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Response of methanotrophs and methane oxidation on ammonium application in landfill soils

Na Yang · Fan Lü · Pinjing He · Liming Shao

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Abstract To test the dose effect of ammonium (NH_4^+) fertilization on soil methane (CH_4) oxidation by methanotrophic communities, batch incubations were conducted at a wide scale of NH_4^+ amendments: 0, 100, 250, 500, and 1,000 mg $N kg_{dry\ soil}^{-1}$. Denaturing gradient gel electrophoresis and real-time quantitative PCR analysis were conducted to investigate the correlation between the CH_4 oxidation capacity and methanotrophic communities. Immediately after the addition of NH_4^+, temporal inhibition of CH_4 oxidation occurred, and this might have been due to the non-specific salt effect (osmotic stress). After a lag phase, the CH_4 oxidation rates of the soils with NH_4^+ fertilization were promoted to levels higher than those of the controls. More than 100 mg $N kg_{dry\ soil}^{-1}$ of NH_4^+ addition resulted in the reduction of type II/type I MOB ratios and an obvious evolution of type II MOB communities, while less than 100 mg $N kg_{dry\ soil}^{-1}$ of NH_4^+ addition induced nearly no change of methanotrophic community compositions. The NH_4^+-derived stimulation after the lag phase was attributed to the improvement of N availability for type I MOB. Compared with the controls, 100 mg $N kg_{dry\ soil}^{-1}$ of NH_4^+ addition doubled the CH_4 oxidation peak value to more than 20 mg $CH_4 kg_{dry\ soil}^{-1} h^{-1}$. Therefore, an appropriate amount of leachate irrigation on the landfill cover layer might efficiently mitigate the CH_4 emissions.

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Introduction

Methane (CH_4) is an important trace gas in atmospheric chemistry for its high global warming potential. The atmospheric concentration of CH_4 has increased from a pre-industrial value of 715 to 1,774 ppb recently, mainly due to human activity (Fletcher et al. 2004). In the presence of oxygen (O_2), soil can act as a sink for CH_4 through the metabolism of methane-oxidizing bacteria (MOB), which use CH_4 as sole carbon and energy source. According to the taxonomy and physiology characteristics, MOB were generally separated into two groups (Hanson and Hanson 1996): type I, including the genera *Methylococcus*, *Methylocaldum*, *Methylomonas*, *Methylomicrobium*, *Methylobacter*, *Methylosphaera*, and *Methylosarcina*, and type II, including the genera *Methylocystis*, *Methylosinus*, *Methylocella*, and *Methylocapsa*, belonging to *gamma*- and *alpha*-subdivision of the phylum *Proteobacteria*. Type I MOB assimilate the formaldehyde for biosynthesis by using the ribulose monophosphate (RuMP) pathway, while type II MOB are using the serine pathway. Since the RuMP pathway was more efficient than the serine one, type I MOB can yield more cells than type II MOB in pure and mixed cultures (de Viries et al. 1990; Graham et al. 1993). Compared with type I, type II MOB can survive in a nitrogen- and copper-limited environment (Graham et al. 1993; Murrell 1994), which may be due to the nitrogen fixation capacity of type II MOB. Type I MOB outcompete type II MOB under high O_2 and low CH_4 conditions,

whereas the latter favor the inverse conditions (Graham et al. 1993). In addition, CH_4 can be co-oxidized by ammonia-oxidizing bacteria (AOB) as well (Hanson and Hanson 1996) due to the broad substrate specificity of ammonia monooxygenase.

As one of the necessary nutrients for microorganisms, nitrogen (N) can affect methanotrophic activities and subsequently interfere with the capacity of soil CH_4 oxidation. Nevertheless, the effect of ammonium (NH_4^+)-based fertilization on CH_4 oxidation is still far from conclusively understood. On one hand, inhibition has been observed in various environments and is generally attributed to the substrate competition between NH_4^+ and CH_4 for the active site of methane monooxygenase (MMO) and the product toxicity of nitrite generated from NH_4^+ oxidation (Steudler et al. 1989; King 1990; King and Schnell 1994). On the other hand, NH_4^+ application can result in stimulation as reported in rice fields (Bodelier et al. 2000b; Kruger and Frenzel 2003; Noll et al. 2008), landfill cover soils (de Visscher et al. 1999), as well as in spruce forest soils (Rigler and Zechmeister-Boltenstern 1999). The metabolism of stimulation was still unclear, and the most accepted explanation was that added NH_4^+ relieved the N limitation status of the investigated methanotrophs. Different amounts of NH_4^+ fertilization contradict the effects on CH_4 oxidation, i.e., 10–60 mM of NH_4^+, corresponding to 14–62 mg $Nkg_{dry\ soil}^{-1}$, stimulated the CH_4 oxidation in soil slurry, but those higher NH_4^+ concentrations were inhibitory (Bender and Conrad 1995). Rigler and Zechmeister-Boltenstern (1999) investigated the effects of NH_4^+ fertilization on two kinds of forest soils. Their results showed that the CH_4 oxidation rate in the deciduous forest soil was negatively correlated with the amount of NH_4^+ added (from 0 to 500 mg $Nkg_{dry\ soil}^{-1}$), while for spruce forest soils, a progressive increase of stimulation of CH_4 oxidation along with an enhanced NH_4^+ concentration (from 0, 10 to 100 mg $Nkg_{dry\ soil}^{-1}$) was observed.

