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Ammonium-dependent regulation of aerobic methane-consuming bacteria in landfill cover soil by leachate irrigation

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Abstract

The impacts of landfill leachate irrigation on methane oxidation activities and methane-consuming bacteria populations were studied by incubation of landfill cover soils with leachate and $(NH_4)_2SO_4$ solution at different ammonium concentrations. The community structures and abundances of methane-oxidizing bacteria (MOB) and ammonia-oxidizing bacteria (AOB) were examined by PCR-DGGE and real-time PCR. Compared with the pure $(NH_4)_2SO_4$ solution, leachate addition was found to have a positive effect on methane oxidation activity. In terms of the irrigation amount, ammonium in leachate was responsible for the actual inhibition of leachate. The extent of inhibitory effect mainly depended on its ammonium concentration. The suppression of the predominant methane-consuming bacteria, type I MOB, was responsible for the decreased methane oxidation activity by ammonium inhibition. Methane-consuming bacteria responded diversely in abundance to ammonium. The abundance of type I MOB decreased by fivefold; type II MOB showed stimulation response of fivefold magnification upon the first addition but lessened to be lower than the original level after the second addition; the amount of AOB was stimulated to increase for 20–30 times gradually. Accumulated nitrate from nitrification strengthened the ammonium inhibition on type I and type II MOB, as a result, repetitive irrigation was unfavorable for methane oxidation.

Key words: greenhouse gas emission; landfill leachate; methane oxidation; methane-oxidizing bacteria (MOB); ammonia-oxidizing bacteria (AOB)

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Introduction

Landfill-originated methane (CH_4) accounts for more than 50% of current greenhouse gases (GHGs) emissions from the sector of post-consumer waste, corresponding to about 6%–12% of the global anthropogenic CH_4 emission (IPCC, 2007; Kumar et al., 2004; Olivier et al., 2005; Stralis-Pavese et al., 2004). Therefore, mitigating CH_4 emission from landfill sites is one of the most practical measures to reduce CH_4 concentration in the atmosphere. When landfill gas penetrates through landfill cover soils, CH_4 emission can be mitigated considerably via microbial oxidation (de Visscher et al., 1999; Scheutz et al., 2009), which is estimated to be 22 Tg of CH_4 per year (Chen et al., 2007). The sulfate-deficient and atmospheric environment of landfill cover soils favor aerobic methane-oxidizing bacteria rather than anaerobic methane-oxidizing archaea. Generally, aerobic methane-oxidizing bacteria (MOB) can utilize CH_4 as their sole source for carbon and energy and can be divided into two distinct groups as type I MOB and type II MOB, which belong to the phylum of Gamma-proteobacteria and Alpha-proteobacteria, re-

spectively (Hakemian and Rosenzweig, 2007; Hanson and Hanson, 1996). In addition to MOB, ammonia-oxidizing bacteria (AOB) can also oxidize CH_4, as their functional enzyme system is not fully specific for ammonium (Bédard and Knowles, 1997).

Irrigation of stabilized landfill leachate to landfill cover soil is a cost-effective operation for leachate treatment, to reduce leachate volume and pollutants (Jones et al., 2006; Zhang et al., 2008a, 2008b, 2010). Much attention has been paid to the influence of leachate irrigation on CH_4 oxidation capacity of the landfill cover soil, but the results seem to be inconclusive. Maurice et al. (1999) reported that leachate irrigation had a positive effect on CH_4 oxidation when plant growth was also stimulated. Nevertheless, Watzinger et al. (2005) found that CH_4 oxidation rate decreased after long-term leachate irrigation. Zhang et al. (2008b) also discovered that CH_4 emissions from the landfill sites with leachate irrigation were much higher than those without leachate irrigation, indicating a negative effect of leachate irrigation on CH_4 oxidation. Besides, it was still uncertain on the major impact factors derived from leachate to CH_4 oxidation. Maurice et al. (1999) attributed the positive effect to the higher nutrients and organics content of leachate, while Watzinger et al.

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(2005) attributed the negative effect mainly to the toxic compounds in landfill leachate primarily by high NaCl concentration. Landfill leachate is usually rich in ammonium (50–2200 mg/L) (Kjeldsen et al., 2002) and ammonium is commonly recognized as one of the key factors to impact CH_4 oxidation in various environments. Unfortunately, the leachate-introduced effect of ammonium on CH_4 oxidation has been seldom documented. As for other nitrogenous fertilizers, the results are often contradictory even from similar ecosystems. Inhibition (Bradford et al., 2001a; Dobbie et al., 1996; Kravchenko et al., 2002), stimulation (Mohanty et al., 2006), or no effect (Gulledge et al., 1997) were observed with the proposed mechanism remaining ambiguous.

To clarify the effect of leachate on CH_4 flux as a kind of ammonium fertilizer, it's necessary to straightforward investigate the effects of ammonium-dependent leachate on methane-consuming bacteria communities, and resultantly how the microbial diversity or abundance influences the CH_4 flux. Culture-independent molecular techniques are increasingly employed to characterize methane-consuming bacteria in landfill cover soils (Cébron et al., 2007; Chen et al., 2007; Stralis-Pavese et al., 2006; Wise et al., 1999), targeting at the impact of environmental perturbation on methane-consuming community. A concept of ecological regulation analysis was recently extended to microbial ecology to address how the MOB population size and cellular activity regulated CH_4 oxidation (Röling, 2007). Watzinger et al. (2008) utilized [^{13}C]-phospholipid fatty acids technique to explore the MOB communities in landfill cover soils treated with leachate irrigation for 1.5 years. Nonetheless, it remained unknown the main factors involved in leachate irrigation that resulted in the organization of MOB community, as well as the relationship of microbial community and the CH_4 flux.

In this study we tested the impact of leachate as a source of ammonium fertilizer on microorganism. The abundance of type I MOB, type II MOB and AOB was quantified by real-time polymerase chain reaction (real-time PCR). The microbial diversity was monitored by PCR-denaturing gradient gel electrophoresis (DGGE). Ecological regulation analysis was introduced to explain the relationship of the population size of methane-consuming bacteria and CH_4 oxidation capacity.

1 Materials and methods

1.1 Soils and leachates

The soils for batch incubation were taken from the upper cover layer (10–30 cm deep) of a municipal solid waste landfill in eastern China, air-dried and sieved through 2 mm-mesh to remove small stones and roots before use. The soils were composed of 31.3% of sand, 49.6% of silt and 19.1% of clay, and the other properties were summarized as: pH 5.19, soil organic carbon 0.35%, organic matter 2.66%, NH_4^+-N 8.1 mg-N/kg dry soil and NO_3^+-N concentration 23 mg-N/kg dry soil. The soils were pre-incubated in Plexiglas cylinders at atmospheric O_2 and 5%

of CH_4 at 25°C for the recovery of CH_4 oxidation for 2 weeks before leachate addition tests. The leachate used for irrigation was partially stabilized and derived from the same landfill site, and its characteristics were summarized as: pH 8.0, COD 1600 mg/L, BOD 100 mg/L, NH_4^+-N 1800 mg/L and NO_3^--N 10 mg/L.

