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Methanotrophic community structure of aged refuse and its capability for methane bio-oxidation

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Abstract

Aged refuse from waste landfills closed for eight years was examined and found to contain rich methanotrophs capable of bio-oxidation for methane. Specially, community structure and methane oxidation capability of methanotrophs in the aged refuse were studied. The amount of methanotrophs ranged 61.97×10^3–632.91×10^3 cells/g (in dry basis) in aged refuse from Shanghai Laogang Landfill. Type I and II methanotrophs were found in the aged refuse in the presence of sterilized sewage sludge and only Type I methanotrophs were detected in the presence of nitrate minimal salt medium (NMS). The clone sequences of the *pmoA* gene obtained from the aged refuse were similar to the *pmoA* gene of *Methylobacter, Methylocaldum*, and *Methylocystis,* and two clones were distinct with known genera of Type I methanotrophs according to phylogenetic analysis. Aged refuse enriched with NMS was used for methane biological oxidation and over 93% conversions were obtained.

Key words: aged refuse; methanotrophs; methane oxidation; landfill

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Introduction

Methane is a well-known greenhouse gas. Atmospheric methane levels have been increasing over the last 300 years, which is thought to be mostly due to human activities (Ward et al., 2004; Lelieveld et al., 1993). According to estimates from International Panel on Climate Change (IPCC), global release of methane was about 3×10^{11} kg in 2000 and this amount has continued to increase. Refuse landfill sites are significant sources of methane release and account for 6%–12% of global methane emissions (IPCC, 2000). Hence, on-site control of methane emissions in refuse landfill sites has been highlighted in recent years.

Methane oxidation capability of landfill covers has been studied, with final cover demonstrating effective reduction of methane release. Scheutz et al. (2004) studied the release of methane and other organic matters on final cover and temporary cover in the Lapouyade landfill in France. Methane emissions were –0.0104~10 g/(m^2·day) on final cover and 49.9 g/(m^2·day) on temporary cover. Further research has focused on environmental and artificial factors influencing methane oxidation capacity of landfill cover, such as moisture content, N fertilizers, vegetation, and additives (e.g., lime) (De Visscher et al., 2001; Hilger et al., 2000a).

Various materials have been investigated for their feasibility in enhancing methane oxidation capacity of landfill cover. Kightley et al. (1995) studied methane oxidation capability of different soils by setting up laboratory-scale soil microcosms. Their results showed the porous, coarse sand soil developed the greatest methanotrophic capacity. Park et al. (2008) proved that amendment with earthworm casts and powdered activated carbon (PAC) stimulated CH_4-oxidizing capacity of landfill cover soil. Abichou et al. (2009) examined performance of bio-covers constructed with a compost layer. Other materials have also been studied, such as sand-compost-perlite (Philopoulos et al., 2009).

Aged refuse has been studied as a natural landfill cover medium for methane emission control. Refuse in landfill becomes stable after many years of biodegradation, and develops into "aged refuse". Previous research examined the properties of aged refuse mined from a closed refuse landfill in the Southern China, and demonstrated that aged refuse was an excellent bioreactor substrate for effective treatment of waste such as leachate and feedlot wastewaters (Zhao et al., 2002b; Zhao and Shao, 2004). Aged refuse is also a potential natural medium suitable for control of landfill CH_4 emissions. Han et al. (2010) studied the effect of bio-column composed of aged refuse on methane abatement in landfill. Maximum methanotrophic

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activity (V_{max}) ranged from 6.4×10^{-3} to 15.6×10^{-3} units, and the half-saturation value (K_M) ranged from 0.85% to 1.67%.

Soils above landfill sites contain methanotrophic populations with the highest methane oxidation capacity (Pavese et al., 2006). The activity and diversity of methanotrophs in landfill cover soil has been studied, but little is known about methanotroph diversity in aged refuse. In this research, methanotrophs amount and community structure in aged refuse were determined, methane oxidation capacity of aged refuse was measured in the laboratory and its influencing factors were analyzed.

