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16S rRNA based T-RFLP analysis of methane oxidising bacteria—Assessment, critical evaluation of methodology performance and application for landfill site cover soils

Nancy Stralis-Pavese [a], Levente Bodrossy [a], Thomas G. Reichenauer [b], Alexandra Weilharter [a], Angela Sessitsch [a,*]

[a] *ARC Seibersdorf Research GmbH, Department of Bioresources, A-2444 Seibersdorf, Austria*
[b] *Department of Environmental Research, A-2444 Seibersdorf, Austria*

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Abstract

Methanotrophic bacteria have a ubiquitous distribution in the environment and play an important role in global climate warming by lowering methane emission into the atmosphere. Globally, landfill sites produce about 10% of the methane entering the atmosphere, and soils above landfill sites have been shown to contain methanotrophic populations with the highest methane oxidation capacity measured.

Landfill site simulating lysimeters were set up in which different vegetation regimes were tested for their effect on methane oxidation. Four different plants (*Miscanthus*, poplar, grass, alfalfa–grass mixture alongside with an unplanted control) were grown with and without landfill gas (LFG) supply. One year after planting, rhizosphere samples in the upper layer of the lysimeters were collected and the methanotroph community was analysed using a cultivation-independent approach. Terminal restriction fragment length polymorphism (T-RFLP) and RNA dot blot hybridisations with primers/probes targeting specifically type I and type II methanotrophs were used. In addition, methanotrophic 16S rRNA gene clone libraries were compared and dominant clones were sequenced. *Methylobacter* and *Methylocystis* were found to be dominant members of the type I and type II methanotroph communities, respectively. The type of plant cover affected both type I and type II methanotrophs, however, biogas had more impact on type I populations displaying higher diversity and abundance in samples supplied with LFG than in the LFG-free controls.

Keywords: Methanotroph; 16S rRNA gene; T-RFLP; RNA; Dot blot hybridisation; Landfill

1. Introduction

Methane oxidising bacteria or methanotrophs have the unique ability of utilising methane as their sole carbon and energy source (Hanson and Hanson, 1996;

* Corresponding author. Tel.: +43 50550 3509; fax: +43 50550 3444.
E-mail address: angela.sessitsch@arcs.ac.at (A. Sessitsch).

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Murrell, 1994). Methanotrophs significantly reduce biological and anthropogenic methane emissions via their activity, thus contributing to the mitigation of global warming (Hanson and Hanson, 1996). Methanotrophs are classified into two groups, type I and type II, differing in many characteristic, including the carbon assimilation pathway and the arrangement of intracellular membranes (Anthony, 1982; Whittenbury and Dalton, 1981). Type I methanotrophs (the family *Methylococcaceae* (Bowman, 2000)) are methylotrophs belonging to the γ-subdivision of *Proteobacteria* and assimilate formaldehyde via the ribulose monophosphate pathway. The family *Methylococcaceae* includes the genera *Methylomonas*, *Methylobacter*, *Methylomicrobium*, *Methylosarcina*, *Methylosphaera*, *Methylococcus*, *Methylocaldum* and "*Methylothermus*" (Bodrossy et al., 1997, 1999; Bowman et al., 1997; Wise et al., 2001). Type II methanotrophs (the family *Methylocystaceae* (Bowman, 2000)) cluster in the α-subclass of *Proteobacteria* and utilise the serine pathway as primary pathway for formaldehyde assimilation. Type II methanotrophs are comprised of the genera *Methylosinus*, *Methylocystis*, *Methylocapsa* and *Methylocella* (Dedysh et al., 2000, 2002).

Landfill methane is produced from a complex process of waste decomposition and subsequent fermentation under anaerobic conditions (Thorneloe et al., 2000). Due to methanogenic activity under anaerobic conditions, landfills produce an estimated 6–12% of the methane entering the atmosphere (Kightley et al., 1995; Houghton et al., 2001; Howeling et al., 1999; Lelieveld et al., 1998; Olivier et al., 1999). However, oxic zones of landfill cover soils were shown to consume significant amounts of the methane produced. They contain methanotroph populations with the highest methane oxidation capacity reported (Jones and Nedwell, 1993; Whalen et al., 1990).

Plants and bacteria have a range of mutually beneficial interactions. Rhizosphere (soil directly influenced by root exudates) appears to be a very heterogeneous habitat for methane oxidising bacteria. Root exudates selectively influence the growth of bacteria that colonize the rhizosphere serving as selective growth substrates for soil microorganisms (De Leij et al., 1994; Lupwayi et al., 1998; Mahaffee and Kloepper, 1997). Furthermore, plant roots loosen the soil structure and channel oxygen in the anoxic portion of landfill soil layers. Because methane oxidation is an aerobic process, plants may exert a significant effect on global methane oxidation and are thus very important in decreasing the overall methane flux to the atmosphere.

