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Effects of copper on methane emission, methanogens and methanotrophs in the rhizosphere and bulk soil of rice paddy

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ABSTRACT

Copper contamination is common in paddy fields due to wastewater irrigation and application of sludge and Cu-containing fungicides. We aimed to study the effects of copper (Cu^{2+}) application on methane emission, methanogens and methanotrophs in both the rhizosphere and bulk soil. The study was conducted in rhizobox in which flooded soil was applied with different Cu^{2+} concentrations. Methane emission was collected with static chamber method and determined by gas chromatography. The diversity and composition of methanogens and methanotrophs were studied using PCR–DGGE and sequencing analysis of methanogenic 16S rRNA and *pmoA* genes. The abundance of methanogens and methanotrophs was determined using quantitative real-time PCR of *mcrA* and *pmoA* genes, respectively. The results showed that Cu^{2+} application decreased methane emission along with the diversity and abundance of methanogens and methanotrophs, although the application of 200 mg kg^{-1} Cu^{2+} did not significantly decrease the diversity of methanogens and methanotrophs in the rhizosphere. In addition, Cu^{2+} decreased methanotrophs diversity more profoundly than methanogens diversity. Methanogens in both the rhizosphere and bulk soil were closely related to *Methanosaeta*, *Methanosarcina*, *Methanobacterium* and *Methanomicrobia archaeon*. Methanotrophs in rhizosphere soil were clustered into four groups (type I methanotrophs, *Methylobacter*, *Methylomonas* and *Methylosarcina*) while those in bulk soil were much less diverse. The addition of 200 to 800 mg kg^{-1} Cu^{2+} did not dramatically change the composition of methanogens; however, for methanotrophs, only one DGGE band belonging to *Methylosarcina* was present after the addition of 800 mg kg^{-1} Cu^{2+}. We conclude that methanotrophs were more sensitive to Cu^{2+} addition than methanogens, and that the rhizosphere environment alleviated Cu^{2+} stress on methanogens and methanotrophs.

1. Introduction

Methane is the one of the most important greenhouse gases (Thielemann et al., 2000). Its annual increase rate in the atmosphere was estimated to be the highest among all greenhouse gases (Khalil, 1999). Anthropogenic activities including rice cultivation, domestic animal grazing, landfillings, coal mining, and oil and gas extraction, contribute more than 60% of global methane emission (IPCC, 2014). Of these anthropogenic activities, rice cultivation is the most significant source. Paddy fields are responsible for approximately 15–20% of global total anthropogenic methane emission (Li et al., 2011).

Copper contamination is common in rice field soil, mainly due to wastewater irrigation, sludge treatment and application of Cu-containing fungicides (Komárek et al., 2010). One study has shown the effect of Cu^{2+} and other heavy metals on soil methane emission (Jiao et al., 2005). However, knowledge about the effect of Cu^{2+} on methanogens and methanotrophs in paddy soil is still limited. Methane emission from paddy fields is the net result of processes including methane production, oxidation and transportation (Cai et al., 2007). More than 80% of total emissions are transported through the aerenchyma system of rice plants (Cheng et al., 2006). A detailed investigation into the influence of Cu^{2+} on methanogens and methanotrophs along with on plant growth might clarify the effects of Cu^{2+} on methane emission. Aquatic plants such as rice plants, survive anoxic conditions by supplying their root system with oxygen from the atmosphere (Chen et al., 2008). The root exudates with released oxygen result in different microbial communities and methane emission flux between rhizosphere and bulk soil (Aulakh et al., 2001). We therefore hypothesized that the effects of Cu^{2+} on methane emission, methanogens and methanotrophs might vary between the rhizosphere and bulk soil.

