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Immediate effects of nitrogen, phosphorus, and potassium amendments on the methanotrophic activity and abundance in a Chinese paddy soil under short-term incubation experiment

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Abstract
Purpose Methane-oxidizing bacteria (methanotrophs) biologically consume and consequently affect the concentration of atmospheric methane (CH_4), the second most prominent greenhouse gas, and therefore play critical roles in the mitigation of global warming effect. Long-term fertilization often affects the methanotrophic community and CH_4 oxidation in various soils. Here, the immediate effects of nitrogen (N), phosphorus (P), and potassium (K) amendments on the CH_4 oxidation activity and methanotrophic community structure were evaluated.
Materials and methods Paddy soil samples were collected from the Taoyuan Experimental Station of the Chinese Academy of Sciences in central Hunan Province of China. A laboratory-based incubation experiment was conducted to investigate the immediate effects of N, P, and K amendments on the methanotrophs in soil. The CH_4 oxidation rates and methanotrophic activities were determined by measuring the dynamic changes of CH_4 concentration in the incubation system. The methanotrophic abundance and community changes in all of the seven treatments with and without nutrients addition were studied using real-time PCR and denaturing gradient gel electrophoresis, respectively.
Results and discussion All of the N, P, and K treatments significantly decreased the CH_4 oxidation activities. Compared with the control, the P and K amendments significantly increased the methanotrophic population size, but the N treatments have no effect on the methanotrophic abundance. A negative correlation was found between methanotrophic activity and methanotrophic abundance. We suggested that methanotrophic activity may not be inferred through the *pmoA* gene copies, especially in the short-term simulation experiments. Investigation of the methanotrophic population size and diversity is not enough to evaluate the soil CH_4 sink accurately.
Conclusions We concluded that the additions of N, P, and K reduce the activity but enhance the abundance of methanotrophs in a Chinese paddy soil through a short-term incubation experiment. Additionally, we found that the CH_4 oxidation activity could be completely inhibited by Cl^- toxicity. Our results implied that caution should be exercised in the types and amounts of fertilizers, especially KCl in agricultural systems to control the instantaneous increase in CH_4 emission from the field.

Keywords Chloride toxicity · Fertilizer · Methane oxidation · Methanotrophs · Nutrient element · *pmoA* · Real-time PCR

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1 Introduction

Soils are an important sink in the global budget of atmospheric methane (CH_4), which is the second most prominent greenhouse gas, contributing roughly 20 % to the observed global warming (IPCC 2007). Methane-oxidizing bacteria

(methanotrophs), which are ubiquitous in various soils (Bodelier et al. 2000; Reay and Nedwell 2004; Zhou et al. 2008a, b; Kolb 2009; Zhang et al. 2010; Semrau 2011; Lü et al. 2012; Zheng et al. 2012), can consume CH_4 with oxygen to produce CO_2 for energy generation, and they utilize the CH_4 carbon for generating new biomass (Conrad and Donald 2007). Methanotrophs are obligate aerobes and are classified into two groups (types I and II) differing in physiology, phylogeny, morphology, and biochemistry characteristics (Hanson and Hanson 1996). Given their critical roles in buffering the global warming effect, a growing body of research is targeting the methanotrophic community structure, their CH_4 oxidation activity, and their responses to environmental factors (Le Mer and Roger 2001; Dumont et al. 2006; Kolb 2009; Bodelier 2011; Zheng et al. 2012).

The ecological distribution, diversity, and CH_4 oxidation activity of methanotrophs are affected by soil characteristics, such as pH, temperature, moisture, and land use (King 1997; Börjesson et al. 1998; Knief et al. 2003; Horz et al. 2005; Mohanty et al. 2007; Menyailo et al. 2008; Singh et al. 2010; Zheng et al. 2012). Chemical fertilizers have been intensively used globally to meet the growing food demands because of continued population growth (Snyder et al. 2009). However, the use of fertilizers in agricultural systems often negatively affects the potential of soils to act as a CH_4 sink, which leads to elevated CH_4 concentrations in the atmosphere (Seghers et al. 2005).

