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Diversity and Dynamics of Methanotrophs within an Experimental Landfill Cover Soil

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Three experimental biocovers were installed during the summer of 2006 at the St-Nicéphore landfill in Québec (Canada). The main objectives of the experimental program were to assess the efficiency of these biocovers in reducing CH_4 emissions and to monitor the evolution in space and time of the dynamics and diversity of methanotrophic bacteria that may be responsible for CH_4 abatement. Dynamics of methanotroph populations derived from most probable number (MPN) counts exhibited different steps (lag, growth, and collapse phases), with mean abundances decreasing somewhat with depth. Methanotroph diversity, as assessed by denaturing gradient gel electrophoresis showed changes over time in the community structure. These changes were dependent on the sampling depth and only Type I methanotrophs belonging essentially to the genus *Methylobacter* were retrieved from our samples. Given the youthful character of the system under study, together with the reported behavior of this functional group of methanotrophs, it was concluded that Type I methanotrophs forming the community structure might reflect pioneer species with a potentially high growth rate (r-strategists) that become numerically dominant reducing the evenness of species distribution. These results contribute to the generally limited body of knowledge on methanotroph diversity in landfill cover soils.

Abbreviations: CCA, canonical correspondence analysis; DDGE, denaturing gradient gel electrophoresis; MDS, multidimensional scaling; MMO, methane monooxygenase; MPN, most probable number; pMMO, particulate MMO; PMOBio, passive methane oxidation biocover; sMMO, soluble MMO.

Methane (CH_4) is a potent greenhouse gas. It absorbs infrared radiation more effectively than CO_2 and has a global warming potential 25 times higher than that of CO_2 (IPCC, 2007). Methane is the product of the anaerobic degradation of organic matter by methanogenic Archeae (Zinder, 1993). In the presence of oxygen, a substantial part of the CH_4 produced can be oxidized by methane-oxidizing bacteria (methanotrophs). Carbon can thus be retained in the system as biomass or emitted as CO_2 (Roslev et al., 1997).

Among the in situ sources of CH_4 contributing to the atmospheric CH_4 budget, emissions from landfills may represent as much as 17% (Wuebbles and Hayhoe, 2002) of the about 70% of CH_4 emissions due to human activities (IPCC, 2007). Therefore, management practices that could help in reducing emissions from landfills are of great importance in connection with the atmospheric CH_4 budget. To date, gas extraction systems have been the principal means of reducing CH_4 emissions from landfills. However, the high cost associated with this technical approach renders it feasible only for landfill sites in the developed world. Even in sites equipped with gas collection systems, significant amounts of CH_4 can still escape as fugitive emissions (Börjesson et al., 2007; Spokas et al., 2006).

It has been shown that when a landfill site is full and capped with soil, vertical migration brings the gas from the anaerobic region of the landfill, where it is formed, into an aerobic environment near the surface, where it may be subjected to microbiological oxidation by methanotrophs (Humer and Lechner, 2001; Jones and Nedwell, 1993; Whalen et al., 1990). Methane oxidation by methanotrophs is catalyzed by methane monooxygenase (MMO), an enzyme that may be either membrane bound, "particulate" MMO (pMMO) or cytoplasmic, "soluble" MMO (sMMO) (Hanson and Hanson, 1996). The sMMO is restricted to Type II methanotrophs (*Methylocystis* and *Methylosinus*) and some Type I representatives from the *Methylomonas* and

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Methylococcus genera. The pMMO is present in all Type I and II methanotrophs and has been used as a gene target for the identification of non-culturable methanotrophs in environmental samples (Murrell et al., 1998), with the exception of all members of the genus *Methylocella* (*M. palustris, M. tundrae and M. silvestris*) (Dedysh et al., 2004; Dunfield et al., 2003). Methanotrophs have been isolated and characterized from a variety of soils (Hanson and Hanson, 1996). Soil-mediated CH_4 oxidation provides an important biological sink for both atmospheric and in situ produced CH_4 (Amaral et al., 1998).

Optimization of CH_4 oxidation by methanotrophs in landfill cover soil may provide an inexpensive management strategy to minimize the emission of CH_4 from landfills to the atmosphere. This prospect prompted an ongoing multidisciplinary project that considered different aspects in assessing the efficiency of landfill cover materials that would act as a passive methane oxidation biocover (PMOBio) to reduce CH_4 emissions from landfills. This type of cover might provide an alternative way to reduce emissions from landfills having lower amounts of CH_4 generation where flaring or energy recovery is uneconomical, or employed as a complementary strategy for gas collection systems to reduce emissions that escape the collection system.

The present study was designed to examine the extent to which methanotrophic microbial communities would thrive in a designed landfill biocover, in response to the presence of CH_4 and hence contribute to CH_4 attenuation. More specifically, our objectives were to examine the temporal and spatial dynamics in methanotrophic activity and community composition to acquire a better understanding of the indigenous microorganisms involved in the observed CH_4 abatement within the experimental landfill cover material. Moreover, since little is known about methanotroph diversity in landfill cover soils, results reported here would contribute to increase knowledge on the relatively unexplored area of the relationship between methanotroph diversity and function.

MATERIALS AND METHODS

Description of the Experimental Field Plot

The experimental plot used in the present study measured 2.75 m (W) by 9.75 m (L) and was constructed during the summer of 2006, at the St-Nicéphore landfill, Quebec, Canada, a waste disposal facility covering approximately 65 ha that receives mainly domestic waste. The majority of this area is already capped with a final cover, mostly constituted of a minimum of 1.2 m of fine-grained soils (usually silt). This studied plot included an 80-cm thick layer of sand-compost mixture underlain by a 10-cm thick transitional layer consisting of 6.4-mm net gravel and a 20-cm thick gas distribution layer (GDL) consisting of 12.7-mm net gravel. Additional details on the design and characteristics of the original sand and compost mixture that were used as well as the construction of this plot (Fig. 1A) can be found in Jugnia et al. (2008).