Methanogens and methanotrophs can be characterized by the 16S rRNA gene or functional genes including *mcrA* and *pmoA*. The *mcrA* gene encodes for the K-subunit of methyl coenzyme M reductase (MCR), the key catabolic enzyme of methanogens (Ramakrishnan et al., 2001). The *pmoA* gene encodes an active subunit of the particulate methane monoxygenase (pMMO). All known methanotrophs possess a *pmoA* gene except *Methylocella* and *Methyloferula* (Dedysh et al., 2000;

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Vorobev et al., 2011). The *mcrA* and *pmoA* genes have been widely used as phylogenetic markers for methanogens and methanotrophs, respectively. The phylogeny of methanogens based on *mcrA* gene is consistent with that based on 16S rRNA gene (Luton et al., 2002). As a result, 16S rRNA is an alternative gene to *mcrA* for the study of methanogens (Watanabe et al., 2009). Phylogenetic studies of methanogens based on methanogenic 16S rRNA or *mcrA* gene and methanotrophs based on *pmoA* gene have revealed community composition of flooded rice soil (Watanabe et al., 2006; Zheng et al., 2008). In the present study, soil methanogens were studied using the 16S rRNA and *mcrA* genes and methanotrophs were studied using the *pmoA* gene. To test our hypothesis, we focused on the effect of Cu^{2+} application on methane emission along with the diversity and abundance of methanogens and methanotrophs in the rhizosphere and bulk soil.

2. Materials and methods

2.1. Soil sampling and physiochemical properties

One soil sample was collected from plowed layer at a depth of 15 cm in a farmland at Wujiang County, Jiangsu Province of China (30°38′N, 120°41′E). Soil sampling was carried out in June 2007, after rice was harvested. The climate is subtropical with an average annual precipitation of 1000 mm and an average temperature of 16 °C. After sampling, the fresh soil was immediately sieved through a 2-mm mesh. Soil aliquots were subjected to measurements of physiochemical properties using routine methods (Lu, 2000); or stored at 4 °C for less than two weeks before experimental setup. The soil physiochemical properties were determined as follows: soil texture, silty loam (9% clay, 30% silt); soil organic C, 20.90 g kg^{-1}; total N, 1.27 g kg^{-1}; total P, 0.31 g kg^{-1}; available P, 4.80 mg kg^{-1}; available K, 87.13 mg kg^{-1}; total Cu, 23.56 mg kg^{-1}; and pH (in 1:2.5 soil:water solution), 6.2.

2.2. Experimental setup

The soil sample was divided into four aliquots with three replicates. Various amount of Cu^{2+} solution ($CuSO_4$, analytical reagent, China National Medicines Inc. Beijing, China) were added to three of the four aliquots to final concentrations of 200 (low concentration treatment, LC), 400 (moderate concentration treatment, MC) and 800 (high concentration treatment, HC) mg Cu^{2+} kg^{-1} soil (dry weight). The aliquot without Cu^{2+} addition served as control (Ct). Each replicate containing 6.5 kg soil was evenly filled in a rhizobox with the dimension of 350 × 200 × 200 (length × width × height, mm). The rhizobox was divided into three compartments with two nylon nets (48 μm mesh size). The central compartment (150 mm in length) was used for plant cultivation, and roots were limited in it. Soil in the central compartment was defined as rhizosphere soil, while soil in the other two compartments (100 mm in length for each) as bulk soil. An aluminum collar with a groove in its upper end was installed on each compartment for methane collection. Soil in the rhizobox was flooded for three days before the planting of rice.

Rice (*Oryza sativa* L.) seeds were sterilized with 75% (*v*/*v*) ethanol and 3% (*v*/*v*) hydrogen peroxide and germinated on moist filter paper in a sterilized box for 30 days. Three rice plants were transplanted to the central compartment of each rhizobox. The rice plants were grown under the greenhouse conditions with temperature ranging from 30 to 36 °C during the daytime and 25 to 30 °C at night. The soil in the rhizobox was kept flooded with water at a level of 4 cm above the soil. To facilitate temporal description, we define day 0 as the day that copper was applied. Methane emitted from the rhizosphere and bulk soil was collected and measured at intervals of 3, 7, 14, 28 and 49 days after Cu^{2+} application. The rhizosphere and bulk soil were collected at a soil depth of 0–5 cm at day 49 for molecular analysis of methanogens and methanotrophs after methane collection.

