA* genes (Alam and Jia, 2012), for *amoA* genes of bacteria and archaea (Lu *et al*., 2012). Real time amplification efficiencies of 101.2% with R^2 value of 0.996, 105.7% with R^2 value of 0.993 and 97.5% with R^2 value of 0.998 were obtained for bacterial *amoA* gene, archaeal *amoA* gene and the *pmoA* gene, respectively. Melting curve was analyzed to evaluate the specificity of amplification products, which gave a single peak for all samples.

Statistics

SPSS software package11.5 was used to perform the Duncan's post hoc tests to calculate the differences within datasets.

Results

CH_4 Oxidation Activity

Methane oxidation kinetics varied greatly in soil microcosms amended with different substrates (Fig. 1). When compared to control microcosm amended only with H_2O (Fig. 1a), the addition of $(NH_4)_2SO_4$ significantly stimulated potential methane oxidation activity (Fig. 1b), and the added methane (~4500 nm CH_4 g^{-1} *d.w.s.)* was almost completely consumed within 3 days of incubation. A total of ~13000 nm CH_4 g^{-1} *d.w.s.* was oxidized within 11 days. Whereas, soil microcosms amended with H_2O and Na_2SO_4 showed almost similar trend of methane oxidation activity and consumed 3 times of added methane (about ~13000 nm g^{-1} *d.w.s.*) after incubation for 28 days (Fig. 1a and 1c). On the contrary, methane oxidation was inhibited in the soil microcosms amended with PB buffer and Na_2CO_3, and only 13% and 7% of the added methane was consumed after incubation for 28 days, respectively (Fig. 1d).

Abundance of MOB, AOB and AOA Communities

The abundance of MOB, AOB and AOA was determined by qPCR targeting *pmoA* and *amoA* genes (Fig. 2). After 28 days of incubation, the highest *pmoA* gene copy number (1.19×10^8 g^{-1}*d.w.s*) was observed in soil microcosms amended with $(NH_4)_2SO_4$, despite being statistically similar with soil microcosms amended with Na_2SO_4 or H_2O (Fig. 2a). In contrast, significantly lower abundance of MOB was observed in soil microcosms amended with PB buffer (3.27×10^7 g^{-1}*d.w.s*), being statistically similar with Na_2CO_3-amended microcosms after 28 days of incubation. It is interesting to note that, *pmoA* gene copy number for different treatments remained largely stable between 14 and 28 days of incubation (Fig. 2a).

The bacterial *amoA* gene copy number was notably influenced by substrate level (Fig. 2b). After 28 days of incubation, the highest bacterial *amoA* gene copy number (8.00×10^7 g^{-1}*d.w.s*) was observed in soil microcosms amended with $(NH_4)_2SO_4$ even though being statistically similar with soil microcosms amended with H_2O. On the other hand, the lowest copy number (1.00×10^7 g^{-1}*d.w.s*) was observed in soil microcosms amended with PB buffer but statistically similar with treatments of Na_2SO_4 and Na_2CO_3. It is noteworthy that bacterial *amoA* gene copy number in soil microcosms with $(NH_4)_2SO_4$ differ greatly between 14 and 28 days of incubation (Fig. 2b). For the native soil, the bacterial *amoA* gene copy number was 1.24×10^8 g^{-1}*d.w.s.*, but after 14 days of incubation bacterial *amoA* gene copy number in soil microcosms with $(NH_4)_2SO_4$ was rapidly increased (1.61×10^8 g^{-1}*d.w.s.*) whereas it decreases remarkably after 28 days of incubation (8.00×10^7 g^{-1}*d.w.s*).

The *amoA* genes abundance of AOA varied among soil microcosms amended with different substrates (Fig. 2c). It is noteworthy that the abundance of archaeal *amoA* genes showed a decreasing trend during microcosm incubations with different substrates (Fig. 2c). The archaeal gene copy number was 1.10×10^8 g^{-1} *d.w.s* in native soil before incubation at day 0. After 28 days of incubation, the archaeal *amoA* gene copy number was significantly highest (8.66×10^7 g^{-1}*d.w.s*) in soil microcosms amended with $(NH_4)_2SO_4$, whereas significantly lowest gene copy number (2.87×10^7 g^{-1}*d.w.s*) was observed in soil microcosms amended with Na_2CO_3 but statistically similar with soil microcosms amended with Na_2SO_4 or Na_2CO_3 or H_2O.