1 Materials and methods

1.1 Methanotrophs cell counts

Aged refuse was excavated from compartments of Shanghai Laogang Landfill closed for eight, ten, and eleven years. Samples for methanotroph cell counts were gained after bulk materials such as stones and plastics were wiped off aged refuse. Xanthozem commonly used as landfill cover material in Shanghai and cover soil from closed landfill were also collected for methanotrophs cell counts. Cover soil were collected about 30 cm below the surface layer of landfill compartments closed for one and eight years where the methane concentrations were tested to be 59% and 2%, respectively. The aged refuse were screened with 1 mm sieves before cell counts and other materials were scrunched and mixed up. All these material were received from Shanghai Laogang Landfill.

Numbering of methanotrophs was carried out using the most probable number (MPN) method as adapted from previous research (Gebert et al., 2003).

1.2 Analysis of community structure of methanotrophs in the aged refuse

Aged refuse was adjusted with CH_4 to appropriate conditions for methanotroph enrichments before DNA extracting. Two samples were prepared, one for incubation with NMS and another for mixing with sterilized sewage sludge.

DNA was extracted from the aged refuse after one-week enrichment using fast DNA spin kit for soil (MP Biomedicals). Extraction and purification procedures were carried out following the manufacturer's instructions of the fast DNA spin kit. DNA was purified while extracted, and was obtained after inspection with agarose gel electrophoresis and stored at –20°C for PCR amplification.

The *pmoA* gene was PCR amplified from total DNA extracted from aged refuse by using primer mb661 in conjunction with primer A189gc. Primers A189gc and mb661 can amplify an approximately 470-bp internal section of *pmoA* and produce strong signals with many methanotrophs (Costello and Lidstrom, 1999). The PCR amplification reactions were carried out using previous laboratory protocols (Auman et al., 2000).

The PCR products were checked on 1% agarose gels. A clone library was constructed for *pmoA* gene, and clones were sequenced and analyzed by Invitrogene Com using an Applied Biosystems automated sequencer.

The GenBank accession numbers for the nucleotide sequences determined in this study were GU233507 to GU233510.

1.3 Methane oxidation capacity of the aged refuse with different placement time

Methane oxidation performance of aged refuse with different placement time was studied through serum bottle experiments. All samples were screened by 4 mm sieves with water content adjusted to about 20%. Approximately 8 g aged refuse was weighed and put into 150 mL serum bottles for all samples. Serum bottles were sealed with rubber stoppers after injection of 20 mL CH_4, and were finally incubated at 30°C.

Enrichments were carried out by two methods, 4 days with nitrate minimal salt medium (NMS) added and 14 days without NMS. In the first method, 2% NMS (dry basis) was added into the aged refuse. Methane concentrations in the serum bottles were analyzed before and after enrichment to determine methane oxidation rates. The same experiments were applied to xanthozem.

1.4 Methane oxidation capacity of domesticated aged refuse

Methane oxidation capacity of aged refuse domesticated with CH_4 was studied by landfill simulating lysimeter experiments. The NMS and sewage sludge were added to aged refuse before adding to lysimeters. Aged refuse with water content about 20% was then added to the lysimeters and ventilated with air and synthetic landfill gas. Synthetic landfill gas was composed of CH_4 and CO_2 with the ratio of 1:1 (V/V). Fluxes of air and landfill gas were 10 and 100 mL/min (Park et al., 2002), respectively. Mixed gas passed through the water before entering the lysimeters to maintain humidity of the aged refuse. Gas samples were collected from the gas inlet and outlet once every day and methane concentration was analyzed to calculate methane oxidation rates.

The experimental rig was a 15 cm (inside diameter) and 80 cm long PVC pillar. From bottom to top, it consisted of gas inlet, ventilated gravel-supporting layer, glass punch panel, and gas outlet (Fig. 1).

1.5 Analytical methods

The pH value, oxidation-reduction potential (ORP), water content, particle size distribution, organic content, and TN were analyzed to characterize the physicochemical properties of aged refuse and other materials. The ORP and pH were measured by a ZD-2 type automatic titrator, water content by electrothermic oven with a drying temperature 65°C and drying time of 24 hr, particle size distribution by 4.00, 0.45, 0.30, 0.2, 0.15 and 0.125 mm sieves, and organic content by dichromate titration and TN by Vario EL III, respectively.

