Response of methanogenic archaeal community to nitrate addition in rice field soil

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Summary
Addition of nitrate strongly inhibits CH₄ production from anoxic soil. The main mechanisms were considered to be the substrate competition and the toxic effect of denitrification intermediates on the methanogenesis. However, it is unclear whether these inhibitory effects are reflected in the structure and dynamics of methanogenic community in the soil. In the present study, the response of methanogenic archaeal community to nitrate addition was determined using terminal restriction fragment length polymorphisms in combination with cloning and sequencing of archaeal 16S rRNA genes. When nitrate was added at the beginning of an anoxic incubation of rice field soil, denitrification occurred rapidly and the denitrification intermediates were detected only for a short time. Total production of CH₄ was reduced, but no obvious effect on the structure of methanogenic community was observed. In contrast, when nitrate was added 20 days after the anoxic incubation, the denitrification intermediates obviously accumulated. CH₄ production was completely suppressed for 7 and 16 days from treatments of 5 and 10 mM nitrate respectively. The dynamics of methanogenic community also diverged greatly from the control. While the hydrogenotrophic methanogens increased and the acetoclastic methanogens decreased with the incubation in the control soil, the structure and abundance of the methanogenic community remained unchanged after the addition of nitrate. Methanogenesis resumed when the denitrification intermediates were depleted in soil. The analysis of carbon isotopic signals revealed that hydrogenotrophic methanogenesis recovered faster than acetoclastic methanogenesis. Our study suggests that the accumulation of denitrification intermediates has a strong inhibitory effect on the activity but not the structure of methanogenic community.

Introduction
Microorganisms in nature are frequently subjected to environmental stresses. One example is the susceptibility of many microbes to the toxic effects of denitrification intermediates. However, only few studies have examined the effect of denitrification on microbial community dynamics in soil. This is surprising, given that denitrifiers are widespread in various environments and denitrification plays a key role in the N cycle of soils (Braker et al., 2001; Kandeler et al., 2006; Bremer et al., 2007).

In rice field soils, most of nitrogen is applied in the form of inorganic, ammonium-based fertilizers. However, nitrate is produced from the oxidation of ammonium in the oxic surface soil and rhizosphere (Arth et al., 1998; Arth and Frenzel, 2000; Nicolaisen et al., 2004). The produced nitrate can readily diffuse into the surrounding anoxic bulk soil where it is prone to denitrification (Arth and Frenzel, 2000; Liesack et al., 2000). The inhibitory effects of nitrate on CH₄ production and emissions have been widely observed in paddy fields (Lu et al., 2000), freshwater sediments, industrial wastewater and municipal solid waste (Percheron et al., 1999; Scholten et al., 2002; El-Mahrouki and Watson-Craik, 2004). The inhibitory mechanisms, however, are poorly understood. On the one hand, the inhibition of methanogenesis can result from the substrate competition by the nitrate reducers. This is because that H₂ partial pressure can be decreased to below the threshold for methanogenesis during the phase of nitrate reduction (Klüber and Conrad, 1998a; Roy and Conrad, 1999). On the other hand, the inhibitory effects can result from the toxic effects of the denitrification intermediates (nitrite, NO, N₂O) (Clarens et al., 1998; Klüber and Conrad, 1998a,b; Roy and Conrad, 1999). The strong inhibitory effect of NO has been demonstrated in a few pure cultures of bacteria (Choi et al., 2006) and methanogens (Klüber and Conrad, 1999b). Nitrite is also inhibitory to many bacterial and archaeal enzymes by forming metal-nitrosyl complexes (Zumft, 1993; Deppenmeier et al., 1996), whereas N₂O can inactivate the cobalamin-dependent enzymes and hence depress the growth and activity of methanogens (Fischer and Thauer, 1990).
Although many studies have demonstrated the suppression of methanogenesis by nitrate addition, only a few have determined the effect on the structure of methanogenic community. In a laboratory incubation of the excised rice roots, it was found that the addition of nitrate suppressed the growth of archaeal populations (Scheid et al., 2004). The activity of Methanosarcina populations was found to be more sensitive than the hydrogenotrophic Rice Cluster I (RC-I) methanogens [now Methanocellales (Sakai et al., 2008)] (Scheid et al., 2003). Recently, the metagenomic data revealed that the RC-I methanogens possess genes encoding a unique set of antioxidant enzymes (Erkel et al., 2006). This feature may facilitate these organisms to cope with O2 (Erkel et al., 2006) as well as nitrosative stresses in the paddy soil.