It is hypothesized that the diverse methanotrophic compositions in different soils and the changes of methanotrophic compositions affected by NH_4^+ fertilization induced the above-mentioned contradictions (Mohanty et al. 2006). Based on denaturing gradient gel electrophoresis (DGGE) analysis, Seghers et al. (2003) investigated the methanotrophic community structures in the soils receiving organic (compost) and mineral (NH_4NO_3) fertilizer, and Zheng et al. (2008) studied the soils without and with N (urea), NK (KCl), NP (superphosphate) K, and NPK+C (recycled crop residues) fertilizer. In their studies, the contradicting effects were in consistent with the distinct methanotrophic community compositions with different types of fertilization. Zheng et al. (2008) also used quantitative polymerase chain reaction (PCR) to detect the MOB abundance during different N fertilization and found that NK and NPK+C fertilizations stimulated the increase of MOB abundance in comparison with the inhibition effect with only N (urea) fertilization. However, few studies have been done to investigate the evolvement of methanotrophic communities in the soils receiving different doses of NH_4^+ fertilizer.

To test the dose effect of NH_4^+ fertilization on soil CH_4 oxidation capacity, we cultured soil samples at a wide range of NH_4^+ concentrations in batch incubation. In the course of incubation, the CH_4 oxidation rates were monitored to represent methanotrophic activities, and real-time quantitative PCR and PCR-DGGE targeting the 16S rDNA gene were applied to evaluate the abundances and community compositions of methanotrophs.

Material and methods

Batch incubation experiments

Soil samples used in this study were collected from the top cover soil (10–30 cm) of a landfill site with subsurface irrigation of leachate (referring to the condition B in Yu et al. 2009) in eastern China in May 2009. After sampling, the soils were air-dried and stored at room temperature until usage. The soil was composed of 32.8% sand, 27.9% silt, and 39.3% clay (sandy loam according to USDA classification). Other properties were as follows: Soil pH was 4.9 (1:2.5 soil–water extraction), organic matter 0.82±0.05% (organic carbon content multiplied by 1.74), total N 0.08% (Kjeldahl method), and 8.2 and 17.9 mg $Nkg_{dry\ soil}^{-1}$ of NH_4^+ and nitrate (NO_3^-), respectively (both extracted by 2 mol L^{-1} of KCl followed by standard analysis methods).

The soils were sieved through 2-mm screens to remove large particulate matters; then, 0, 100, 250, 500 to 1,000 mg $Nkg_{dry\ soil}^{-1}$ of NH_4^+ were applied to the soil samples (denoted as CON, N100, N250, N500, and N1000, respectively) by rewetting them to water content of 16% ($w\ w^{-1}$) with appropriate $(NH_4)_2SO_4$ solution. Three hundred grams of soil samples was transferred to 1-L serum bottles, which were sealed by rubber stoppers and injected with CH_4 to obtain 5% in the headspace. The headspace of incubation bottles was flushed daily with fresh air and re-injected with CH_4. When the CH_4 oxidation rate was higher than 12 mg $CH_4kg_{dry\ soil}^{-1}h^{-1}$, the flush-reinjection process was conducted every 12 h. All experiments were carried out in duplicate at 25°C. Meanwhile, to test the abiotic methane oxidation, a negative control experiment was set up with autoclaved soils in parallel.

The CH_4 oxidation rates were measured to represent methanotrophic activities using the method modified from Seghers et al. (2003). In brief, after the injection of CH_4, the CH_4 concentrations in the headspace of the serum

bottles were monitored five times during the next 24 h, and when the CH_4 oxidation rates were higher than 12 mg CH_4 $kg_{dry\ soil}^{-1}h^{-1}$, the CH_4 concentrations were monitored four times during the next 12 h. Then the CH_4 oxidation rates were determined by the regression of concentrations against time. The concentrations of CH_4 were measured with a gas chromatograph (GC112A, Shanghai Precision & Scientific Instrument Co., Ltd, Shanghai, China) equipped with a flame ionization detector and a 2-m stainless steel column packed with Porapak Q (60/80 mesh). The temperatures of the injector, oven and detector were set at 80°C, 60°C, and 180°C, respectively. The carrier gas was N_2 with a flow rate of 40 mL min^{-1}.

After each test of the CH_4 oxidation rate, 3–5-g soil samples were obtained from the incubation bottles and used to detect the concentrations of NH_4^+ and NO_3^-. The pH of the soil was determined on the 1st day and 72nd day and slightly reduction (about 0.1) was observed, which could be considered stable during the incubation period.