As a major nutrient element controlling biological productivity in terrestrial systems, nitrogen (N) also has an important function in CH_4 oxidation. However, there is still no consistent point of view concerning methanotrophic communities under N fertilizer applications. The mainstream viewpoint presents that N application is inhibitory (Steudler et al. 1989; Hütsch et al. 1994; Reay and Nedwell 2004), whereas other studies reported stimulatory effects (Bodelier et al. 2000; Yang et al. 2011) and no effects on methanotrophs (Dunfield et al. 1995; Dan et al. 2001). On the other hand, phosphorus (P) fertilizers were suggested to have an apparent effect on microbial composition and biomass (He et al. 2008; Liu et al. 2012), as well as on CH_4 production and oxidation activity (Lu et al. 1999; Conrad and Klose 2005). Furthermore, potassium (K) amendment seems to stimulate the methanotroph population in rice field soil (Babu et al. 2006).

A clear distinction was found between the short- and long-term effects of fertilization (Hütsch 2001). For instance, the short-term application of N fertilizers results in an immediate inhibition of CH_4 consumption because of competition for methane monoxygenase (MMO) (Schimel 2000). In the long-term application, however, N fertilizers alter the methanotrophic composition, resulting in inhibited CH_4 oxidation (Hütsch 2001; Seghers et al. 2005). Many studies have determined the effect of long-term fertilization on methanotrophs in field conditions (Seghers et al. 2003, 2005; Gulledge et al. 2004; Zheng et al. 2008). However, few reports have assessed the short-term effects of fertilization on methanotrophic activity or community (Jang et al. 2011), especially investigations on methanotrophic activity and communities under short-term nutrient additions.

In the present study, we determined the CH_4 oxidation activity of a Chinese paddy soil to the immediate addition of N, P, and K through a short-term, laboratory-based incubation experiment. The community structure of methanotrophs in the soil incubated was also studied using real-time PCR and denaturing gradient gel electrophoresis (DGGE). The objectives were to detect and compare the immediate effects of N, P, and K amendments on CH_4 oxidation activity and methanotrophic community structure in a short-time incubation system.

2 Materials and methods

2.1 Experimental soil

Soil samples (0–20 cm in depth) were collected from a nonfertilized plot in a long-term fertilization experimental field at the Taoyuan Experimental Station (28°55′ N, 111° 26′ E) of the Chinese Academy of Sciences in central Hunan Province of China. The description of the station is available in Zheng et al. (2008). Soil was sieved through a 2-mm mesh to remove fine roots and large organic debris and was stored at 4 °C prior to the incubation experiment. The selected soil characteristics are listed in Table 1.

2.2 Methane oxidation

The soil incubation experiment was conducted according to the method described by Zheng et al. (2012). Briefly, 22 g of fresh soil (equivalent to 20 g dry weight) was transferred into a 250-mL culture serum bottle. An empty bottle (no soil added) served as blank to measure the gas tightness of bottles. Then, 3 mL of aqueous N [as $(NH_4)_2SO_4$ and urea],

Table 1 Selected physicochemical properties of the tested soil

Item	Value
pH (H_2O)	4.94[a]
Clay content (%)	30.5
Silt content (%)	53.5
Sand content (%)	16.0
Soil organic matter (g/kg)	29.2
Total nitrogen (g/kg)	3.53
Available phosphorus (mg/kg)	19.1
Available potassium (mg/kg)	47.1

[a]The data of pH, soil organic matter, total nitrogen, available phosphorus, and potassium were reported previously (Zheng et al. 2008)

P (as NaH_2PO_4 and KH_2PO_4), and K (as KCl and K_2SO_4) was uniformly added into the corresponding bottles using a 10-mL syringe, and the chemicals and soil samples in bottles were carefully mixed by hand. After addition of solutions, the amounts of the N, P, and K were 150, 220, and 332 mg/kg dry weight, respectively (fertilizer levels were widely adopted in practical agriculture) (Table S1, Electronic supplementary material). Sterilized water (3 mL) was applied as the zero nutrient control. Each treatment contained 21 culture serum bottles for 7 sampling times. The bottles were sealed with thick butyl rubber stoppers and covered with aluminum caps. All bottles were supplemented with CH_4 at an initial concentration of approximately 11 $\mu L L^{-1}$ (ppm) and incubated at 25 °C in the dark. After incubated for 0, 24 (12 for P treatments and its control, the same as below), 48 (36), 96 (60), 144 (108), 240 (204), and 336 (324)h, three culture serum bottles for each treatment were randomly taken out and used for analyzing the CH_4 concentrations, respectively.