The objective of the present study was to determine the effects of nitrate addition on the activity and structure of methanogenic archaeal community in an anoxic rice soil. Nitrate was applied to the anoxic soil at the beginning and 20 days after the start of an anoxic incubation. We found that inhibitory effects on the structure and activity of methanogens differed greatly with the time of nitrate application.

**Results and discussion**

**Experiment I: addition of nitrate at the beginning of anoxic incubation**

The rice soil was collected from a paddy field in the southeastern China. The soil characteristics have been described previously (Peng et al., 2008; Qiu et al., 2008). Soil slurries were anaerobically incubated at 25°C under dark condition.

In experiment I, nitrate was added at the beginning of the anoxic incubation. The concentration of nitrate and denitrification products was below the detection limit throughout the incubation in the control soil (Fig. S1). In the nitrate treatments, nitrate, nitrite and nitrous oxide (N2O) were detected between day 1 and day 3. The partial pressure of H2 in the headspace accumulated until day 5 (Fig. S2A). The concentrations of acetate and propionate in soil slurries increased gradually, remained high until day 12, and then decreased rapidly (Fig. S2B and C). Sulfate and Fe(III) [indicated by the increase of Fe(II)] were reduced in 10 days (Fig. S2E and F). Addition of nitrate significantly reduced H2 partial pressure in the headspace and the concentrations of fatty acids in soil slurries. The dynamics of sulfate and Fe(III), however, were not affected. Methane production was detected on day 3 in all treatments. The period of rapid CH4 production corresponded with the rapid decrease of acetate (Fig. S2B and D). The rates of CH4 production increased in the order of control > 5 mM nitrate > 10 mM nitrate (Fig. S2D).
Terminal restriction fragment length polymorphism (T-RFLP) analysis of the archaeal 16S rRNA genes was used to determine the dynamics of archaeal populations in soil slurries (Fig. 1). Seven terminal restriction fragments (T-RFs; 77, 85, 93, 187, 285, 382 and 394 bp) were detected as major peaks in T-RFLP profiles from both experiments I and II (the results of experiment II will be discussed below). Two clone libraries retrieved from soil samples in experiment II (Table S1) revealed that the archaeal community in soil consisted of Methanosarcinaceae, Methanosaetaceae, Methanomicrobiales and RC-I methanogens, and of the following yet uncultured archaeal lineages: RC-II, RC-III, RC-V, LDS (for Lake Dagow sediment) cluster (Glissmann et al., 2004), crenarchaeotal group 1.1b (equivalent to RC-VI) (Jurgens et al., 2000; Wu et al., 2006), and group 1.3 (equivalent to RC-IV). In silico analysis of 109 sequences (Table S1) indicated that the 77 bp T-RF could be assigned to euryarchaeotal RC-V, 85 bp to Methanomicrobiales, 93 bp to Methanobacteriales, 285 bp to Methanosaetaceae and 382 bp to RC-III. The 187 bp T-RF indicated mostly Methanosarcinaceae but sometimes also crenarchaeotal group 1.1b. The 394 bp T-RF predominantly represented RC-I but occasionally also Methanomicrobiales.

The T-RFLP patterns in the nitrate treatments were almost identical to the control throughout the incubations (Fig. 1). The community itself, however, showed obvious shift over the 30-day incubation. T-RFLP patterns remained similar within the first 8 days of the incubation. From day 8 to day 15, the relative abundance of 187 bp T-RF substantially increased, while the 394 and 285 bp T-RFs decreased. From day 22 to day 30, the relative abundance of the 187 bp T-RF decreased again while the 394 bp T-RF relatively increased.