DNA extraction and quantification analysis by real-time PCR

Soil DNA was extracted from 0.4 g of soil samples by the modified phenol chloroform and ethanol precipitation methods (Ye et al. 2007). The abundances of type I MOB and type II MOB and AOB were analyzed by real-time PCR. Real-time PCR amplification was performed on a Realplex thermo-cycler (Eppendorf, Hamburg, Germany) in 20-μL reaction mixtures using SYBR® Premix Ex Tay™ as described by the suppliers (Takara Bio, Otsu, Shiga, Japan). Two reverse primers of MethT1bR (5′-GAT TCY MTG SAT GTC AAG G-3′) and MethT2bR (5′-CAT CTC TGR CSA YCA TAC CGG-3′) (Wise et al. 1999) and their common forward primer 533f (5′- GTG CCA GCA GCC GCG GTA A-3′) (Weisburg et al. 1991) were used to determine the 16S rDNA gene copy numbers of type I and type II MOB, respectively. As to AOB, the primers *amoA*-1F (5′-GGG GTT TCT ACT GGT GGT-3′) and *amoA*-2R (5′-CCC CTC KGS AAA GCC TTC TTC-3′) (Rotthauwe et al. 1997) targeting *amoA* gene were used. Real-time PCR assay was carried out with the protocol as follows: 95°C for 2 min; 40 cycles of 15 s at 95°C; 20 s at 57°C, 60°C, and 58°C for type I MOB, type II MOB, and AOB, respectively; 15 s at 72°C for reading the plate, followed by a melting curve analysis to confirm PCR product specificity by measuring fluorescence continuously over the temperature increase of 55–95°C. All tests were done in triplicate and data analysis was carried out with the Realplex software (version 2.2), in which the parameter C_t (threshold cycle) was determined as the cycle number at which a statistically significant increase in the reporter fluorescence was detected.

One standard curve was generated for each assay, using serial 10-fold dilutions of purified DNA solutions in sterilized water, for which DNA fragments were the PCR products obtained by using the same primers. The concentrations of the DNA solutions were determined spectrophotometrically. The slopes of standard curves were −3.435, −3.148, and −3.294, corresponding to the efficiencies of the PCR reaction of 0.95, 1.08, and 1.01 for type I MOB, type II MOB, and AOB, respectively. The corresponding regression coefficient values (R^2) were 1.000, 0.995, and 0.998.

PCR-DGGE analysis

For DGGE analysis of type II MOB, a GC clamp (CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC C) was attached to the 5′-end of the forward primer. PCR amplification was performed in a total 50-μL volume containing 0.25 μmol L^{-1} of each primer, 200 μmol L^{-1} of each dNTP, 5 μL 10× PCR buffer, 2 μmol L^{-1} of $MgCl_2$, 5 U of Taq polymerase, and 5 μL of template DNA. PCR amplification conditions were as follows: pre-denaturation at 94°C for 5 min; 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 45 s, and extension at 72°C for 90 s; and a final extension at 72°C for 5 min ended the amplification cycle.

DGGE was performed essentially as described by Muyzer et al. (1993): 40 μL of PCR product and 15 μL of 6× loading buffer were loaded onto 6% polyacrylamide gels (acrylamide/bisacrylamide, 37.5:1) with denaturing gradients of range 40–60%. The gel was electrophoresed in 1× TAE buffer at 100 V for 17 h at 60°C. After electrophoresis, the gels were stained by ethidium bromide for 15 min, followed by photography using UV transillumination.

Sequencing and sequence analysis

Dominant bands were excised from DGGE gels and transferred to 100 μL of Tris-EDTA buffer for 8 h at 4°C for DNA elution. After that, the eluted DNA was re-amplified using the original PCR primers and programs and confirmed by another DGGE. Only pure bands were amplified with primers without GC clamp and then sequenced on an ABILR-377 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were aligned to related sequences available in the NCBI databases using the BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST). Phylogenetic trees were calculated and drawn using the Neighbor-Joining algorithm and Mega 3.0 software. The sequences have been deposited at GenBank with accession numbers: HM209447 to HM209451 and JF769887 (16S rDNA gene sequences).

Shannon diversity index

The community diversity was assessed by Shannon diversity index (H), which was calculated based on DGGE data. Each detected band was defined as a specific phylotype and labeled on Fig. 3a. The pixel intensity for the phylotype was detected by the QUANTITY ONE® software (Version 4.6.2, Bio-Rad, USA). The Shannon diversity index was calculated by Eq. 1.

$$H = -\sum_{i=1}^{S} (N_i/N)\log(N_i/N) \quad (1)$$

in which N_i is the pixel intensity of the ith phylotype, N is the total pixel intensity of all phylotypes, and S is the number of phylotypes.

Statistical analysis

All statistic analyses were performed utilizing SPSS 16.0 (SPSS Inc., Chicago, IL, USA). The differences presented were confirmed by t test at a 95% confidence level.

Results

Effect of ammonium addition on soil methane oxidation capacities

As shown in Fig. 1, the CH_4 oxidation peak values of the soils with the treatments of CON, N100, N250, N500, and N1000 were 12.2, 19.8, 16.6, 23.5, and 15.5 mg $CH_4 kg_{dry\ soil}^{-1} h^{-1}$, respectively. The CH_4 oxidation rates were similar to the results previously reported in mineral landfill soils (15–26 mg $CH_4 kg_{dry\ soil}^{-1} h^{-1}$) but lower than that in organic-rich cover soils (128–400 mg $CH_4 kg_{dry\ soil}^{-1} h^{-1}$) (Borjesson et al. 2004).