The CH_4 concentration was determined by extracting 1 mL of gas samples from each bottle and measured using a gas chromatograph (Agilent 6820, Agilent Technologies) equipped with a flame ionization detector. The carrier gas for the chromatograph was N_2 (30 $mL min^{-1}$), and the injector, oven, and detector temperatures were 100, 80, and 180 °C, respectively. The flame gases, including H_2 and compressed air, were introduced at 20 and 30 $mL min^{-1}$, respectively. At the end of incubation, the soil samples at 336 (324)h were collected for DNA extraction and subsequent real-time PCR analysis.

2.3 DNA extraction

Soil DNA was extracted using the FastDNA® Spin Kit for Soil according to the manufacturer's instructions. The concentration and quality of the extracted DNA were analyzed through spectroscopic analysis (NanoDrop Technologies). The DNA samples were diluted 10-, 100-, and 1,000-fold to test the possible inhibitory effects of humic substances via PCR. Consequently, the 10-fold diluted DNA samples (i.e., 10–20 $ng \mu L^{-1}$) were selected as templates.

2.4 Real-time PCR

Methanotrophic abundance was analyzed based on the copy numbers of *pmoA* gene, which encode subunits of particulate methane monooxygenase, using real-time PCR, which was performed on an iCycler iQ5 thermocycler (Bio-Rad). Specific primer pairs A189 and mb661 (Costello and Lidstrom 1999; Holmes et al. 1999) were used to amplify the methanotrophic *pmoA* gene fragments. Amplification was performed using SYBR® Premix Ex Taq™, as described by the suppliers (TaKaRa). The detailed real-time PCR assay was performed through the protocols described by Zheng et al. (2010). All PCR assays were performed at least in triplicate. Data analysis was carried out using iCycler software (version 1.0.1384.0 CR).

2.5 Denaturing gradient gel electrophoresis

The extracted DNA was used as templates to produce PCR products for subsequent DGGE. The primers A189-gc and mb661 were selected to amplify the methanotrophic *pmoA* gene fragment. The composition of the PCR mixtures and the touchdown thermocycling conditions were described previously (Zheng et al. 2008). The PCR products were loaded onto 6 % (*w*/*v*) polyacrylamide gel (37.5:1, acrylamide/bisacrylamide) with a denaturing gradient of 40–60 %, where 100 % denaturant contains 7 M urea and 40 % (*v*/*v*) formamide. Electrophoresis was conducted at 60 °C, starting at 150 V for 10 min and then at 120 V for 6 h. The gel was then stained for 30 min in SYBR green gold nucleic acid gel stain (1:10,000) and photographed with a GBOX/HR-E-M (Gene Company Limited, Syngene, UK).

2.6 Statistical analyses

The CH_4 consumption was plotted using SigmaPlot software (version 10.0). Statistical and correlation analyses were carried out using SPSS software (version 15.0). One-way ANOVA was used to determine significant differences in potential CH_4 oxidation rates and *pmoA* gene copy numbers among the seven treatments at the $P<0.05$ level.

3 Results and discussion

A clear reduction in head-space CH_4 concentration was found in the treatments containing soil during the 2-week incubation (Fig. 1). Overall, CH_4 was consumed faster in the controls than in the nutrient amendments irrespective of N, P, and K. More CH_4 (83.6 %) was oxidized in the controls and the NaH_2PO_4 treatment (82.9 %) than in any of the other treatments (5.4–74.6 %). The highest methane oxidation rate (MOR) was found in the control, followed by the treatments of NaH_2PO_4 and KH_2PO_4 during the first 144 h. Then, the MORs in all of the treatments decreased (Fig. 2), although the MORs in the treatments of $(NH_4)_2SO_4$ and urea increased gradually during the 96th to 144th hour (see Fig. 2). Overall, the addition of N, P, and K inhibited the potential CH_4 oxidation activity to different extents (Fig. S1, Electronic supplementary material).

