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Diversity of methanotrophs in a simulated modified biocover reactor

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Abstract

A simulated landfill biocover microcosm consisting of a modifying ceramsite material and compost were investigated. Results show that the mixture can improve the material porosity and achieve a stable and highly efficient (100%) methane oxidation over an extended operating period. The diversity of the methanotrophic community in the microcosm was assessed. Type I methanotrophs were enhanced in the microcosm due to the increased air diffusion and distribution, whereas the microbial diversity and population density of type II methanotrophs were not significantly affected. Moreover, the type I methanotrophic community structure significantly varied with the reactor height, whereas that of type II methanotrophic communities did not exhibit a spatial variation. Phylogenetic analysis showed that type I methanotroph-based nested polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) resulted in the detection of eight different populations, most of which are related to *Methylobacter* sp.,whereas that of type II resulted in the detection of nine different populations, most of which are related to Methylocystaceae. Methanotrophic community analysis also indicated that a number of new methanotrophic genera not closely related to any known methanotrophic populations were present.

Key words: landfill biocover; methane oxidation; methanotrophs; PCR-DGGE; ceramsite

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Introduction

Methanotrophs, which are Gram-negative bacteria that utilize methane (CH_4) as their sole carbon and energy source, play an important role in the balance of atmospheric methane. CH_4 oxidation by methanotrophs in soil environments is estimated to account for 80% of the CH_4 consumed globally (Reeburgh et al., 1993).

CH_4 emission from landfills is the 4th highest anthropogenic CH_4 emission in the world and needs efficient technology for *in situ* oxidation (IPCC, 2007). Landfill cover soil has been proven capable of oxidizing CH_4 to produce carbon dioxide (CO_2) via methanotrophic bacteria (Perdikea et al., 2008; Whalen et al., 1990) in response to changes in landfill CH_4 management.

Traditionally, methanotrophs are classified into two general groups (types I and II) based on several characteristics, such as cell morphology, membrane arrangement, carbon assimilation pathway, and predominant phospholipid fatty acids (Hanson and Hanson, 1996). Type I methanotrophs, which belong to γ-Proteobacteria, consist of *Methylomonas, Methylococcus, Methylomicrobium, Methylosarcina*, *Methylosphaera, Methylothermus, Methylosoma, Methylohalobius, Methylocaldum,* and *Methylobacter* (Hanson and Hanson, 1996; Rahalkar et al., 2007). They can be further divided into two different groups, types Ia and Ib; the latter is composed of *Methylococcus, Methylocaldum,* and *Methylothermus*, whereas the former consists of the rest of the type I methanotrophs (Lee, 2008). The type II methanotrophs belong to α-Proteobacteria, including *Methylosinus, Methylocella, Methylocapsa,* and *Methylocystis*.

The growth of type I methanotrophs is significantly influenced by O_2. They also grow faster than the type II bacteria and are more sensitive to the environment, such as O_2 concentration and nitrogen. By contrast, higher CH_4 concentrations favor the growth of type II methanotrophs. Crossman et al. (2004) confirmed that type I methanotrophs are responsible for CH_4 oxidation in the top section of the landfill cap, whereas the type II methanotroph population is responsible for CH_4 oxidation in the deepest section of the landfill.

The application of landfill cover layers for CH_4 oxidation is mostly influenced by the available O_2 concentration (Nikiema et al., 2007). A number of researchers prefer the use of multilayer beds (Berger et al., 2005; Scheutz et al., 2009), compost (Streese and Stegmann, 2003), and soil (Parker et al., 2002) to reduce problems related to O_2 diffusion in landfill covers and open biofilters. These porous materials can improve O_2 distribution. However, they cannot overcome the problem of insufficient O_2 concentration because of the limited diffusion of atmospheric O_2. Generally, an oxygenated zone of only 0.60–0.80 m is observed (Crossman et al., 2004; Kallistova et al., 2005; Perera et al., 2002; Tagaris et al., 2003).

Most studies focused on single compost as landfill

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biocover material. CH_4 oxidation varies widely among different composts, such as leaves, domestic waste, and sludge (Einola et al., 2008; Wilshusen et al., 2004). Modifying the biocover material to improve CH_4 oxidation by methanotrophs in landfills is a feasible strategy.