The application of molecular biological tools has greatly facilitated the study of methanotroph communities in natural environments. Methanotroph 16S rRNA and other genes encoding enzymes carrying out defined function have been successfully applied as a phylogenetic marker to assess the ecology and diversity of methanotrophs (Murrell et al., 1998). Molecular approaches such as terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE), thermal gradient gel electrophoresis (TGGE), single strand conformation polymorphism (SSCP) (Schwieger and Tebbe, 1998) and concomitant sequence analysis of 16S rRNA gene clones, have been used as a method for the rapid analysis of microbial diversity (Dunbar et al., 2000; Muyzer et al., 1993; Smalla et al., 2001). Most bacterial community analyses rely on DNA, whereas rRNA is used as a target if highly active populations are to be analysed. The average rRNA content per cell is directly correlated with the growth rate of active bacteria, and therefore changes in cellular rRNA content reflect changes in the metabolic activity within the respective population (Wagner, 1994).

In this study, the objective was to analyse the population structure of methanotrophic bacteria in the rhizosphere of planted landfill site simulating lysimeters in order to improve methane oxidation, as well as to investigate the environmental factors affecting the ecology of these organisms in landfill site cover soils. In general, little is know about the functioning of methanotrophic bacteria in complex environments such as landfills. Therefore, more knowledge about the role of methane-utilising microbes in regulating methane emissions to the atmosphere in this dynamic ecosystem is essential to facilitate the development of optimized landfill management strategies. Cultivation-independent approaches including T-RFLP, cloning and sequence analysis of 16S rRNA genes and Northern blot hybridisation with probes specific for type I and type II methanotrophs have been applied.

2. Materials and methods

2.1. Lysimeter experiments

Small lysimeters with a volume of 200 l, and a depth of 65 cm were set up to simulate landfill site environments with different plant covers. Lysimeters contained a 15 cm drainage layer of gravel at the bottom, followed by a fleece to stop small particles from being washed out into the drainage layer. Finally, lysimeters were filled up with mature compost from sewage sludge. Half of the lysimeters received artificial landfill gas (LFG) (CH_4:CO_2 = 3:2) from the bottom at a constant rate of 100 l CH_4 day^{-1} m^{-2}. For each plant cover, five lysimeters with LFG supply and four LFG free control lysimeters were set-up. The following plant covers were tested: a mixture of grass (10% *Lolium perenne*, 20% *Festuca rubra commutata*, 15% *Festuca rubra trichophylla*, 15% *Festuca rubra genuina*, 10% *Festuca ovina*, 15% *Festuca ovina duriuscula*, 15% *Poa pratensis*) and alfalfa (GA+ and GA−); grass only (G+ and G−); *Miscanthus* (M+ and M−); poplar (P+ and P−) and as negative control bare soil (BS+ and BS−), where + and − refer to lysimeters with LFG supply and LFG free controls, respectively. Lysimeters were wrapped in a 6 cm thick insulation mat that was covered with an aluminium-foil on the outside (Telwolle, Isover, Austria). Lysimeters contained gas drainage lines at 10, 20, 30, 40 and 50 cm depths for gas sampling. The compost used to fill the lysimeters had a pH of 7.0, 38% water content, 12.2% total C, 0.8% total N and a C/N ratio of 14.9. Methane concentrations at 10 cm depth in planted, landfill gas supplied lysimeters were reduced from 60% to a value of 0–3%. Bare soil lysimeters oxidised about half of the methane provided, decreasing its concentration from 60% to 30–35%.

2.2. Collection of samples

Twelve months after start of the lysimeter experiment, three replicate rhizosphere soil samples were collected from each treatment. As the experiment was continued sampling was performed in a non-destructive way by sampling the soil adjacent to plant roots at a depth of 10–20 cm. Rhizosphere soil samples were mixed, sieved and stored at −20 °C for subsequent analyses.

2.3. DNA extraction

DNA isolation was performed as previously described by Sessitsch et al. (2001). Briefly, soil samples was performed by suspending 0.4 g soil and 0.3 g glass in extraction buffer (0.12 M sodium phosphate pH 8.0) and treated with lysozyme/β-mercaptoethanol. Cells were disrupted by bead beating and cells lysis was completed by incubation in SDS for 30 min at 80 °C. Impurities were removed by phenol-chloroform extraction and precipitation of humic acids by potassium acetate. DNA precipitation was completed by addition of iso-propanol, washed in 70% ethanol and air dried. The pellets were dissolved in 40 μl TE containing 1 mg ml^{-1} RNase H. The crude DNA extract was purified using sepharose CL-6B (Pharmacia) impregnated with 20 mg polyvinyl-pyrrolidinone (Sigma, 20 mg ml^{-1}) in a column and checked on conventional agarose gel electrophoresis. DNA obtained by this treatment was used for PCR.