Apparently, although total production of CH$_4$ was reduced probably due to competitive consumption of electron donors by nitrate reducers, the structure of methanogenic community remained unaffected by nitrate addition (Fig. 1). The dynamics of Methanosarcinaceae as indicated by the 187 bp T-RF coincided with the acetate concentration in soil slurries, which was consistent with the finding that Methanosarcina spp. was stimulated with the increasing of acetate concentration in soil (Kruger et al., 2005; Peng et al., 2008). The relative change of the hydrogenotrophic RC-I and/or Methanomicrobiales (as indicated by the 394 bp T-RF) was possibly the result of both the change in the relative abundance of Methanosarcinaceae and their adaptation to H$_2$ dynamics (Lu et al., 2005; Sakai et al., 2007; Peng et al., 2008). Since the denitrification took place rapidly and the denitrification intermediates (nitrite and N$_2$O) were detected only for a short time, the toxic effect of the denitrification intermediates, if it existed, might be too short to influence the structure of methanogenic community in the soil.

**Experiment II: addition of nitrate at 20 days after anoxic incubation**

In experiment II, nitrate was added 20 days after the start of the anoxic incubation period. As in experiment I, the concentrations of nitrate, nitrite and N$_2$O were below the detection limit in the control soil. However, in the 5 and 10 mM nitrate treatments, nitrate remained detectable until day 7 and day 13 respectively (Fig. 2A). Nitrite accumulated and reached the maxima between day 2 and day 4 (Fig. 2B). In the treatment of 10 mM nitrate, nitrite kept at higher concentration and remained detectable until day 21. N$_2$O also accumulated in the headspaces and displayed two peaks on day 3 (for both treatments) and day 11 (for 10 mM nitrate) respectively (Fig. 2C).

Acetate remained low throughout the incubation in the control soil (Fig. 2E), but accumulated markedly in the nitrate treatments, starting from day 7 and day 16 and reaching the maxima at day 27 and day 40 for 5 and 10 mM treatments respectively. Propionate had a lower concentration but the same time-course trends as acetate (Fig. 2F). H$_2$ was below the detection limit in this experiment (data not shown). The concentration of sulfate remained close to the detection limit (60 µM). A slight increase, however, was detected after nitrate addition, particularly for the 10 mM treatment (Fig. 2G). The concentration of Fe(II) stayed constant between 100 and 120 µmol g$^{-1}$ dry weight soil and was not affected by nitrate addition (Fig. 2H). The concentration of CH$_4$ increased steadily in the control soil. However, CH$_4$ production was completely suppressed for a period of 7 and 16 days for the treatments of 5 and 10 mM nitrate respectively (Fig. 2H, insert). Thereafter, the production of CH$_4$...
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resumed, but significant increase did not occur until around day 20 and day 33 for the treatments of 5 and 10 mM nitrate respectively (Fig. 2H).

Apparently, the addition of nitrate in experiment II strongly suppressed methanogenesis, which seemed not recovered until all denitrification intermediates were depleted. This result is consistent with the previous studies in Italian rice soil (Klüber and Conrad, 1998a; Roy and Conrad, 1999), in which even the addition of substrate (rice straw, propionate, acetate or H2) to provide more electron donors did not prevent the suppression by the denitrification intermediates.

Interestingly, acetate did not accumulate immediately although CH4 production was completely suppressed during the denitrification phase. One possibility was that acetate was consumed by nitrate reducers and denitrifiers during this period. In addition, it could be also possible that syntrophic acetate oxidation occurred before the recovery of acetoclastic methanogenesis (Schink, 1997). Nitrate reducers and hydrogenotrophic methanogens might serve as the syntrophic partners to keep H2 at an adequate level for the recovery of acetoclastic methanogenesis (Schink, 1997).