In the current study, a modified biocover material with high CH_4 oxidation efficiency was used. The CH_4 oxidation efficiencies were assessed over an extended operating period. The methanotrophic population structure and activity were also investigated to determine the effect of O_2 on the microbial population. The study aimed to provide a sufficient air supply in the landfill biocover to promote the growth of methanotrophic bacteria and reduce the CH_4 emission from landfill sites.

The current work presents an investigation of the diversity of methanotrophic communities in response to changes in the CH_4:O_2 ratio (V/V) in a simulated modified biocover microcosm via the polymerase chain reaction (PCR) amplification of 16S rRNA with subsequent denaturing gradient gel electrophoresis (DGGE).

1 Materials and methods

1.1 Experimental setup

The experimental setup of the simulated biocover microcosm (Fig. 1) consisted of a 0.35 m × 0.20 m (height and inner diameter) PVC pipe. A 0.04 m layer of gravel was placed at the bottom of the column to ensure a homogenous distribution of the CH_4 gas pumped from a gas cylinder to simulate the landfill CH_4 produced from buried garbage.

The 0.30 m high (from the top of the reactor to the gravel layer) filling material was prepared from leaves mixed with chicken manure and soil at a ratio of 10:45:45 by weight and was allowed to mature for more than 6 months.

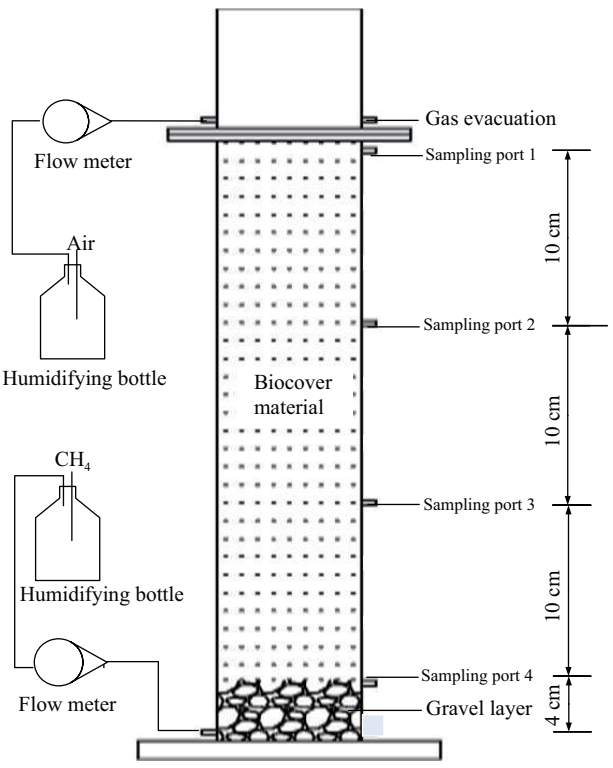

Fig. 1 Experimental setup.

The oxygen (O_2) consumption rate of the compost met the standard of the U.S. Composting Council (< 0.4 mg/hr) (1997). Before filling, the material was passed through a sieve with a 2-mm pore size to provide a fine and homogenous matrix with improved mass transfer. Furthermore, a 2-mm diameter ceramsite agitator was mixed with the compost at a ratio of 10:90 (m/m) to improve the porosity of the matrix. The bulk density of the mix biocover material was 310.0 kg/m^3.

The microcosm was equipped with a blower at the top to simulate natural wind blowing at the top of a landfill, with the air flow rate set at 200.0 mL/min. The flow rate of CH_4 pumping in through a gas inlet below the gravel layer was set at 4.0 mL/min. Both air and CH_4 were humidified before flowing into the microcosm to maintain the moisture of the biocover material. The microcosm had four gas-sampling ports at 10 cm intervals and an outlet port at the top. The gas was sampled twice a day to measure CH_4, O_2, and CO_2 levels using a gas chromatograph (Agilent 6890N, Agilent Technologies, Palo Alto, CA, USA) with a thermal conductivity detector. The detection program consisted of a carrier gas (N_2) with a 40 mL/min flow rate, a 120°C oven temperature, a 160°C injector temperature, and a 160°C detector temperature. The lowest detectable limit was ϕ (O_2) = 0.2%, ϕ (CH_4) = 0.1%, and ϕ (CO_2) = 0.1%.

The physical and chemical properties of the biocover material, including moisture content, pH, organic matter, and Cu^{2+}, were characterized before and after the experiment using standard methods (Soil and Plant Analysis Council, 1999).