2.4. PCR amplification of 16S rRNA

To amplify type I 16S rDNA sequences (922 bp) from methanotrophic bacteria, the forward primer Meth T1dF (5′-CCTTCGGGMGCYGACGAGT-3′) and the reverse primer Meth T1bR (5′-GATT-CYMTGSATGTCAAGG-3′) (Wise et al., 1999) were used. Type II 16S rRNA gene (1009 bp) was amplified using the forward primer: 8f (5′-AGAGTTT-GATCCTGGCTCAG-3′) (Edwards et al., 1989) and the reverse primer Meth T2R (5′-CATCTCTGRC-SAYCATACCGG-3′) (Wise et al., 1999). All reactions were carried out in 50 μl (total volume) mixtures containing approximately 100 ng of soil DNA, 1× reaction buffer (Gibco, BRL), 200 μM each dATP, dCTP, dGTP and dTTP, 3 mM $MgCl_2$, 0.15 μM of each primer and 2.5 U Taq DNA polymerase (Gibco, BRL). The reactions were performed in a (PT-100™, MJ Research Inc.) thermal cycler by using an initial denaturation step of 95 °C for 5 min followed by 30 cycles consisting of 95 °C for 30 s, 52 °C annealing (type I)/56 °C (type II) for 1 min, 72 °C for 2 min, and a final extension step of 72 °C for 4 min. PCR products were checked on agarose gels, and amplified 16S rDNAs were used for construction of clone libraries. For T-RFLP analysis the same PCR primers and conditions as described above were used. Though, the

forwards primers Meth T1dF and 8f were labelled at the 5′ end with 6-carboxyfluorescein (6-Fam; MWG).

2.5. Terminal restriction fragment length polymorphism (T-RFLP) analysis

Approximately 200 ng fluorescently labelled PCR amplification products were digested with the restriction enzymes *Msp*I (Gibco, BRL) for type I methanotrophs and *Hha*I (Gibco, BRL) and *Rsa*I (Gibco, BRL) individually for type II methanotrophs. Aliquots of 1.0 μl were mixed with 1 μl of loading dye buffer (Fluka) and 0.3 μl of the DNA fragment length standard (Rox 500; PE Applied Biosystems Inc., Faster City, CA, USA). This mixture was then denatured at 92 °C for 2 min and immediately chilled on ice prior to electrophoretic separation on 5% polyacrylamide gels. Fluorescently labelled terminal restriction fragments were measured using an ABI 373A automated DNA sequencer (PE Applied Biosystems Inc.) and the ABI PRISM Big Dye terminator cycle sequencing kit (Perkin-Elmer) in the GeneScan mode. Lengths of labelled fragments were determined by comparison with the internal standard using the GeneScan 2.5 software package (PE Applied Biosystems Inc.).

2.6. Analysis of T-RF profiles

Considering that a difference of ±2 bp in the sizes of T-RFs is possible to occur due to the nature of the gel separation with our automated DNA sequencer, T-RFs that differed by less than 2 bp were clustered. Additionally, to avoid detection of primers and uncertainties of size determination, terminal fragments smaller than 35 bp were excluded from the analysis. T-RFLP profiles were normalized according to a procedure suggested by Dunbar et al. (2000). First, to standardise the DNA quantities, the sum of peak heights in each profile of a sample was calculated as a representation of the total DNA quantity, and the peak intensity was adjusted to the smallest DNA quantity within a treatment. For example, given two profiles with total fluorescence values of 942 and 2000, respectively, each peak in the latter profile would be multiplied by a correction factor of 0.47 (i.e., a quotient of 942/2000). After adjustment, terminal fragments (T-RFs) were only scored positive, when they showed intensities higher than 30 fluorescence units. Representative community profiles comprised average fluorescence intensities (≥30 fluorescence units) of T-RFs represented in normalized replicate profiles. Cluster analysis based on Euclidian distance was performed by comparing peak heights in representative samples by using the Statistica software.