Soil Environment, Physicochemical Variables, and Methanotroph Counts

Probes for the measurement of temperature and gas concentration were installed at four separate points along the downhill longitudinal axis of the experimental plot and at four different depths (six in the case of gas probes) in each profile (Fig. 1B). Meteorological data, including precipitation and atmospheric pressure, were recorded continuously by a weather station installed near the experimental plot. The temperature probes (HOBO U12, from Onset) were connected to data loggers (HOBO U12, from Onset) and set to record values every 30 min.

Substrate samples were collected at three different points from the uppermost part of the PMOBio (0–10, 10–20, 20–30, and 30–40 cm) using PVC coring tubes (inner diameter = 5 cm). Thereafter, subsamples for physicochemical analyses and methanotroph counts were placed in air-tight plastic bags and kept at 4°C until analyzed in the laboratory, which was done within 24 h. For molecular biology analyses, 30-g samples were collected in 50-mL polypropylene conical tubes (BD Falcon, BD Sciences, San Jose, CA) and stored at −20°C.

The physicochemical analyses (Page et al., 1982) included gravimetric water content (by overnight drying at 105°C), organic matter

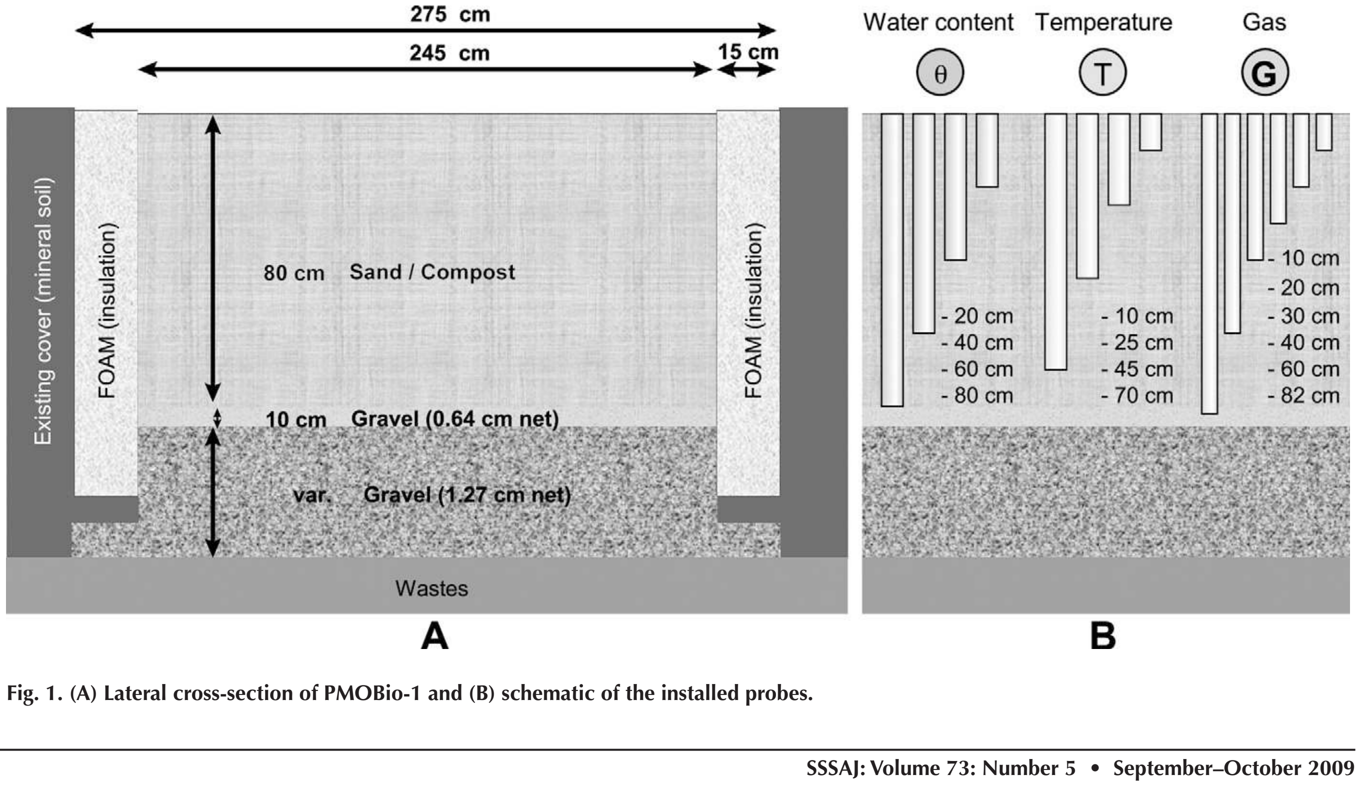

Fig. 1. (A) Lateral cross-section of PMOBio-1 and (B) schematic of the installed probes.

content (by loss-on-ignition at 550°C for 2 h), pH (determined in distilled water 1:3 v/v).

Counts of methanotrophic bacteria were performed following the most probable number method, with soil slurries that were serially diluted in 96-well microtiter plates. In brief, fresh soil (5 g) was suspended in 45 mL of a mineral salts medium (Heyer et al., 2002) and shaken for 1 h. Two hundred microliters of this suspension were placed in the first well of a 96-well microtiter plate. Serial 10-fold dilutions were performed from the second well to the eleventh well by transferring 20 μL to the next well, which contained 180 μL of mineral medium, using a multichannel pipettor. The 12th well contained only mineral medium and was used as the control. The plates were incubated for 4 wk at 25°C in gastight jars with air containing 3% CO_2 and 18% CH_4. After incubation, all plates were read using a microplate reader to determine positive or negative growth of bacteria. The MPN was corrected for the positive bias that is characteristic of published MPN tables (Salama et al., 1978) and calculated from the dilution factor and dry weight of the soil.