1.2 Soil incubation with leachate addition

Soil sample of 400 g was transferred from the Plexiglas cylinder into 1.5-L bottles that were closed with rubber stoppers. Appropriated amount of leachate and water was sprayed into the soil to obtain 18% of water content. To evaluate the effects of ammonium concentrations, the leachate was applied at three ammonium levels, 50, 100, and 150 mg NH_4^+-N/kg dry soil. To investigate the effects of ammonium sources, the treatments with inorganic compound $(NH_4)_2SO_4$ were established as well for comparison purpose. That was, the treatments of 100 mg NH_4^+-N/kg dry soil used 100% leachate (marked as LL100), 50% leachate plus 50% $(NH_4)_2SO_4$ solution (LN100), and 100% $(NH_4)_2SO_4$ solution (NN100). The treatments of 50 and 150 mg NH_4^+-N/ kg dry soil used 100% leachate as the solo ammonium source (LL50 and LL150). On day 15 of incubation, the additions of ammonium liquors were conducted again for all treatments. Another treatment (CON) was set up as control without ammonium addition.

All soil incubations were supplemented with CH_4 and CO_2 both up to concentration of 2.5%, in order to simulate real landfill fields. Every day, the headspace of the sealed bottles was flushed well with air, and CH_4 and CO_2 were re-added again. Each treatment of incubations was conducted in duplication at 25°C.

1.3 Determination of methane oxidation rate

The incubated soil of 20 g was placed loosely at the bottom of 250-mL serum bottle and then the bottles were sealed with butyl rubber stoppers. During the next 24-hr incubation for CH_4 oxidation rate testing, the concentration of CH_4 and CO_2 in the headspace and temperature were the same as batch incubation. CH_4 concentration in the headspace was monitored by analyzing 1-mL gas sample using gas chromatograph (GC) at 0, 2, 6, 10 and 24 hr after CH_4 and CO_2 addition. The GC instrument (GC112A, Shanghai Precision & Scientific Instrument Co., Ltd., China) was equipped with a flame ionization detector and the carrier gas was N_2 with a flow rate of 40 mL/min. The temperatures of the injector, oven and detector were set at 80, 60 and 180°C, respectively. For the CH_4 oxidation followed zero order kinetics, the CH_4 oxidation rate could be calculated via linear regression of the CH_4 concentrations against time.

1.4 Characteristic analyses for soils and leachates

The moisture contents of soils were determined gravimetrically by drying for 24 hr at 105°C. The soil pH was determined in a 1:2.5 (W/V) soil-water mixture. NH_4^+-N, NO_2^--N and NO_3^--N in soils were extracted by shaking 10 g of fresh soil sample with 50 mL of KCl solution (2 mol/L) for 1 hr before filtering, and their concentrations

were then analyzed using the standard methods (APHA et al., 1998). Total nitrogen (TN) was analyzed using the Kjedahl method (Lu, 2000). The organic matters content in soil was determined based on the organic carbon content multiplied by 1.732 (Lu, 2000). The particle size distribution of soil was evaluated by the hydrometer method (Lu, 2000).

Leachate was monitored for pH, NH_4^+-N, chemical oxygen demand (COD) and 5-day biochemical oxygen demand (BOD_5). The pH was measured with a digital pH meter (PHS-25, Shanghai Precision & Scientific Instrument Co., Ltd., China). NH_4^+-N was measured using a distillation method. COD and BOD_5 were measured using standard methods (APHA et al., 1998).

1.5 Total DNA extraction of soils

DNA extraction was modified from two reported methods (Griffiths et al., 2000; Yeates et al., 1997). Briefly, 0.5 g of soil was mixed with 600 μL of extraction buffer (100 mmol/L Tris-HCl (pH 7.0), 100 mmol/L sodium EDTA (pH 8.0), 1.5 mol/L NaCl) and 0.5 g of glass beads (0.1 mm diameter), and then the mixture was blended in a bead-beater (XW-80A, JINGKE, Shanghai, China) for 5 min. After addition of 60 μL sodium dodecyl sulfate (20% W/V) and 5 sec of blending, the mixture was incubated at 65°C for 1 hr followed by 10 min of centrifugation at 10,000 $\times g$. Another round of extraction was performed to recover more DNA from settled pellets by 200 μL of extraction buffer. The supernatant in two round of extraction was collected together and then mixed with an equal volume of polyethylene glycol 6000 (30%, W/V)/NaCl (1.6 mol/L). After 2 hr of incubation at 4°C, the solution was centrifuged at 16,000 $\times g$ for 30 min. Next, pelleted nucleic acid was re-suspended in 540 μL of TE buffer (10 mmol/L Tris-HCl, 1 mmol/L sodium EDTA, pH 8.0) and 60 μL of sodium acetate (3 mol/L, pH 5.3) was added to obtain a final concentration of 0.5 mol/L. In order to precipitate proteins and polysaccharides, the mixture was transferred to an ice-bath for 5 min and then centrifuged at 16,000 $\times g$ at 4°C for 30 min. Afterwards, the aqueous phase was extracted with an equal volume of phenol/chloroform, then chloroform/isoamyl alcohol twice separately. DNA was precipitated by adding 0.6 volume of isopropanol and incubated for 2 hr on the ice. Then DNA was pelleted by centrifugation at 16,000 $\times g$ for 30 min, and washed by ice cold 70% (V/V) ethanol. Finally, air dried DNA was re-suspended in 50 μL of TE buffer.

1.6 Quantification of type I MOB, type II MOB and AOB by real-time PCR

DNA samples from each treatment were amplified and monitored by real-time PCR method using Bio-Rad MiniOpticon (Bio-Rad, USA). Amplification of real-time PCR was performed in 20 μL of reaction mixtures by using SYBR green PCR master mix as described by the suppliers (Takara Bio, Japan). The primer sets used to quantify separately the 16S rDNA of type I MOB and type II MOB and the functional gene $amoA$ of AOB in the soil samples are listed in Table 1. The PCR temperature program was as follows: 95°C for 30 sec, followed by 45 cycles of 95°C for 5 sec, 60°C for 10 sec and 72°C for 5 sec. Standard curve for each primer set was constructed by using a mixture of DNA fragments of equal length, which was derived from a group-specific PCR amplification with the same primer set used for the real-time PCR amplification. The amount of DNA in the mixture was determined spectrophotometrically and the weight of one gene copy was determined. Dividing the amount of DNA in the standard curve by the weight of one gene copy yielded the copy number present in the standard curve. The obtained coefficient of determination R^2 values between the threshold cycle and the number of target gene copies were greater than 0.99 for each standard curve. The amplification efficiency of each primer set was between 0.90 and 1.10. The gene copy numbers per gram of soil were calculated and analyzed for significance by general liner model analysis.