2.7. Construction of 16S rRNA gene libraries and restriction fragment length polymorphism (RFLP)

Type I and type II methanotroph 16S rDNA PCR products were purified with NucleoTrap CR kit (Macherey-Nagel), ligated into the pGEM-T vector (Promega) following manufacturers' instructions and transformed into *Escherichia coli* DH5α competent cells. Individual colonies containing inserts of the appropriate size were suspended in 80 μl of TE pH 8.0 and boiled for 10 min. Cell debris was removed by centrifugation and 1 μl portions of the supernatant were used as template in PCR mixtures to reamplify the 16S rRNA gene inserts. PCR primers and conditions were the same as those described. The reamplified product was used in restriction digestions with tetrameric restriction enzymes. The 16S rRNA genes were digested with 5U of endonucleases *Msp*I (Gibco, BRL) and *Hha*I (Gibco, BRL) for type I and type II methanotrophs, respectively, for 3 h at 37 °C. Digested 16S rRNA genes were resolved on 2.5% agarose gels and grouped manually based on the restriction patterns.

2.8. Sequence analysis

For sequence identification of methanotrophs, PCR products were purified using the NucleoTrap CR kit (Macherey-Nagel) according to the manufacturer's instructions and used as templates in sequencing reactions. Partial DNA sequencing was performed with the M13(-20)fw (5′-GTAAAACGACGGCCAG-3′) and M13rev (5′-CAGGAAACAGCTATGAC-3′) primers targeting the multicloning site of the vector as well as with the 16S rRNA gene sequencing primer 518r (5′-ATTACCGCGGCTGCTGG-3′) (Liu et al., 1997), by the dideoxy chain termination method (Sanger et al., 1977) using an ABI 373A automated DNA sequencer and the ABI PRISM Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems Inc.). The ARB software package

(Ludwig et al., 2004) was used for phylogenetic sequence analysis. The nearest neighbor method was used to construct phylogenetic trees.

2.9. Ribosomal RNA extraction

Lyophilized soil was treated twice with 1% active diethylpyrocarbonate (DEPC) and once with 0.1% overnight at 37 °C and autoclaved DEPC. RNA isolation was based on initial ribosome isolation from rhizosphere soil and was performed as described previously (Felske et al., 1996). RNA from reference strains (*Methylomonas methanica* S1 and *Methylosinus trichosporium* OB3b for type I and type II methanotrophs respectively) was isolated by using the Rneasy Mini Kit of QIAGEN (Hildenberg, Germany) according to the manufacturer's instructions.

2.10. Dot blot hybridisation

RNAs were immobilised on Hybond N+ membranes (Amersham, Slough, England) by UV light using standard techniques (Sambrook et al., 1989). Blots were prepared with 25, 50, 100, 250, 500, 1000 ng of RNA of reference strains side by side with 1000 ng of rRNA rhizosphere samples. The oligonucleotides probes and annealing temperature determinations used in this study were carried out as described by Gulledge et al. (2001). The probes used for the detection of type I and type II methanotrophs and hybridisation temperatures are listed in Table 1. The numbering used in probe designation represents the forward position of the homologous base in the *E. coli* 16S rRNA gene. Oligonucleotides probes were labelled at the 5′-end by using phage T4 polynucleotide kinase (Promega) and 30 μCi of [^{32}P]ATP (3000 Ci/mmol; Amersham) (Cunningham, 1995). Prehybridisation, hybridisation and wash solutions used were described previously (Tsien et al., 1990). The membranes were incubated for 2 h in prehybridisation buffer (0.9 M NaCl, 50 mM sodium phosphate [pH 7.0], 50 mM EDTA [pH 7.2], 0.5% SDS, 10× Denhardt's solution (Sambrook et al., 1989) at the temperature specific (T_d) for each probe (Table 1). Hybridisation was carried out overnight in hybridisation buffer (0.9 M sodium phosphate [pH 7.2], 1% SDS, 1% bovine serum albumin, 1 mM EDTA [pH 8.0]) with 20 pmol of appropriate labelled probe. Subsequently, the membranes were washed once at room temperature and once at hybridisation temperature for 30 min in hybridisation buffer. The last wash step was in wash solution (40 mM sodium phosphate [pH 7.2] with1% SDS and 1 mM EDTA) for 10 min at room temperature. Hybridised membranes were incubated with a detection screen (Molecular Dynamics, Sunnyvale, California) and fluorescent signals were detected and quantified with a Phosphor Imager SF (Molecular Dynamics).

2.11. Sequence accession numbers

The nucleotide sequences determined in this study have been deposited in the GenBank database under accession numbers AY599181 to AY599194.