DNA Extraction from Soil Samples and PCR Amplification

Total DNA was extracted directly from samples using mechanical shaking and an enzymatic lysis procedure as outlined in Fortin et al. (2004). The crude DNA extracts were purified using Sephacryl/PVPP spin columns. The PVPP resin was prepared as described by Holben et al. (1988). The Sephacryl S-400 HR (GE Healthcare Bio-Sciences, Piscataway, NJ) was equilibrated in TE pH 7.6 as follows: batches of 25 mL of resin were washed with 25 mL TE (10 mM Tris-HCl pH 7.6; 1 mM Na_2EDTA), then centrifuged for 1 min at 524 × *g*. The supernatant was removed and the resin was washed again with fresh TE, pH 7.6. The resin was subjected to a total of five washes as described above. The crude DNA was prewarmed at 37°C for 10 min before the purification step. The spin columns were layered with 400 μL of PVPP, then 400 μL of Sephacryl S-400 HR, and were centrifuged for 2 min at 799 × *g* before and after the addition of 50 μL of crude DNA.

Primers complementary to conserved regions of the particulate methane monooxygenase (*pmoA*) gene were used to amplify a 500 bp fragment. The forward primer sequence contained a GC-clamp, to stabilize the melting behavior of the amplified fragments during denaturing gradient gel electrophoresis (Sheffield et al., 1989). The sequence of the GC-clamp was 5’-GCG-GGC-GGG-GCG-GGG-GCA-CGG-GGG-GCG-CGG-CGG-GCG-GGG-CGG-GGG. The primer sequences were as follows: A189f: 5′-GGNGACTGGGACTTCTGG and mb661r: 5’-CCGGMGCAACGTCYTTAC, with non specific bases (N, M, Y) replaced by I (for inosin). The PCR mixture (50 μL) contained 10 μL of total DNA (undiluted, 1:10 or 1:50, initial DNA concentrations ranging from 5 to 45 ng μL^{-1}), 25 *p*mol of each primer, 8 μL of 1.25 mM of dNTP, 5 μL of 25 mM $MgCl_2$, 0.13 μg μL^{-1} bovine serum albumin (BSA) and 2.5 units of Taq polymerase (Invitrogen, Burlington, ON). The samples were denatured for 5 min at 96°C before the addition of the Taq polymerase. A touchdown PCR was performed in which the annealing temperature was set at 60°C and decreased by 0.5°C every cycle until it reached 50°C. Denaturation was performed at 94°C for 1 min followed by an annealing time of 1 min and primer extension at 72°C for 3 min. Fourteen additional cycles were performed at 50°C, for a total of 35 cycles. An aliquot of the PCR product was electrophoresed on a 1.4% agarose gel, with several dilutions of the 100-bp DNA marker from MBI Fermentas (Amherst, NY). The products were quantified by SYBR Safe staining and spot densitometry using a ChemiImager (Alpha Innotech Corp., San Leandro, CA).

Denaturing Gradient Gel Electrophoresis Analysis

Denaturing gradient gel electrophoresis was performed with a Bio Rad DCode Universal Mutation Detection system. Electrophoresis was performed on 0.75 mm thick 8% polyacrylamide gels (ratio of acrylamide to bisacrylamide, 37.5:1) in 1X TAE buffer (40 mM Tris, 40 mM acetic acid, 1 mM EDTA; pH 7.4) at 60°C. Approximately 450 ng of PCR product (*pmoA* gene) was applied to individual lanes of the gel. Electrophoresis conditions were 16 h at 80V in a linear 30 to 60% denaturant gradient (100% denaturant was 7 M urea and 40% deionized formamide). The gel was stained for 30 min in Vistra Green nucleic acid stain (GE Healthcare Bio-Sciences) and visualized with a fluorImager system model 595 (Molecular Dynamic, Sunnyvale, CA). The gel image was processed using Adobe Photoshop 5.0.

Denaturing Gradient Gel Electrophoresis Fragment Isolation and Sequencing

Taxonomic identification was performed by excising and sequencing the DGGE bands. Briefly, selected bands were excised from the gel and eluted in 60 μL of MilliQ water at 4°C overnight. Fifty microliters of the eluted DNA was purified with the QIAquick PCR purification kit (QIAGEN Inc. Mississauga, ON). One microliter of purified DNA was used as template for the reamplification of the DGGE fragment using the same primers without the GC clamp. The reaction mixture was the same as mentioned above. For PCR cycling, an initial denaturation of 5 min at 96°C was followed by twenty-five cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The PCR products were combined and concentrated by ethanol precipitation. The precipitated product was run on a 1.4% agarose gel. The appropriate band was cut, purified with the Illustra GFX purification kit (GE Healthcare Bio-Sciences) and quantified by spot densitometry, as outlined above. Sequencing was performed by the “Laboratoire de Synthèse et d’Analyse d’Acide Nucleiques” of Laval University (Ste-Foy, QC) with a capillary ABI Prism 3100 sequencer.

Statistics

The DGGE gel photographs were screened for the presence (1) or absence (0) of *pmoA* bands. A similarity matrix was generated with this binary data using the Jaccard coefficient and analyzed with non-metric multi-dimensional scaling (MDS) using XLSTAT software. The distance measure used for the MDS ordination was the Bray–Curtis coefficient. To determine the configuration that produced the lowest stress solution, which measures the goodness-of-fit of the ordination, non-metric MDS analysis was run 100 times, completing 41 random restarts of the iteration to determine the minimum stress level.

Canonical correspondence analysis (CCA) was further used for revealing relationships between community structure, as determined by DGGE profiles, and environmental variables. The purpose of this approach was to find the combination of explanatory variables that best explained the variation of the DGGE matrix. The significance of the constrained ordination process was tested with the Monte Carlo permutation test (999 permutations). The CCA was performed with the software CANOCO for Windows 4.53 (Biometris, the Netherlands).

