

# Tobermolite effects on methane removal activity and microbial community of a lab-scale soil biocover

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**Abstract** Three identical lab-scale biocovers were packed with an engineered soil (BC 1), tobermolite only (BC 2), and a mixture of the soil and tobermolite (BC 3), and were operated at an inlet load of 338–400 g-CH<sub>4</sub> m<sup>-2</sup> d<sup>-1</sup> and a space velocity of 0.12 h<sup>-1</sup>. The methane removal capacity was 293 ± 47 g-CH<sub>4</sub> m<sup>-2</sup> d<sup>-1</sup> in steady state in the BC 3, which was significantly higher than those in the BC 1 and BC 2 (106 ± 24 and 114 ± 48 g-CH<sub>4</sub> m<sup>-2</sup> d<sup>-1</sup>, respectively). Quantitative PCR indicated that bacterial and methanotrophic densities (6.62–6.78 × 10<sup>7</sup> 16S rDNA gene copy number g-dry sample<sup>-1</sup> and 1.37–2.23 × 10<sup>7</sup> *pmoA* gene copy number g-dry sample<sup>-1</sup> in the BC 1 and BC 3, respectively) were significantly higher than those in the BC 2. Ribosomal tag pyrosequencing showed that methanotrophs comprised approximately 60 % of the bacterial community in the BC 2 and BC 3, while they only comprised 43 % in the BC 1. The engineered soil favored the growth of total bacteria including methanotrophs, while the presence of tobermolite enhanced the relative abundance of methanotrophs, resulting in an improved habitat for methanotrophs as well as greater methane mitigation performance in the mixture. Moreover, a batch experiment indicated that the soil and tobermolite mixture could display

a stable methane oxidation level over wide temperature (20–40 °C, at least 38 μmol g-dry sample<sup>-1</sup> h<sup>-1</sup>) and pH (5–8, at least 61 μmol g-dry sample<sup>-1</sup> h<sup>-1</sup>) ranges. In conclusion, the soil and tobermolite mixture is promising for methane mitigation.

**Keywords** Methane mitigation · Biocover · Soil · Tobermolite · Methanotrophs

## Introduction

Landfills are a significant anthropogenic source of methane (CH<sub>4</sub>) with global emission estimates of approximately 500–800 Mt CO<sub>2</sub>-eq year<sup>-1</sup> [3]. Landfill-derived methane is primarily generated by the anaerobic decomposition of landfill solid waste via methanogenesis, which was historically the largest source of global methane emissions from the waste sector [4, 8]. Landfill methane mitigation technologies include gas collection, incineration, and biotic methane mitigation systems (e.g., landfill biocover and biofilters) [4]. Biocover systems have been used to mitigate methane mediated with biological oxidation by methanotrophs. Microbial methane oxidation is recognized as being globally important, accounting for approximately 80 % of global methane consumption [30]. Earlier field studies have demonstrated that methane oxidation rates can be >200 g-CH<sub>4</sub> m<sup>-2</sup> d<sup>-1</sup> in engineered landfill biocover systems [4, 5]. Recently, the approach of enhancing the methanotrophic activity has been proposed for greater performance of the systems.

The biocover performance is dependent on the packing materials, temperature, pH, moisture content, methane and oxygen concentrations, and nutrients [31]. Methanotrophs must become well established and active in landfill

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biocover systems to establish successful methane mitigation. It is generally accepted that the physico-chemical properties of the packing materials are identified as the key parameters of the packing materials for optimum methane consumption by methanotrophs [6, 16, 31]. Conventional biocover systems have been operated using landfill cover soils manipulated with inorganic or organic materials such as mature compost, earthworm cast, powdered activated carbon, saporlite, and granular-activated carbon (GAC) to improve the establishment and activity of methanotrophs [17, 27, 31]. Although the previous studies reported that the methane oxidation and methanotrophic population levels were enhanced by introducing those materials into soils, biocover systems still face the problems of how to improve both the microbial establishment and the systematic performance that can determine their success. Thus, an alternative material is required to satisfy those factors for great methane mitigation in biocover systems.

In the present study, three lab-scale biocover systems were prepared using the soil mixture (BC 1), tobermolite (BC 2), and soil and tobermolite mixture (BC 3) to develop an efficient biocover system for methane removal. Tobermolite was used as a novel packing material and its quality was evaluated using the removal efficiency and the capacity of the methane by comparing these values with those of a conventional biocover system (BC 1). To investigate the bacterial and methanotrophic stability and establishment, their populations were analyzed using quantitative real-time PCR (qRT-PCR) and the relative abundance and community structure were assessed using pyrosequencing assay. In addition, batch-scale experiments were employed to evaluate the suitability of a packing material with regard to environmental changes such as temperature and pH, which should be considered when using biocover materials in the field.