Table 1
Oligonucleotide probes targeting methanotrophic bacteria applied in this study

Probe name Abbreviation	Probe sequence (5′–3′)	Target group(s)	Reference strain(s)		T_d (°C)
			Positive control(s)	Negative control(s)	
Am 445	CTTATCCAGGTACCGTCATTATCGTCCC	Type II methanotrophs	*Methylosinus trichosporium* OB3b	*Rhizobium fredii* 61A201 (*α-Proteobacterium*)	57
Am 976	GTCAAAAGCTGGTAAGGTTC	Type II methanotrophs	*Methylosinus trichosporium* OB3b	*Rhizobium fredii* 61A201 (*α-Proteobacterium*)	51
Gm633	AGTTACCCAGTATCAAATGC	*Methylobacter* and *Methylomicrobum*	*Methylomonas Methanica* S1	*Escherichia coli* (*γ-Proteobacterium*)	50
Gm705	CTGGTGTTCCTTCAGATC	Type I methanotrophs except *Methylocaldum*	*Methylomonas Methanica* S1	*Escherichia coli* (*γ-Proteobacterium*)	51

3. Results

3.1. Landfill site simulation experiments

Landfill site simulating lysimeters with different plant covers were monitored for 1 year and analyzed with respect to their methane oxidation potential. The different vegetations covering these simulated landfill site lysimeters investigated were poplar, *Miscanthus*, grass, a mixture of grass and alfalfa and bare soil as control.

All plant covers tested improved the methane oxidation potential of the soil significantly as compared to bare soil. The methane oxidation capacity of the soils is best indicated by the disappearance of methane and the concomitant increase in the concentration of carbon dioxide, with decreasing depth. This is reflected in the methane to carbon dioxide ratios, as shown in Fig. 1. Typical gas concentration profiles for landfill site simulating and control (LFG free) lysimeters are also shown in Fig. 1. While all plant covered lysimeters oxidised most of the methane supplied, thus their maximal methane oxidation capacity was not measured, the methane to carbon dioxide profiles showed strong differences. The highest methane oxidation was found in soils with a grass and alfalfa (GA) plant cover. Soils with poplar (P) and with *Miscanthus* (M) oxidised methane at a similar rate. Lysimeters with a grass only (G) plants cover oxidised methane the least, however, methane oxidation was considerably better than observed with bare soil cover (BS).

All LFG supplied lysimeters with plant cover oxidised most of the methane supplied, equaling a methane oxidation rate of 35 g methane m^{-2} day^{-1} or higher. While the highest methane oxidation capacity of the lysimeters was not tested, these results still correlate well with the extremely high methane oxidation rates reported for landfill site cover soils earlier, 45 g methane m^{-2} day^{-1} (Whalen et al., 1990) and 166 g methane m^{-2} day^{-1} (Kightley et al., 1995). In contrast, bare soil lysimeters oxidised significantly less methane, at a rate of 25 g methane m^{-2} day^{-1} or less. The difference between the methane oxidation capacity of the planted and bare soil lysimeters is even higher than indicated by these data. Bare soil lysimeters had methane concentrations of 30–35% in the top 10 cm layers, indicating that they reached their maximum oxidation capacity. In contrast, planted lysimeters oxidised all or almost all the methane supplied and thus higher maximum methane oxidation rates can be extrapolated for them.

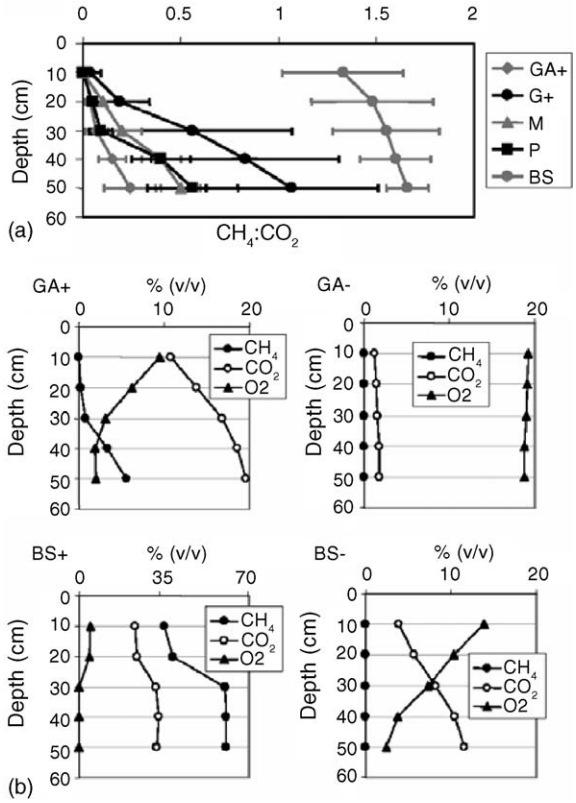

Fig. 1. (a) Depth profiles of methane to carbon dioxide ratios in the lysimeters. The figure shows results obtained before dismantling the lysimeters. Data shown represent the average of results from five parallel lysimeters. Very similar results were obtained throughout the three summer months preceding dismantling. The ratio of methane to carbon dioxide in the original artificial biogas mix introduced in the bottom of the lysimeters was 3:2. (b) Typical gas concentration profiles for landfill site simulating and control lysimeters. GA+, biogas-plus lysimeter with grass–alfalfa mixture cover; GA−, biogas-free lysimeter with grass–alfalfa mixture cover; BS+, biogas-plus lysimeter without plant cover; BS−, biogas-free lysimeter without plant cover.