Terminal restriction fragment length polymorphism profiles in experiment II revealed that nitrate addition had a profound influence on the dynamics of archaeal community (Fig. 3). In the control soil, the acetoclastic Methanosarcinaceae (187 bp T-RF) displayed a high abundance at the early period but markedly decreased while the hydrogenotrophic RC-I and/or Methanomicrobiales (394 bp T-RF) gradually increased in the later stages (Fig. 3A). This tendency which already initiated at the end of experiment I was obviously due to the decrease of acetate concentration in the later stages. The increase of RC-I and/or Methanomicrobiales was also consistent with the previous observation that RC-I methanogens became more abundant in the later stages, possibly due to the low H2 partial pressure in the incubations (Peng et al., 2008). In contrast, in the treatment of nitrate addition, T-RFLP patterns remained unchanged with the Methanosarcina-like T-RF (187 bp) being dominant during most period of the incubations (Fig. 3B and C). Only at the end, the 187 bp T-RF slightly decreased while the 394 bp T-RF relatively increased. In addition, the T-RFLP patterns were almost identical between the treatments of 5 and 10 mM nitrate. Thus, it appeared that the methanogenic community in the nitrate treatments stopped shifting in the way as in the control soil (Fig. 3).

The total copy number of archaeal 16S rRNA genes in experiment II was determined by quantitative (real-time) PCR. The 16S rRNA genes ranged between 1.1 × 108 and 3.1 × 108 copies g⁻¹ soil and appeared relatively higher at day 16 and day 33 compared with day 1 and day 58, but these differences were not statistically significant (Fig. 4). Taking into account the shift of community structure in the control as revealed by T-RFLP profile (Fig. 3A), the results suggested that the number of RC-I populations increased while that of Methanosarcina decreased in the later stages of control soil. Unexpectedly, the addition of nitrate at both concentrations did not significantly influence the copy number of 16S rRNA genes compared with the control. Since the structure of methanogenic community in the nitrate treatments did not change during most periods of the incubations (Fig. 3B and C), the results suggested that neither differential growth nor death of methanogens occurred in soil slurries after nitrate addition.

Carbon-13 isotopic signals of CH4 and CO2 were determined during the recovery phase in experiment II. Since CH4 production from CO2 reduction displays a greater 13C isotopic discrimination than the cleavage of acetate, the 13C-isotopic signal of CH4 and CO2 can be used to predict the contribution of hydrogenotrophic methanogenesis relative to acetoclastic methanogenesis to total CH4 production in anoxic soil (Fey et al., 2004; Conrad, 2005; Penning and Conrad, 2007). In the control soil, the δ13CH4 decreased from −40‰ at day 1 to −68‰ at day 16 and then remained at low values until the end of incubation (Fig. 5). This result indicated that the activity of hydrogenotrophic methanogenesis increased in the later stages. This conclusion was in agreement with the increase of RC-I methanogens and/or Methanomicrobiales relative to acetoclastic Methanosarcinaceae in the incubations (Fig. 3A). In the nitrate treatments, the δ13CH4 values also decreased. However, a lag phase was detected before the decreases of δ13CH4 values. This lag phase (10 and 28 days for the treatments of 5 and 10 mM nitrate respectively) corresponded to the accumulation of the denitrification intermediates and the complete suppression of CH4 production (Fig. 2A–C and H). The lag phase was longer for the treatment of 10 mM (28 days) than 5 mM nitrate (10 days). The δ13CO2 values in the control soil (ranging from −25‰ to −12‰) were greater than in the nitrate addition treatment (−26‰ to −20‰). Out of the two nitrate treatments, the values were slightly higher for 5 mM nitrate than 10 mM nitrate.

The decrease of the δ13CH4 values during the recovery phase indicated that hydrogenotrophic methanogenesis recovered faster than acetoclastic methanogenesis. Notably, during this period of recovery, the acetate had already accumulated to a high concentration (Fig. 2E) and should not be limited to the activity of the acetoclastic Methanosarcina spp. Furthermore, the relative abundance of Methanosarcinaceae remained high in the nitrate-amended soils (Fig. 3B and C). Therefore, our results suggest that the activity rather than the population
abundance of the acetoclastic methanogens was strongly inhibited in the nitrate treatments. A previous study also indicated that nitrate addition resulted in more serious suppression to the activity of *Methanosarcina* spp. compared with the hydrogenotrophic RC-I and/or *Methanomicrobia* (Scheid et al., 2003). The mechanisms are currently unclear. One plausible reason, however, is likely