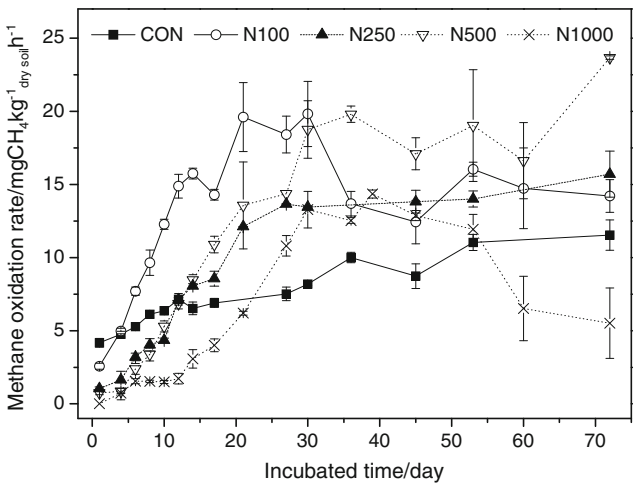

Fig. 1 The CH_4 oxidation capacities of the soils with 0, 100, 250, 500 to 1,000 mg $N kg_{dry\ soil}^{-1}$ of NH_4^+ application (denoted as CON, N100, N250, N500, and N1000, respectively) during incubation

Compared with the CON, where the CH_4 oxidation rates increased gradually from 4.2 to 12.2 mg $CH_4 kg_{dry\ soil}^{-1} h^{-1}$ during the 72-day incubation, NH_4^+-amended soils exhibited different patterns of CH_4 oxidation along with time. On the first day, the CH_4 oxidation rates of experiments N100, N250, N500, and N1000 were 2.6, 1.0, 0.7, and 0 mg $CH_4 kg_{dry\ soil}^{-1} h^{-1}$, respectively, which were lower than that of CON (4.2 mg $CH_4 kg_{dry\ soil}^{-1} h^{-1}$) and negatively correlated with the NH_4^+ concentration. After different levels of lag phase, the CH_4 oxidation rates of the NH_4^+-amended soils increased rapidly to the peak values with the similar increasing rates, i.e., 0.45–0.85 mg $CH_4 kg_{dry\ soil}^{-1} h^{-1}$ per day, which were higher than 0.16 mg $CH_4 kg_{dry\ soil}^{-1} h^{-1}$ per day for CON. The CH_4 oxidation rates of N100 increased with no lag, exceeded that of CON after the 4th day with the oxidation rates both of 4.8 mg $CH_4 kg_{dry\ soil}^{-1} h^{-1}$, and achieved the peak value of 19.8 mg $CH_4 kg_{dry\ soil}^{-1} h^{-1}$ on the 21th day. For N250 and N500, the lag time before significant enhancement of the CH_4 oxidation was 4 days, and the oxidation rate was 7.1 mg $CH_4 kg_{dry\ soil}^{-1} h^{-1}$ when they overbore CON on the 12th day. The peak values of the CH_4 oxidation rates were 13.5 and 18.8 mg $CH_4 kg_{dry\ soil}^{-1} h^{-1}$ for N250 and N500, respectively, which were achieved on the 30th day and increased slightly afterward. The lag time of N1000 was the longest, lasting for 12 days, and the oxidation rate was 10.8 mg $CH_4 kg_{dry\ soil}^{-1} h^{-1}$ when it overbore that of CON on the 27th day. After maintaining above this level for 26 days, the CH_4 oxidation rate decreased rapidly to 5.5 mg $CH_4 kg_{dry\ soil}^{-1} h^{-1}$ on the 72th day.

Throughout the experimental period, the NH_4^+ and NO_3^- concentrations in the soils did not show a significant change. In the negative control experiment with autoclaved soils, the CH_4 concentrations in the headspace remained constant over the entire experimental period indicating the absence of abiotic CH_4 oxidation.

Effect of ammonium addition on methanotrophic abundance

In the original soils, the 16S rDNA gene copy numbers of type I and type II MOB were 0.1 and 2.8×10^7 copies $g_{dry\ soil}^{-1}$, respectively, while the copy numbers of AOB were two orders of magnitude less (4.0×10^5 copies $g_{dry\ soil}^{-1}$) than that of type II MOB. Therefore, type II MOB were the predominant methanotrophs.

No significant trend of the 16S rDNA copy numbers of MOB was observed along with the increased amount of NH_4^+ added (Fig. 2a). Herein, the ratio of type II/type I MOB was used to evaluate their relative abundance. In the