## Materials and methods

### Packing material preparation for lab-scale biocover systems

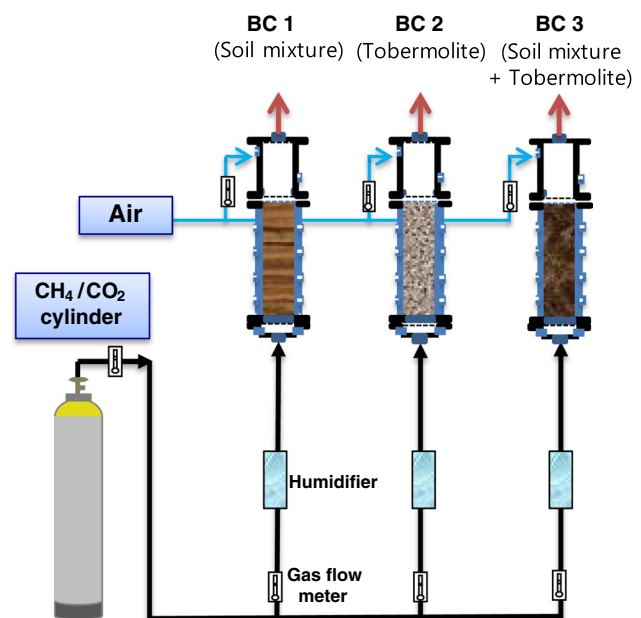
Soil was collected from landfill cover soil at a depth of 10–30 cm from the surface in Gapyeong-gun, Gyeonggi-do, South Korea [20]. Earthworm cast was obtained from the Nangi Municipal Sewage Treatment Plant, Goyang, Gyeonggi-do, South Korea [25]. The landfill cover soil and earthworm cast were sieved with a 2-mm mesh prior to use. GAC with a diameter of 4–8 mm was purchased from Jaeil Carbon, Co. Ltd., Yong-In, South Korea. Commercial saporlite with a diameter of 3 mm was obtained from Kyungseo Co. Ltd., Seoul, South Korea. Tobermolite with a diameter of 3–8 mm was purchased from JawooBio, Daejeon, South

Korea. The tobermolite was washed 4–5 times with tap water before use. All of the packing materials were stored at room temperature prior to use.

Three mixtures of the packing materials were prepared to develop the efficient biocover systems. For BC 1 and BC 3, the earthworm cast was added to the biocovers as inoculums of methanotrophs. BC 1 was filled with a soil mixture of landfill soil, GAC, earthworm cast, and saporlite (4:2:1:1, w/w) to simulate a conventional biocover system, while BC 3 was packed with a mixture of tobermolite, landfill soil, and earthworm cast (2:1:1, w/w). For BC 2, the earthworm cast was mixed with distilled water (1:1, v/v) and its supernatant was used as an inoculum of the methanotrophs for BC 2. The washed tobermolite was suspended with the supernatant to inoculate the methanotrophs into the BC 2 for 1 day and the inoculated tobermolite was packed into BC 2. All of the packing mixtures were completely mixed before adding into the biocovers.

### Lab-scale biocover systems

Three identical biocover systems were set up with acrylic columns to simulate landfill cover environments. Figure 1 shows a schematic diagram of the biocover systems. The lab-scale biocover systems consisted of two sections (the packing and ventilating sections) with an inner diameter of 8 cm. The packing height and volume of the packing section were 50 cm and 2.5 L, respectively. It had a perforated plate at the bottom. A gas inlet port was located at the bottom of the biocover. The ventilating section (with



**Fig. 1** The schematic diagram of the methane-removing biocover systems

a height of 15 cm) was assembled on the packing section and it had air inlet and gas outlet ports. A synthetic gas of methane/carbon dioxide (2:3, v/v) was commercially purchased from Seoul Special Gas Inc., Seoul, South Korea, and it was passed through a 50-cm long humidifier before introducing it into the biocover. The synthetic gas was controlled with a gas flow meter, but it could have fluctuated since the cylinder has high pressure of gas. Thus, we measured inlet and outlet gas concentrations to obtain correct results. The gas was continuously fed into the biocover systems at a flow rate of 5 ml min<sup>-1</sup> (with a space velocity of 0.12 h<sup>-1</sup>). Compressed air entered into the top of the ventilating section at a flow rate of 20 ml min<sup>-1</sup>. The biocovers were operated for 134 days at room temperature. The methane oxidation efficiencies and capacities were calculated from the inlet and outlet methane fluxes of the biocover systems. The methane fluxes were calculated from the flow rates and the methane concentrations at the inlet and outlet. The operating and maintenance conditions were previously described by Kim et al. [18]. The moisture content of the biocovers was adjusted to 20 % with distilled water.

#### Gas analysis

The methane concentration was monitored at the inlet and outlet for 39 days using gas chromatography (GC, 6850 N, Agilent Technologies Inc., Santa Clara, USA) equipped with a flame ionization detector (FID) and a wax column (30 m × 0.32 mm × 0.25 μm, Supelco Inc., Bellefonte, PA, USA), as previously described by Lee et al. [20].

#### Nucleic acid extraction and qRT-PCR

After a 39-day operation period, the packing mixtures were collected in duplicate from the top (0–10 cm) layer of the surface of each biocover for analysis of the bacterial and methanotrophic community structures. The collected packing mixtures were immediately stored at -70 °C prior to use. Genomic DNA was extracted from 0.5 g of the packing mixture from each biocover in duplicate. DNA was extracted using a NucleoSpin Soil kit (Macherey–Nagel GmbH & Co. KG, Düren, Germany) with a modification wherein the samples were disrupted using a BeadBeater-8 system (BioSpec Inc., Bartlesville, OK, USA) at 5,000 rpm for 30 s. The DNA was suspended in 100 μL of the elution buffer and stored at -20 °C before use. The DNA was quantified using an ASP-2680 spectrophotometer (ACT-Gene Inc., Piscataway, NJ, USA).

qRT-PCR was used to quantify the total bacteria and methanotrophs using the primer sets of 304F & 805R and A189f & mb661r, respectively. The 304F and 805R primer set targets 16S rDNA for total bacteria [18] and the A189f and mb661r primer set targets the α-subunit of

the particulate methane monooxygenase gene (*pmoA*) for methanotrophs [29].

*Escherichia coli* and *Methylobacter luteus* (NCIMB11914) were used to establish standard curves for the quantitative detection of the total bacteria and methanotrophs, respectively. The PCR mixtures and reaction conditions were previously described by Kim et al. [19]. The 16S rDNA and *pmoA* copy numbers were calculated by measuring the DNA concentrations. The DNA standards of the total bacteria and the methanotrophs were 10<sup>4</sup>–10<sup>8</sup> and 10<sup>3</sup>–10<sup>7</sup> copies, respectively. qRT-PCR was performed in duplicate.