Table 1: Effect of CH_4 on nitrification activity and gene copy numbers of AOA, AOB and MOB after 7 days of incubation

Specification	Incubation time		
	Day 0*	Day 7	
		Without CH_4	With CH_4
NH_4^+-N (μg g^{-1} *d.w.s.*)	1.1±0.05	65.2±2.86	26.2±0.26
NO_3^--N+ NO_2^--N(μg g^{-1} *d.w.s.*)	4.34±0.05	35.1±3.13	98.7±4.42
AOA gene copies (g^{-1} *d.w.s.*)	$1.10\times10^8\pm1.69\times10^7$	$4.09\times10^7\pm1.25\times10^6$	$6.30\times10^7\pm1.87\times10^6$
AOB gene copies (g^{-1} *d.w.s.*)	$1.24\times10^8\pm1.42\times10^7$	$4.14\times10^7\pm5.34\times10^5$	$4.04\times10^7\pm1.77\times10^6$
MOB gene copies (g^{-1} *d.w.s.*)	$1.15\times10^8\pm1.9\times10^6$	$4.63\times10^7\pm1.30\times10^6$	$4.87\times10^7\pm7.99\times10^5$

*: data at day 0 was background value determined immediately before addition of 1.0 mM of $(NH_4)_2SO_4$ to soil
Note: the data presented are mean values of the triplicate microcosms± standard deviation

Effect of Methane on Soil Nitrification

The results revealed that nitrification activity was stimulated in the presence of CH_4 (Table 1). Significantly higher nitrification activity was observed in soil microcosms treated with CH_4 (98.7 μg nitrate + nitrite N g^{-1} *d.w.s.*), when compared with that in soil microcosms without CH_4 addition (35.1 μg nitrate plus nitrite N g^{-1} *d.w.s.*) after 7 days of incubation (Table 1). This was further supported by the fact that the ammonium consumed was recovered in almost stoichiometric amount to the produced nitrate and nitrite in soil microcosm after incubation for 7 days (Table 1). In the meantime, the native soil contained only 4.34 μg (nitrate + nitrite) N g-^{1}*d.w.s*, while the ammonium concentration was as low as 1.1μg NH_4^+-N g^{-1} *d.w.s*. lending strong support for the stimulated activity of nitrification in microcosms upon ammonium fertilizations. It is however noteworthy that the *pmoA* gene copy number and bacterial *amoA* gene copy number remained largely constant for the soil microcosms with and without CH_4, although archaeal *amoA* gene copy number was considerably higher (6.30×10^7 g^{-1}*d.w.s.*) in soil microcosms with CH_4 as compared to the soil microcosms without CH_4 (4.09×10^7 g^{-1}*d.w.s.*) after 7 days of incubation (Table 1).

Discussion

Results of the study demonstrated that potential methane oxidation activity was influenced by different substrates during the microcosm incubation. It clearly demonstrated stimulatory effect of ammonium on methane oxidation activity in the form of $(NH_4)_2SO_4$. However, it is generally accepted that the methane oxidation is inhibited by nitrogenous fertilizers in various soil or sediment habitats including agricultural soil and forest soils (King and Schnell, 1994a; Van der Nat *et al*., 1997; Sitaula *et al*., 2000). Pairwise comparisons between soil microcosms amended with $(NH_4)_2SO_4$ and Na_2SO_4 clearly demonstrated that it is ammonium rather than sulfate anion that resulted in the stimulated activity of methane oxidation in the paddy soil tested (Fig. 1a, b, c). Stimulation of methane oxidation by ammonium fertilization might result from the deficiency of mineral nitrogen that facilitated an inactive and probably non-growing methanotrophic community. Therefore, addition of nitrogenous fertilizers thus would remove the nitrogen-deficient conditions and stimulate methane oxidation activity. It is speculated that the soil used in our study was limited by the availability of N for the adequate growth of the active microbial community. Therefore, the stimulation of methane oxidation activity was observed