In the laboratory, CH_4 was analyzed in a 200 μL sample by gas chromatography (GC-14B, Shimadzu, Japan) with a stainless steel column packed with Carbosive SII (diameter

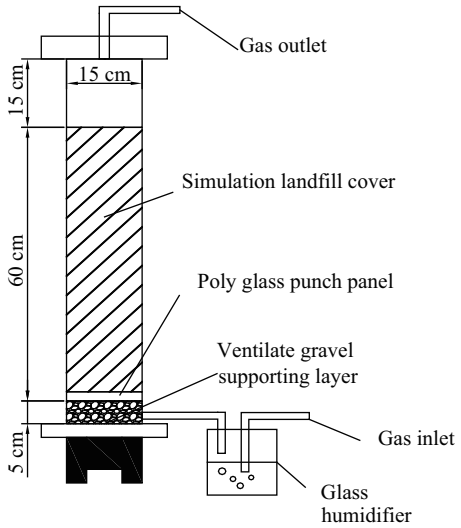

Fig. 1 Schematic form of landfill simulating lysimeter.

of 3.2 mm and 2.0 m length) and thermal conductivity detector (TCD). The temperature of the injection, column and detector was set at 40, 80 and 90°C, respectively. The carrier was nitrogen and the flow rate used was 30 mL/min.

In the landfill site, CH_4 concentration in the cover layer of closed landfill was analyzed with a portable gas analyzer (GA-PLUS2000, TEC, England).

2 Results and discussion

2.1 Methanotrophs amounts of aged refuse

Data in Table 1 show that the aged refuse contained rich methanotrophs, 61.97×10^3–632.91×10^3 cells/g dw. Methanotroph amount in the aged refuse was higher than that of xanthozem and cover soil in low CH_4 concentration environments.

Table 2 presents bacteria amounts between the aged refuse and various soils. Amounts of methanotrophs and bacteria in the aged refuse were compared, with methanotrophs considered the preponderate community. The growth of methanotrophs was induced by the presence of landfill gas and high landfill gas content within closed landfill contributed to the abundance of methanotrophs in aged refuse.

Table 1 Methanotrophs amounts and physicochemical properties comparison between the aged refuse and other materials

Sample	Cell amount ($\times 10^3$/g dw)	Organic content (%)	pH	ORP	TN (%)
Aged refuse (8 years)	453.33	10.36	7.49	–34	0.54
Aged refuse (10 years)	61.97	9.69	7.96	–62	0.49
Aged refuse (12 years)	632.91	10.13	7.57	–38	0.80
Xanthozem	1.28	1.40	8.63	–98	0.14
Cover soil (1 year)	1148.57	3.78	8.14	–71	0.35
Cover soil (8 years)	2.96	7.47	8.42	–78	0.45

ORP: oxidation-reduction potential; TN: total nitrogen.

Table 2 Bacteria comparison between the aged refuse and soils taken from various locations

Sample (location)	Bacteria amount ($\times 10^6$ cells/g dw)
Aged refuse (13 years, Shanghai)	8.63
Aged refuse (9 years, Shanghai)	9.02
Krasnozem (Hangzhou)	11.03
Laterite (Xuwen)	5.07
Paddy soil (Suzhou)	32.30
Dark chestnut earth (Manzhouli)	9.05

2.2 Community structure of methanotrophs in aged refuse

2.2.1 Phylogenetic analyses

The *pmoA* sequences obtained in this study were aligned manually with *pmoA* and *amoA* sequences obtained from the GenBank database. The *pmoA* sequences were subjected to phylogenetic analyses using MEGA3.1.

2.2.2 Aged refuse with NMS

Database searches indicated that the clone sequences of the *pmoA* gene obtained from aged refuse with NMS were similar to that of *Methylobacter* and *Methylocaldum*. The sequence identity of the aged refuse clones with these species was high (about 99%), except for clone M2-85 (93%). Figure 2 shows the phylogenetic analysis of the aged refuse clones and their relationship with the characterized methanotrophs of genera such as *Methylococcus*, *Methylomonas*, *Methylomicrobium*, *Methylosphaera* and *Methylocystis*. Some uncharacterized strains were also included in the phylogenetic tree. According to Fig. 2, clones M2-8, M2-23, M1-049, and M1-054 were similar to *Methylocaldum*. Clones M1-46 and M1-68 were most closely related to a group containing *Methylobacter* sp. BB5.1. No clones clustered closely with any characterized Type II methanotrophs.

