Soil Biology & Biochemistry 85 (2015) 31–38

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

The differential effects of ammonium and nitrate on methanotrophs in rice field soil

Ang Hu [a], Yahai Lu [a, b, *]

[a] College of Resources and Environmental Sciences, China Agricultural University, Beijing 100193, China
[b] College of Urban and Environmental Sciences, Peking University, Beijing 100871, China

ARTICLE INFO

Article history:
Received 22 October 2014
Received in revised form
24 February 2015
Accepted 26 February 2015
Available online 14 March 2015

Keywords:
Methanotrophs
Methane oxidation
Nitrogenous fertilizer
Rice field soil

ABSTRACT

It has been known that nitrogenous fertilizers can either stimulate or inhibit methane oxidation in soils. The mechanism, however, remains unclear. Here we conducted laboratory incubation experiments to evaluate the effects of ammonium versus nitrate amendment on CH_4 oxidation in a rice field soil. The results showed that both N forms stimulated CH_4 oxidation. But nitrate stimulated CH_4 oxidation to a greater extent than ammonium per unit N base. The 16S rRNA genes and the *pmoA* genes were analyzed to determine the dynamics of total bacterial and methanotrophic populations, respectively. The methanotrophic community consisted of type I and type II methanotrophs and was dominated by type I group after two weeks of incubation. Nitrate promoted both types of methanotrophs, but ammonium promoted only type I. DNA-based stable isotope probing confirmed that ammonium stimulated the incorporation of $^{13}CH_4$ into type I methanotrophs but not type II, while nitrate caused almost homogenous distribution of $^{13}CH_4$ in type I and type II methanotrophs. Our study suggests that nitrate can promote CH_4 oxidation more significantly than ammonium and is probably a better N source for both types of methanotrophs in rice field soil. More investigations, e.g. using ^{15}N labeling, are necessary to elucidate this possibility.
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1. Introduction

Approximately half of the methane produced in anoxic environments is oxidized by methanotrophs before it escapes into the atmosphere (Thauer, 2010). The activity and growth of methanotrophs in soils are crucial in balancing the atmospheric CH_4 concentration. Various environmental and agricultural factors like soil type, water management, and fertilization can affect CH_4 oxidation (Bodelier et al., 2000; Ma et al., 2010, 2013; Ma and Lu, 2011). In Asian rice field soils, nitrogen is a yield-limiting factor of rice production (Cassman et al., 1998) and the application of nitrogenous fertilizers is expected to increase in the next decades due to the food demand of the increasing population (Glibert et al., 2006). It is imperative to determine the effect of nitrogenous fertilizers on CH_4 oxidation in order to better predict and mitigate CH_4 emission from rice field soils.

Aerobic methanotrophs in rice field soils consist mainly of proteobacterial lineages, while verrucomicrobial methanotrophs are generally restricted to extreme environments (Op den Camp et al., 2009). The proteobacterial methanotrophs are conventionally classified into type I (*Gammaproteobacteria*) and type II (*Alphaproteobacteria*) groups based on their cellular morphology, chemistry and physiology (Hanson and Hanson, 1996; Luke and Frenzel, 2011). The *pmoA* gene that encodes the subunit of membrane-bound methane monooxygenase (MMO) is present in all known bacterial methanotrophs except *Methylocella* and *Methyloferula* (Dedysh et al., 2000; Vorobev et al., 2011) and can be used as a phylogenetic marker for the ecological studies of methanotrophs (McDonald et al., 2008; Ma et al., 2013).

A number of studies revealed the stimulation of CH_4 oxidation by ammonium in paddy field soils (Bodelier et al., 2000; Krueger and Frenzel, 2003; Noll et al., 2008). It has been hypothesized that the stimulating effect is related to N limitation in rice field soil (Schimel, 2000), where N uptake by rice plants depletes available N in the rooting zone and rhizosphere. Albeit a crucial nutrient for methanotrophs, high ammonium concentrations can inhibit CH_4 oxidation through either competition for methane monooxygenase (MMO) or generation of toxic hydroxylamine and nitrite from ammonium oxidation (Steudler et al., 1989; King and Schnell, 1994; Schimel, 2000; Reay and Nedwell, 2004; Saari et al., 2004; Stein and Klotz, 2011).