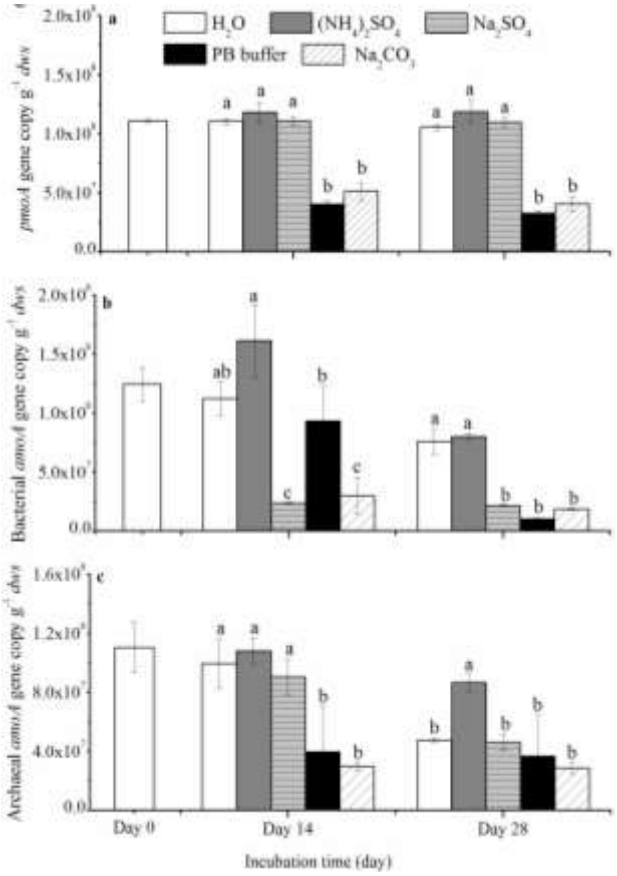

Fig. 2: Change in the abundance of *pmoA* genes of methane-oxidizing bacteria (a), of *amoA* genes of *Bacteria* (b) and *Archaea* (c) in soil microcosms amended with different substrates over an incubation course for 28 days. Error bars represent the standard deviation of triplicate microcosms and the same letter above the columns refers to no statistically significant difference among treatments ($P > 0.05$). All designations are the same as those in Fig. 1

upon the addition of $(NH_4)_2SO_4$. The *pmoA* gene copy number of our study largely supports this observation as the highest *pmoA* gene copy number was observed in soil microcosms amended with $(NH_4)_2SO_4$, after 28 days of incubation.

The inhibition of methane oxidation was observed by phosphate buffer (Fig. 1d). Furthermore, this observation was supported by the *pmoA* gene copy numbers as the lowest *pmoA* gene copy number was observed in soil microcosms amended with PB buffer after 28 days of incubation. The addition of Na_2CO_3 also showed inhibitory effect on CH_4 oxidation activity. The *pmoA* gene copy number after 4 weeks of incubation support our observation where significantly lower gene copy number was found in soil microcosms amended with Na_2CO_3. Jones and Morita (1983) reported that increasing concentration of the carbonate in a solution caused a corresponding decrease in the amount of $^{14}CH_4$-C incorporated into cellular material indicating lower methane consumption in presence of carbonate. Thus we speculate that carbonate concentration is important for the methane oxidation activity and the applied carbonate concentration in our study was accountable for the observed inhibition of methane oxidation activity.

The effect of CH_4 on microbial nitrification activity is much more debated but rarely tested. Results of our study indicated that methane has stimulatory effect on ammonia oxidation. The effect of methane on ammonia oxidation is complex, and culture studies indicated that both stimulation and inhibition apparently being involved (O'Neill and Wilkinson, 1977). King and Schnell (1994b) have also shown that methane enhances ammonia oxidation by *M. trichosporium* OB3b and *Methylobacter albus*. Schnell and King (1994) showed consistent results with a model in which methane stimulates ammonia oxidation by methanotrophs, with the resultant nitrite causing toxicity. In support of this interpretation, exogenous nitrite was a more effective inhibitor of methane consumption than ammonium. However, the robust experimental evidence is still missing for nitrite toxicity linked to methanotrophic communities in complex soil environments. Research findings also indicated that MOB could switch from methane oxidation to ammonia oxidation upon fertilizer addition by using stable C and N isotope probing (Acton and Baggs, 2011). However, a consistent pattern of interaction between these processes in the environment has failed to emerge, with reports of both inhibition (Megraw and Knowles, 1987; Roy and Knowles, 1994) and stimulation (Bodelier and Frenzel, 1999) of nitrification activity by methanotrophs. Methanotrophic suppression or stimulation of nitrification depends on various factors. The most likely outcome of methanotrophic suppression or stimulation of nitrification will depend on the in situ CH_4 concentrations. Nitrification activity in Hamilton Harbour sediment slurries were clearly stimulated by intermediate concentrations of CH_4 (Roy and Knowles, 1994). This is similar to the CH_4-dependent nitrification reported for *Methylosinus trichosporium* OB3b (Knowles and Topp, 1988). Thus we guessed that the methane concentration in our study was favorable enough to stimulate the nitrification activity.

In crux, $(NH_4)_2SO_4$ stimulated the methane oxidation activity, while the microbial oxidation of ammonia was enhanced by methane as well. These results were further supported by enumerating the population sizes of methane and ammonia oxidizing microorganisms using quantitative real-time polymerase chain reaction. As per our present understanding, this study represents the first attempt to investigate methane effect on ammonia oxidation and ammonium effect on methane oxidation simultaneously in a single soil microcosm. Our results implicated that the interaction between microbial methane and ammonia oxidizers can extensively shape the carbon and nitrogen turnovers in paddy soil and provide strong hints that underlying microbial mechanisms are far more complicated than previously recognized. The rapid advancement of culture-independent techniques, such as next-generation sequencing and stable-isotope probing, holds great promises for deciphering the microbial mechanisms underlying the complicated interactions between ammonia and methane oxidizers.

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