#### 16S rRNA-based analysis of bacterial communities using pyrosequencing

The pyrosequencing analysis was performed in duplicate using the samples collected from the top layer of each biocover after a 39-day operation period. For PCR, the primer set of 340F and 805R was used to amplify the 340–805 region of the 16S rRNA gene (positions based on *Escherichia coli*) containing the V3 and V4 regions. Six different composite primer sets were made based on the 340 F-805R set for multiplex pyrosequencing; the composite forward primer **340F** 5'-CCATCTCATCCCTGCGTGTCT CCGAC-TCAG-NNNNNNNNNN-TCCTACGGGAGG CAGCAG-3' and the composite reverse primer **805R** 5'-CC TATCCCCTGTGTGCCTTGGCAGTC-TCAG-GACTACHV GGGTATCTAATCC-3'. The first sequence of 26-bp is the Genome Sequencer FLX Titanium adaptors A and B. The sequence in italics is the four-base library key. NNNNNNN NNN is the 10-bp multiplex identifier tag (provided from Macrogen co. Ltd, Seoul, Korea), and the last is the primer targeting the 16S rRNA gene [18]. For each sample, four independent PCR mixtures were prepared in parallel to avoid PCR bias. The PCR mixtures and conditions were previously described in detail by Kim et al. [18]. The purified DNA concentrations were measured using an ASP-2680 spectrophotometer (ACTGene Inc.). Equal amounts of the purified DNAs were combined in a single tube and sent to Macrogen Incorporation (Seoul, South Korea) to be run on a Genome Sequencer 454 FLX Titanium system (Roche Diagnostics Inc., Mannheim, Germany).

#### Pyrosequencing data analysis

For high-quality sequences, the primer sites of the sequences were trimmed and the low-quality and chimera sequences were excluded. The primer sites and low-quality sequences (length <400 nt, average quality score <25, and with an ambiguity) were removed using the RDP pyrosequencing pipeline [7]. Any possible chimeras were removed

using Black Box Chimera Check software [11] with the default setting. In this study, the RDP pyrosequencing pipeline was used to analyze the pyrosequencing data. The most abundant sequence of each OTU (97 % similarity) was BLAST searched against the RDP database (Release 10) to determine the phylogeny of the OTU and then the Chao1 richness estimates (Chao1) and Shannon–Weaver diversity indices ( $H'$ ) were calculated. Pyrosequencing reads were taxonomically assigned using the RDP classifier of the pipeline with bootstrap values more than 80 %. The RDP classifier provides rapid and independent taxonomic classifications from domain to genus [18]. The classification results were analyzed using MEGAN software version 4.50 [13] for community analysis at the genus level. Bacterial communities were compared using the function of “Goodall-normalized” in the MEGAN software. The sequences were identified at the species level when the similarity was greater than 99 %. The pyrosequencing reads obtained in this study were deposited into the DNA DataBank of Japan (DDBJ) Sequence Read Archive (<http://trace.ddbj.nig.ac.jp/dra>) under accession no. DRA001850.

#### Statistical analysis

Relative abundances of OTUs were used for principal component analysis (PCA), and redundancy analysis (RDA) with UniFrac [21] and CANOCO 4.5 software for Windows (SCIENTIA software, New York, NY, USA). A statistical analysis of variance (ANOVA) was performed for validation of the qRT-PCR abundance and methane oxidation rates of each group using the SPSS 20 for Windows (SPSS Inc., Chicago, USA) program. The level of significance was  $p = 0.05$  in all of the statistical analyses.

#### Effects of environmental changes on the methane oxidation of the biocovers

To evaluate the effects of environmental changes such as temperature and pH on the methane oxidation of BC 3, the packing mixtures were collected at the end of the experimental period. The packing mixtures were completely mixed before sampling. To investigate the effects of the temperature on the methane oxidation of BC 3, 20 g of the sample was added into 600-ml serum bottles containing 10 ml of a nitrate mineral salt (NMS) medium. The bottles were sealed with butyl rubbers and parafilm and then methane was injected into each bottle at a final concentration of 5 % (v/v) from a methane cylinder (99 %, Seoul Special Gases, Seoul, Korea). The bottles were incubated at 15, 20, 25, 30, 35, and 40 °C with an agitation of 150 rpm. The NMS medium consisted of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  1 g,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  0.295 g,  $\text{KNO}_3$  1 g,  $\text{KH}_2\text{PO}_4$  0.26 g,  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  0.41 g, and  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  0.0025 g per 1-L; plus trace

elements of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  500  $\mu\text{g}$ ,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  400  $\mu\text{g}$ ,  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  20  $\mu\text{g}$ ,  $\text{H}_3\text{BO}_3$  15  $\mu\text{g}$ ,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  50  $\mu\text{g}$ ,  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$  10  $\mu\text{g}$ , and EDTA 250  $\mu\text{g}$ .

To evaluate the effects of the pH on the methane oxidation of BC 3, the bottles were prepared as described above except that NMS media were adjusted with 0.2 M  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  and 0.2 M  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  solutions for the desired pH levels (pH 5, 6, 7, and 8). The serum bottles were incubated at 30 °C with an agitation of 150 rpm. The concentration of methane in the headspace was periodically monitored using a gas-tight syringe. All of the experiments were performed in triplicate.