The *pmoA* gene sequences were aligned using MacVector 7.2 software and compared with the EMBL Nucleotide Sequence Database using the FASTA search program (Pearson and Lipman, 1988). The Phylogenetic tree (neighbor-joining algorithm with Jukes-Cantor) was constructed using the MacVector 7.2 software package. The robustness of inferred topologies was later tested by 1000 bootstrap resamplings of the neighbor-joining data.

Accession Numbers

Sequences from this study have been deposited in the GenBank database under Accession Numbers EU292151-EU292168.

RESULTS

Depth profiles of temperature, pH, gravimetric water content, organic matter content, and methanotroph counts within the cover soil are presented in Fig. 2. Temperature varied from 4.2 to 27.2°C (mean ± SD = 14.9 ± 6.7°C) and exhibited variations with depth and time, the highest thermal amplitude being associated with the uppermost part of the cover. Until August 2006, the temperature generally decreased with depth throughout the cover. In September it remained almost constant and, from October, the temperature started to increase with depth until the end of the study.

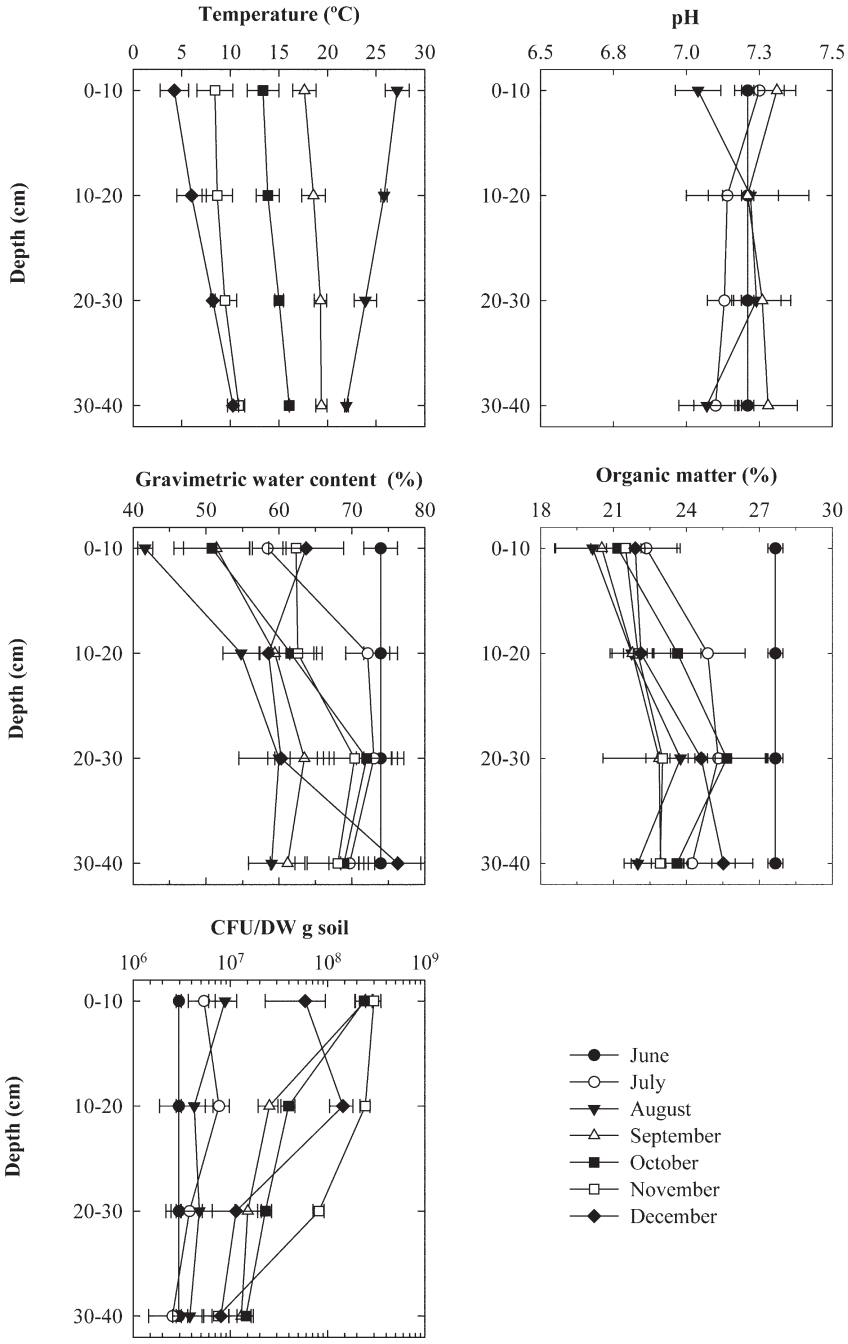

Fig. 2. Temporal evolution of temperature, pH, gravimetric water content, organic matter content and colony forming unit (CFU) of methanotrophs within the passive methane oxidation biocover (PMOBio). No data was available for the temperature in June. Also, the constancy of the pH, soil moisture, organic matter, and counts with depth in June was due to the fact that data were from the original sand and compost mixture that we used during the construction of the experimental plot. Data are averages of triplicates with error bars equivalent to 1 S.D.

A different trend was observed for the pH of the soil; it ranged from 7.0 to 7.3 (Mean ± SD = 7.2 ± 0.1) and varied only slightly with time and depth throughout the study period. For all the samples analyzed, the water content ranged from 41.7 to 76.3% (Mean ± SD = 67.1 ± 8.5%), while the organic matter content fluctuated between 20.1 and 27.7% (Mean ± SD = 23.6 ± 2.2%). The values for these two variables increased slightly with depth, whereas with time, only fluctuations in gravimetric water content near the surface (0–10 cm) appeared significant.