![Fig. 3. Structure dynamics of archaeal community in anoxic paddy soil in experiment II. Shown are the relative abundances of T-RFs from the soils of control (A), 5 mM (B) and 10 mM (C) nitrate treatments (means – SD, n = 3). In experiment II, nitrate was injected at 20 days after the anoxic incubation. Thus, the ‘day 0’ on the x-axis corresponds to ‘day 20’ in the entire incubation period. The sample collection, DNA extraction, PCR and T-RFLP analysis followed the protocols as described in the legend of Fig. 1. Mb, *Methanobacteriales*; Maa, *Methanosaetaceae*; Mr, *Methanosarcinaceae*; Mm, *Methanomicrobiales*; Cren, crenarchaeotal group.]

![Fig. 4. Total copy numbers of archaeal 16S rRNA genes in experiment II. Shown are the copy number per gram of soil in the control, 5 and 10 mM nitrate treatments. Nitrate was added at 20 days after the anoxic incubation. The ‘day 0’ on the x-axis corresponds to ‘day 20’ in the entire incubation period. Data are means ± SD (n = 3). The copy number of archaeal 16S rRNA genes was determined using quantitative (real-time) PCR. DNA was extracted from soil samples (500 mg) using Fast DNA Spin Kit for Soil (BIO 101; Q-Biogene, Heidelberg) according to the manufacturer’s protocol. The archaeal 16S rRNA genes were quantified in a 7500 real-time PCR system (Applied Biosystems), following the protocol as described by Kemnitz and colleagues (2005). A standard curve was prepared from purified plasmid DNA carrying the archaeal 16S rRNA gene insert with the concentration ranging from 1 ¥ 10^3 to 5 ¥ 10^8 copies ml^-1. d.w., dry weight.]

![Fig. 5. The isotopic signal of CO₂ and CH₄ in the anoxic incubation of paddy soil in experiment II. Shown is the time-course of δ¹³C natural abundances in control, 5 and 10 mM nitrate-amended soils (mean ± SD, n = 3). Nitrate was added at 20 days after the anoxic incubation. The ‘day 0’ on the x-axis corresponds to ‘day 20’ of the entire incubation. The headspace of all bottles was re-flushed with N₂ at ‘day 0’. Gas samples were collected as described in legend of Fig. 2. Stable isotope ratios of δ¹³C /δ¹²C of CH₄ and CO₂ were determined using gas chromatograph-combustion-isotope ratio mass spectrometry (GC-C-IRMS) system (Thermo Finnigan, Germany). The natural abundance of heavy isotopes was expressed as part per thousand relative to the international standard Pee-Dee belemnite (PDB) using delta unit (δ).]
related to the defence mechanisms of methanogens to the oxidative stresses. Microorganisms are known to use similar mechanisms to defend the oxidative and nitrosative stresses (Freitas et al., 2004; Redding et al., 2006; Overton et al., 2008). The faster recovery of the activity of RC-I methanogens relative to *Methanosarcinaeae* is likely due to the fact that RC-I methanogens possess a unique set of antioxidant enzymes (Erkel et al., 2006), which may facilitate the better survival under O$_2$- as well as nitrate-induced stresses.

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**References**


Response of methanogens to nitrate in rice soil

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Concentrations of nitrate (A), nitrite (B) and N\(_2\)O (C) in control, 5 and 10 mM nitrate treatments in experiment I. Nitrate was added at the beginning of anoxic incubation. Data are means ± standard deviations (SD) (n = 3).

**Fig. S2.** Concentrations of H\(_2\) (A), acetate (B), propionate (C), CH\(_3\)COO\(^-\) (D), SO\(_4^{2-}\) (E) and Fe(II) (F) in control, 5 and 10 mM nitrate treatments in experiment I. Nitrate was added at the beginning of anoxic incubation. Data are means ± SD (n = 3).

**Table S1.** Composition of archaeal clone libraries retrieved from control and 5 mM nitrate treatment at day 58 in experiment II. Nitrate was added after 20 days of anoxic incubation of rice field soil.

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