3.2. Validation of T-RFLP analysis for methanotrophs

Methanotroph communities in landfill site simulating lysimeters with different plant regimes were analysed by determining the number and abundance of T-RFs observed in digests of PCR amplified

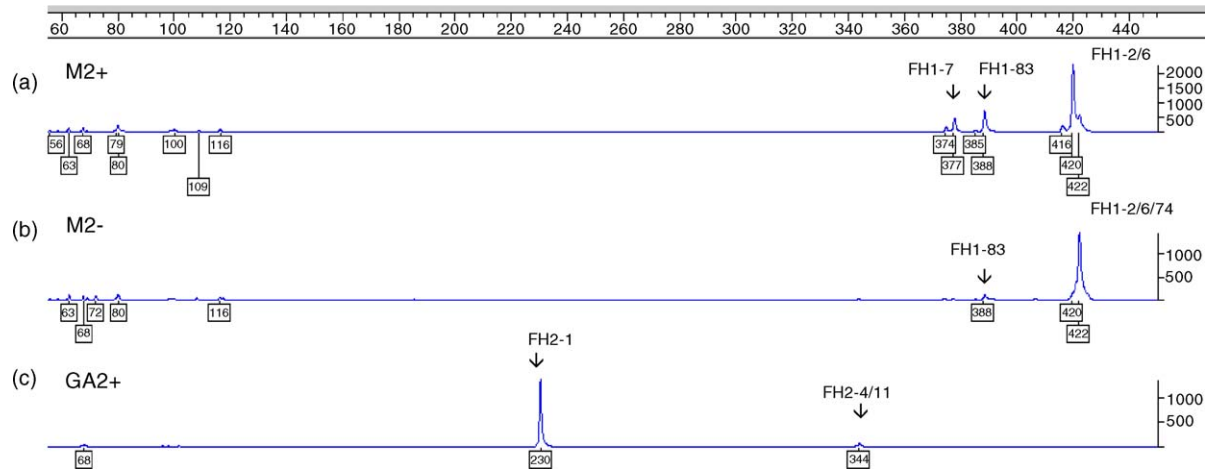

Fig. 2. 16S rDNA-based *Msp*I (M2+ M2−) and *Hha*I (GA2+) T-RFLP fingerprint patterns of rhizosphere samples of *Miscanthus* pot 2 with and without LFG and of rhizosphere grass–alfalfa mixture pot 2 with LFG. (a and b) Sequenced type I methanotroph clones FH1-2; FH1-6; FH1-7; FH1-74 and FH1-83 are associated with their correspondent T-RFs peaks. (c) Sequenced type II methanotrophic clones FH2-1; FH2-2; FH2-4 and FH2-11 are associated with their correspondent T-RFs peaks.

16S rDNAs (Fig. 2). Degenerate methanotroph-specific 16S rRNA PCR primers were used to assess methanotroph diversity. Two primer pairs (MethT1dF-MethT1bR and 8f-MethT2R) were applied (Wise et al., 1999) targeting type I and type II methanotrophs, respectively. The primer pairs did not cover the more recently discovered genera of *Methylocaldum* and *Methylosphaera* of the type I methanotrophs as well as *Methylocapsa* and *Methylocella* of the type II methanotrophs, respectively. Three restriction endonucleases were identified as those producing the highest numbers and best size distribution of T-RFs, *Msp*I for type I methanotrophs and *Hha*I and *Rsa*I (applied individually) for type II methanotrophs. T-RF fragment sizes were predicted for known methanotroph 16S rDNA using the ARB phylogenetic software package (Ludwig et al., 2004). A summary table showing T-RFLP peaks (fragment sizes) predicted for known type I and type II methanotrophs are displayed in Table 2.

