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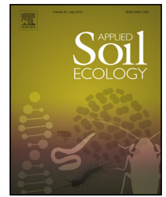

Methane oxidation and response of *Methylobacter/Methylosarcina* methanotrophs in flooded rice soil amended with urea

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ABSTRACT

Nitrogen, which is one of the most important factors controlling methane oxidation, affects rice production and methane emission from rice fields. Therefore, in this study, we aimed to investigate the effects of urea fertilization on methanotrophic communities and methane oxidation. Chinese rice soil was incubated with urea at concentrations of 0, 200, 400, and 800 mg N kg^{-1} dry soil, and methanotrophic communities were analyzed by terminal-restriction fragment length polymorphism (T-RFLP) of the particulate methane monooxygenase gene (*pmoA*). Metabolically active methanotrophs were identified by combining T-RFLP analysis of *pmoA* transcripts and rRNA stable isotope probing. The results showed that a higher concentration of urea delayed the initiation of methane oxidation but stimulated the final methane oxidation rate of the rice soil. As more urea fertilizers were applied, acceleration of the methane oxidation rate occurred more rapidly. These effects were cumulative and were not directly related to the ammonium concentration in rice soil. Moreover, the activity of the *Methylobacter/Methylosarcina* group was stimulated by urea fertilizer, and the composition of the dominant and active methanotrophic community changed only slightly after 90 h. Our results indicated that urea fertilization generally stimulated methane oxidation and methanotrophic activity, primarily by stimulating the relative abundance and activity of the *Methylobacter/Methylosarcina* group.

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1. Introduction

The aerobic oxidation of methane contributes to the reduction of microbial methane emission in rice field soil. Up to 40% of the produced methane is oxidized by methanotrophs before its emission (Frenzel, 2000). The rate of methane oxidation generally depends on several factors, including methane, oxygen (Cho et al., 2012), and chemical fertilizers (Zheng et al., 2012), among which nitrogenous fertilizers are critical. Because the availability of nitrogen is the yield-limiting factor for plants in most rice agro-ecosystems (Cassman et al., 1998), nitrogenous fertilizers have been intensively used to meet the increasing demands for rice. However, nitrogenous fertilizers, especially ammonium-based N-fertilization, affect methane oxidation because of the similarities between the key enzymes catalyzing the first step of CH_4 oxidation and ammonium oxidation (Mancinelli, 1995). Methanotrophs are thought to be able to switch the substrates from CH_4 to ammonia when a large amount of ammonia-N is applied to the soil (Dunfield and Knowles, 1995). Interestingly, ammonium-based N-fertilization has been shown to have inhibitory effects (Alam and Jia, 2012; Steudler et al., 1989), stimulatory effects (Bodelier et al., 2000; Noll et al., 2008), or no effects (Dan et al., 2001; Liikanen and Martikainen, 2003) on methane oxidation in forest soils (Steudler et al., 1989), rice field soils (Alam and Jia, 2012; Bodelier et al., 2000; Dan et al., 2001; Noll et al., 2008), and lake sediments (Liikanen and Martikainen, 2003).

Aerobic methane oxidizing bacteria (MOB), also called methanotrophs, are characterized by their ability to utilize methane as the sole source of carbon and energy. Phylogenetically, aerobic methanotrophs belong primarily to *Alphaproteobacteria* (type II MOB) and *Gammaprote obacteria* (type I MOB). The alphaproteobacterial methanotrophs, including five described genera, can be further divided into the families *Beijerinckiaceae* and *Methylocystaceae*, whereas the gammaproteobacterial methanotrophs, including 18 described genera, belong to the families *Methylococcaceae* and *Methylothermaceae* (Deutzmann et al., 2014; Hirayama et al., 2014; Hoefman et al., 2014). Type I methanotrophs are subdivided into the *Methylobacter/Methylosarcina* group (type Ia) and the *Methylococcus* group (type Ib) (Kolb et al., 2003). A few studies have found that

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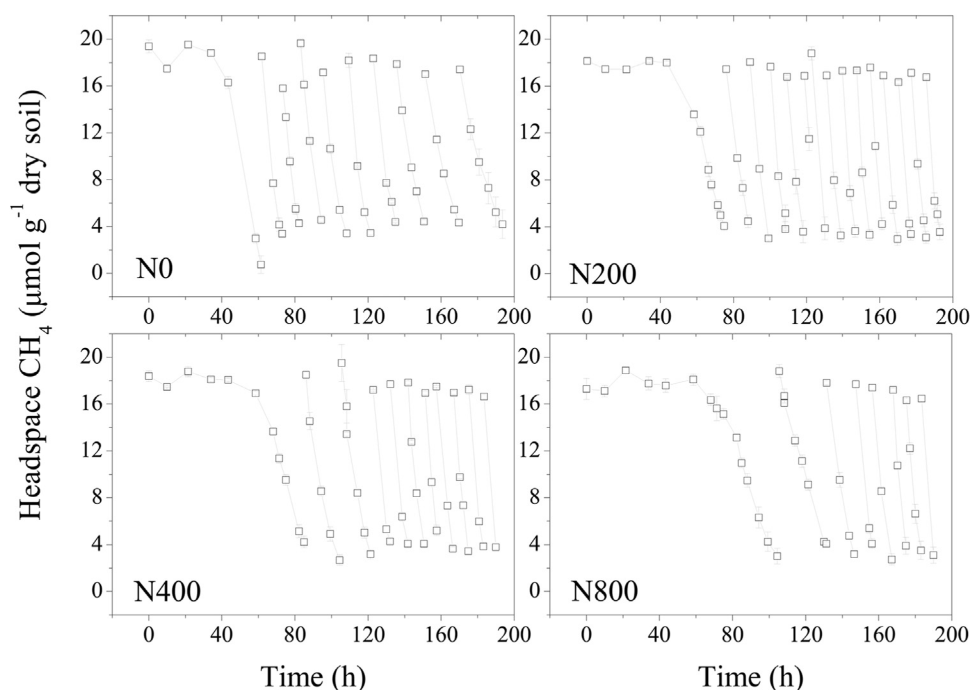