1.2 Sampling and DNA extraction

Residues were collected from the top to the bottom layer at 10 cm intervals after a two-month operation at 25°C, which is the optimum temperature for methanotroph growth and CH_4 oxidation (Börjesson et al., 2004). Each sample had three replicates.

DNA extraction was performed using a FastDNA SPIN Kit for Soil (MP Biotechnology, USA) according to the manufacturer's protocol. The product from the DNA extraction was verified via electrophoresis in 1% agarose. The resultant DNA was stored at –20°C for further analysis.

1.3 PCR and DGGE analysis

The MethT1bR and MethT1dF primers (Table 1) were used to amplify the 16S rRNA sequences of type I methanotrophs present in the environmental DNA. The 16S rRNA sequences of type II methanotrophs were amplified using MethT2R and the bacteria-specific primer 27F (Table 1). All primers were at a final concentration of 0.2 μmol/L and the DNA templates were approximately 10 ng. PCR was performed on a thermocycler (TC-3000, High System, Barloworld Scientific Ltd., UK) under the following conditions: 2.5 min at 94˚C, followed by 10 cycles at 94°C for 30 sec, 56°C for 45 sec, and 72°C for 1 min, then

Table 1 Methanotroph-specific primers

Primer	Sequence[a]	Target genera	Reference
MethT1dF	5′-CCTTCGGGMGCYGACGAGT-3′	Type I methanotrophs	Wise et al., 1999
MethT1bR	5′-GATTCYMTGSATGTCAAGG-3′		Wise et al., 1999
27F	5′-AGAGTTTGATCMTGGCTCAG-3′	Type II methanotrophs	Lane and Stackebrandt, 1991
MethT2R	5′-CATCTCTGRCSAYCATACCGG-3′		Wise et al., 1999
358F	5′-CCTACGGGAGGCAGCAG-3′	All bacteria	Murray et al., 1996
517R	5′-ATTACCGCGGCTGCTGG-3′		Murray et al., 1996

[a] Y represents C or T; R represents A or G; M represents A or C; S represents C or G.

subsequently for 10 cycles at 94°C for 30 sec, 56°C for 1 min, and 72°C for 1.5 min. This was followed by 13 cycles at 94°C for 30 sec, 56°C for 1.25 min, and 72°C for 2.25 min. The final extension step was at 72°C for 7.5 min. The presence and size of the amplification products were determined by agarose gel electrophoresis and ethidium bromide staining.

A nested-PCR approach was used to profile the types I and II methanotrophic communities via DGGE. The PCR-amplified sequences were used as templates, and GC358F (with a 40 bp GC clamp added to the 5′ end) and 517R were used as primers (Table 1), which span the variable region 3 (V3 region) of the 16S rRNA gene (Murray et al., 1996). The thermocycling program for touchdown PCR was as follows: initial denaturation was performed at 94°C for 2.5 min and then at 94°C for 40 sec, followed by touchdown primer annealing from 72 to 55°C (the annealing temperature was decreased by 1°C for each cycle for the first 17 cycles to the touchdown temperature at 55°C). This was followed by an extension at 72°C for 1 min for each of the 17 cycles, 10 cycles at 94°C for 40 sec, 55°C for 1 min, and 72°C for 1.5 min. Ten more cycles were performed at 94°C for 40 sec, 55°C for 1.25 min, and 72°C for 2 min. The final extension step was 72°C for 7.5 min.

The nested-PCR products were analyzed on 1% agarose gels to confirm the presence of a single amplification of the expected size. DGGE analysis was conducted using a DCode system (170-9083, Bio-Rad, USA). Samples of the PCR product (30 μL) were loaded onto 6.5% (*W*/*V*) polyacrylamide gels in a 1× TAE buffer (40 mmol/L Tris base, 20 mmol/L sodium acetate, 1 mmol/L EDTA). The denaturing gradient was from 20% to 70% (for type I sequences) and 30% to 60% (for type II sequences). The denaturant was prepared using 7 mol/L urea and 40% deionized formamide. Gels were run in 1× TAE buffer at 100 V for 12 hr at 60°C and visualized via SYBR-Gold staining. The gel was photographed using a gel photo system (GelDoc 2000, BioRad, CA, USA). The photographs were analyzed using a BioRad Quantity One software package.