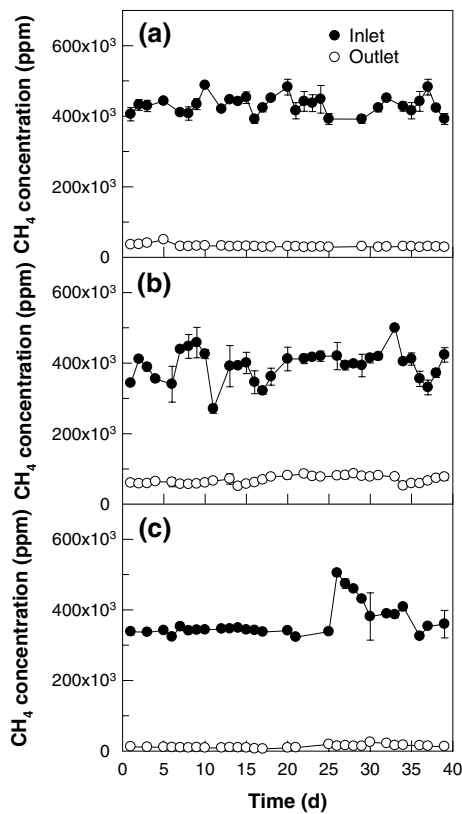
## Results

### Methane removal performance of lab-scale biocover systems

Lab-scale biocover systems were set up to simulate methane-removing landfill cover environments and three packing mixtures were used to develop an efficient biocover system for methane removal. Figure 2 shows the inlet and outlet methane concentrations and the removal efficiencies of the biocover systems for 39 days. All of the biocover systems immediately removed methane without an acclimation period and they were stably operated during a 39-day experimental period (Fig. 2). The average methane removal efficiencies of BC 1, BC 2, and BC 3 were  $27 \pm 9$ ,  $30 \pm 13$ , and  $67 \pm 11$  %, respectively, and their maximum methane removal efficiencies were 37, 50, and 83 %, respectively, at an inlet methane concentration of 40 % (v/v) and a space velocity of  $0.12 \text{ h}^{-1}$  (a residence time of 8.3 h). The methane removal capacities of BC 1, BC 2, and BC 3 were  $106.0 \pm 24.3$ ,  $111.4 \pm 48.0$ , and  $292.6 \pm 46.9 \text{ g-CH}_4 \text{ m}^{-2} \text{ d}^{-1}$ , respectively, at an inlet load of  $337.8\text{--}399.6 \text{ g-CH}_4 \text{ m}^{-2} \text{ d}^{-1}$  in a steady state condition.

### Comparison of the bacterial and methanotrophic populations in the biocover systems

To evaluate the dynamics of the bacterial and methanotrophic populations in the biocover systems with different packing mixtures, the qRT-PCR method was employed for quantitative analyses of their population levels after the 39-day experimental period. The qRT-PCR results showed that the bacterial and methanotrophic population levels changed with different packing mixtures of the systems (Fig. 3). The bacterial populations of BC 1, BC 2, and BC 3 were  $6.78 \pm 1.74$ ,  $0.67 \pm 0.08$ , and  $6.62 \pm 2.67 \times 10^7$  16S rDNA gene copy number g-dry sample<sup>-1</sup>, respectively, showing that the bacterial densities of BC 1 and BC 3 were significantly higher than that

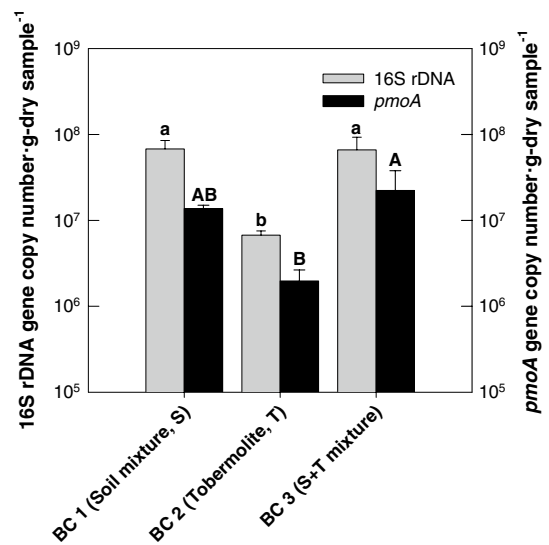


**Fig. 2** Removal efficiencies of methane for the lab-scale biocover systems during a 39-day operation period. **a** BC 1. **b** BC 2. **c** BC 3

of BC 2 ( $p < 0.05$ ). In addition, the methanotrophic population levels of BC 1, BC 2, and BC 3 were  $1.37 \pm 0.13$ ,  $0.20 \pm 0.07$ , and  $2.23 \pm 1.56 \times 10^7$  *pmoA* gene copy number g-dry sample<sup>-1</sup>, respectively, indicating that BC 1 and BC 3 had higher methanotrophic densities than that of BC 2 ( $p < 0.05$ ).

**Analysis of the bacterial communities in the biocover systems**

The bacterial community structures in the biocover systems with different packing mixtures were investigated using pyrosequencing assay. Table 2 lists the number of observed OTUs, Chao1,  $H'$ , and evenness indices at a 3 % dissimilarity level. The number of OTUs, Chao1, and evenness indices of the bacterial communities was 258–352, 424–584, and 0.61–0.67, respectively, and there were similar levels among the biocover systems ( $p > 0.05$ ). However, the  $H'$  indices of the bacterial communities were  $3.94 \pm 0.04$ ,  $3.39 \pm 0.04$ , and  $3.58 \pm 0.05$  in BC 1, BC 2, and BC 3, respectively, and those values were significantly different among the systems ( $p < 0.05$ ). A total of five bacterial genera (*Dokdonella*, *Pseudoxanthomonas*, *Sphingomonas*, *Methylocaldum*, and *Methylobacter*) were primarily

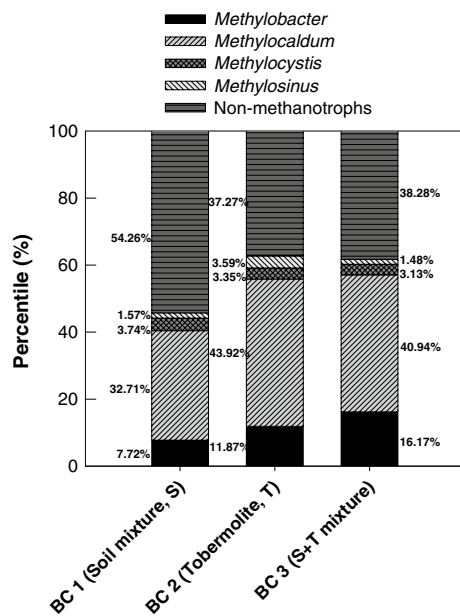


**Fig. 3** Abundance of the bacterial and methanotrophic populations of the methane-removing biocover systems with different packing mixtures. The different letters (A-B/a-b) indicate the statistical significance ( $p < 0.05$ )