Counts of methanotrophic bacteria varied from 1.50×10^6 to 1.83×10^8 CFU gdw^{-1} (per gram of dry weight soil), with a mean ± SD across all sampling dates of $3.40 \pm 2.68 \times 10^7$ CFU gdw^{-1}. On average, the number of methanotrophic bacteria decreased somewhat with depth from $7.83 \pm 5.42\ 10^7$ CFU gdw^{-1} near the surface (0–10 cm) to $4.12 \pm 2.68 \times 10^7$ CFU gdw^{-1} at the 10- to 20-cm depth and $1.21 \pm 1.03 \times 10^7$ CFU gdw^{-1} between 20 and 30 cm and reached a minimum of $4.49 \pm 2.93 \times 10^6$ CFU gdw^{-1} at 30 to 40 cm. Temporal evolution of depth profiles of counts of methanotrophs could be divided into three phases (Fig. 2): The lag phase included the first three sampling campaigns (June to August), when the abundance profiles were essentially identical. During the second phase (August to November), counts of methanotrophic bacteria increased with time, particularly at the surface, to reach the maximum values observed during this study. In the third phase that started in December, a drop in methanotroph counts was observed, particularly in the 0- to 10-cm depth.

Figure 3 presents the results of the DGGE analyses. Visual inspection of the fingerprint—where banding patterns represent changes in microbial diversity—suggested substantial species variability over time and depth in the CH_4 oxidizing bacterial community. More precisely, the DGGE patterns indicated that although a few dominant bands (such as Bands D, E, and V) were detected in almost every sample, the number of bands as well as their intensities increased considerably over time near the surface (0–10 cm; Fig. 3). At this depth, the intensity of several bands increased steadily from the third sampling campaign onward with appearance in some cases of new bands such as F, K, M, and R. On the other hand, changes in intensity and banding pattern observed within and between the 10- to 20- and 30- to 40-cm sampling depths were less significant, with no clear trend over time, as compared to the 0- to 10-cm sampling depth. This observation was substantiated by the multidimensional scaling (MDS) statistical analysis, which indicated that time-related differences in community structure varied from one another between the 0- to 10-cm, 10- to 20-cm, and 30- to 40-cm sampling depths (Fig. 4).

The relationship between the community composition and soil environmental variables was tested by CCA (Fig. 5). The first canonical axis (eigen value = 0.113, F = 3.078, p = 0.018) and all canonical axes (sum of all canonical eigen values = 0.219, F = 2.11, p = 0.003) were statistically significant in this analysis. Phylotype-environmental correlations were high, especially for Axes 1 and 2 (0.937 and 0.665), indicating a relationship between species and environmental variables. The first two axes accounted for 66% of the total variance and the first axis alone explained 45%.

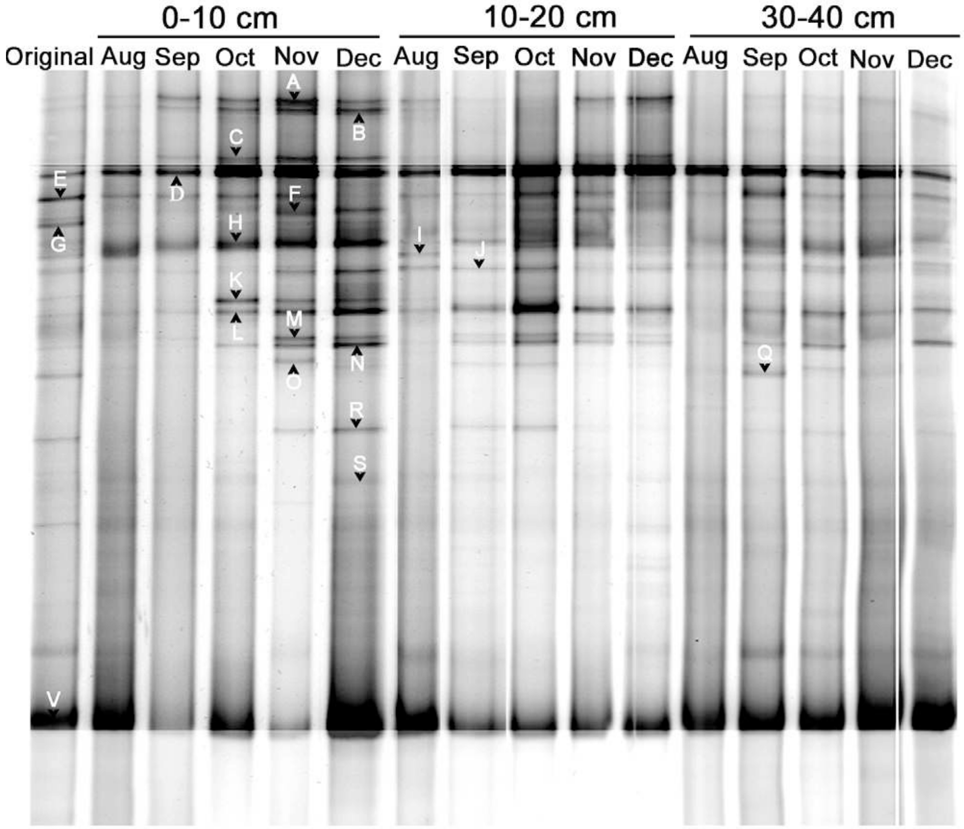

Fig. 3. Negative image of denaturing gradient gel electrophoresis (DGGE) patterns obtained from samples from 0–10 cm, 10–20 cm and 30–40 cm sampling depths. The first lane (Original) represent the community structure of methanotrophs in the mixture of sand and compost used as substrate during the construction of the passive methane oxidation biocover (PMOBio). The arrows show the position of the bands that were excised and sequenced.