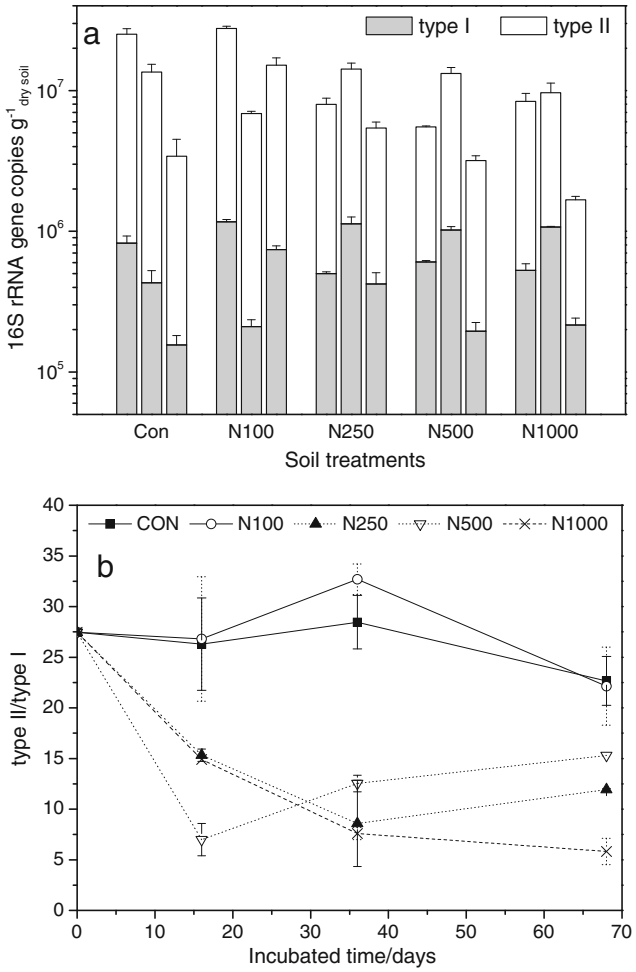

Fig. 2 Quantification of type I and type II methane-oxidizing bacteria (MOB) by real-time PCR amplification targeting 16S rDNA gene fragments (CON, N100, N250, N500, and N1000 represent the soils receiving 0, 100, 250, 500, and 1,000 mg $Nkg_{dry\ soil}^{-1}$ of NH_4^+, respectively). **a** Copy numbers of type I and type II MOB with incubation time (three bars in every treatment represent cell counts on the 16th, 36th, and 68th day from *left* to *right*, respectively). **b** The ratios of the copy numbers of type II/type I MOB. *Error bars* indicate the range of two measurements

high NH_4^+-amended samples (N250, N500, and N1000), the type II/type I MOB ratios decreased rapidly during the first 16 days (Fig. 2b) and then maintained constant in the last period, while the ratios in the other two treatments (CON and N100) fluctuated around the initial levels. In addition, Pearson correlation analysis was conducted but no significant correlation ($P>0.05$) was found among the CH_4 oxidation rates and the abundances of type I MOB or type II MOB or the ratios of type II/type I MOB.

During the incubation, 40% of the AOB copy numbers were undetected in all the soil samples (data not shown). In the other samples, 70% of the detected AOB copy numbers were less than 5,000 copies $g_{dry\ soil}^{-1}$, and the highest value was only 3.1×10^4 copies $g_{dry\ soil}^{-1}$. Furthermore, no correlation existed between the AOB copy numbers and the CH_4 oxidation rates.

Effect of ammonium addition on the community compositions of type II methane-oxidizing bacteria

The DGGE profile (Fig. 3a) showed that the communities of type II MOB varied by the treatments. It was clear that the samples with high NH_4^+ amendment (N250, N500, and N1000) have distinct community compositions from the CON and N100, as the extra bands 3 and 4 were predominant but bands 1 and 2 diminished in the former ones. The CON and N100 treatments exhibited no obvious evolvements of the DGGE band patterns during the 72-day incubation. This was further supported by principal component analysis of the DGGE profile (Fig. 3b), which showed that the samples of CON and N100 at all the three sampling times clustered together with the original soil, which represented the CON sample at time 0. However, for higher NH_4^+-amended samples, the methanotrophic commu-

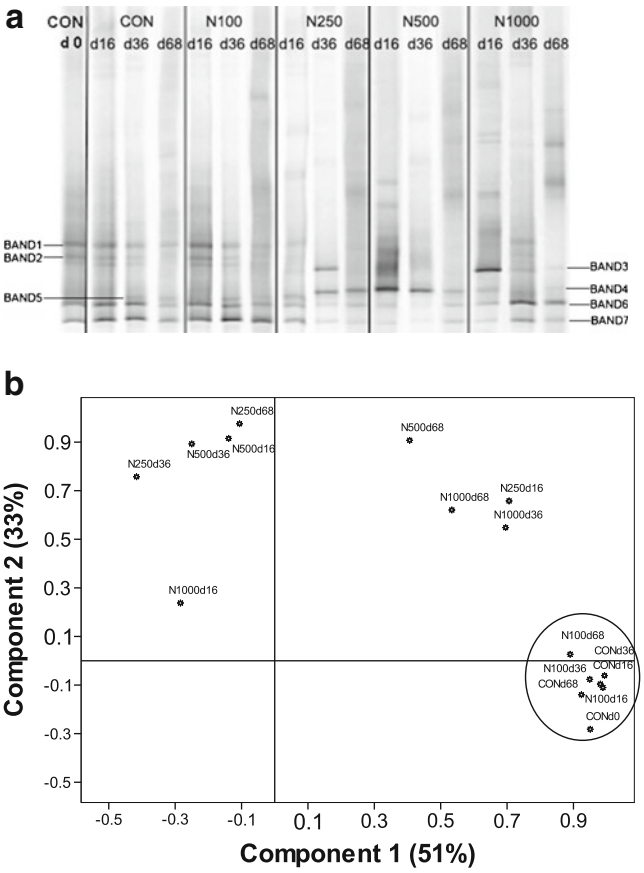

Fig. 3 DGGE profile analysis of type II methane-oxidizing bacteria targeting 16S rDNA gene fragment (CON, N100, N250, N500, and N1000 represent the soils receiving 0, 100, 250, 500, and 1,000 mg N $kg_{dry\ soil}^{-1}$ of NH_4^+, respectively). **a** DGGE profile. **b** Principal component analysis of the DGGE profile, in which only the seven labeled bands were used to do the analysis

nities evolved greatly over time with the conversion of predominant species. BAND 3 existed in the soil samples with N250 and N1000 treatment. It became predominant on the 36th day in the N250, while this time for N1000 was at least 20 days earlier. As to the N500 treatment, the only dominant specie was BAND 4 during the entire incubation period.