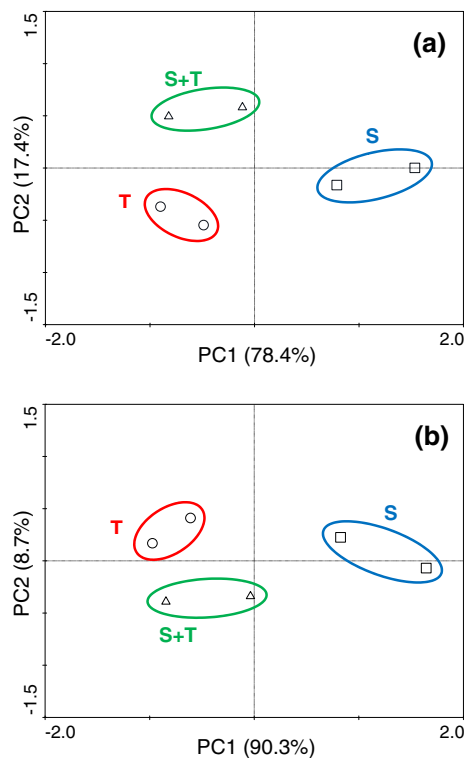
observed in all of the bacterial communities of the systems with differences in the dominance (Supplementary Table 1). The genus *Methylocaldum* dominated the bacterial communities in BC 2 and BC 3, followed by *Methylobacter*, *Pseudoxanthomonas*, *Sphingomonas*, and *Dokdonella*, while *Methylocaldum* dominated the bacterial community in BC 1, followed by *Sphingomonas*, *Pseudoxanthomonas*, *Dokdonella*, and *Methylobacter*. Eight bacterial genera (*Bacillales*, *Bosea*, *Bradyrhizobium*, *Brevundimonas*, *Burkholderiales*, *Devosia*, *Phenylobacterium*, and *Xanthobacteraceae*) were detected only in or relatively higher in BC 1 and BC 3, while four bacterial genera (*Acidobacteria*, *Methylosinus*, *Polyangiaceae*, and *Vampirovibrio*) were present only in or relatively higher in BC 2. The PCA results showed that the bacterial communities of BC 2 and BC 3 were closely grouped in comparison to that of BC 1 (Fig. 5a).

**Analysis of the methanotrophic communities in the biocover systems**

The methanotrophic communities were analyzed using pyrosequencing reads derived from the bacterial libraries. The numbers of OTUs, Chao1, evenness, and  $H'$  values of the methanotrophic communities are listed in Table 2. Defining a species as 1 % dissimilarity, the numbers of OTUs, Chao1, and  $H'$  of the methanotrophic communities were 358–475, 563–791, and 3.33–4.05, respectively, and they showed similar values among the biocover systems ( $p > 0.05$ , Table 2). Only the evenness indices differed significantly among the biocover systems ( $p < 0.05$ , Table 2). The evenness values of the methanotrophic communities



**Fig. 4** Comparison of the methanotrophic and non-methanotrophic community compositions in the methane-oxidizing biocovers with different packing mixtures: relative abundance of the methanotrophs in the bacterial communities

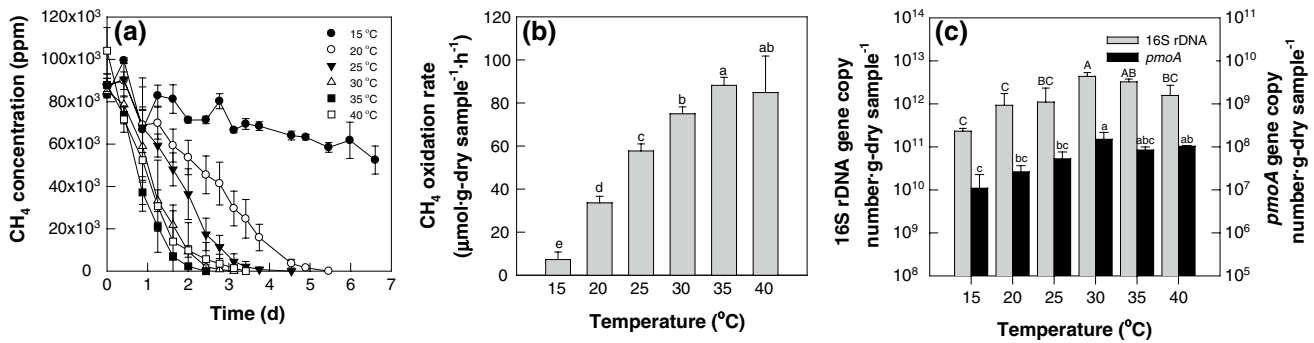


**Fig. 5** Principal component analysis (PCA) of relative abundances of OTUs from the 16S rRNA pyrosequencing data for comparison of the bacterial (a) and methanotrophic communities (b) in the methane-oxidizing biocover systems with different packing mixtures. S (open square), soil mixture; T (open circle), tobermolite; S + T (open triangle), soil mixture + tobermolite