Methane appeared to have a relatively strong impact on community profile of type I methanotrophs in rhizosphere sample of *Miscanthus* and of type II methanotrophs in grass–alfalfa mixture. However, in general, a smaller diversity of type II methanotroph was observed by T-RFLP, thus only limited number of type II methanotroph clones were analysed. Therefore, 16S rRNA gene fragments from three soil samples (M2+, M2− for type I and GA2+ for type II) were analysed by cloning and sequencing to confirm the presence of methanotrophs, the presence of which was indicated by the T-RFLP peaks. Clone libraries were first analysed by RFLP and representatives of each RFLP pattern represented by more than one clone were sequenced (Table 3). Eighty-six clones of type I methanotrophs from the M2+ and 81 clones from the M2− sample were analysed, resulting in the identification of 23 and 45 RFLP pattern groups, respectively. Twelve clones of type II methanotrophs from the GA2+ sample fell into of six distinct groups as defined by RFLP patterns.

Sequence data were analysed and phylogenetic trees were constructed (Fig. 3) using the ARB software package. Clones FH1-6 and FH1-7, representing 74% of the M2+ (LFG supplied) and 28% of the M2− (LFG free control) libraries, respectively, were related to the type I methanotrophs *Methylobacter* spp. LW12 and LW14 (Table 3). The less abundant RFLP types found in the M2+ library were most closely related to *Methylosarcina quisquilliarum* AML-C10 (clone FH1-2) and *Methylobacter* sp. LW1 (clone FH1-83). Furthermore, less abundant RFLP types found only in M2− showed no close relatedness to type I methanotrophs. For peaks higher than 117 bp, T-RFLP and clone library results were congruent. The high abundance of RFLP group G1-6 corresponded

Table 2
Predicted T-RF fragment sizes for type I and type II methanotrophs (16S rRNA digested with *Msp*I, *Hha*I and *Rsa*I, respectively)

Genus	Species	T-RF
Type I		*Msp*I
Methylomonas	*methanica*	374–376
	methanica	386
	scandinavica	374
	fodinarium, *lutea*, *rubra*	375
	aurantiaca	376
Methylomicrobium	*agile*, *album*	375
	pelagicum	376
	alcaliphilum, *buryaticum*	418
Methylosarcina	*fodinarium*, *quisquilliarum*	386
Methylobacter	*capsulatus*, *luteus*	373
	psychrophilus, *whittenburyi*, strain BB5.1	375
	bovis, *vinelandii*, other strains	383–386
	strains LW12/LW14	418
Methylococcus	*capsulatus*, *thermophilus*	419
Type II		*Hha*I
Methylocystis	*parvus*	229–231
Methylocystis	*echinoides and pyriformis*	339–340
Methylosinus	*trichosporium*	65
Methylosinus	*sporium*	340–343
Type II		*Rsa*I
Methylocystis	*parvus*	422–425
Methylocystis	*echinoides and pyriformis*	420
Methylosinus	*trichosporium*	111–114
Methylosinus	*sporium*	421–425

well with the peak at 420. Similarly, group G1-7, abundant in the M2+ library had a calculated T-RF value of 375 which was found in the corresponding T-RFLP profile. Lower abundance RFLP groups were generally detected at their respective calculated T-RF sizes. T-RF peaks of 110 bp or smaller were not associated with methanotrophs based on in silico sequence analysis. The analysis of a small type II clone library (Table 3) from GA2+ showed that the dominant group of the clones identified by RFLP analysis and sequencing corresponded to the major peak of the T-RFLP pattern. This group (G2-1), represented by clone FH2-1 with a predicted T-RF peak of 231 bp, was most closely related to *Methylocystis* sp. LW5, while clone FH2-8 to *Methylocystis* sp. AML-A6. We obtained a small number of clones, which were not related to 16S rDNA sequences of methanotrophs (see Table 3), indicating that the PCR primers applied were not completely specific to type I and type II methanotrophs, respectively. This shortcoming could also explain the presence of some small peaks in T-RFLP, which were not found in the clone libraries.

3.3. T-RFLP community analysis of lysimeter experiment

T-RFLP was used to analyse 24 lysimeter samples (4 different plant covers; 3 replicates; lysimeters treated with LFG and LFG-free controls) for type I and type II methanotroph diversity. For type I methanotrophs, a total of 12 peaks were detected in the different samples (Fig. 4). Rhizosphere communities from most of the LFG free lysimeters produced more fragments smaller than 117 bp, while in LFG fumigated lysimeters, a higher diversity of restriction fragments in the range of 117–421 bp was found. In addition, three T-RFs of 117, 419 and 421 bp were present in all samples. The T-RF of 421 bp exhibited high abundance across all samples, but higher fluorescence intensities were found in LFG free lysimeters, whereas the T-RF of 419 bp had greatly higher intensities in LFG fumigated lysimeters than in those without fumigation. In LFG treated samples some peaks (fragments 375, 377, 385, 416 bp) were exclusively found, while others (fragments 117, 388, 419 bp) showed increased signals in comparison to control treatments. For T-RFLP analysis of type II methanotrophs two restriction endonucleases (*Rsa*I and *Hha*I) were chosen to cut amplified 16S rRNA genes. T-RF profiles obtained with *Rsa*I endonuclease (Fig. 5) showed that T-RFs of 424 bp (GA+) and of 421 bp (G+, P+) had higher intensities in LFG treated samples than in untreated ones. Using *Hha*I digestion Fig. 6), we found two peaks to be specifically induced in the presence of LFG—a 231 bp fragment in soil samples obtained from GA+ and a 341 bp fragment from P+ and G+.