Specific PCR-DGGE bands were manually excised from the gel, suspended in 50 μL sterile water and incubated overnight at 4°C. The PCR-DGGE protocol was then repeated using the excised bands as templates until only a single band was detected. The last PCR cycle was performed without the GC-clamp attached to the forward primer 358F and 517R. The resulting PCR products were sent to SinoGenoMax Co., Ltd. (Beijing, China) for purification and sequencing.

1.4 Sequence similarity and phylogenetic analysis

Sequence similarity searches were performed using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) program (http://www.ncbi.nlm.nih.gov/BLAST/). Reference sequences were obtained from the GenBank database.

Band patterns from PCR-DGGE fingerprints were analyzed via the unweighted pair group method with arithmetic mean (UPGMA) using DNAMAN v. 4.1 (Lynnon Biosoft, USA). The scanned gel images were analyzed using the Labwork 4.6 software (Media Cybernetics, USA). Binary sequences were generated from individual fingerprints by determining the number and position of the bands compared to the total number of band positions using the Labwork software.

The richness (R) and the Shannon-Weaver index of general diversity (H) were calculated from the DGGE band profiles to evaluate the microbial community at different reactor heights. The R for all the DGGE band patterns was calculated using the respective band data analyzed by Labwork 4.6. R is equal to the total number of species in the DDGE profile because each band in the profile is likely derived from one phylogenetically distinct population.

The index H was calculated on the basis of the bands on the gel lane that were applied to generate the dendrograms using the band intensities, as determined by the peak heights in the densitometric curves (Ampe and Miambi, 2000; Nübel et al., 1999). The following equation (Shannon and Weaver, 1963) was used:

$$H = -\sum (P_i \times \ln(P_i)) \quad (1)$$

where, P_i is the relative probability of the bands in a gel lane, which can be calculated from:

$$P_i = n_i/N \quad (2)$$

where, n_i is the height of peak i and N is the sum of all peak heights in the densitometric curve.

The relative abundance of each band was considered as a variable. Similarity matrices of the methanotrophs revealed by the DGGE patterns were calculated with the squared Euclidean distance coefficient using the Statistical Package for Social Science (SPSS) 13.0 software (SPSS Inc., Chicago, USA).

1.5 Accession numbers for the nucleotide sequences

The partial sequences of the 16S rRNA genes of the methanotrophic bacteria obtained in the current study are

available in the NCBI database under accession numbers HM601439–HM601452.

2 Results and discussion

2.1 Methane oxidation capacity in a simulated landfill biocover reactor

In the simulated biocover microcosm, the CH_4 concentration in the upper part of the reactor decreased from 6.13% to 0% after a 25-day operating time. The concentration was stable thereafter, indicating that the CH_4 oxidation efficiency reached 100% after a one-month operation (data not shown).

The average O_2 concentration detected from all layers (except the bottom layer) was higher than 10% and hardly fluctuated at different layers. The CO_2 concentration in the biocover reactor layers varied from 3.1% to 13.5% from top to bottom, indicating an enhanced oxidation of CH_4 to CO_2 because of the sufficient O_2 provided to the biocover microcosm (Table 2). The biocover was found capable of overcoming the problem of limited O_2 transport using high porosity materials such as compost and a ceramsite agitator, among others, thereby allowing methanotrophic populations to thrive in the microcosm.

Physicochemical analysis shows that the pH at different layers changed slightly from the beginning of the operation (pH 7.99) to the end of the experiment (pH 8.07–8.58), and shows an increasing trend from the bottom to the top layer (Table 3). Therefore, CH_4 oxidation by methanotrophs can change the pH of biocover soils. Moreover, because the concentration of CH_4 at the bottom was higher than at the top, a higher amount of CH_4 was oxidized and more CO_2 was produced, leading to a decrease in pH from top to bottom. pH largely depends on the matrix used as well as the metabolic pathway of specific microorganisms. The recommended pH for methanotrophs in literature ranges from 5.5 to 8.5, with the optimum levels at 6.5–7.5 (Hanson and Hanson, 1996; Le Mer and Roger, 2001).

The organic matter (OM) content in the biocover matrix had no significant change compared with that of the original material (28.1%). It varied within a range of 27.2%–27.9% from the bottom to the top layer. This high OM content is a good promoter of CH_4 oxidation (Kightley et al., 1995).