were  $0.68 \pm 0.03$ ,  $0.57 \pm 0.03$ , and  $0.55 \pm 0.02$  in BC 1, BC 2, and BC 3, respectively, thus BC 1 showed a higher evenness level than BC 2 and BC 3. The methanotrophs were more abundant in BC 2 and BC 3 than in BC 1 (Fig. 4). Four methanotrophic genera in total (*Methylobacter*, *Methylocaldum*, *Methylocystis*, and *Methylosinus*) were observed in all of the systems, but *Methylocaldum* was the most abundant. The abundances of *Methylobacter* and *Methylocaldum* in the bacterial communities were relatively higher in BC 2 and BC 3 than BC 1. *Methylocystis* and *Methylosinus* were present in all of the methanotrophic communities and their abundances in the bacterial communities were comparable among the biocover systems. The PCA results also indicated that the methanotrophic communities of BC 2 and BC 3 were more closely grouped than with that of BC 1 (Fig. 5b). The methanotrophs comprised approximately 63 and 62 % of the bacterial communities in BC 2 and BC 3, respectively, while they comprised only 46 % of the bacterial community in BC 1 (Fig. 4). The methanotrophs dominated the bacterial communities in BC 2 and BC 3.

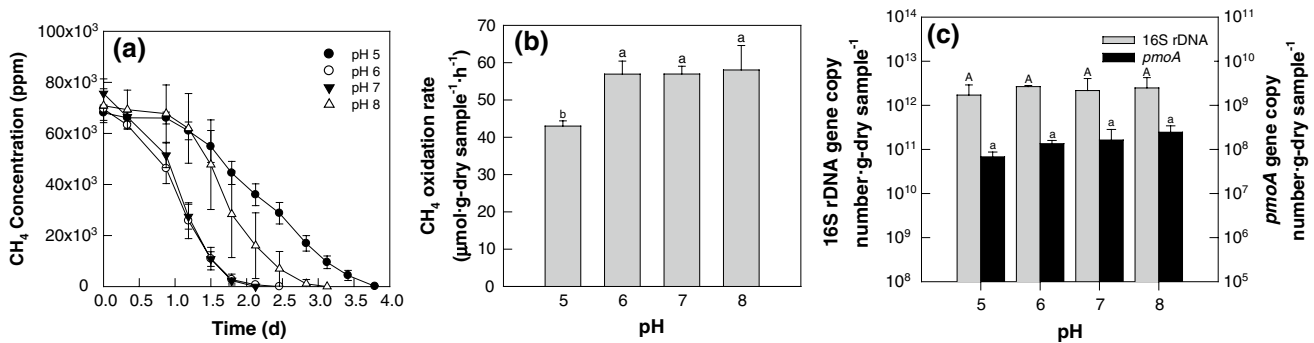
Effects of environmental changes on the performance of BC 3

To assess the effects of environmental conditions such as temperature and pH changes on the performance of the methane-removing biocover systems, the packing mixture that showed the greatest methane removal efficiency and capacity was collected from BC 3. Batch-scale experiments were applied to estimate the effects of the environmental changes on the performance of BC 3. qRT-PCR was employed for the quantitative analysis of the bacterial and methanotrophic population changes with respect to the environmental conditions. Methane oxidation was observed over a temperature range of 15–40 °C, although it showed different methane oxidation rates (Fig. 6). Methane was immediately oxidized without a lag period at all of the temperatures, but the methane oxidation declined rapidly at 15 and 20 °C, as indicated by the methane oxidation rates (Fig. 6a). The maximum methane oxidation rate was  $88 \pm 3.8 \mu\text{mol g-dry sample}^{-1} \text{ h}^{-1}$  at 35 °C, followed by  $85 \pm 17 \mu\text{mol g-dry sample}^{-1} \text{ h}^{-1}$  and  $75 \pm 3.2 \mu\text{mol g-dry sample}^{-1} \text{ h}^{-1}$  at 40 and 30 °C, respectively (Fig. 6b). The methane oxidation rates declined to approximately 8, 38, and 65 % of the maximum oxidation rate at 15, 20, and 25 °C, respectively. However, the qRT-PCR showed that the bacterial and methanotrophic populations were not significantly changed with changes in the temperature (Fig. 6c). Both the bacterial and methanotrophic population levels were slightly lower at 15 and 20 °C, but they remained at the levels of  $10^{11}$  16S rDNA gene copy number g-dry sample<sup>-1</sup> and  $10^7$  *pmoA* gene copy number g-dry sample<sup>-1</sup> over a range of 15–40 °C, respectively.



**Fig. 6** Effects of temperature changes on the methane oxidation and bacterial and methanotrophic populations of the S + T mixture collected from BC 3. Time profiles of the methane oxidation (a), the

methane oxidation rates (b), and the abundance of the bacterial and methanotrophic populations (c) at different temperatures. The different letters (A-C/a-e) indicate the statistical significance ( $p < 0.05$ )



**Fig. 7** Effects of pH changes on the methane oxidation and the bacterial and methanotrophic populations of the S + T mixture collected from BC 3. Time profiles of the methane oxidation (a), the methane

oxidation rates (b), and the abundance of the bacterial and methanotrophic populations (c) at different pH levels. The same letters (A or a) indicate statistical non-significance ( $p > 0.05$ )

The effects of varied pH levels on the methane oxidation of the packing mixture from BC 3 incubated at 30 °C were examined. Methane was oxidized with a 1 day lag period at pH levels of 5 and 8, whereas no lag period was observed for methane oxidation at pH levels of 6 and 7 (Fig. 7a). The methane oxidation rate was approximately 57 μmol g-dry sample<sup>-1</sup> h<sup>-1</sup> at pH levels between 6 and 8, while it declined to 75 % of the maximum methane oxidation rate at a pH level of 5 (Fig. 7b). Both the bacterial and methanotrophic population levels remained with the densities of 10<sup>12</sup> 16S rDNA gene copy number g-dry sample<sup>-1</sup> and 10<sup>8</sup> pmoA gene copy number g-dry sample<sup>-1</sup> over a range of pH levels from 5 to 8, respectively (Fig. 7c). The optimum conditions for the methane oxidation of the packing mixture from BC 3 were 30–40 °C and pH levels between 6 and 7.