Fig. 1. Effects of the rate of urea-N on methane concentrations over a 193-h incubation. Data are the mean values ± SEs ($n = 3$).

type I methanotrophs are mainly present in rice roots (Shrestha et al., 2010; Wu et al., 2009) and rhizospheres (Qiu et al., 2008) and become active after the application of nitrogenous fertilizer (Bodelier et al., 2000; Noll et al., 2008). However, these studies were based on a single concentration of nitrogenous fertilizers or a single sampling point. Therefore, it is unclear whether the CH_4 oxidation activity and the community composition of metabolically active methanotrophs are shaped differently with different nitrogenous fertilizer levels at different time points. The possibility of inhibition at the biochemical level exists when the amount of ammonium is in greater larger excess than the concentration of methane in methane monooxygenase (Schimel, 2000); therefore, the quantitative limit of nitrogenous fertilization must be investigated. However, not all members of the methanotrophic community are equally active in the methane oxidation process. Stable isotope probing (SIP) is a powerful approach for identifying any unknown active microbial populations. Depending on the assimilation of CH_4, an experiment can specifically label methanotroph biomass using $^{13}CH_4$, after which the nucleic acids of the MOB can be retrieved by equilibrium centrifugation, as shown in DNA-SIP (Morris et al., 2002). Because RNA-SIP has advantages for detecting microorganisms that actively synthesize ribosomes from the substrate carbon owing to the high turnover of rRNA (Lueders et al., 2004), this method was used to follow the activity changes in MOB upon fertilization in an Italian rice field soil (Noll et al., 2008).

Although the effects of nitrogenous fertilizers on methane oxidation have been extensively studied in the past two decades, it is still unclear whether CH_4 oxidation activity and the community composition of metabolically active methanotrophs are shaped differently with different levels of nitrogenous fertilizers at different time points. Accordingly, in this study, we evaluated the oxidation of CH_4, which is influenced by different levels of nitrogenous fertilizers in rice soil, in a laboratory-based incubation experiment. Changes in the community structures of active methanotrophs with different incubation times and ammonium concentrations were also investigated. Furthermore, methanotrophs that would react specifically upon fertilization with nitrogen were investigated using SIP.

2. Materials and methods

2.1. Soil and incubation

Rice field soil was collected from the surface at 0–15 cm depth of a farmed rice field (29°45′55″N, 121°53′01″E) in Zhejiang Province of China in the spring of 2011. The rice field, which was used for the double cropping of rice for 15 years, was improved from mud flat wetlands. The soil was rice paddy soil and was classified as Endoaquepts according to soil taxonomy (Soil Survey Staff, 2014). The soil was silt loam, with 4.48% sand, 83.51% silt, and 12.01% clay. The collected soil was first air-dried and then passed through a 2-mm sieve to remove fine roots and large organic debris for homogenization. The following characteristics of the soil sample were measured (per kg of soil): pH_{H2O}, 7.88; organic carbon, 27.28 g; total nitrogen, 1.77 g; total phosphorus, 0.5521 g; available phosphorus, 3.195 mg; and available potassium, 239.93 mg.

The experiment followed a completely randomized block design with four replicates that had the following four treatments of nitrogenous fertilizer: 0 mg N kg^{-1} (N0), 200 mg N kg^{-1} (N200), 400 mg N kg^{-1} (N400), and 800 mg N kg^{-1} (N800); and two treatments of methane: $^{12}CH_4$ and $^{13}CH_4$. Three g of dry soil was added to 125-mL glass bottles, followed by 3 g of double-distilled water to obtain soil slurries. The bottles were sealed with butyl rubber stoppers and pre-incubated horizontally, followed by shaking at 160 rpm and 25 °C in the dark for 1 day to initiate microbial activity. Because Cl^- is toxic and may inhibit CH_4 consumption in rice soil (Shrestha et al., 2010; Zheng et al., 2012), urea was selected as a slow-release source of NH_4^+ in this study. One milliliter of urea solution was added to each bottle of soil slurries to obtain the expected nitrogenous fertilizations concentrations. Soil samples with urea were again incubated under ambient air for 3 days, after which 10000 ppmv (~18 μmol g^{-1} dry soil) of methane was injected into the headspace of the bottle for methane oxidation. The headspace was refilled with fresh air and methane when the methane concentration in the headspace fell below 5 μmol g^{-1} dry soil. Methane oxidizing incubation was carried out for 8 days. In

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