* Corresponding author. Urban and Environmental Sciences, Peking University, Beijing 100871, China. Tel./fax: +86 10 62755683.
E-mail address: luyh@pku.edu.cn (Y. Lu).

http://dx.doi.org/10.1016/j.soilbio.2015.02.033
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The actual effect of N on CH_4 oxidation depends on methanotroph composition. Since most of type II methanotrophs possess N_2 fixation machinery as backup for N nutrition, type I methanotrophs have been considered more sensitive to and benefiting more from N fertilization in rice field soils (Bodelier et al., 2000; Noll et al., 2008; Qiu et al., 2008). In an isotopic labeling experiment, Mohanty et al. (2006) showed that the type I methanotrophs were stimulated by N whereas the type II group was inhibited in forest and rice field soils. Studies on pure cultures revealed that methanotrophic strains even within the same type differed markedly in their ability to metabolize ammonia and tolerate the negative effects from ammonia oxidation and nitrite production (Nyerges and Stein, 2009; Nyerges et al., 2010).

Another factor controlling the N effect can be the chemical form of nitrogen in soil. Nitrogen is present in different forms and their turnover is highly dynamic in rice field soil (Kirk and Olk, 2000). Most of previous studies have used urea and ammonium as N sources to investigate the effect on CH_4 oxidation (Bodelier et al., 2000; Cai and Mosier, 2000; Noll et al., 2008; Qiu et al., 2008; Shrestha et al., 2010; Alam and Jia, 2012). These N sources represent the most common fertilizers applied in rice fields. However, transformations of N species occur readily when N fertilizers enter the soil (Yuan and Lu, 2009; Ke and Lu, 2012). Nitrate can be generated via nitrification in the rice rooting zone and rhizosphere. Very little, however, is known about the differential effects of N forms on CH_4 oxidation in rice field soils (Bodelier, 2011). In contrast to ammonium, nitrate as a nutrient may have the advantage not serving as a competing substrate for CH_4 oxidation.

The objectives of the present study were: i) to compare the effects of ammonium and nitrate on CH_4 oxidation over a range of N application rates; ii) to determine the responses of bacterial and methanotrophic communities to the application of two N forms; and iii) finally to understand the stimulating effects of N on CH_4 oxidation in rice field soils.

2. Material and methods

2.1. Preparation of soil incubation

Rice field soil was collected from a long-term (the fifth year) fertilizer experiment field at Zhejiang Agricultural Institute in Hangzhou, China (30°26′N, 120°24′E). Soil samples were collected from the control plot in an area of 78 m^2 without N application. Twenty cores of top soil (0–15 cm) were taken in the spring of 2009 using the s-shape sampling method. The field had been left fallow for about four months since the rice harvest in previous fall. Soil samples were air dried, sieved (2 mm) and stored at room temperature for three months before the use in present experiment. The soil had the following characteristics as measured by standard methods (Page et al., 1982): pH 6.7, organic C of 13.9 g/kg and total N of 0.41 g/kg. Sterile demineralized water was added to create soil water content of 15% (w/w) for the incubations. 120-ml serum bottles were filled with 10 g of soil (d.w.), closed with butyl stoppers and sealed with aluminum crimps. The initial methane concentration in the headspace was set between 1.5 and 2% (v/v). No extra oxygen was added as the initial O_2 to CH_4 ratio was >10, which was five times higher than the need based on the stoichiometry of CH_4 oxidation. Since the headspaces were exchanged frequently whenever CH_4 was consumed to lower than 1000 ppm_v, we assumed that O_2 was not limiting during the incubation. All bottles were incubated at 25 °C for a total of about two weeks in darkness. Each treatment was carried out in four replicates.