**Discussion**

Three biocover systems were operated with different packing mixtures and their performance and bacterial

communities were evaluated to develop the most efficient system for methane removal. The removal efficiencies and capacities of methane varied with the packing mixtures of the biocover systems, although all of the systems were stably operated for 39 days without an acclimation period (Fig. 2). BC 3 (soil mixture + tobermolite, S + T mixture) showed the greatest performance of the methane removal efficiency and capacity with values of 67 % and 292.6 g-CH<sub>4</sub> m<sup>-2</sup> d<sup>-1</sup>, respectively, at a space velocity of 0.12 h<sup>-1</sup>, while BC 1 (soil mixture, S) and BC 2 (tobermolite, T) showed lower methane removal efficiencies and capacities of 27–30 % and 106–111.4 g CH<sub>4</sub> m<sup>-2</sup> d<sup>-1</sup>, respectively. These results indicate that the addition of tobermolite to the soil mixture improved the biocover system performance and this system is more efficient for methane oxidation than those of previous studies using mixed packing materials. Park et al. [27] reported that the methane removal capacity was 233.6 g-CH<sub>4</sub> m<sup>-2</sup> d<sup>-1</sup> at a space velocity of 0.027 h<sup>-1</sup> from a biocover with a soil and earthworm cast mixture (3:2, w/w) and this capacity level was greater than that from a biocover with a soil and

**Table 1** Physico-chemical properties of the packing mixtures

Biocover	BC 1 (Soil mixture, S)	BC 2 (Tobermolite, T)	BC 3 (S + T mixture)
Materials	Landfill soil, GAC, earthworm cast, saprolite	Tobermolite	Tobermolite, landfill soil, earthworm cast
Mixing ratio (w/w)	4:2:1:1	1	2:1:1
Organic matter content (% w/w)	21.14 ± 0.90	4.79 ± 0.35	18.30 ± 2.06
Water holding capacity (% w/w)	46.94 ± 6.61	83.55 ± 4.44	54.00 ± 2.92
Bulk density (g/ml)	0.73 ± 0.01	0.26 ± 0.01	0.48 ± 0.01

**Table 2** Operational taxonomic units (OTUs), species richness, evenness, and diversity estimates of the bacterial and methanotrophic libraries

Community <sup>a</sup>	Sample	Dissimilarity	OTUs	Chao1	H'	Evenness
Bacteria	BC 1	3 %	352 ± 33	584 ± 126	3.94 ± 0.04	0.67 ± 0.00
	BC 2		258 ± 50	424 ± 24	3.39 ± 0.04	0.61 ± 0.01
	BC 3		295 ± 40	547 ± 179	3.58 ± 0.05	0.63 ± 0.02
Methanotrophs	BC 1	1 %	381 ± 74	791 ± 175	4.05 ± 0.33	0.68 ± 0.03
	BC 2		358 ± 148	563 ± 182	3.33 ± 0.09	0.57 ± 0.03
	BC 3		475 ± 71	638 ± 59	3.37 ± 0.05	0.55 ± 0.02

<sup>a</sup> Total numbers of reads were equalized with the lowest number of reads (1,382 reads)

powdered activated carbon mixture (3:2, w/w). Kettunen et al. [15] found that the methane removal capacity was 31.4 g-CH<sub>4</sub> m<sup>-2</sup> d<sup>-1</sup> at a space velocity of 0.012–0.016 h<sup>-1</sup> from a biocover using a mixture of mature sewage sludge compost, de-inking waste, and sand (4:2:4, w/w). Perdikea et al. [28] reported that a biocover with compost and sawdust (9:1, w/w) showed a methane removal capacity of 11.3 g-CH<sub>4</sub> m<sup>-2</sup> d<sup>-1</sup> at a space velocity of 0.001 h<sup>-1</sup>. In comparison with the results of the previous studies, BC 3 showed a greater or comparable removal capacity of methane at a space velocity of at least fourfold faster, indicating greater system performance of methane removal by BC 3. Physical gas adsorption was not observed in the systems containing the packing mixtures, indicating that methane was biologically consumed by methanotrophs (data not shown).

The methane removal capacity is associated with the properties of the packing materials. In general, the porosity, coarseness, water holding capacity, and organic matter content are considered as key factors of the packing materials for the optimum methane consumption of methanotrophs [6, 16, 31]. Tobermolite showed those properties with a high porosity and a water holding capacity of 72.1 % (v/v) and 83.5 % (w/w), respectively, but it had a low organic matter content of 4.79 ± 0.35 % (w/w) (Table 1) [14]. In contrast, the soil mixture had a high organic matter content of 21.14 ± 0.90 %, but it showed a low water holding capacity and a high bulk density of 38.66 % and 0.73 g ml<sup>-1</sup>, respectively. Therefore, tobermolite combined with the soil mixture synergistically improved the physico-chemical properties with respect to the high porosity, water holding capacity, and low bulk density as well

as organic matter supply. Kightley et al. [16] reported that methane oxidation rate was higher of coarse sand (10.4 mol of CH<sub>4</sub> m<sup>-2</sup> d<sup>-1</sup>) than that of fine sand or clay (7 mol of CH<sub>4</sub> m<sup>-2</sup> d<sup>-1</sup>). Bender and Conrad [1] also showed that larger particles had greater specific activities of methane oxidation than smaller particles. Molecular ecological approaches were employed to examine how the packing mixtures influence the bacterial and methanotrophic communities in the biocover systems. The bacterial and methanotrophic population densities varied with the packing mixtures of the biocover systems, both of them were higher in BC 1 and BC 3 than BC 2 (*p* < 0.05, Fig. 3). These significant increases in the populations were only observed in the biocover systems amended with the soil mixture (Table 1). BC 1 and BC 3 containing the soil mixture had high organic matter contents of 18.30–21.14 %, presumably derived from the earthworm cast (a C:N ratio of 40 and an organic matter content of 56.29 %), and this may have influenced the bacterial and methanotrophic populations [18]. Our results were in accordance with those of previous studies. Humer et al. [12] explained that the organic matter content is vitally important in methane oxidation, because organic matter primarily serves as a substrate for microorganisms and improves the properties of soil. Martineau et al. [22] reported that the methanotrophic activity and populations were enhanced by nutrient amendment. Kightley et al. [16] found that the methane oxidation of soil was significantly stimulated by adding sewage sludge.