1.7 PCR-DGGE analysis

For DGGE analysis, a GC clamp was attached to the 5′ end of forward primer. The group-specific PCR amplification for type I MOB and type II MOB was performed in a 50 μL reaction mixture containing 0.5 μmol/L, 200 μmol/L of each dNTP, 5 μL 10× PCR buffer, 2 μmol/L $MgCl_2$, 2.5 U of Taq polymerase and 1 μL of template DNA. The PCR amplification conditions were as follows: 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 sec, anneal at 55°C for 30 sec, and extension at 72°C for 90 sec; and a final extension at 72°C for 5 min ended the amplification cycle. PCR amplification of $amoA$ gene fragment was performed in total 50 μL volume containing 0.2 μmol/L of each primer, 200 μmol/L of each dNTP, 5 μL 10× PCR buffer, 3 mmol/L $MgCl_2$, 4 U of Taq polymerase and 5 μL of template DNA. PCR amplification conditions modified after Rotthauwe et al. (1997) were as follows: 94°C for 5 min; 35 cycles of denaturation at 94°C for 45 sec, anneal at 55°C for 30 sec, and extension at 72°C for 1 min; and a final extension at 72°C for 6 min ended the amplification cycle. The 5 μL of each PCR product was analyzed by

Table 1   PCR primer sequences used in this study

Name	Target	Primer sequence (5′-3′)	Reference
533f	All bacteria	GTGCCAGCAGCCGCGGTAA	Weisburg et al., 1991
MethT1bR	MOB I	GATTCYMTGSATGTCAAGG	Wise et al., 1999
MethT2bR	MOB II	CATCTCTGRCSAYCATACCGG	Wise et al., 1999
$amoA$-1F	$amoA$	GGGGTTTCTACTGGTGGT	Rotthauwe et al., 1997
$amoA$-2R	$amoA$	CCCCTCKGSAAAGCCTTCTTC	Rotthauwe et al., 1997

electrophoresis on 1.0% agarose gel and visualized by ethidium bromide staining.

DGGE was performed essentially as described by Muyzer et al. (1993). Briefly, PCR products were loaded onto 8% (V/V) polyacrylamide gels in $1\times$ TAE (20 mmol/L Tris, 10 mmol/L acetate, 0.5 mmol/L EDTA at pH 7.5). The polyacrylamide gels were made with a denaturing gradient ranging from 40% to 60% for type I MOB and type II MOB, and from 25% to 70% for AOB. The electrophoresis was run overnight for 17 hr at 60°C at 100 V. After the electrophoresis, the gels were stained for 15 min in water containing 0.5 μg/mL ethidium bromide. Images were photographed on a UV trans illumination table.

The computerized images of DGGE profiles were analyzed with the Quantity One software, Version 4.5.2 (Bio-Rad Laboratories, USA). Shannon-weaver index of general diversity (H) was used to analyze the DGGE banding pattern, which was calculated on the basis of the bands on the gel tracks, using densitometric curves. The equation is: $H = -\sum (n_i/N)\ln(n_i/N)$, where n_i is the height of the peak and N is the sum of all peak heights of the densitometric curve.

2 Results

2.1 Methane oxidation rates

The irrigation with either leachate or $(NH_4)_2SO_4$ solution at all ammonium concentrations caused the reduction of soil CH_4 oxidation rate along with time (Fig. 1). The CH_4 oxidation rate of CON maintained at 0.23 μmol/(g dry soil·hr) during the incubation. After the first round of addition, irrigation caused a significant decrease of CH_4 oxidation of all samples on 1 day regardless of ammonium sources and concentration. The CH_4 oxidation rate under ammonium concentration of 50 mg NH_4^+-N/kg dry soil (LL50) decreased to 63.3% of the initial value, the sample with 100 mg NH_4^+-N/kg dry soil (LL100, LN100, NN100) deceased to 57.1% and 150 mg NH_4^+-N/kg dry soil (LL150) decreased to 50.5%. After day 1, the CH_4 oxidation rate increased again and reached a peak value from day 3 to day 6. Then, the CH_4 oxidation rates of leachate-added samples reached a relatively stable stage until day 15, while the solo $(NH_4)_2SO_4$-added treatment (NN100) posed decrease of CH_4 oxidation rate continuously. After the second round of addition, CH_4 oxidation rate of all experiments decreased progressively with no increasing stage like the first addition along with incubation time and depended on the amount of ammonium. During the first 15 days after the second addition, the CH_4 oxidation rates of LL100 and LL150 decreased rapidly to 0.06 and 0.04 μmol/(g dry soil·hr), respectively, whereas that of LL50 was higher of 0.12 μmol/(g dry soil·hr). As for the treatments with same ammonium concentration, the solo leachate-added samples (LL100) exhibited a much higher CH_4 oxidation rate at the end of the incubation than the other two ammonium sources of irrigation treatments (LN100 and NN100), 0.06 μmol/(g dry soil·hr) vs. 0.02
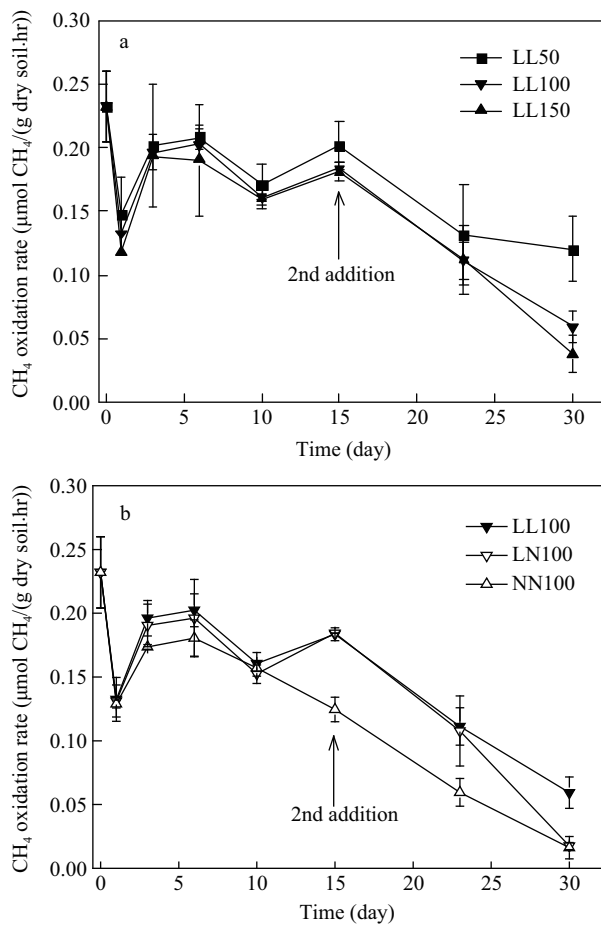
Fig. 1 Methane oxidation rates during the incubation. (a) samples were added with leachate to different ammonium concentration; (b) samples were added with different fertilizers to an ammonium concentration of 100 mg NH_4^+-N/kg dry soil ; Error bars indicate the range of two measurements.