The moisture content of the biocover reactor layers (40.1%–46.7%) after the experiment was slightly higher than at the beginning of the experiment (40.0%) because of CH_4 humidification. Scheutz and Kjeldsen (2004) studied the factors affecting CH_4 oxidation in landfill cover soil, and reported that the optimum moisture for CH_4 oxidation is 18%–25%. The efficiency of CH_4 oxidation is greatly decreased when the moisture is above 35% because of the decreased air capacity of the soils. However, we obtained a high CH_4 efficiency under a relatively high moisture content (above 40%), which is beneficial to the growth of microorganisms. The difference between the two studies may be due to the significant difference in OM content between the soil and the compost; in the former, OM was only 1%–2%. The difference in OM content certainly resulted in a big difference in the field capacity. Therefore, using the field capacity as an index during matrix optimization for CH_4 bio-oxidation would be reasonable. The efficiency of CH_4 oxidation would be increased by amending $CuCl_2$ to paddy soils. Cu^{2+} influences the ratio of the membrane-bound (particulate) CH_4 monooxygenase enzyme (pMMO) to the cytoplasmic (soluble) CH_4 monooxygenase enzyme (sMMO) in methanotrophs (Auman and Lidstrom, 2002; Mohanty et al., 2000). The result shows that the Cu^{2+} concentration changed little during the experiment, indicating that it was not leached due to the high pH in the reactor.

Table 2 Characteristics of gas samples in different layers of the microcosm

Height (cm)	O_2 (%)	CO_2 (%)	CH_4 (%)
0	19.3 ± 1.0	3.1 ± 0.7	0 ± 0.00
10	16.5 ± 0.8	5.6 ± 0.4	2.42 ± 1.20
20	10.6 ± 0.8	8.9 ± 0.5	5.87 ± 0.90
30	0 ± 0.1	13.5 ± 0.9	9.59 ± 1.10

Values are given as mean ± standard deviation (n = 5).

2.2 Diversity and community structure of the methanotrophs

The bacterial diversity of methanotrophs at different heights of the simulated biocover reactor was characterized via DGGE of 16S rRNA. Figure 2 shows the microbial community structure of the methanotrophs at different microcosm heights after a one-month operation.

A total of 23 bands (R = 23) for the type I methanotrophs and 25 bands (R = 25) for the type II methanotrophs (Table 4) were retrieved, indicating that 23 species of type I methanotrophs and 25 species of type II methanotrophs were present in the tested samples. The type I population varied with the location in the cover layers, given that most of the prominent bands were obtained at the 10 cm height.

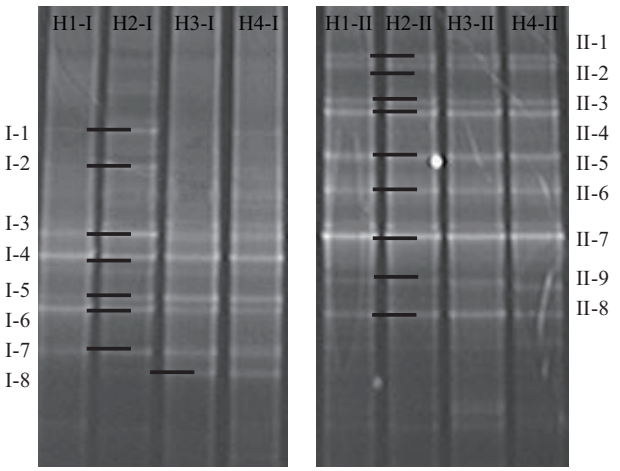

Fig. 2 DGGE band profiles of the amplified 16S rDNA fragments from four samples in the reactor. The labels of H1-I to H4-I, and H1-II to H4-II at the top of the lanes represent communities of type I and II methanotrophs from samples at heights from 0 to 30 cm, respectively. Black bands labeled on the gels as I-1 to I-8, and II-1 to II-9 were excised and subjected to sequencing and identification.