2.2.3 Aged refuse with sterilized sewage sludge

Clone sequences of the *pmoA* gene obtained from the aged refuse with sewage sludge were closely related to *Methylobacter, Methylocaldum* and *Methylocystis*. The sequence identity of clones W1-6 and W2-96 with *Methylobacter* sp. BB5.1 was high (> 99%). Figure 3 shows W2-60, W2-86, and W2-88 grouped with *Methylocystis parvus*, W1-8, W1-88, W1-2, and W2-68 were close with *Methylocaldum* sp. The sequence identity of clones W2-26, W2-67 with known methanotrophs were low, then they were distinct with these given genera of Type I methanotrophs.

In general, the *pmoA* gene sequences grouped within the range of previously described methanotrophs sequences. Stralis-Pavese et al. (2006) analyzed methane oxidizing bacteria of landfill site cover soils with different plant covers, and their results showed that the dominant methanotrophs belonged to *Methylobacter* and *Methylocystis*. Uz et al. (2003) detected Type I methanotrophs *Methylobacter* and Type II methanotrophs *Methylocystis* and *Methylosinus* from landfill samples 3 m below the surface. Wise et al. (1999) studied the diversity of the methanotrophic community in mildly acidic landfill cover soil and indicated that Type I clone sequences most closely related to gene of *Methylobacter* sp. strain BB5.1 and Type II clones showed high identity percentages to *Methylocystis echinoides*, *Methylocystis parvus* and *Methylocystis* sp.