μmol/(g dry soil·hr). On day 30, the CH_4 oxidation rate of LN100 and NN100 was only 0.02 μmol/(g dry soil·hr).

2.2 Conversion of nitrogen compounds

The profiles of the inorganic N content (NH_4^+-N and NO_3^--N) of the soils are shown in Fig. 2. For the three treatments of dosing leachate at different ammonium concentrations, after the first addition, all three soils showed a rapid decline of NH_4^+-N content, and reached a state of lower than 10 mg NH_4^+-N/kg dry soil until day 15. After the second addition, the NH_4^+-N contents also declined rapidly, and reached a state of less than 30 mg NH_4^+-N/kg dry soil until day 30. As to NO_3^--N, the concentration ascended slightly at the first six days but rapidly afterwards, suggesting the nitrification of ammonium after day 6. With regard to the increase of CH_4 oxidation rate from day 2 to day 6 (Fig. 1), it was inferred that ammonium was utilized mainly as the nitrogen source for MOB at the initial period. Afterwards, the nitrification led to the accumulation of NO_3^--N (Fig. 2a, b), which could noncompetitively inhibit MOB and result in the falloff of the CH_4 oxidation rates (Fig. 1). It was observed that higher ammonium addition led to higher NO_3^--N content on day 15. The content of NO_2^--N was three order of magnitude lower than that of NO_3^--N during the incubation (results not shown).

Table 2   Soil pH during incubation

Treatment	pH on day 15	pH on day 30
LL50	6.48 ± 0.28	6.61 ± 0.07
LL100	6.33 ± 0.01	6.58 ± 0.64
LL150	6.32 ± 0.40	6.67 ± 0.21
LN100	5.82 ± 0.08	6.51 ± 0.60
NN100	5.58 ± 0.03	5.65 ± 0.11

which was then negligible. The pH evolutions of the three treatments were similar and kept at around 6.5 (Table 2), implying the leachate addition did not change the pH over time. The conversion profiles of NH_4^+-N and NO_3^--N after the second addition were similar to those of the first addition (Fig. 2a, b).

For the treatments of adding different ammonium sources at the same ammonium concentration (Fig. 2c, d), the trends of NH_4^+-N and NO_3^--N were similar regardless of the proportion of leachate, i.e., NH_4^+-N descended gradually to constant and NO_3^--N ascended quickly after six days. Nonetheless, the NO_3^--N levels in the three treatments were the same, while the NH_4^+-N levels were diverse. Although the same loadings of ammonium were added to the soils, the extractable amounts were different for leachate and $(NH_4)_2SO_4$. The 58, 75 and 96 mg NH_4^+-N/kg dry soil were determined for LL100, LN100 and NN100 on day 1, respectively, possibly due to the combination of ammonium with leachate organic matter. As the result, the relevant higher CH_4 oxidation from

leachate treatment can be for the sake of less available free ammonium.

2.3 Abundance and diversity of methane-consuming bacteria revealed by real-time PCR and PCR-DGGE

The abundance of type I MOB and type II MOB and AOB in soils was measured by group-specific real-time PCR analysis. Figures 3 and 4 show the 16S rDNA gene copies for type I MOB and type II MOB and *amoA* gene copies for AOB. The initial number of type I MOB was almost 50 times higher than those of type II MOB. After addition of leachate and $(NH_4)_2SO_4$, two types of MOB showed different reflections in abundance. The abundance of type I MOB with the treatments of LL50, LL100, LL150, LN100 and NN100 decreased sharply of 52.4%, 48.1%, 18.3%, 50.4% and 40.5% on day 1, respectively (Fig. 3a), mainly dependent on the ammonium concentration. Afterwards the type I MOB copies gradually declined and reached a relatively stable stage of about 2×10^7 copies/g soil until day 30, i.e. about 20% of the original copies. No conclusive difference in abundance among samples for the different ammonium sources was observed (Fig. 4a).