From the biplot of the CCA result with interspecies distances (Fig. 5), significant correlation to the species variation was observed by three parameters, namely temperature, organic matter, and gravimetric water content. The latter two variables were placed in the same quadrant, showing a strong correlation among them. A group made of Bands A, B, D, E, H and V had a strong correlation with temperature, while Bands G, J, N, and R were associated with high organic matter and gravimetric water content. Several bands including C, K, O, M, F, which could not be grouped with the other bands, showed little influence of the above mentioned three factors and appeared to be derived principally from the 0- to 10-cm sampling depth.

An examination of the CCA plot reflected differences mentioned above between the investigated sampling depths. The 0- to 10-cm and 30- to 40-cm depths were opposed, while the 10- to 20-cm sampling depth was placed in the middle of the plot forming a different group (Fig. 5). Also, samples collected during relatively warm periods (August and September) grouped opposite to those collected during relatively cold periods (November and December)

All of the major bands in the DGGE gel that were excised and sequenced (indicated by arrows, Fig. 3) were related to the *pmoA* gene from Type I methanotrophs in the *Gamma*-subdivision of the *Proteobacteria* (Fig. 6). More precisely, sequences from Bands J, K, L M, N and R grouped among the Type I methanotrophs, closest to previously described uncultured bacterium (EF212356; 90–93%) from landfill cover soil. With the exception of Bands V (PMOB-V) and O (PMOB-O) that grouped with landfill cover soil uncultured *Methylocaldum sp.* (AY195659; 99%) and *Methylomonas sp* ML64 (AF510080; 92%), respectively, sequence analysis revealed that most DGGE bands showed similarity with the *pmoA* gene from *Methylobacter* strain isolated from diverse habitats including landfill cover soil (Fig. 6).

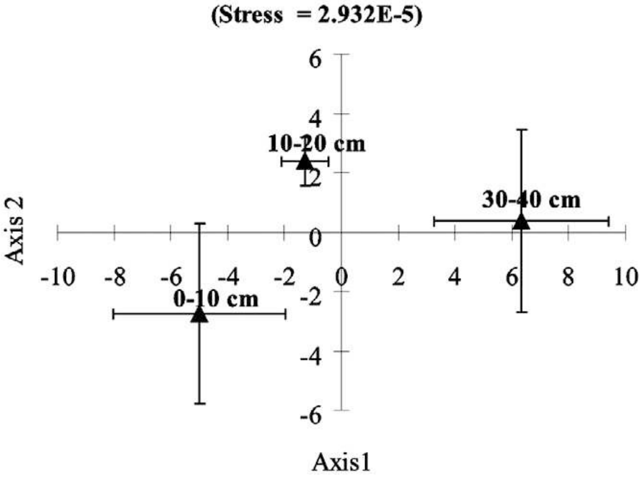

Fig. 4. Non-metric multidimensional scaling (MDS) analysis of *pmoA* derived DGGE patterns. All symbols represent mean values ± SE calculated using sampling dates as replicates.

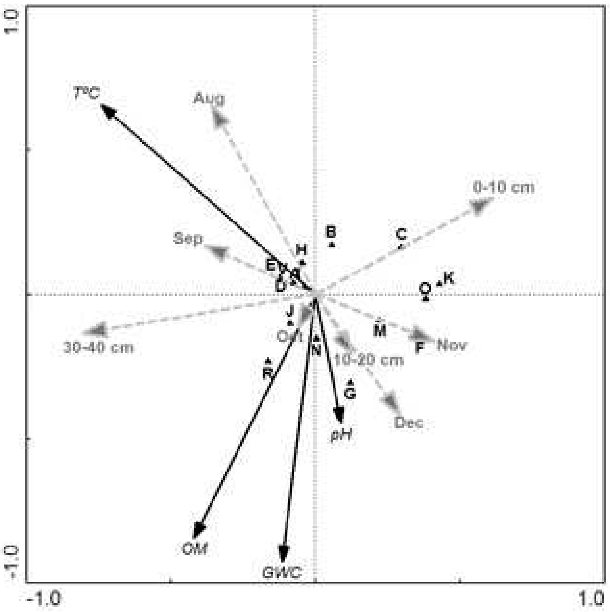

Fig. 5. Canonical correspondence analysis (CCA) ordination biplot of DGGE bands (black triangle) and environmental variables (represented by bold arrows). GWC = gravimetric water content, T°C = temperature, OM = organic matter. Dashed arrows represent supplementary variables from sampling month and depth data (not involved in calculation) and that were included in the graph to show the relative partition by month and depth with respect to bands from the denaturing gradient gel electrophoresis (DGGE) fingerprints.

DISCUSSION

Preliminary results with the sand-compost substrate mixture used in this study demonstrated the effectiveness of this material for CH_4 abatement, and more details on this during the study period are presented in Jugnia et al. (2008). Our objectives here were to examine the spatiotemporal dynamics and diversity of the indigenous methanotrophs involved in the observed CH_4 abatement within the experimental landfill cover material.

Gas Profiles and Methane Abatement

The different patterns of representative O_2 and CH_4 concentration profiles showed that CH_4 abatement does occur and was more pronounced within the uppermost part of the biocover (0–10 cm). However, the zone of optimum CH_4 abatement moved during the study period and was sometimes found at lower depths (Jugnia et al., 2008). This is a common and well known feature in landfill cover soil (Berger et al., 2005; Humer and Lechner, 2001; Visvanathan et al., 1999; Whalen et al., 1990) that has been linked to factors such as temperature, water content of the soil, differential pressure between the waste mass, the atmosphere and the concentrations of O_2, and CH_4. Temperature and water content (which directly affect O_2 availability) of the substrate have been identified as the two variables influencing CH_4 abatement within this PMOBio (Jugnia et al., 2008), in agreement with the results of a recent study by Einola et al. (2007).