3.1 Effect of nitrogen

For the N treatments, a significant inhibition of CH_4 consumption in acidic rice field soil was observed in the two N

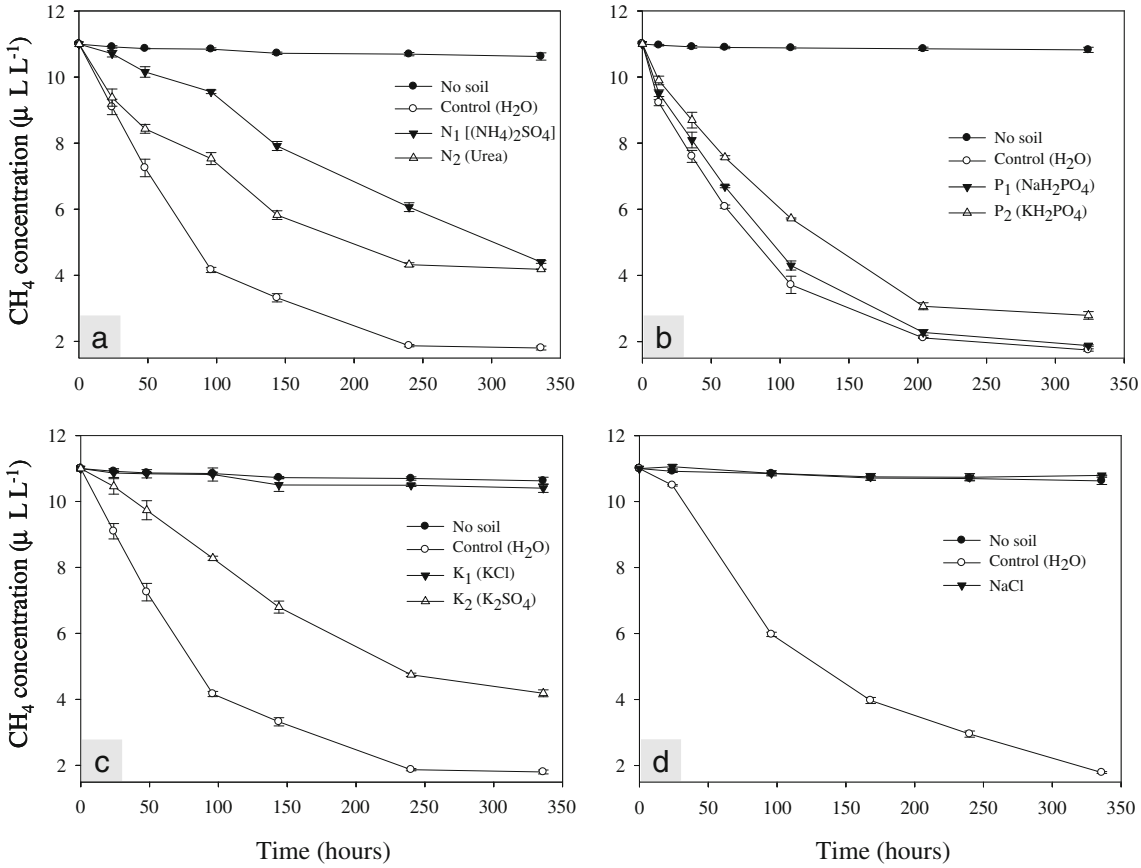

Fig. 1 Effects of the addition of N (**a**), P (**b**), K (**c**), and NaCl (**d**) on CH_4 consumption in a Chinese paddy soil

treatments, $(NH_4)_2SO_4$ and urea. Similarly, an immediate reduction in CH_4 oxidation rate of acidic grassland soil by adding $(NH_4)_2SO_4$ was reported previously (Tlustos et al. 1998). N is typically considered an inhibiting factor of CH_4 consumption in soils (Bodelier and Laanbroek 2004; Aronson and Helliker 2010). The competitive inhibitor of MMO and the toxicity of intermediates and end products (i.e., hydroxylamine and nitrite) were together attributed to decreased CH_4 oxidation activity (Schnell and King 1995; Hütsch 2001). However, Yang et al. (2011) reported that an appropriate amount of NH_4^+ addition can enhance the CH_4 oxidation in the landfill cover soil due to the NH_4^+/CH_4 ratio (< 0.1). Apparently, much higher NH_4^+/CH_4 ratio (about 760) may result in the inhibition of CH_4 oxidation