Shown by the Shannon diversity index (Table 1), the community diversity of type II MOB experienced a reduction along with incubation time in lower NH_4^+ amendments (N100 and N250). Comparatively, other samples had obvious fluctuated diversities. Furthermore, Pearson correlation analysis showed that the CH_4 oxidation rates were significantly negatively correlated with the Shannon diversity indexes of type II MOB (R=−0.364, P<0.05).

Within the seven dominant bands in the DGGE profile, only six of them were successfully purified by the second round of DGGE process and subsequently sequenced and BAND 2 was excluded (Fig. 4). On the phylogenetic tree for 16S rDNA gene sequences of type II MOB, the bands 1, 3, 6, and 7 clustered with *Methylocystis* sp., while BAND 5 was similar to *Methylosinus* sp. The specie similar to BAND 1 was *Methylocystis heyeri* sp., which was previously isolated from acidic (pH 4.2–4.3) *Sphagnum* peat and tropical forest soil (Dedysh et al. 2007); at pH 4.9, our soil samples were of similar acidity. In addition, BAND 4 possessed 97–98% similarity to sequences of several species of uncultured *alpha proteobacteria*, which have been detected in rock biofilm from gold mines (NCBI accession numbers: FM 253641 and FN 594678) and pasture soils (NCBI accession number: AY395334).

Discussion

Effect of ammonium addition on methane oxidation

The CH_4 oxidation rates of the NH_4^+-amended soils were low on the 1st day but increased quickly to exceed the oxidation rates of the CON after a lag phase. Larger quantities of NH_4^+ fertilizations resulted in lower CH_4 oxidation rates on the 1st day and longer lag time. The highest CH_4 oxidation rates achieved were positively correlated with the initial NH_4^+ levels, except for the N500. The effect patterns of NH_4^+ fertilization can be described as temporal inhibition-delayed stimulation of CH_4 oxidation capacity.

The positive correlation between the inhibition levels and the initial NH_4^+ levels made it seem that substrate competitive inhibition of the CH_4 oxidation occurred. However, no increase of soil NO_3^- concentrations was observed in this study, indicating that NH_4^+ oxidation was not significant. Similarly, Tlustos et al. (1998) found that the NH_4^+ concentrations in the grassland soils remained constant after the addition of $(NH_4)_2SO_4$, and no NO_3^- was detectable. It was generally accepted that the favorite pH for nitrification was around 6.6–8.0, and the nitrification would completely stop when the pH was lower than 4.5 (Maier et al. 2008). We attributed the insignificant NH_4^+ oxidation to the acidic soils used in our (pH=4.9) and Tlustos' (pH=4.67) studies, under which conditions ammonium (pKa=9.25) occurred mostly in the protonated form and was not suitable as a substrate for monooxygenase (Kowalchuk and Stephen 2001). In this case, NH_4^+ could not be oxidized by MOB through competing the active point of MMO with CH_4, and no toxic products (e.g., nitrite) could be generated and act on MOB. The only possible explanation lasted for the temporal inhibition derived from NH_4^+ could be a general salt effect (osmotic stress). Saari et al. (2004) found that the osmotic stress derived from K_2SO_4 addition could inhibit atmospheric CH_4 oxidation.

Compared with the soil samples with no fertilization (CON), the CH_4 oxidation rate was promoted by NH_4^+ addition after a lag phase. For the NH_4^+-derived stimulation of CH_4 oxidation, diverse explanations can be summarized from previous literatures. Park et al. (1992) and Bodelier et al. (2000a) commented that nitrogen addition might interfere directly with the CH_4 oxidizing enzyme system, as immediate CH_4 consumption induced by either NH_4^+ or NO_3^- addition. Nevertheless, this was not valid in the present study due to the delayed rather than immediate stimulation of CH_4 oxidation. Cai and Mosier (2000)

Table 1 Shannon indexes of type II methane-oxidizing bacteria in all the treatments using DGGE bands pattern data

Soil treatments	CON	N100[a]	N250[b]	N500[b]	N1000[b]
Day 0	0.66±0.02	0.66±0.02	0.66±0.02	0.66±0.02	0.66±0.02
Day 16	0.61±0.02	0.63±0.01	0.67±0.00	0.51±0.01	0.47±0.00
Day 36	0.58±0.05	0.56±0.02	0.54±0.04	0.30±0.01	0.53±0.00
Day 68	0.58±0.00	0.49±0.06	0.47±0.01	0.53±0.01	0.55±0.01

Means±SD are given (n=2)