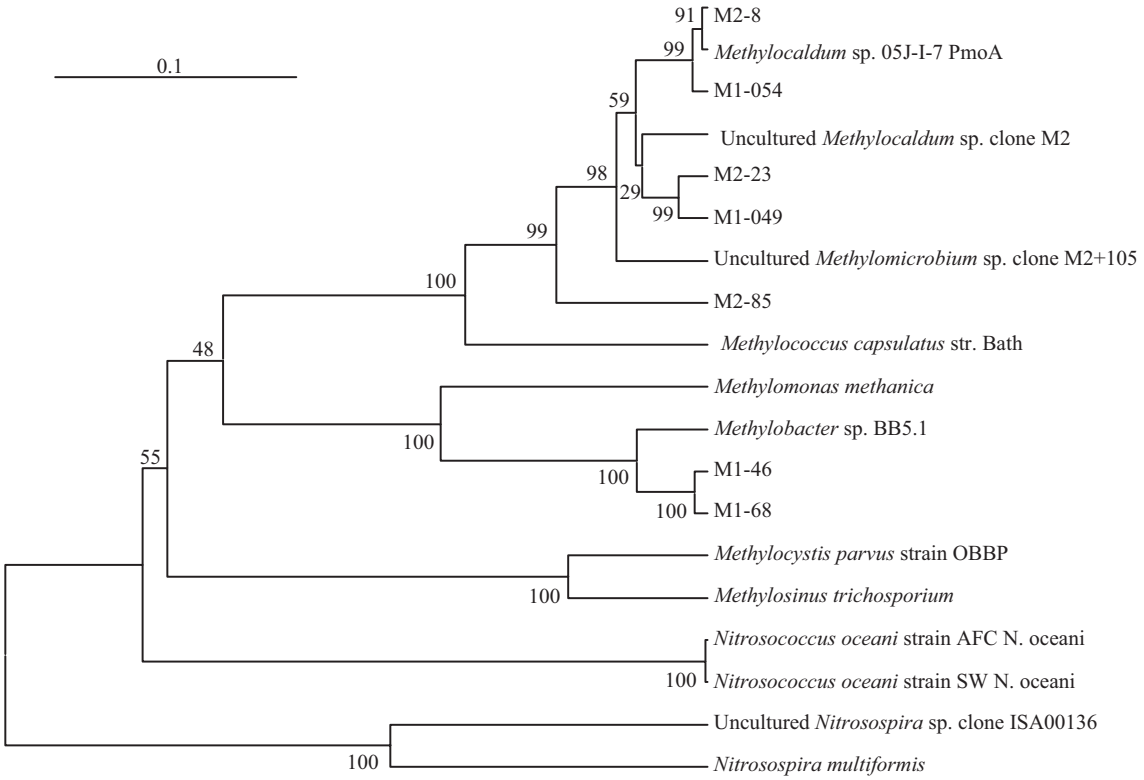

Fig. 2 Phylogenetic analysis of *pmoA* gene clone sequences retrieved from the aged refuse with NMS addition. Bootstrap values are shown near the clades. The bar indicates the estimated number of base changes per nucleotide sequence position.

Our phylogenetic analyses showed Type I methanotrophs were found in aged refuse with NMS and sterilized sewage sludge, but Type II was only detected in the latter. This was caused by different responses of Type I and Type II methanotrophs to nutrient condition changes. Jugnia et al. (2009) and Wu et al. (2009) found Type I methanotrophs grew faster than Type II in feasible conditions for methane oxidation and became the dominant community. This might reflect pioneer species with a potentially high growth rate (r-strategists) that became numerically dominant and reduced the evenness of species distribution. Henckel et al. (2000) also found Type I methanotrophs responded faster and with pronounced shifts in population structure, dominated the activity. Much research works have indicated Type II methanotrophs can grow well in poor nourishment environments and distribute widely, such as deep ocean environments (Zelenkina et al., 2009; Malashenko et al., 2000; Orcutt et al., 2005). Graham et al. (1993) demonstrated soils rich in organic matter may favor the growth of Type II methanotrophs. Wise et al. (1999) found NMS addition stimulated Type I methanotrophs growth, the nutrient-rich 1 × NMS selected for Type I methanotrophs, while the nutrient-poor 0.2 × NMS tended to enrich for Type II methanotrophs.

Therefore, Type I methanotrophs responded more quickly than Type II for inducement of CH_4, and organic matter was effective for rapid growth of Type II. The NMS sample stimulated Type I methanotrophs growth, allowing it to become the predominant community, which was disadvantageous for detection of Type II. For another sample, organic matter stimulated growth of Type II and both methanotrophs were detected.

2.3 Methane oxidation capacity of aged refuse

Table 3 shows methane oxidation capacities of the aged refuse with different placement time and xanthozem in serum bottle experiments. For 14 days enrichment without NMS, methane conversion rates of aged refuse were higher than that of xanthozem except for aged refuse 14 years old. The addition of NMS stimulated methane oxidation activity of the aged refuse obviously. For 14 and 18 years old aged refuse, methane conversion rates were 56.75% and 93.96% after 4 days with NMS, compared to –2.91% and 24.09% after 14 days without NMS. The stimulation of NMS for methane oxidation was not obvious for the xanthozem sample.

Physicochemical properties of aged refuse were analyzed to study their influences on methane oxidation capacity. Table 4 lists correlations between each character and methane conversion rates of different aged refuse in serum bottle experiments, with no obvious correla-

Table 3 Methane oxidation capacity of the aged refuse and xanthozem in serum bottle experiments

Placement time of aged refuse (years)	Methane conversion rate (%)	
	Without NMS for 14 days	With NMS for 4 days
18	24.09	93.96
17	38.23	94.20
16	56.72	93.94
14	–2.91	56.75
13	11.07	62.45
11	36.63	74.48
10	4.05	52.93
8	39.76	58.58
Xanthozem	2.07	5.63