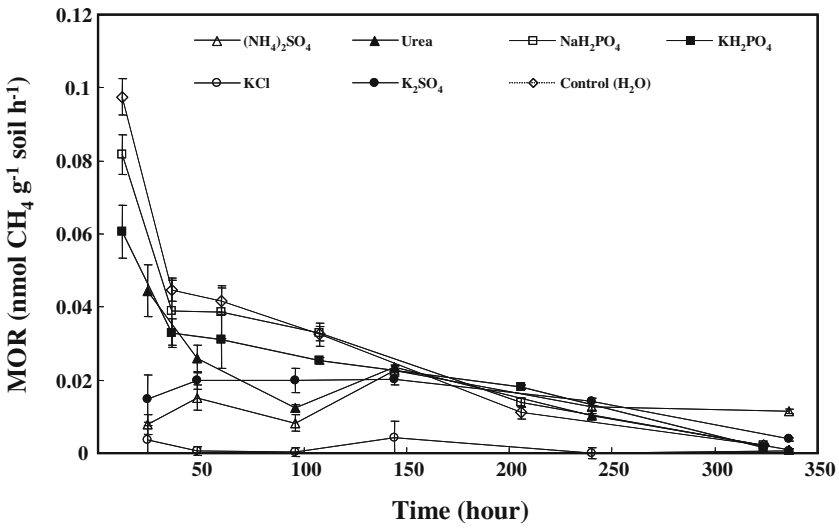

Fig. 2 Methane oxidation rates (MORs) during the incubation with N, P, and K amendments in a Chinese paddy soil

in this study. In addition, compared with the urea treatment, $(NH_4)_2SO_4$ showed stronger inhibition of CH_4 oxidation. Similarly, in the rice microcosm, the CH_4 oxidation rates were much lower in the NH_4^+ than that in the urea treatment (Shrestha et al. 2010). Collectively, our results indicated that the additions of N fertilizers inhibited CH_4 consumption in acidic rice soil.

3.2 Effect of phosphorus

As a major limiting nutrient for plant growth, P fertilizers are used in intensive agricultural systems to overcome soil P deficiency and thus achieve higher global food production (Richardson et al. 2011). In the current study, the amendments of P also negatively affected CH_4 oxidation, although the inhibited strength was lower than the N amendments caused. This result differs from the study by Conrad and Klose (2005), in which P fertilization stimulated the potential methanotrophic activity in the rhizosphere. Given that the effects of P application on lower CH_4 emission in paddy soil were potentially ascribed to plants and/or rhizosphere (Lu et al. 1999; Rath et al. 2005), however, P additions negatively affecting CH_4 oxidation in this study could be interpreted as follows. Without planting rice in our incubation experiment, we cannot detect the effect of the rhizosphere on P assimilation and the subsequent stimulation of CH_4 consumption because the rhizosphere induces a spatial pattern in the distribution of methanotrophs and is a potential microsite of intense CH_4 oxidation (Dubey and Singh 2000).

3.3 Effect of potassium

Approximately 57 % decrease in CH_4 occurred before 240 h of incubation in the K_2SO_4 treatment, whereas a small amount of CH_4 (0.6 $\mu L L^{-1}$) was oxidized until the end of incubation. However, nearly no change in CH_4 concentration was found in the KCl treatment. Compared with the control, the applications of K showed inhibitory effect on CH_4 oxidation. In a field study, Conrad and Klose (2005) pointed out that the net effect of potassium phosphate on stimulated CH_4 emission is mainly from CH_4 production and ventilation rather than CH_4 oxidation. However, another field study found that K amendment effectively reduces CH_4 emission from flooded soil partly by stimulating the methanotrophic bacterial population (Babu et al. 2006). The difference between these field studies and the current results possibly resulted from the consideration on the function of plant and its rhizosphere effect (Lee et al. 2011). Another possible explanation for this discrepancy could be that the soil water conditions (e.g., flood or drainage) in the field is distinctly different from the soil incubation conditions in the laboratory because increased CH_4 oxidation activity was recently demonstrated from continuous flooding to drainage (Ma and Lu 2011).