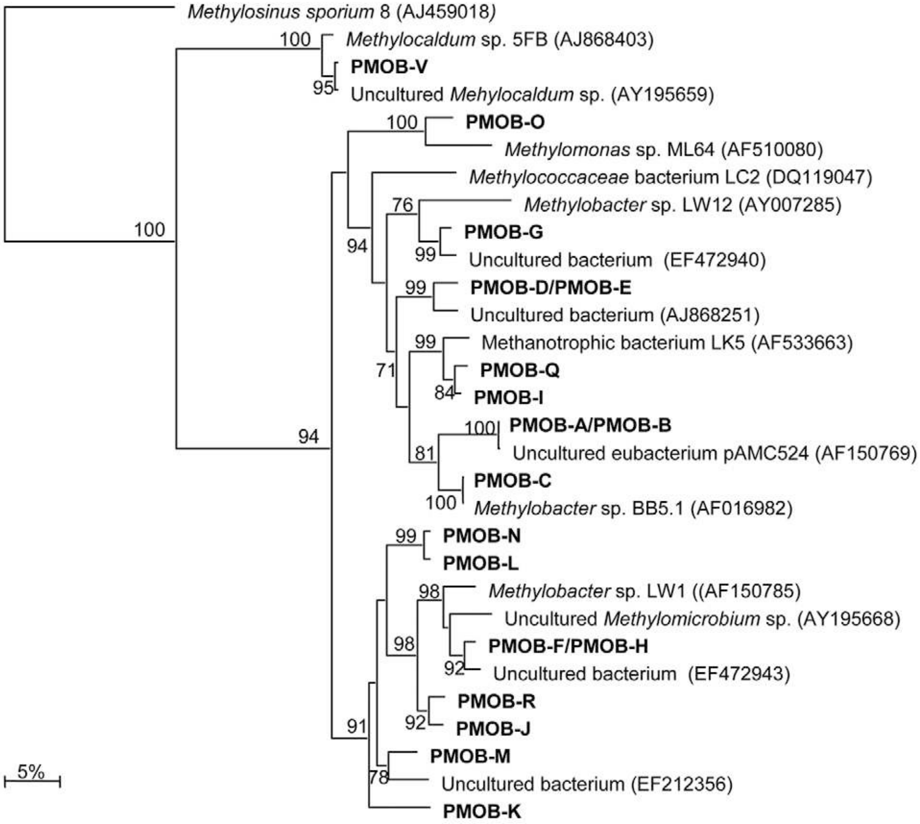

Fig. 6. Phylogenetic analysis of the deduced sequence of pmoA genes from methanotrophs. Total community DNA from samples was amplified with the primer set A189/mb661. The tree was constructed with the pmoA sequence of Methylosinus sporium 8 as outgroup. Bootstrap probability (%) values are represented at the nodes (when more than 50%). The bar represents a 5% sequence divergence, as determined by measuring the length of the horizontal line connecting any two species.

Soil and Environmental Variables in Relation to Methane Abatement and Methanotrophs

Since there was a sufficient supply of CH_4 throughout the study, pH, O_2 availability and temperature are the other factors that could have affected CH_4 oxidation. However, the fluctuation in soil pH throughout the study remained within a narrow range of values generally reported as optimal for the growth of methanotrophs (Jugnia et al., 2008). In contrast, temperature exhibited variations with depth throughout the monitoring period, the highest thermal amplitude being associated with the uppermost part of the cover where most of the CH_4 oxidation occurred. This suggests a relationship between CH_4 oxidation and temperature in this part of the cover. It has been shown that CH_4 consumption by methanotrophs changes with temperature (Börjesson et al., 2004; Einola et al., 2007; Kettunen et al., 2006; Park et al., 2005) when the water content, among other factors, is not limiting (Einola et al., 2007).

Significant fluctuations in the gravimetric water content observed near the surface (0–10 cm) was related to the variations in temperature and CH_4 oxidation activity, and the water content of the cover soil identified as a key factor contributing to limit deep CH_4 oxidation by preventing O_2 from reaching deeper zones within the cover (Jugnia et al., 2008). On the other hand, throughout the study period, it is within the first 30 cm from the surface that the organic matter content exhibited its highest variability. A significant decrease over time in organic matter content near the surface, as compared with deeper layers was due to more active aerobic respiration within this zone. It appears that the development of an active heterotrophic community, including methanotrophs, in the substrate within the uppermost zone of the cover, contributed to higher rates of O_2 consumption and limited its penetration into the PMOBio.

There is a possibility that non-methanotrophic bacteria contributed to the results of our MPN counts. Therefore, methanotroph counts presented here may have overestimated the actual number of culturable methanotrophic bacteria in our samples. However, spatiotemporal variations in these counts might reflect dynamics of methanotrophs that occurred during our study. Methanotrophic bacterial populations developed well within the PMOBio and a similarly high population of methanotrophs was detected by Jäckel et al. (2005) in the upper layer of a mature compost material (1.8×10^6 to 3.2×10^7cells per g). Population sizes of 10^6 cells g^{-1} soil were detected previously in wetland and landfill soils using the MPN method (Jones and Nedwell, 1993; Kallistova et al., 2007; Svenning et al., 2003). As for the temporal evolution of depth profiles of methanotroph counts, the lag phase followed the construction of the experimental cell and included the first three sampling campaigns, during which abundance profiles were essentially identical (Fig. 2). Kightley et al. (1995) reported that under laboratory conditions (19°C), it took 1 month to establish the steady-state conditions needed for growing methanotrophs in a soil column. Our results indicated that the establishment of methanotrophs probably takes much longer under field conditions. This observation corroborates a report from a previous study in a landfill cover soil by Börjesson et al. (1998). During the second phase, which extended from August to October 2006, counts of methanotrophs increased with time, particularly at the surface, to reach the maximum values observed during this study. The low number of methanotrophs determined by MPN in December within the cover soil layer above 40 cm was probably due to the lower temperatures associated with the onset of winter.