[a] Significantly different among incubation time at P<0.05

[b] Significantly different among incubation time at P<0.01

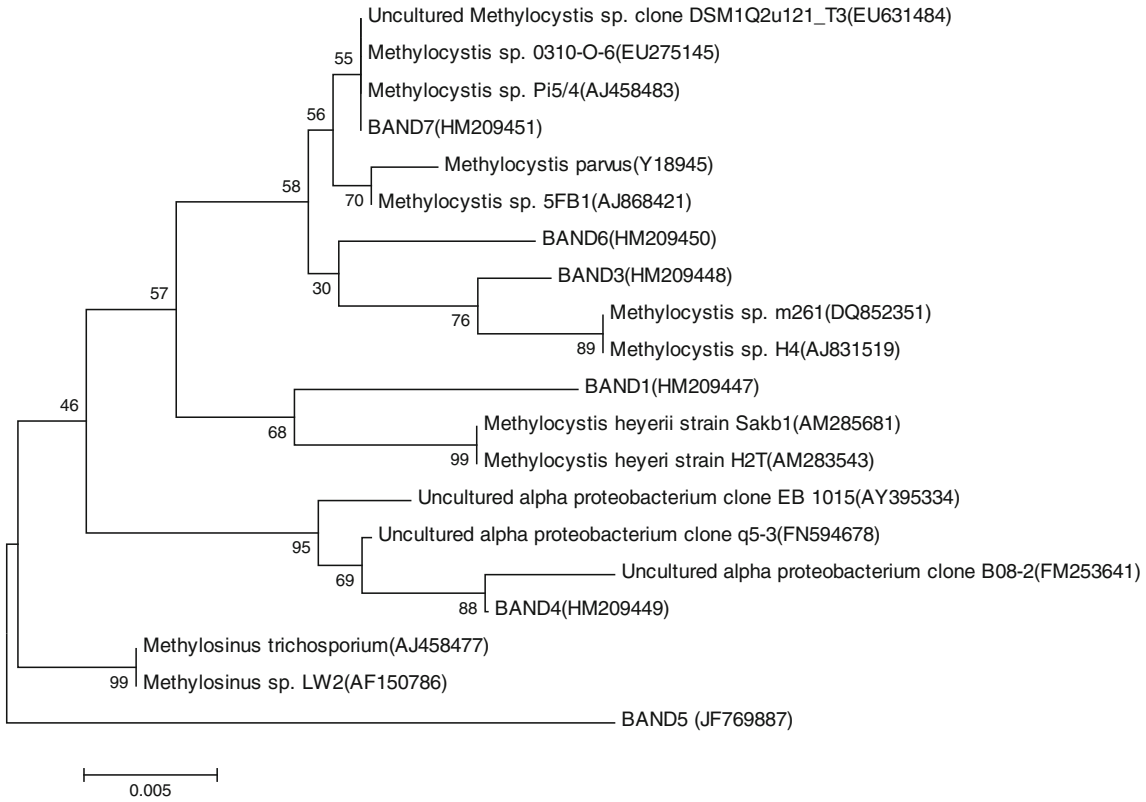

Fig. 4 Neighbor-joining phylogenetic tree of the 16S rDNA gene sequences (463 bp) of type II methane-oxidizing bacteria from incubated landfill soils. The *bar* represents 0.002 substitutions per nucleotide position

ascribed the promoted CH_4 consumption owing to enhancement of AOB. However, in the present study, the observed stimulation effects were regarded not to be associated with AOB for two reasons: The copy numbers of AOB were not comparable with those of MOB, as the former were about 1/70 of the latter in the original soils and decreased about two magnitudes along with incubation and the CH_4 oxidation rate of AOB was only 1/5 of the slowest MOB in a pure culture experiment (Hanson and Hanson 1996). Besides, Bender and Conrad (1995), de Visscher et al. (1999), and Papen et al. (2001) found that NH_4^+-based fertilization could relieve the N limitation of methanotrophs rather than stimulate CH_4 oxidation process. As a necessary nutrient for methanotrophs, 0.25 mol of nitrogen is required for 1 mol of carbon assimilation (Anthony 1982). If 1 mol of CH_4 consumption is assumed to lead to 0.4 mol of carbon assimilation, then the ratios of NH_4^+/CH_4 should be >0.1 to ensure no N limitation happened (Bodelier and Laanbroek 2004). In this study, the NH_4^+/CH_4 ratio of the original soils was 0.096 near the N limitation threshold for methanotrophs.

We hypothesized that the mechanisms of NH_4^+ utilization and CH_4 oxidation in this study were as follows: The bioavailable NH_4^+ could be inherent, mineralized from organic N and externally added as $(NH_4)_2SO_4$, and was regulated by the adsorption or desorption from the high cation-exchanging capacity clay particles. The NH_4^+ oxidation process was impeded at low pH conditions. Considering the process of CH_4 oxidation, the NH_4^+ in the soils could be assimilated in the cells of MOB, which could reduce the NH_4^+ concentrations in the soil solution. Then, the adsorbed NH_4^+ could be released from the clay particles to the soil solution, and this might explain the observation that the extractable NH_4^+ concentrations in the soils displayed no significant variation.

Effect of ammonium addition on the methanotrophic communities

The CH_4 oxidation rates were negatively correlated with the Shannon diversity indexes of type II MOB, but were not correlated with methanotrophic abundances. Compared with the DGGE profile, when the CH_4 oxidation rates of high NH_4^+-amended samples reached the peak values on the 36th day (Fig. 1), the predominant species correspondingly appeared. This was consistent with the work of Seghers et al. (2005), who observed that long-term herbicide application altered the methanotrophic community structures, but not significantly affected either the soil CH_4 oxidation rates or methanotrophic populations. It was demonstrated that methanotrophic community structures

rather than the methanotrophic quantities were more sensitive to outside environmental factors.