3.4 Effect of chloride

Interestingly, CH_4 consumption was completely inhibited with the addition of KCl. Given that the same K concentrations were applied in both K_2SO_4 and KCl treatments, an additional incubation experiment was then conducted to verify the effect of Cl^-. As predicted, similar result was observed when adding the same concentration of chloride as NaCl (see Fig. 1). We thus suggest that Cl^- inhibited microbial CH_4 oxidation during soil incubation. The toxicity of chemical (e.g., halogenated hydrocarbons) on methanotrophs expressing particulate MMO (pMMO) was also examined previously (Han et al. 1999). Additionally, CH_4 oxidation could also be inhibited by organic acids and ethanol either through organic compound toxicity or their preferred utilization (Wieczorek et al. 2011). The current results suggest that the main microbial CH_4 oxidation was inhibited by Cl^- toxicity. However, the methanotrophic community composition would change; some methanotrophs would adapt to the Cl^- stress and become as dominant groups. This could be interpreted that different groups have different physiological responses and environmental adaptations to chemicals (Nyerges and Stein 2009).

Indeed, the community structure of methanotrophs was changed under KCl amendment, as well as other treatments (Fig. S2, Electronic supplementary material), as indicated by specific DGGE bands (i.e., marked from B-1 to B-10), which would be immediately induced by the N, P, K, and Cl^- amendments. Cluster analyses also showed that the differences in methanotrophic community patterns resulted from the nutrient addition treatments because the changes could be distinguished between treatments (Fig. S3, Electronic supplementary material). However, we cannot identify which methanotroph(s) changed during the treatments because the subsequent DGGE sequencing experiments failed.

3.5 Methanotrophic abundance under N, P, and K amendments

In the current study, real-time PCR was used to quantify the methanotrophic *pmoA* gene copies in the soil samples collected after a 2-week incubation. As shown in Fig. 3, the highest abundance of methanotrophs was found in the KCl treatment (8.9×10^7 copies per gram of soil), followed by K_2SO_4 (7.6×10^7), NaH_2PO_4 (6.0×10^7), and KH_2PO_4 5.4×10^7) treatments. The methanotroph abundance found in all four treatments was significantly higher than that in the control (2.8×10^7). Firstly, no significant difference was observed between the two N treatments [$(NH_4)_2SO_4$ and urea;

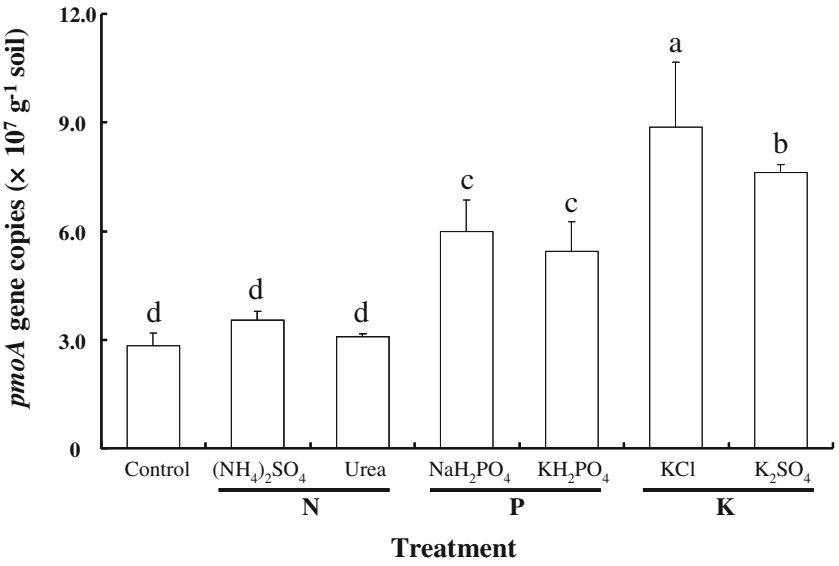

Fig. 3 Quantification of methanotrophic *pmoA* gene copy numbers in a Chinese paddy soil under different nutrient treatments. The *different letters above the bars* indicate significant differences among treatments at $P<0.05$

3.5×10^7 and 3.1×10^7, respectively] and the control. This result is in agreement with another report, which demonstrated that methanotroph abundance was not affected by N application (animal urine) in six grazed grassland soils (Di et al. 2011). Given that the CH_4 oxidation in the N treatments differed from the control, the results could be explained based on physiological responses, and perhaps environmental adaptations, of methanotrophs, which may have N-specific species (Nyerges and Stein 2009).