In contrast, the abundance of type II MOB was promoted at the first 10 days of the first addition, and was inhibited greatly by the addition at the second time, decreasing to 64.8%, 28.3% and 28.6% of their original abundance at day 30 for LL50, LL100 and LL150, respectively
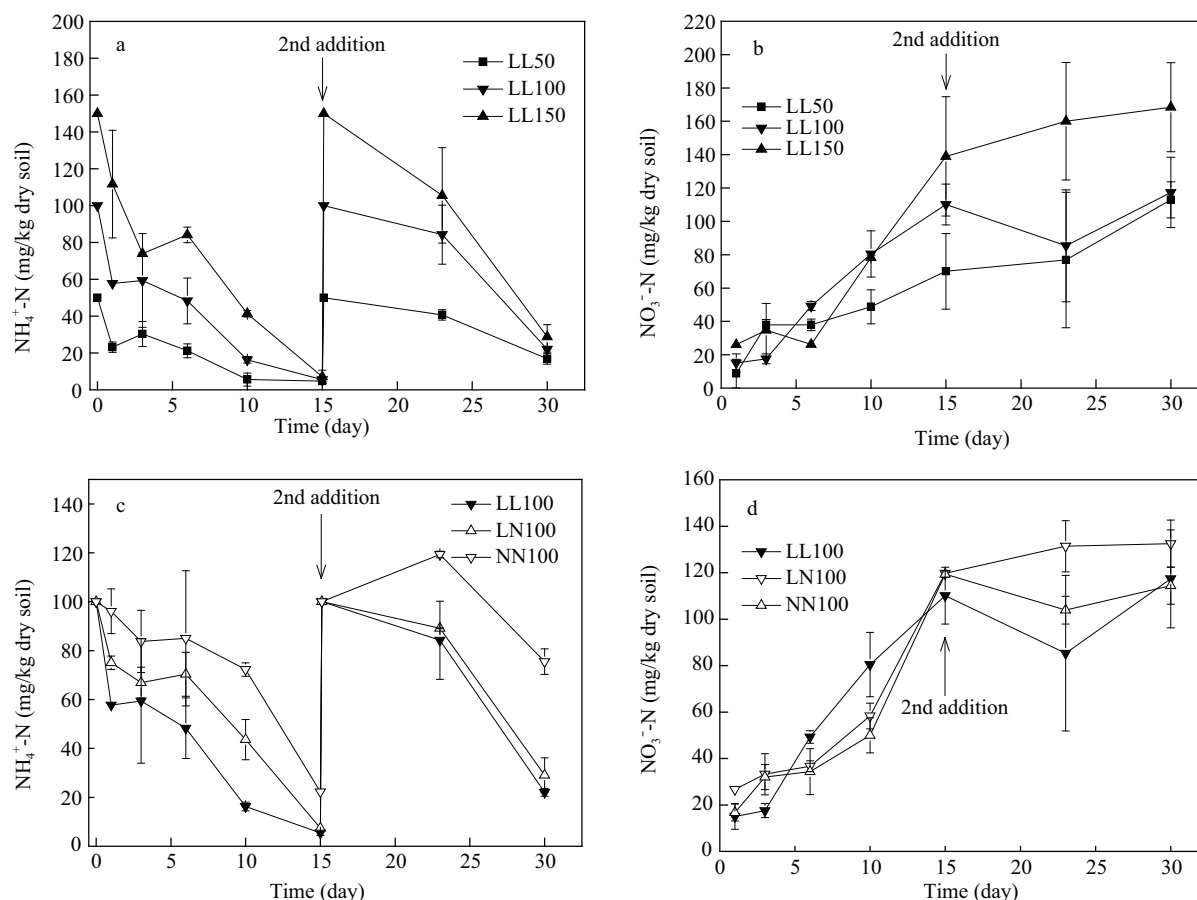
Fig. 2   Changes in NH_4^+-N and NO_3^--N concentrations when leachate was added to different ammonium concentrations (a, b) or different fertilizers were added to an ammonium concentration of 100 mg NH_4^+-N/kg dry soil (c, d). Error bars indicate the range of two measurements.
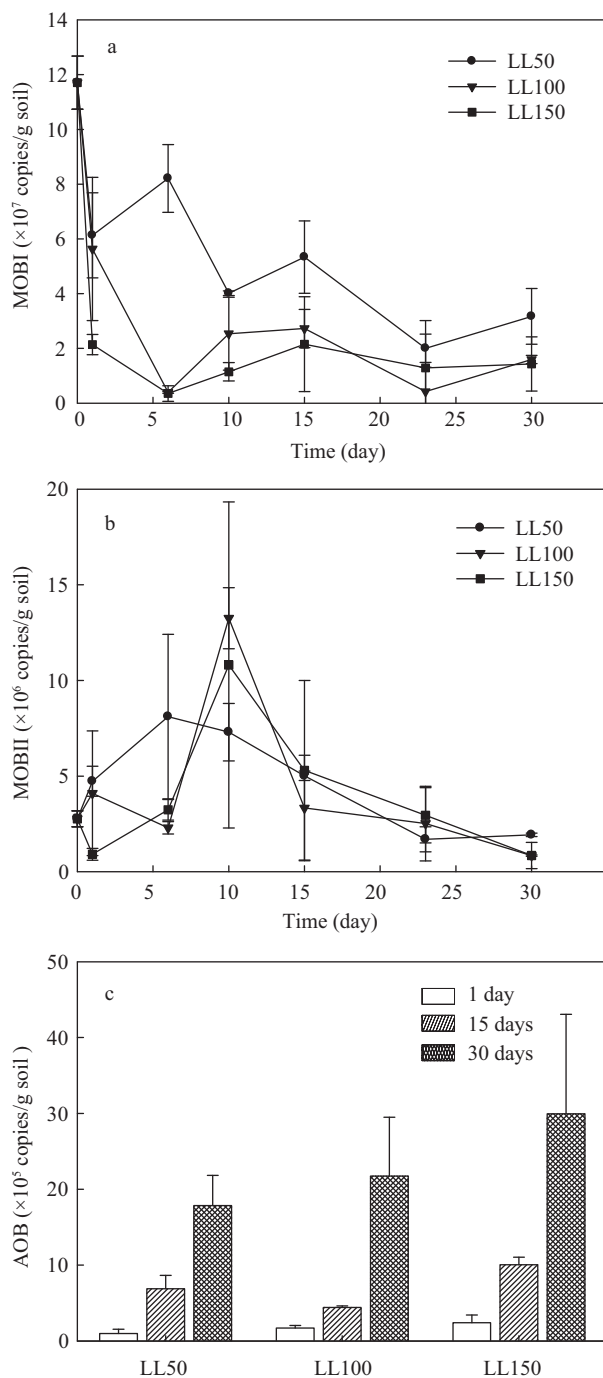
Fig. 3 Quantification of 16S rDNA gene copy numbers from type I and type II methane-oxidizing bacteria and *amoA* gene copy numbers from ammonia-oxidizing bacteria when leachate was added to different ammonium concentrations. (a) type I MOB; (b) type II MOB; (c) AOB. Error bars indicate the range of two measurements.
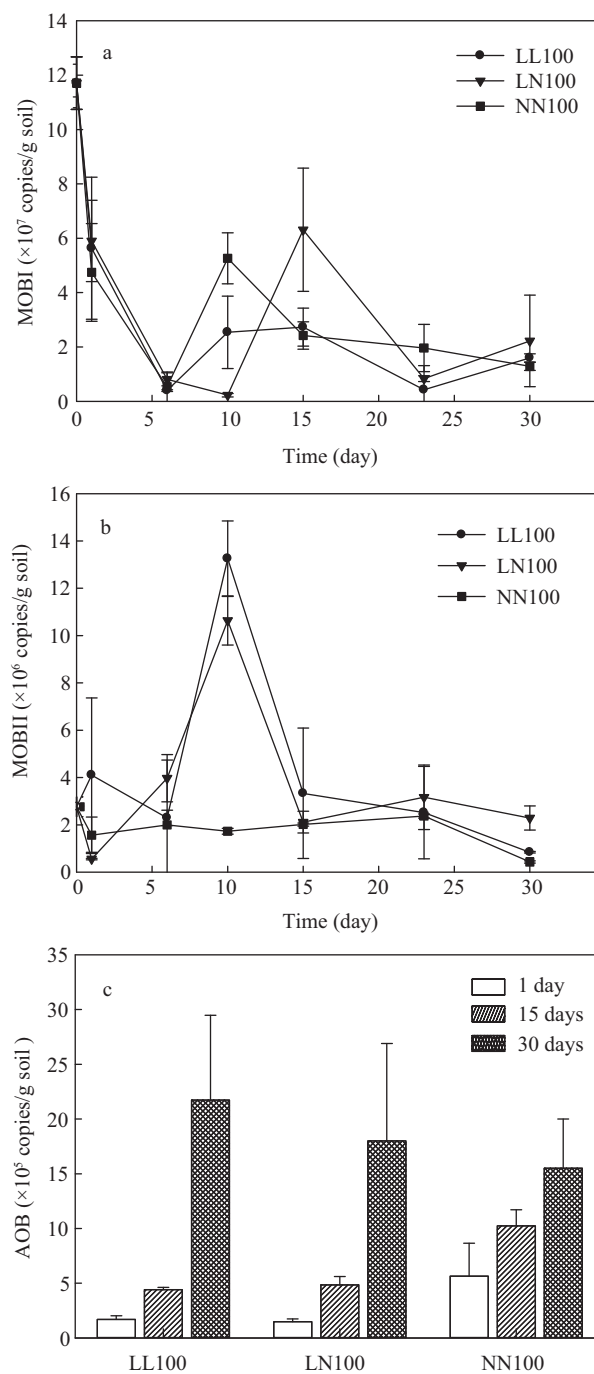
Fig. 4 Quantification of 16S rDNA gene copy numbers from type I and type II methane-oxidizing bacteria and *amoA* gene copy numbers from ammonia-oxidizing bacteria when different fertilizers was added to an ammonium concentration of 100 mg NH_4^+-N/kg dry soil. (a) type I MOB; (b) type II MOB; (c) AOB. Error bars indicate the range of two measurements.