Considering the responses of methanotrophs on NH_4^+ addition, two kinds of effect patterns can be summarized: (1) Lower NH_4^+ amendments (CON and N100) induced relatively constant community compositions and (2) higher NH_4^+ application (N250, N500, and N1000) posed decreased ratios of type II/type I MOB and significant community evolutions of type II MOB. It was generally believed that type II MOB can survive better in nutrient-limited environments, but type I MOB were more competitive than type II MOB in nutrient-rich environments (Amaral and Knowles 1995; Borjesson et al. 1998; Wise et al. 1999). Microarray analysis (Lee et al. 2009) of methanotrophs in landfill soils revealed that 16.5 mg $Nkg_{dry\ soil}^{-1}$ of NH_4^+ amendment led to increased signals of type I MOB but relatively constant signals of type II MOB. Thus, we hypothesized that for the N-limited soils (as in this study), lower NH_4^+ application (≤100 mg $Nkg_{dry\ soil}^{-1}$) can relieve the N limitation status of MOB and subsequently promote the activities of all MOB with no significant competitions among methanotrophic species. Whereas, >100 mg $Nkg_{dry\ soil}^{-1}$ of NH_4^+ fertilization was higher enough to favor the growth of part of the MOB species, i.e., type I MOB, kinds of *Methylocystis*-like species (BAND 3 and BAND 4 in the DGGE profile, Fig. 3a), and altered the methanotrophic community structures. The only *Methylosinus*-like species (BAND 5 in the DGGE profile, Fig. 3a) detected in this study diminished under high NH_4^+ amendments.

Possible threshold level distinguishing ammonium-derived inhibition or stimulation

Many researchers have found that NH_4^+ application could stimulate CH_4 oxidation at high CH_4 mixing ratios, while at low CH_4 concentrations, NH_4^+ acted as an inhibitor. For example, de Visscher et al. (2001) incubated landfill cover soils with 3% CH_4 and found that the CH_4 uptake rates of the soils were initially stimulated when mixed with sugar beet leaves rich in ammonium, while Boeckx et al. (1996) observed an inhibition of CH_4 oxidation derived from NH_4^+ application in a similar experiment at 10 ppm of CH_4. Bodelier et al. (2000a, b) and Mohanty et al. (2006) have done many studies to investigate the influence of NH_4^+ fertilization on CH_4 oxidation in rice fields, either in situ or in lab scale, and most of their studies showed stimulation effects. From their experimental conditions, with 1% CH_4 and 40–400 kg Nha^{-1} of NH_4^+ added, we calculated that the NH_4^+/CH_4 ratios during the experiments were 0.4–4, within the above-mentioned N limitation threshold of 0.1 and the maximum value of 9.6 in our study. As to the upland soil, fed with atmospheric CH_4 (about 1.8 ppm), NH_4^+ fertilization always inhibited CH_4 oxidation. With the NH_4^+ fertilization of 0.1–20 mg $Nkg_{dry\ soil}^{-1}$ (Gulledge et al. 1997), the NH_4^+/CH_4 ratios in upland soils were 30–600, much greater than the value of 9.6 in the present study and the thresholds of N inhibition observed in littoral sediments (Bosse et al. 1993) and planted intertidal marshes (van der Nat et al. 1997) at 190 and 30, respectively.

Accordingly, we attribute the influence patterns of NH_4^+ application on CH_4 oxidation to the ratios of NH_4^+/CH_4. For the environment with high NH_4^+/CH_4 ratio (>30 and mainly at the atmospheric CH_4 concentrations), nitrogen content is sufficient for assimilation and added NH_4^+ would compete with CH_4 or introduce more toxic byproducts during its oxidation. However, at lower NH_4^+/CH_4 ratios (<0.1 and mainly at percent magnitude CH_4 mixing ratios), inherent N is not sufficient for the higher requirement of more CH_4 assimilation and appropriate NH_4^+ application can promote the CH_4 oxidation rate. The latter situation often occurs in rice fields, wetlands, and landfill cover soils. Considering the adjustability, landfill may be one of the most practical places to enhance the CH_4 oxidation capacity by the addition of NH_4^+.

Ammonium addition to reduce methane emission from waste landfill

As discussed before, in N-limited environment, an appropriate amount of NH_4^+ addition could stimulate the CH_4 oxidation capacity. In this study, the CH_4 oxidation peak value of the landfill cover soils nearly doubled from 12.2 mg $CH_4kg_{dry\ soil}^{-1}h^{-1}$ in CON to 19.8 mg $CH_4m^{-2}\ h^{-1}$ in N100, equaling 3,800 to 6,200 mg $CH_4m^{-2}\ h^{-1}$ in field scale. Considering the sampling site with the largest CH_4 flux of about 4500 mg $CH_4m^{-2}\ h^{-1}$ (Zhang et al. 2008), if we assume that other environmental conditions are as favorable as in this study and the highest CH_4 oxidation rate can be maintained, appropriate amount of NH_4^+ addition could convert the CH_4 emission to a net sink. Compared with costly commercial mineral NH_4^+-based fertilization, landfill leachate irrigation, a process to reduce the liquid quantity and the pollutants by solar evaporation and soil absorption, respectively (Jones et al. 2006), may be a good option for NH_4^+ application. By investigating CH_4 emissions from landfill sites with or without landfill leachate irrigation, Shao et al. (2009) demonstrated that the average CH_4 flux from test sites with 1 mm day^{-1} leachate irrigation (1,000 1,340 mg NL^{-1} of NH_4^+) was 1/10 of that with no irrigation. However, besides the mitigation of CH_4 emission, leachate irrigation may lead to some other environmental problems owing to increased NH_4^+ and water content in the cover soils, such as larger emissions of nitrous oxide (Watzinger et al. 2005), which should always be kept in mind.

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