The bacterial and methanotrophic communities also differed considerably with the packing mixtures of the biocover systems, but their changes were distinct from those in the populations. The addition of tobermolite induced a



substantial change in the bacterial and methanotrophic community structures. BC 2 and BC 3 amended with tobermolite exhibited lower Chao1,  $H'$ , and evenness index levels of the bacterial and methanotrophic communities than those levels in BC 1 (Table 2). The PCA results demonstrated replicate homogeneity as well as differences among the biocover systems with different packing mixtures. The PCA results also showed that both of the bacterial and methanotrophic communities of BC 2 and BC 3 were more closely clustered than those of BC 1 (Fig. 5).

The observed methanotrophs in this study were *Methylobacter*, *Methylocaldum*, *Methylocystis*, and *Methylosinus*. *Methylocaldum* was most abundant in all of the systems (Fig. 4). Interestingly, the abundances of *Methylocaldum* and *Methylobacter* in the bacterial communities increased in BC 2 and BC 3. This result is consistent with the fact that methanotrophs were shown to comprise approximately 60 % of the bacterial communities in BC 2 and BC 3, which is higher than that of BC 1 (Fig. 4). These results indicate that the addition of tobermolite may enhance the methanotrophic abundance of the bacterial communities and influence their structures. Plessis et al. [10] reported that known methane-oxidizing bacteria were not retrieved from a biofilter with a mixture of compost pine bark and perlite, since the intrinsic complexity of the compost may be ascribed to low selectivity for the growth of methanotrophs. Kim et al. [18] also reported that methanotrophs comprised 29 % of the active community (DNA) in a lab-scale methane-degrading biocover.

Compared to previous studies, tobermolite might provide a good environment for the growth of methanotrophs. Redundancy analysis (RDA) was performed to investigate the relationship between the bacterial community composition and the packing mixtures. RDA revealed that all of the methanotrophs (*Methylobacter*, *Methylocaldum*, *Methylocystis*, and *Methylosinus*) were positively correlated with tobermolite, while *Dokdonella* and *Sphingomonas* were negatively correlated with tobermolite (data not shown). Those six bacterial genera (*Methylobacter*, *Methylocaldum*, *Methylocystis*, *Methylosinus*, *Dokdonella*, and *Sphingomonas*) comprised at least 60 % of all of the bacterial communities, indicating that tobermolite primarily influenced the bacterial communities of BC 2 and BC 3. Although eight bacterial genera (*Bacillales*, *Bosea*, *Bradyrhizobium*, *Brevundimonas*, *Burkholderiales*, *Devosia*, *Phenylobacterium*, and *Xanthobacteraceae*) were positively correlated with the soil mixture, their changes were insignificant due to the minor proportion of the bacterial communities that they comprised (data not shown). These results also support the fact that tobermolite significantly changed the bacterial communities and enhanced the methanotrophic proportion in particular. Therefore, these changes possibly accounted for the greatest performance of BC 3 for methane removal.

To evaluate the suitability of the S + T mixture as a packing material in the field conditions, we examined the effects of environmental variables such as temperature and pH changes on the methane oxidation of the S + T mixture collected from BC 3. The methane oxidation of the S + T mixture varied significantly as a function of the incubation temperatures ranging from 15 to 40 °C (Fig. 6). Batch tests showed a significant response to temperature changes between 15 and 20 °C, whereas they showed a minimal response to pH changes, as indicated by the methane oxidation rates (Figs. 6, 7). The methane oxidation rates declined rapidly at 15 and 20 °C, perhaps due to the metabolic instability at low temperatures. This result suggests that the S + T mixture might be strongly dependent on the temperature rather than the pH changes. However, the methane oxidation remained at least 65 % of the maximum oxidation at mesophilic temperatures (25–40 °C) and at a range of pH levels between 5 and 8. Our findings are consistent with those of previous studies that showed most of the methanotrophs maintained their methane oxidation activity over a range of 20–37 °C and a pH range of 5.5–8.5 with a difference in the optimum conditions [2, 9, 12, 24, 26].

To the best of our knowledge, this is the first report to evaluate tobermolite as a packing material in landfill biocover systems for methane removal. Our findings indicate that the biocover performance for methane removal was greatly improved by adding tobermolite to the soil mixture. Due to its high porosity, tobermolite offers more surfaces for methanotrophic colonization as compared to the soil mixture without tobermolite and it also improves the gas diffusion by increasing the contact area. In addition, its high porosity induces high gas permeability [23] and this greater gas penetration is advantageous for methane removal due to the fact that it allows oxidation to occur in the deeper soil.

## Conclusions

Significant enhancement of the methane removal capacity could be achieved in the soil mixture biocover amended with tobermolite. The soil and tobermolite mixture synergistically improved their physico-chemical properties with respect to the high porosity, water holding capacity, and low bulk density as well as organic matter supply. The soil mixture increased the bacterial biomass and tobermolite served as a suitable habitat for the selective growth of the methanotrophs as well as improving gas permeability. Since the mixture of soil and tobermolite showed greater methane removal capacity (293 g-CH<sub>4</sub> m<sup>-2</sup> d<sup>-1</sup>), this mixture is a highly competitive packing bed material for methane-removing biocover systems.

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