(Fig. 3b). Nonetheless, the difference between influences of ammonium concentration on type II MOB abundance was not significant. However, the abundance of type II MOB in the samples added with $(NH_4)_2SO_4$ solution only was not promoted in the first 10 days (Fig. 4b).

The abundance of AOB was promoted dramatically both by addition of leachate and $(NH_4)_2SO_4$ solution along with the time (Figs. 3c and 4c). The promotion was basically positively related to the amount of leachate addition.

As to the community structures, similar banding patterns were observed in the DGGE profiles of type I and type

II MOB with all the treatments regardless of ammonium concentration or sources, whereas the dominant AOB member has shifted during 30-day incubation, especially for $(NH_4)_2SO_4$ added ones. The Shannon-weaver index calculated from PCR-DGGE profiles (Table 3) shows that the diversities of type I and type II MOB from LL100 and LL150 were higher than LL50, whereas the difference among LL100, LN100 and NN100 was not significant. Comparatively, the diversity of AOB increased with leachate ammonium concentration and incubation.

Table 3 Comparison of Shannon-weaver index (H) calculated from 16S rDNA-DGGE profiles

Organism	Time (day)	LL50	LL100	LL150	LN100	NN100
Type I MOB	1	0.77	0.52	0.62	0.56	0.56
	15	0.60	0.61	0.34	0.63	0.58
	30	0.59	0.30	0.57	0.48	0.53
Type II MOB	1	0.60	0.32	0.39	0.31	0.36
	15	0.69	0.46	0.32	0.47	0.42
	30	0.40	0.39	0.23	0.44	0.46
AOB	1	0.44	0.46	0.49	0.47	0.62
	15	0.45	0.49	0.55	0.53	0.52
	30	0.79	0.78	0.95	0.77	0.68

3 Discussion

3.1 Ammonium-dependent influence of leachate irrigation on methane oxidation activity

To illustrate the dependency of CH_4 oxidation on ammonium level, the linear regression of CH_4 oxidation rate vs. NH_4^+-N concentration was done for LL50, LL100 and LL150 at different sampling times. The slopes of the linear regression equations were determined to be –0.288 (R^2 = 1.000), –0.083 (R^2 = 0.967), –0.175 (R^2 = 0.958), –0.115 (R^2 = 0.825), –0.210 (R^2 = 0.836), –0.186 (R^2 = 0.705) and –0.821×10^{-6} μmol CH_4/(mg NH_4^+-N·hr) (R^2 = 0.931), respectively for day 1, 3, 6, 10, 15, 23 and 30. It demonstrates that the CH_4 oxidation rate was negatively linear-correlated with the ammonium levels. Furthermore, the slope was higher on day 30, implying that the sample after the second addition was more sensitive to ammonium turbulence. NO_3^- accumulation (Fig. 2b, d) may be the possible reason for the rapid decreased of CH_4 oxidation after the second addition of ammonium, since NO_3^- was described as an important inhibitor for CH_4 oxidation (Dunfield and Knowles, 1995).

The inhibition patterns of leachate have the similar trends as those of $(NH_4)_2SO_4$ solution. Since sulfate was reported to be relative nontoxic (Bradford et al., 2001a, 2001b), it was suggested that ammonium was an important factor in leachate that was responsible for the actual inhibition of leachate and the extent of inhibitory effect of leachate mainly depended on its ammonium concentration. Compared with the solo $(NH_4)_2SO_4$ treatment (NN100), leachate treatments (LL100 and LN100) were less inhibitory. It was attributed to that the combination of ammonium to leachate organic matters led to the reduced availability of ammonium and resultantly led to the reduced inhibition. Furthermore, the pH of LL100 kept at around 6.5 (Table 2), the pH of LN100 decreased to below 6.0 in the first addition, but gradually increased to 6.5 after the second addition. Whereas the soils with NN100 treatment kept acid with the pH around 5.5 due to the acidity of $(NH_4)_2SO_4$ solution. It implied that leachate undertook a function of pH buffer, which might be attributed to the richness of organic matters.

3.2 Ammonium-dependent regulation on microbial ecology and methane oxidation flux

Our real-time PCR assays showed that type I MOB was the absolutely predominant methane-consuming bacteria

during the whole incubations regardless of ammonia treatments. The ratio of DNA-fragment copies for type I MOB, type II MOB and AOB was around 1200:50:1 initially. The ratios changed to around 25:2.5:1 on day 15 and 15:1:1 on day 30. Among these three bacteria, type I MOB was most sensitive to ammonium exposure, which was indicated by the sharp decrease immediately after the first addition of ammonium. Upon the whole, the abundance of type I MOB decreased by leachate or $(NH_4)_2SO_4$ treatments, type II MOB showed stimulation response to leachate treatments upon the first addition but lessened to be lower than the original level after the second addition, AOB was stimulated for 20–30 times.

Concerning the CH_4 oxidation abilities of type I MOB, type II MOB and AOB, AOB was reported to present the lowest ability. In this context, when presuming in our research framework that type I and type II MOB presented the same capacity for methane oxidation per gene copy, the absolute predominance of type I MOB and its sensitivity to ammonium inhibition would explain the eventual decline of CH_4 oxidation rates in Fig. 1. The suppression of type I MOB in large amounts could also be related to the acute inhibition on CH_4 oxidation on day 1. Comparatively, the transitory ammonium stimulation on type II MOB and the durative stimulation on AOB could contribute the recovery of CH_4 oxidation rates on day 2 to day 6.