Rice root and shoot dry weights were estimated at day 49. The plant shoot and root were harvested by cutting rice at 5 cm above the soil. The soil was removed from the root system by washing on a sieve, and the shoot and root were dried at 80 °C for two days and weighed. After weighing, the shoot was ground in a stainless steel mill and digested with concentrated HNO_3 and H_2O_2. The Cu^{2+} concentrations in shoot were determined by an atomic absorption spectrometer (Hitachi 180, Japan).

2.3. Methane collection

Soil methane flux was measured using a static chamber technique combined with gas chromatography (Bárcena et al., 2010). Two chambers were used in this study to simultaneously collect methane from the rhizosphere and from the bulk soil. Soil methane flux was measured by attaching a chamber top into grooves of a rhizobox. The groove was filled with water to prevent gas leakage. Gas samples (60 mL) were withdrawn at 0, 15 and 30 min using air-tight nylon syringes and stored in 100-mL gas-bags. Methane concentration was analyzed by gas chromatography within 24 h of collection (Maljanen et al., 2004). Certified methane concentration standards (China National Research Center for Certified Reference Materials, Beijing) containing 2030 ppbv CH_4 in N_2 were used for calibration. Methane flux (mg m^{-2} h^{-1}) was estimated by using the following equation (Nouchi et al., 1994):

$$F = H \times (M_w/M_v) \times (273.2/273.2 + T) \times (dC/dt). \quad (1)$$

Where H is the height of the chamber (m), M_w is the molar mass of methane (16.123 × 10^3 mg), M_v is the molare volume of methane (22.41 × 10^{-3} m^3), T is the air temperature in the chamber (°C), and dC/dt is the change in methane concentration (μL L^{-1}) per unit of time (h).

2.4. DNA extraction

Total DNA was extracted using the FastDNA SPIN kit for soil (Bio 101) according to the manufacturer's instructions. DNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). The A260/A280 ratios were all found to be above 1.8. The recovered DNA was eluted in 10 mM Tris–HCl buffer (pH 7.5), and aliquots were stored at −80 °C.

2.5. Quantitative real-time PCR analysis

The methanogenic *mcrA* gene and methanotrophic *pmoA* gene were quantified by real-time PCR using an Opticon 2 continuous fluorescence detection system (MJ Research, Waltham, MA, USA). The 25-μL reaction mixtures included 0.5 μL of template DNA, 12.5 μL of SYBR Premix Ex Taq (TaKaRa Bio Inc., Shiga, Japan), 5 nmol of the primers *mcrA*-f (5′-GGT GGT GTM GGA TTC ACA CAR TAY GCW ACA GC-3′) and *mcrA*-r (5′-TTC ATT GCR TAG TTW GGR TAG TT-3′) for *mcr*A gene (Vianna et al., 2008), and A189-f (5′-GGN GAC TGG GAC TTC TGG-3′) and mb661-r (5′-CCG GMG CAA CGT CYT TACC-3′) for *pmo*A gene (Kolb et al., 2003). The protocol for *mcrA* amplification was as follows: 94 °C for 1 min, and 30 cycles consisting of 94 °C for 5 s, 55 °C for 15 s, and 72 °C for 20 s. For *pmoA* gene amplification, the protocol was the same except that the annealing temperature was increased to 60 °C. Standard calibration curves were created with purified plasmid DNA carrying either *mcrA* gene inserts or *pmoA* gene inserts by producing a ten-time dilution series from 10^8 to 10^3 target gene copies μL^{-1}.

2.6. PCR–DGGE analysis

The amplification of methanogenic 16S rRNA gene for DGGE was done by using primer set 1106 F (5′-TTW AGT CAG GCA ACG AGC-3′)