Prior to N amendment, soils were preincubated for three days with two complete consumptions of the added methane. Two batches of incubations were then prepared in which nitrogen was applied in two dosages. To the first batch, aliquots of NH_4Cl solutions (0.5 ml) were added to generate the final concentration of 39, 130 and 260 mg NH_4^+-N kg^{-1} s.d.w. (soil dry weight) and to the second batch $NaNO_3$ solution (0.5 ml) was added to have a final concentration of 8.5, 17, 25.5, 85 and 170 mg NO_3^--N kg^{-1} s.d.w., respectively. The latter had a wider range of N levels, but was not tested at the highest level as of ammonium (260 mg N kg^{-1} s.d.w.), as this concentration of nitrate hardly occurred in the farmer's fields. The control was carried out with the addition of the same volume of water. Methane in the headspace was monitored at intervals from 1 h to 18 h depending on the rate of CH_4 oxidation. When methane concentration dropped to <1000 ppm_v, the bottles were reopened for a few seconds and then replenished by injection of CH_4 to the concentration of 1.5–2% again in the headspace. The second dosage of nitrogen was applied when the rate of CH_4 oxidation had decreased to about 1 μmol CH_4 $h^{-1}g^{-1}$ s.d.w. The rates of the second dosage were the same as the first ones. Soil samples were collected at end of the experiment (14 days).

For the time-course observation of methanotrophic community, additional incubations were prepared for the treatments of 130 mg NH_4^+-N kg^{-1} s.d.w. and 170 mg NO_3^--N kg^{-1} s.d.w and the control without N application. Thirty-two bottles were prepared for each treatment and control. Destructive soil sampling was performed eight times within the total period of 11 days.

For the DNA stable isotope probing (DNA-SIP) experiment, incubations were prepared with treatments of 100 mg NH_4^+-N and 100 mg NO_3^--N kg^{-1} soil, respectively. ^{13}C fully labeled CH_4 was administered at a concentration of 5% (v/v) at the beginning of the incubation. A parallel control was prepared with administration of $^{12}CH_4$ instead of $^{13}CH_4$. Each treatment was performed in triplicate. Soil samples were collected at 55 h and 180 h, respectively.

2.2. Chemical analyses

Gas samples (500 μl) were taken from the headspace using a Pressure-Lok precision analytical syringe (Valco Instruments Co. Inc.). The concentration of methane was analyzed using GC-7890A (Agilent Technologies, USA) equipped with a thermal conductivity detector (TCD). The detection limit of the TCD detector is 50 ppm_v. A calibration curve was prepared from a CH_4 standard covering a range from 961 ppm_v to 2.98% of CH_4 (v/v). Multiple injections from standards or samples gave consistent results. Since only 1.5 ml of gas sample at the maximum was removed during each closed period from 120 ml incubation bottle, this removed volume was not replaced with air and the pressure change was not taken into account in calculation of CH_4 concentration. Ammonium (NH_4^+), nitrate (NO_3^-) and nitrite (NO_2^-) were extracted by mixing 5 ml of 1 M KCl with 0.5 g of soil samples and measured by DX-120 ion chromatography (Dionex, America) following the protocol as described previously (Yuan and Lu, 2009).

2.3. Microbial molecular analyses

2.3.1. DNA extraction

Total DNA was extracted from soil samples (0.5 g) using the FastDNA SPIN kit for soil (BIO 101; Q-Biogene, Heidelberg) according to the manufacturer's protocol. DNA extracts were stored at −20 °C.

2.3.2. T-RFLP analysis

Amplifications of bacterial 16S rRNA and functional genes of *pmoA* were performed using the primer sets 27f/907r and A189f/mb661r, respectively. The forward (27f, A189f) primers were 5′-end labeled with 6-carboxyfluorescein. PCR products were purified by TIAN quick midi purification kit (Tiangen, China) and digested for

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