Our study showed that type I MOB thrived better than type II MOB in landfill cover soil. Similar results were found in wetland (DeJournett et al., 2007), forest soils (Mohanty et al., 2006), lake sediments (Rahalkar et al., 2009) and landfill cover soil (Kallistova et al., 2007) recently, suggesting that type I MOB was able to compete effectively with type II MOB in high-methane low-oxygen environments. Type I MOB outnumbered type II MOB in our research system notwithstanding that the uncertainty existing for dead DNA also could be measured by real-time PCR assays. Nevertheless, the transitory stimulation of type II MOB by leachate ammonium (Fig. 3b) also confirmed that type II MOB should be more robust against environmental turbulence. Compared with solo $(NH_4)_2SO_4$ (NN100) addition with no stimulation of type II MOB (Fig. 4b), nutrients like organic matter in leachate were the likely factor supporting growth of type II MOB in the first 10 days (Fig. 4b).

Population of AOB increased rapidly following the additions of leachate or $(NH_4)_2SO_4$, and mainly associated with the ammonium concentration (Figs. 3c and 4c). Cavagnaro et al. (2008) also found in agricultural soil, AOB

population sizes doubled following $(NH_4)_2SO_4$ addition, suggesting that NH_4^+ was a key factor to promote growth of AOB.

CH_4 oxidation rate depends on microorganism cell numbers and cellular activity. In order to address the relationship of the community of methane-consuming bacteria and CH_4 oxidation rate response to ammonium addition, ecological regulation analysis was used in the present work, which was useful to quantify how biogeochemical fluxes were regulated by the microorganisms performing the process and the degree to which changes in fluxes were due to changes on population size and to changes in cellular activity (Röling, 2007). The population regulation coefficient ρ_p was obtained by corresponding to the slope of a double logarithmic plot of cell numbers vs. flux, while the cellular regulation coefficient ρ_c was the complementary number of ρ_p. For LL50 treatment, ρ_p was much higher than 0 (Table 4), thus, the changes in CH_4 oxidation rates in this condition were primarily regulated at the changes in cell numbers. For LL100 and LL150, ρ_c was indistinguishable from 0 and 1. Thus, it suggested that CH_4 oxidation rates were regulated both by cell numbers and cellular activity.

Table 4 Population (ρ_p) and cellular regulation coefficients (ρ_c), with standard error of mean (SEM), for CH_4 oxidation process

Treatment	ρ_p	SEM	> 0*	< 1*	ρ_c
LL50	1.95	0.55	+	–	–0.95
LL100	0.78	1.01	–	–	0.22
LL150	0.5	0.6	–	–	0.5

* Indicates whether ρ_p was significantly different ($p < 0.05$) from 0 (CH_4 oxidation completely regulated by cellular activity) or 1 (flux completely regulated by population size), with '+' indicating a significant difference.

3.3 Effect of repetitive irrigation

The CH_4 oxidation rates kept falling after the second addition of leachate or $(NH_4)_2SO_4$ solution. Contrastively, there experienced a recovery period from the acute inhibition after the first addition. Correspondingly, the abundance of both type I MOB and type II MOB kept decreasing after the second addition. It was deduced that MOB were seriously impaired when subjected to a second run of ammonium addition. The accumulated nitrate could play a crucial role in this exacerbated inhibitory effect for both type I MOB and type II MOB. As to AOB, the microbial abundance kept increasing in the first and second addition, indicating the independence on nitrate.

4 Conclusions

Ammonium in leachate was responsible for the actual inhibition of leachate. The extent of inhibitory effect mainly depended on its ammonium concentration. The suppression of the predominant type I MOB was responsible for the reduced CH_4 oxidation activity by ammonium inhibition. Organic matter in leachate was favorable to lessen available ammonium content and to promote the growth of type II MOB. Thereby, in the sense of carbon

sequestration, leachate could be a better nitrogen fertilizer than inorganic chemical ones. Nevertheless, the application of leachate should be controlled at an appropriate ammonium concentration, to alleviate ammonium inhibition. Furthermore, continuously repetitive irrigation is not recommended, and a fallow period after leachate irrigation is suggested to avoid the accumulative toxicity of nitrate originated from ammonium nitrification. Real-time PCR quantitatively attested that type I MOB, type II MOB and AOB responded diversely to environmental regulation. Hence, to clarify the non-conclusive debate on ammonium effects, it was important fundamentally to investigate the composition and diverse responses of methane-consuming bacteria in environmental samples.

References

APHA, AWWA, WEF, 1998. Standard Methods for the Examination of Water and Wastewater (20th ed.). American Public Health Association, Washington DC.

Bédard C, Knowles R, 1997. Some properties of methane oxidation in a thermally stratified lake. *Canadian Journal of Fisheries and Aquatic Sciences*, 54(7): 1639–1645.

Bradford M A, Ineson P, Wookey P A, Lappin-Scott H M, 2001a. The effects of acid nitrogen and acid sulphur deposition on CH_4 oxidation in a forest soil: a laboratory study. *Soil Biology and Biochemistry*, 33(12-13): 1695–1702.

Bradford M A, Wookey P A, Ineson P, Lappin-Scott H M, 2001b. Controlling factors and effects of chronic nitrogen and sulphur deposition on methane oxidation in a temperate forest soil. *Soil Biology and Biochemistry*, 33(1): 93–102.

Cavagnaro T R, Jackson L E, Hristova K, Scow K M, 2008. Short-term population dynamics of ammonia oxidizing bacteria in an agricultural soil. *Applied Soil Ecology*, 40(1): 13–18.

Cébron A, Bodrossy L, Chen Y, Singer A C, Thompson I P, Prosser J I et al., 2007. Identity of active methanotrophs in landfill cover soil as revealed by DNA-stable isotope probing. *FEMS Microbiology Ecology*, 62(1): 12–23.

Chen Y, Dumont M G, Cébron A, Murrell J C, 2007. Identification of active methanotrophs in a landfill cover soil through detection of expression of 16S rRNA and functional genes. *Environmental Microbiology*, 9(11): 2855–2869.

de Visscher A, Thomas D, Boeckx P, van Cleemput O, 1999. Methane oxidation in simulated landfill cover soil environments. *Environmental Science and Technology*, 33(11): 1854–1859.

Dobbie K E, Smith K A, Prieme A, Christensen S, Degorska A, Orlanski P, 1996. Effect of land use on the rate of methane uptake by surface soils in northern Europe. *Atmospheric Environment*, 30(7): 1005–1011.

Dunfield P, Knowles R, 1995. Kinetics of inhibition of methane oxidation by nitrate, nitrite, and ammonium in a Humisol. *Applied and Environmental Microbiology*, 61(8): 3129–3135.