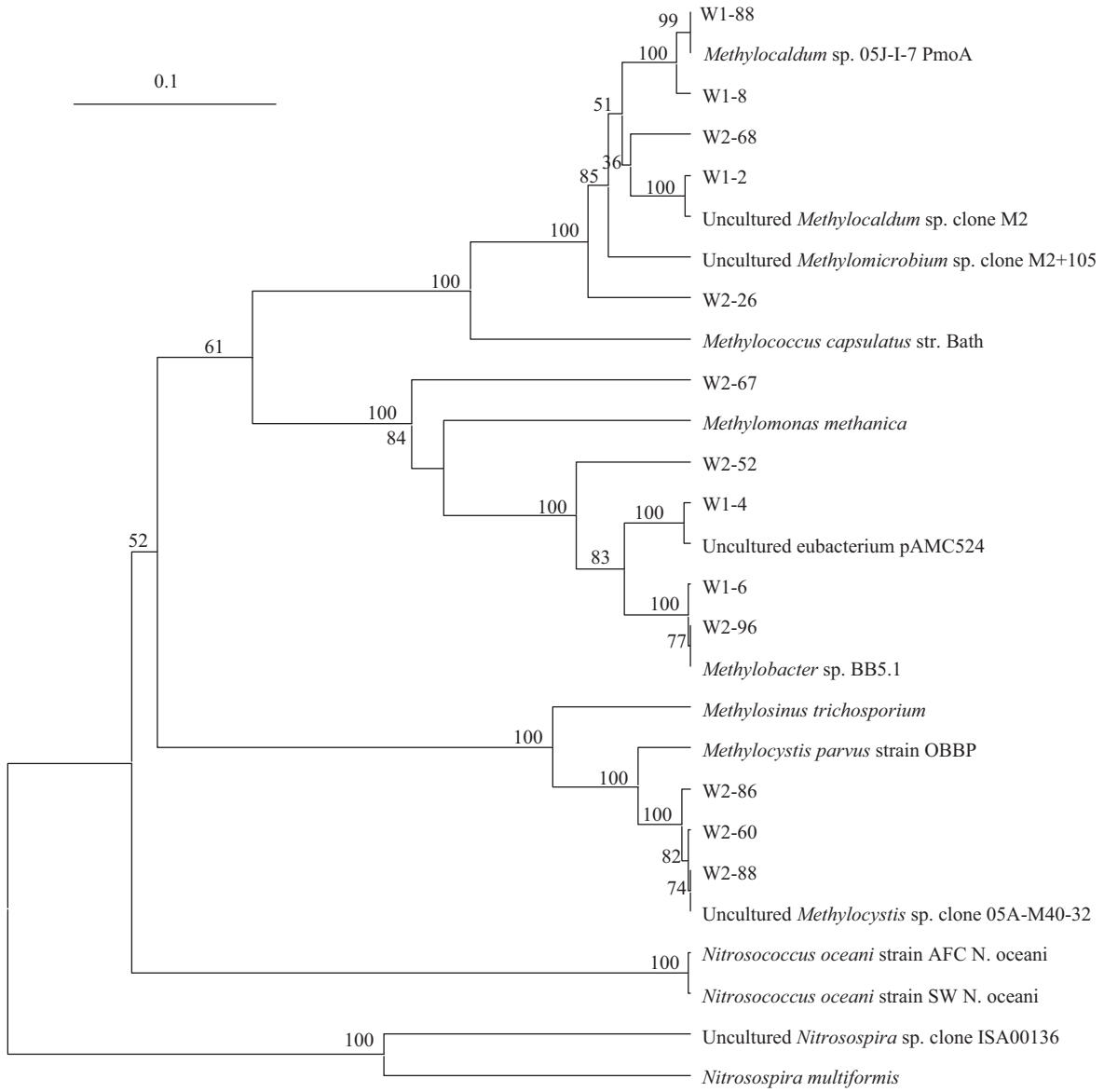

Fig. 3 Phylogenetic analysis of *pmoA* gene clone sequences retrieved from aged refuse with sterilized sewage sludge. Bootstrap values are shown near the clades. The bar indicates the estimated number of base changes per nucleotide sequence position.

Table 4 Correlations (r) between physicochemical properties and methane conversion rates of the aged refuse

Correlations		Without NMS	With NMS
Moisture content		0.252	0.039
pH		0.043	–0.025
ORP		–0.334	–0.083
Organic content		–0.424	–0.127
Particle size distribution	> 4 mm	0.531	0.279
	0.45–4 mm	–0.623	–0.486
	0.3–0.45 mm	–0.295	0.146
	0.2–0.3 mm	–0.316	–0.162
	0.15–0.2 mm	–0.117	0.155
	0.125–0.15 mm	–0.642	–0.358
	0.125 mm	–0.032	0.102

tions observed between them. On the whole, aged refuse with longer placement time had better methane oxidation capacity. Methane conversion rates of aged refuse with placement time of 18, 17 and 16 years were higher than others, especially with NMS (Table 3).