Moreover, in contrast to a previous study, which showed that P was not a key factor controlling the abundance of methanotrophs in paddy soil under long-term fertilization regimes (Zheng et al. 2008), the P treatments applied in the current study resulted in clearly enhanced methanotrophic abundance than the control. It is possible that the effect of P amendments on the growth or metabolism of methanotrophs was more direct and intensive during the short-term incubation process than their long-term effects in the field plots. However, further research is needed to clarify the short-term stimulation of methanotrophs with P addition.

Unexpectedly, significantly higher abundances were detected in the KCl and K_2SO_4 treatments than those in the control. This result was consistent with another study, in which K fertilizer application seemed to lead to increased methanotrophic abundance in paddy field soil (Zheng et al. 2008). Particularly in the present study, a negative correlation was found between methanotrophic activity and methanotroph abundance ($n=21$, $P<0.01$, Fig. 4). It was likely that acute chemical-induced stresses changed the ratio of type I to type II methanotrophs and thus resulted in converse shifts in activities and abundance. Future studies are necessary to determine how type I and II methanotrophs adapted to the immediate nutrient and/or chemical stresses. Hence, we suggest that methanotrophic activity cannot be solely inferred through the gene copy numbers of methanotrophs, especially in the short-term simulation experiments. Investigating the size and diversity of the methanotrophic community is not enough to evaluate the soil CH_4 sink accurately. The overall CH_4 oxidation activity should be taken into consideration.

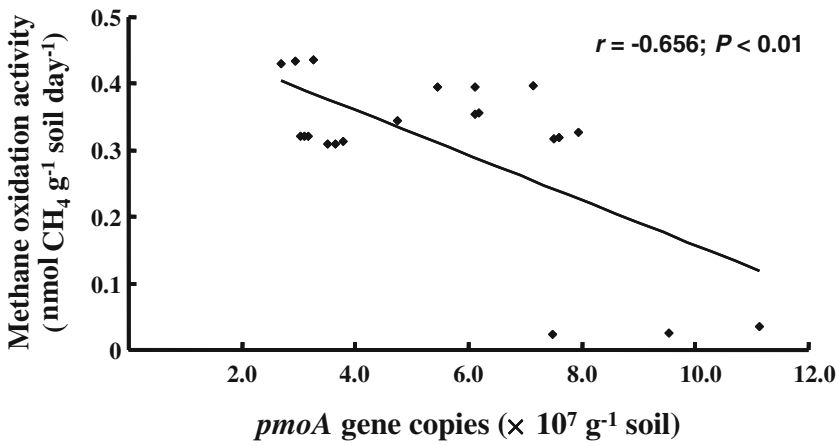

Fig. 4 Relationships between methane oxidation activities and methanotrophic *pmoA* gene copy numbers in a 2-week incubation experiment with different N, P, and K amendments

4 Conclusions

In the present study, we compared the immediate effects of N, P, and K on the CH_4 oxidation and methanotrophic community structure in a Chinese paddy soil through the short-term incubation experiment without planting. N, P, and K amendments clearly reduced the CH_4 oxidation activities. Compared with N and K, the P treatments showed a relatively weak inhibitory effect on the CH_4 oxidation. In addition, the P and K treatments significantly increase methanotrophic abundance, but the N treatment minimally affected the methanotrophic population size. We further found that CH_4 consumption could be completely inhibited by Cl^- toxicity. This study revealed that the fertilization could cause immediately negative effects on CH_4 consumption and thus potentially enhanced the CH_4 emission from paddy soil. Given the same fertilization level, we suggest that increasing the times of fertilization and decreasing the amounts of fertilizers might avoid the acutely negative effect on the CH_4 oxidation activity. Additionally, as the current results imply, caution should also be exercised in the application of KCl fertilizer in agricultural systems to control the increase in CH_4 emission from the rice field.

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