DeJournett T D, Arnold W A, LaPara T M, 2007. The characterization and quantification of methanotrophic bacterial populations in constructed wetland sediments using PCR targeting 16S rRNA gene fragments. *Applied Soil Ecology*, 35(3): 648–659.

Griffiths R I, Whiteley A S, O'Donnell A G, Bailey M J, 2000. Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. *Applied and Environmental Microbiology*, 66(12): 5488–5491.

Gulledge J, Doyle A P, Schimel J P, 1997. Different NH_4^+-inhibition patterns of soil CH_4 consumption: A result of distinct CH_4-oxidizer populations across sites? *Soil Biology and Biochemistry*, 29(1): 13–21.

Hakemian A S, Rosenzweig A C, 2007. The biochemistry of methane oxidation. *Annual Review of Biochemistry*, 76(1): 223–241.

Hanson R S, Hanson T E, 1996. Methanotrophic bacteria. *Microbiological Reviews*, 60(2): 439–471.

IPCC, 2007. Climate Change 2007, the Fourth Assessment Report (AR4) of the United Nations. Intergovernmental Panel on Climate Change (IPCC).

Jones D L, Williamson K L, Owen A G, 2006. Phytoremediation of landfill leachate. *Waste Management*, 26(8): 825–837.

Kallistova A Y, Kevbrina M V, Nekrasova V K, Shnyrev N A, Einola J K M, Kulomaa M S et al., 2007. Enumeration of methanotrophic bacteria in the cover soil of an aged municipal landfill. *Microbial Ecology*, 54(4): 637–645.

Kjeldsen P, Barlaz M A, Rooker A P, Baun A, Ledin A, Christensen T H, 2002. Present and long-term composition of MSW landfill leachate: A review. *Critical Reviews in Environmental Science and Technology*, 32(4): 297–336.

Kravchenko I, Boeckx P, Galchenko V, Van Cleemput O, 2002. Short- and medium-term effects of NH_4^+ on CH_4 and N_2O fluxes in arable soils with a different texture. *Soil Biology and Biochemistry*, 34(5): 669–678.

Kumar S, Mondal A N, Gaikwad S A, Devotta S, Singh R N, 2004. Qualitative assessment of methane emission inventory from municipal solid waste disposal sites: A case study. *Atmospheric Environment*, 38(29): 4921–4929.

Lu R K, 2000. Methods for Soil Agrochemistry Analysis. China Agricultural Science and Technology Press, Beijing.

Maurice C, Ettala M, Lagerkvist A, 1999. Effects of leachate irrigation on landfill vegetation and subsequent methane emissions. *Water, Air, and Soil Pollution*, 113(1-4): 203–216.

Mohanty S R, Bodelier P L E, Floris V, Conrad R, 2006. Differential effects of nitrogenous fertilizers on methane-consuming microbes in rice field and forest soils. *Applied and Environmental Microbiology*, 72(2): 1346–1354.

Muyzer G, de Waal E C, Uitterlinden A G, 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology*, 59(3): 695–700.

Olivier J G J, Van Aardenne J A, Dentener F J, Pagliari V, Ganzeveld L N, Peters J A H W, 2005. Recent trends in global greenhouse gas emissions:regional trends 1970–2000 and spatial distribution of key sources in 2000. *Environmental Sciences*, 2(2-3): 81–99.

Rahalkar M, Deutzmann J, Schink B, Bussmann I, 2009. Abundance and activity of methanotrophic bacteria in Littoral and Profundal sediments of Lake Constance (Germany). *Applied and Environmental Microbiology*, 75(1): 119–126.

Röling W F M, 2007. Do microbial numbers count? Quantifying the regulation of biogeochemical fluxes by population size and cellular activity. *FEMS Microbiology Ecology*, 62(2): 202–210.

Rotthauwe J H, Witzel K P, Liesack W, 1997. The ammonia monooxygenase structural gene *amoA* as a functional marker: Molecular fine-scale analysis of natural ammonia-oxidizing populations. *Applied and Environmental Microbiology*, 63(12): 4704–4712.

Scheutz C, Kjeldsen P, Bogner J E, De Visscher A, Gebert J, Hilger H A et al., 2009. Microbial methane oxidation processes and technologies for mitigation of landfill gas emissions. *Waste Management & Research*, 27(5): 409–455.

Stralis-Pavese N, Bodrossy L, Reichenauer T G, Weilharter A, Sessitsch A, 2006. 16S rRNA based T-RFLP analysis of methane oxidising bacteria-assessment, critical evaluation of methodology performance and application for landfill site cover soils. *Applied Soil Ecology*, 31(3): 251–266.

Stralis-Pavese N, Sessitsch A, Weilharter A, Reichenauer T, Riesing J, Csontos J et al., 2004. Optimization of diagnostic microarray for application in analysing landfill methanotroph communities under different plant covers. *Environmental Microbiology*, 6(4): 347–363.

Watzinger A, Reichenauer T G, Blum W E H, Gerzabek M H, Zechmeister-Boltenstern S, 2005. The effect of landfill leachate irrigation on soil gas composition: methane oxidation and nitrous oxide formation. *Water, Air, & Soil Pollution*, 164(1-4): 295–313.

Watzinger A, Stemmer M, Pfeffer M, Rasche F, Reichenauer T G, 2008. Methanotrophic communities in a landfill cover soil as revealed by [^{13}C] PLFAs and respiratory quinones: Impact of high methane addition and landfill leachate irrigation. *Soil Biology and Biochemistry*, 40(3): 751–762.

Weisburg W G, Barns S M, Pelletier D A, Lane D J, 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology*, 173(2): 697–703.

Wise M G, Mcarthur J V, Shimkets L J, 1999. Methanotroph diversity in landfill soil: Isolation of novel type I and type II methanotrophs whose presence was suggested by culture-independent 16S ribosomal DNA analysis. *Applied and Environmental Microbiology*, 65(11): 4887–4897.

Yeates C, Gillings M R, Davison A D, Altavilla N, Veal D A, 1997. PCR amplification of crude microbial DNA extracted from soil. *Letters in Applied Microbiology*, 25(4): 303–307.

Zhang H H, He P J, Shao L M, 2008a. N_2O emissions from municipal solid waste landfills with selected infertile cover soils and leachate subsurface irrigation. *Environmental Pollution*, 156(3): 959–965.

Zhang H H, He P J, Shao L M, 2008b. Methane emissions from MSW landfill with sandy soil covers under leachate recirculation and subsurface irrigation. *Atmospheric Environment*, 42(22): 5579–5588.

Zhang H H, He P J, Shao L M, 2010. Ammonia volatilization, N_2O and CO_2 emissions from landfill leachate-irrigated soils. *Waste Management*, 30(1): 119–124.

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