Table 3 Characteristics of solid samples in different layers of the microcosm

Height (cm)	pH	Moisture content (%)	Organic matter (%)	Cu^{2+} (mg/kg)
0	8.07 ± 0.4	42.3 ± 2.1	27.2 ± 0.5	54.24 ± 1.5
10	8.23 ± 0.5	42.5 ± 1.0	27.4 ± 0.9	53.52 ± 1.2
20	8.57 ± 0.3	40.1 ± 1.2	27.9 ± 0.7	53.72 ± 0.9
30	8.58 ± 0.7	46.7 ± 1.3	27.5 ± 1.0	51.81 ± 0.1
Original*	7.99 ± 0.4	40.0 ± 1.2	28.1 ± 0.8	53.20 ± 2.0

Values are given as mean ± standard deviation (n = 3).
* The original soil was sampled before filling the microcosm.

Table 4 Comparison of richness and the microbial diversity of methanotrophs

Lane No.	Richness (R)	Microbial diversity (Shannon-Weaver index, H)
H1-I	16	2.59
H2-I	21	2.84
H3-I	19	2.83
H4-I	23	3.02
H1-II	25	2.86
H2-II	25	2.88
H3-II	25	2.95
H4-II	24	2.85

As for type II, no significant difference in the number of bands as well as in the location was observed, revealing that most type II methanotrophs have wide adaptability under landfill biocover conditions while type I methanotrophs are sensitive to O_2 and CH_4 concentrations.

The analysis indicates that sufficient O_2 promoted the growth and influenced the structure of type I methanotrophs but had little effect on the type II methanotrophic structure and population density. O_2 is a limiting factor for type I methanotrophs, which grow faster than type II; thus, type I methanotrophs are more sensitive to the environment (Amaral et al., 1995; Graham et al., 1993). Both type I and type II methanotrophs produce pMMO, which is thought to proliferate under low CH_4:O_2 ratios (V/V) (Auman and Lidstrom, 2002; Costello et al., 2002; Hanson and Hanson, 1996). By contrast, most type II methanotrophs produce not only the pMMO enzyme but also a more reactive sMMO, which explains the fast acclimation of type II methanotrophs to the environment and their existence in a wide range of CH_4:O_2 ratios (V/V) (Costello et al., 2002). Börjesson et al. (2004) also proved that most type I methanotrophs favor a low CH_4 concentration (< 0.016%, V/V) and a high O_2 concentration (close to that of air), whereas type II methanotrophs favor a 1%–7% (V/V) CH_4 concentration and 15%–18% (V/V) O_2 concentration. Our study confirmed that conclusion as almost no spatial variation for type II methanotroph classes was observed. Meanwhile, a high O_2 concentration and low CH_4 concentration favor more classes of type I methanotrophs, for instance at the height of 10 cm.

The H index showed no significant change in type II methanotrophs (ranging from 2.85 to 2.95), but greatly changed in type I methanotrophs (ranging from 2.59 to 3.02), indicating that a greater significant difference in the microbial diversity of type I populations was observed compared with those of type II at different reactor heights.

2.3 Cluster analysis

Cluster analysis (Fig. 3) reveals that the average branch length for the type I population was longer than that of type II, indicating that the CH_4 and O_2 concentrations at different heights exerted a greater effect on the type I populations. Furthermore, for type I, lanes H3-I (20 cm) and H4-I (30 cm) show a high level of similarity, whereas H2-I (10 cm) shows the least similarity with the other three layers. For the type II population, H1-II and H2-II show the same similarity level, and they then combine with H3-II and H4-II. This result indicates that the changes in the type I and type II methanotrophic communities with height (and thus, with different CH_4:O_2 ratios (V/V)) did not follow the same trend (Fig. 3). Most of the type II population was found to favor the landfill conditions and only slightly changed, whereas the change in the landfill cover greatly affected the type I classes. However, dominant type I methanotrophs were also found in the landfill cover soil. This result is different from that reported by Börjesson et al. (2004), who proved that most type I methanotrophs favor a low CH_4 concentration (< 0.016%, V/V) and high O_2 concentration.

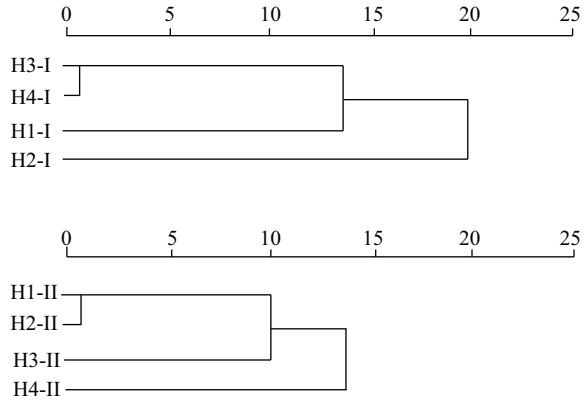

Fig. 3 Hierarchical cluster analysis results of the DGGE profiles reflecting the structural diversity of the methanotrophic bacterial community.