Figure 4 shows changes in methane oxidation rate of aged refuse domesticated with CH_4 in landfill simulating lysimeters. Methane oxidation rates of simulating lysimeters experienced rapid increases during 35 days. From day 20 to 24, methane oxidation rates of columns increased quickly, before experiencing a rapid decline. This fluctuation was more obvious for the lysimeter with NMS, with methane oxidation rates up to 209–254 g/(m^2·day) during day 22–24, but 157 g/(m^2·day) on day 25, and finally about 230 g/(m^2·day). Methane oxidation capability of the aged refuse reached 200–240 g/(m^2·day) after 35 days. Aged refuse showed better methane oxidation performance compared with previous reports.

Fluctuations in methane oxidation rates have also been mentioned in other research. Hilger et al. (2000b) hypothesized that a gradual accumulation of exopolymeric substances (EPS) contributed to decrease of methane uptake by clogging soil pores or limiting gas diffusion. Glucose concentrations of soil in columns sparged with synthetic landfill gas averaged 426 mg/kg dry soil, while average glucose concentration was 3.2 mg/kg dry soil for

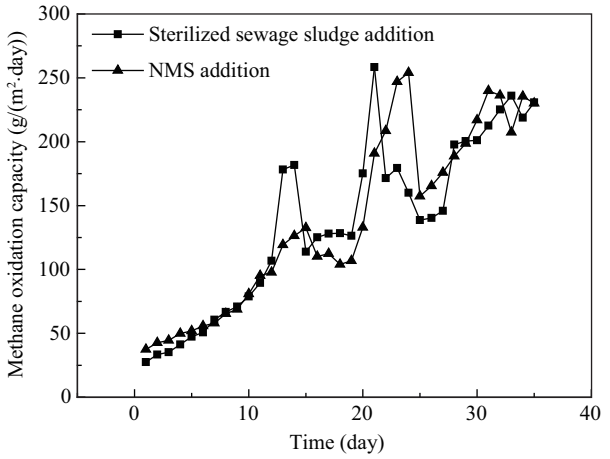

Fig. 4 Methane oxidation capabilities of landfill simulating lysimeters filled with aged refuse.

the column sparged with air. Highly viscous extracts were also measured from landfill gas sparged soil. Dedysh et al. (2005) found acetate addition inhibited methane oxidation over the short time, but the longer-term consequences were stimulatory on methane oxidation.

Both NMS and sterilized sewage sludge addition effectively enhanced methane oxidation ability of the aged refuse in this study. Methane oxidation rates of lysimeter with NMS were higher than that with sewage sludge at the beginning period. Sewage sludge addition resulted in more complicated nutrition condition for microbes, and a greater fluctuation in methane oxidation rates were observed. Both methods stimulated specific methanotroph growth with sparging landfill gas over the short time, NMS stimulated Type I methanotrophs growth, and organic matter tended to enrich Type II methanotrophs.

3 Conclusions

Methanotrophs were abundant in the aged refuse and were the dominant community. Both Type I and Type II methanotrophs were found in the aged refuse, and the clone sequences of the *pmoA* gene were similar to the *pmoA* gene of *Methylobacter, Methylocaldum* and *Methylocystis*. Two types of methanotrophs behaved differently under different nutrient conditions, with Type II methanotrophs only found in the presence of sterilized sewage sludge rather than NMS. Aged refuse with different placement time exhibited methane oxidation ability over the short time, with methane oxidation rates between 52.93% and 93.96% after 4 days with NMS addition compared to xanthozem with 5.63%. Landfill simulating lysimeter experiments suggested both NMS addition and sewage sludge addition enhanced methane oxidation ability of aged refuse effectively, and methane oxidation capabilities reached 200–240 g/(m^2·day) after 35 days.

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