Denaturing Gradient Gel Electrophoresis Analysis and Sequencing: Methanotrophs Diversity

Molecular techniques represent a significant step forward in studies of soil microorganisms, as they allow the assessment of the total population, independent of culturing. Theoretically, culture-independent analysis provides improved coverage of community richness, however, factors such as DNA extraction bias, PCR bias, and relative abundance of target genes may affect the fingerprints obtained. While DGGE fingerprinting does not provide complete coverage of soil bacterial diversity, since only the most dominant microbial populations (approximately the top 10%) can be resolved in any fingerprint (Muyzer and Smalla, 1998; Nikolausz et al., 2005), it is a suitable approach in comparative analyses of the dominant members of communities. As in previous studies (Bodelier et al., 2005; DeJournett et al., 2007; Henckel et al., 2001; Wartiainen et al., 2003), we used the presence and pattern of different bands in the DGGE to elucidate diversity in the community of methanotrophs among the three sampling depths considered in this study.

Changes observed over time in the number of bands, particularly between the original sample (the sand and compost mixture used during the construction of the experimental plot) and those collected following the construction, reflected changes in species to those more adapted to the conditions prevailing at the time for each of the different sampling depths considered. Multidimensional scaling statistical analysis revealed that time-related differences in community structure occurred at all sampling depths (0–10, 10–20, and 30–40 cm) in this study. This can be interpreted as depth-related factor(s) shaping the methanotroph community structure, and/or different responses of the methanotrophs present at these depths to changing environmental conditions. At the 0- to 10-cm depth, significant differences in band intensities and numbers occurred, paralleling the greatest fluctuations in temperature and gravimetric water content at this depth where CH_4 oxidation was reported to be more significant (Jugnia et al., 2008). This emphasizes the importance of this layer, which contains the highest methanotrophic population density and diversity, as being the most susceptible to environmental changes that can have the greatest impact on methane oxidation activity. Previous studies (Eller and Frenzel, 2001; Horz et al., 2002; Stralis-Pavese et al., 2004) reported that changes in the methanotrophic community structure had an impact on the level of CH_4 oxidation activity.

Although the DGGE banding patterns suggested that there were changes in the methanotrophic community, all the sequences from the major DGGE bands shared similarity to Type I methanotrophs. Phylogenetic analyses showed that these changes occurred principally with species related to *Methylobacter*, as most of the sequences related to the methanotrophs clustered within this genus. This indicates that members of the genus *Methylobacter* might be dominant in the system studied, in addition to the other members of Type I methanotrophs, *Methylocaldum* and *Methylomonas* that were identified. The dominance of Type I methanotrophs in this study is consistent with a recent observation by Chen et al. (2007) who reported the prevalence of Type I methanotrophs in landfill cover soil using A189/mb661 primers. Stralis-Pavese et al. (2006) also noted the dominance of Type I methanotrophs in landfill cover soil, where landfill gas had a positive effect on the presence of *Methylobacter*. However, our inability to detect Type II methanotrophs in this study may not rule out their presence. Type II methanotrophs *Methylosinus* and *Methylocystis* have been detected in other landfills using different molecular methods, such as 16S RNA gene libraries (Chen et al., 2007; Uz et al., 2003), catalyzed reporter deposition-FISH (CARD-FISH) (Kallistova et al., 2007) or indirect immunofluorescence (Kallistova et al., 2005). Type II methanotrophs were probably much less abundant in our samples than those from these other sites.

Results from previous environmental studies and continuous culture experiments (Hanson and Hanson, 1996; Henckel et al., 2000; Macalady et al., 2002; Stralis-Pavese et al., 2004) have led to the commonly cited hypothesis that Type II methanotrophs predominate in high CH_4 concentration environments, while Type I methanotrophs dominate in environments with low CH_4 concentrations. Given that methanotrophs in landfill cover soil are expected to encounter high CH_4 concentrations, similar to those recorded (~550,000 ppmv) at the bottom of our experimental plot, we anticipated the prevalence of Type II methanotrophs. Yet, only Type I methanotrophs were retrieved from our samples, although the primer pair (A189/mb661) we used has been shown to amplify the majority of both Type I and Type II methanotrophs (Bourne et al., 2001).

The above discrepancy supports the growing body of evidence suggesting that there is no clear prevalence of Type I or Type II populations at different regimes of CH_4 concentration (DeJournett et al., 2007; Dunfield et al., 1999; Henckel et al., 2000; Knief et al., 2006; Singh et al., 2007), and that no clear delineations can be made about the niches occupied by Type I vs. Type II species: these groupings may be too broad for such generalization, and any observed trends may be ecosystem-specific (Knief and Dunfield, 2005; Knief et al., 2006).

Previous studies reported that Type I methanotrophs dominate at rapidly changing growth conditions (Graham et al., 1993; Macalady et al., 2002). According to Torsvik et al. (2002), stable conditions are generally associated with increased diversity, while unpredictable or disturbed environments lead to the outgrowth of dominant, adapted populations. In ecology, both unpredictable environments and/or rapidly changing growth conditions are generally among other characteristics of newly formed systems, where changes in the community structure is shaped by species with a high growth rate (r-strategists). Given that the experimental PMOBio investigated in the present study was relatively young (less than 1-yr old), it can be hypothesized that the system had not yet reached maturity, and a climax community, typical of established environmental conditions had probably not yet been attained. Accordingly, our results can be interpreted as changes in methanotroph community structure within an immature PMOBio, and Type I methanotrophs in this study can be viewed as pioneer species with a potentially high growth rate that become numerically dominant and reduced the evenness of species distribution.

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