2.4 Analysis of phylogenetic relationships

As shown in Fig. 2, eight bands were manually excised from the denaturing gels containing the 16S rRNA gene fragments of type I methanotrophic bacteria and sequenced. The detected populations are related to several methanotrophic genera, such as *Methylobacter* sp., which is the most prevalent species. Band I-1 represents *Methylasarcina* sp., and band I-7 and I-8 represent *Methylomicrobium* sp. Some bands are not related to any exact genus but still belong to the type I methanotroph family

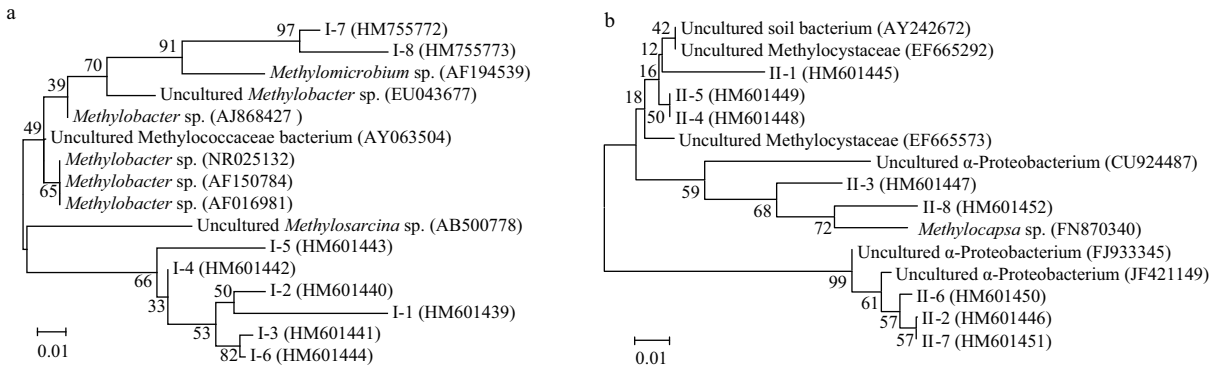

Fig. 4 Neighbor-joining tree depicting the phylogenetic relationships of the type I (a) and type II (b) methanotrophic populations detected in the reactor via nested PCR-DGGE.

Methylococcaceae (Fig. 4a).

Similarly, nine different bands were manually excised from the denaturing gels containing 16S rRNA gene fragments of type II methanotrophic bacteria and sequenced. The results show that these species have high phylogenetic similarities. Band II-8 is *Methylocapsa* sp. Bands II-1, II-4 and II-5 are related to the Methylocystaceae family but do not belong to any exact genus. Methylocystaceae was the most predominant type II methanotroph family identified (Stralis-Pavese et al., 2006). The rest belong to the α-Proteobacterium family or uncultured soil bacterium (Fig. 4b).

The most abundant type I population is *Methylobacter* sp., whereas that of type II belongs to Methylocystaceae bacterium. Other studies also found that these two species are extremely abundant in the top layer of landfill covers where O_2 is sufficient. Approximately 94.4%–96.1% of the type I methanotrophs are *Methylobacter* sp., whereas Methylocystaceae account for 97.9%–99.1% of the total type II methanotrophs (McDonald and Murrell, 1997; Stralis-Pavese et al., 2006; Vallaeys et al., 1997). Although the material applied in the current simulated biocover microcosm was specially made to improve the O_2 transfer for better CH_4 oxidation, the dominant populations for both type I and type II show no significant differences from those of landfill covers consisting of soil or clay.

3 Conclusions

We used ceramsite and compost as composite landfill biocover. The mixture can improve the porosity of the material to achieve good and effective oxidation. Moreover, ceramsite has a low density and can be reused. The addition of ceramsite to the material is therefore expected to be more cost-effective than using compost alone.

The high CH_4 oxidation rate in the modified biocover reactor can perhaps be attributed to the vast metabolic diversity of methanotrophs, as evidenced by the predominance of both types I and II methanotrophs in the system.

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