Considering the results of T-RFLP profiles of type I methanotrophs (Fig. 4), methane had a strong impact on community profiles in all treatments, most pronounced in the peaks between 375 and 419 bp. In general, methanotrophic species richness was higher in lysimeters with LFG than in those without LFG. Additionally, members of type I *Methylobacter* (potentially also *Methylococcus* and *Methylomicrobium*) (see Table 2) appeared to be present in all

Table 3
Distribution of RFLP types among 16S rRNA gene clones obtained from the treatments of rhizosphere *Miscanthus* with and without landfill gas supply (M2+ and M2−, respectively) using *Msp*I digestion and of rhizosphere alfalfa–grass mixture with landfill gas supply (GA2+) using *Hha*I digestion

Group	Sequenced representative clones	Theoretical T-RF	Closest NCBI match	%Homology	No. of Clones M2+[a]	No. of clones M2−[a]
M2+						
G1-6	FH1-6	418	*Methylobacter* spp. LW12 and LW14 (AY599181)	97	32	22
G1-7	FH1-7	375	*Methylobacter* spp. LW12 and LW14 (AY599182)	96	32	1
G1-2	FH1-2	418	*Methylosarcina quisquilliarum* AML-C10 (AY599183)	95	2	6
G1-83	FH1-83	386	*Methylobacter* sp. LW1 (AY599184)	97	1	3
M2−						
G1-70	FH1-70	405	*Thiobacillus prosperus* (AY599185)	93	0	6
G1-54	FH1-54	70	*Thioalkalivibrio thiocyanodenitrificans* (AY599186)	91	0	2
G1-74	FH1-74	419	*Thermomonas haemolytica* (AY599187)	94	0	2
G1-159	FH1-159	70	*Thiobacillus prosperus* (AY599188)	91	0	2
	Total				86	81
					No. of clones	
GA2+						
G2-1	FH2-1	231	*Methylocystis* sp. LW5 (AY599189)	98	5	
G2-4	FH2-4	339	*Paracoccus denitrificans* (AY599192)	87	1	
G2-7	FH2-7	423	*Methylocystis* sp. AML-A6 (AY599190)	93	2	
G2-8	FH2-8	511	*Methylocystis* sp. AML-A6 (AY599191)	99	1	
G2-11	FH2-11	341	*Bosea minatitlanensis* (AY599193)	98	2	
G2-13	FH2-13	520	*Bosea thiooxidans* (AY599194)	96	1	
	Total				12	

[a] Only clones found more than once in the clone libraries are shown.

samples. The dominance of the type I *Methylobacter* was confirmed by previous clone library analysis (RFLP typing and sequencing of representative clones) from M2+. Phylogenetic sequence analysis of the cloned 16S rRNA genes indicated that novel, so far uncultured methanotrophs relatively distantly related to *Methylosarcina* and different groups of *Methylobacter* were the dominant members of the type I methanotroph community in the M2+ and M2− lysimeters (Fig. 3).

The analysis of type II methanotrophs indicated high abundance of *Methylocystis* in GA2+ samples by both individual cleavages, also supported by comparative sequence analysis using *Hha*I digestion (Fig. 3). The analysis of type II methanotrophs was subsequently based on the *Hha*I digestions. The peaks associated with LFG addition under grass and poplar plant covers (in G+ and P+ lysimeters) arose most likely from *Methylocystis echinoides/Methylocystis pyriformis* related methanotrophs.

A cluster analysis based on T-RFLP profiles (peak height information) demonstrated the degree of differences of type I methanotroph population structures between planted lysimeters with and without LFG treatment (data not shown). Rhizosphere communities from LFG treated samples formed a clearly separated cluster from the LFG free controls. All planted lysimeters that received LFG provided higher T-RF peaks indicating higher abundance. In contrast, LFG did not have the same effect on type II methanotrophs as observed for type I methanotrophs. The different population structures from type II appeared to be more closely associated with the type of plant cover than with the presence of LFG.

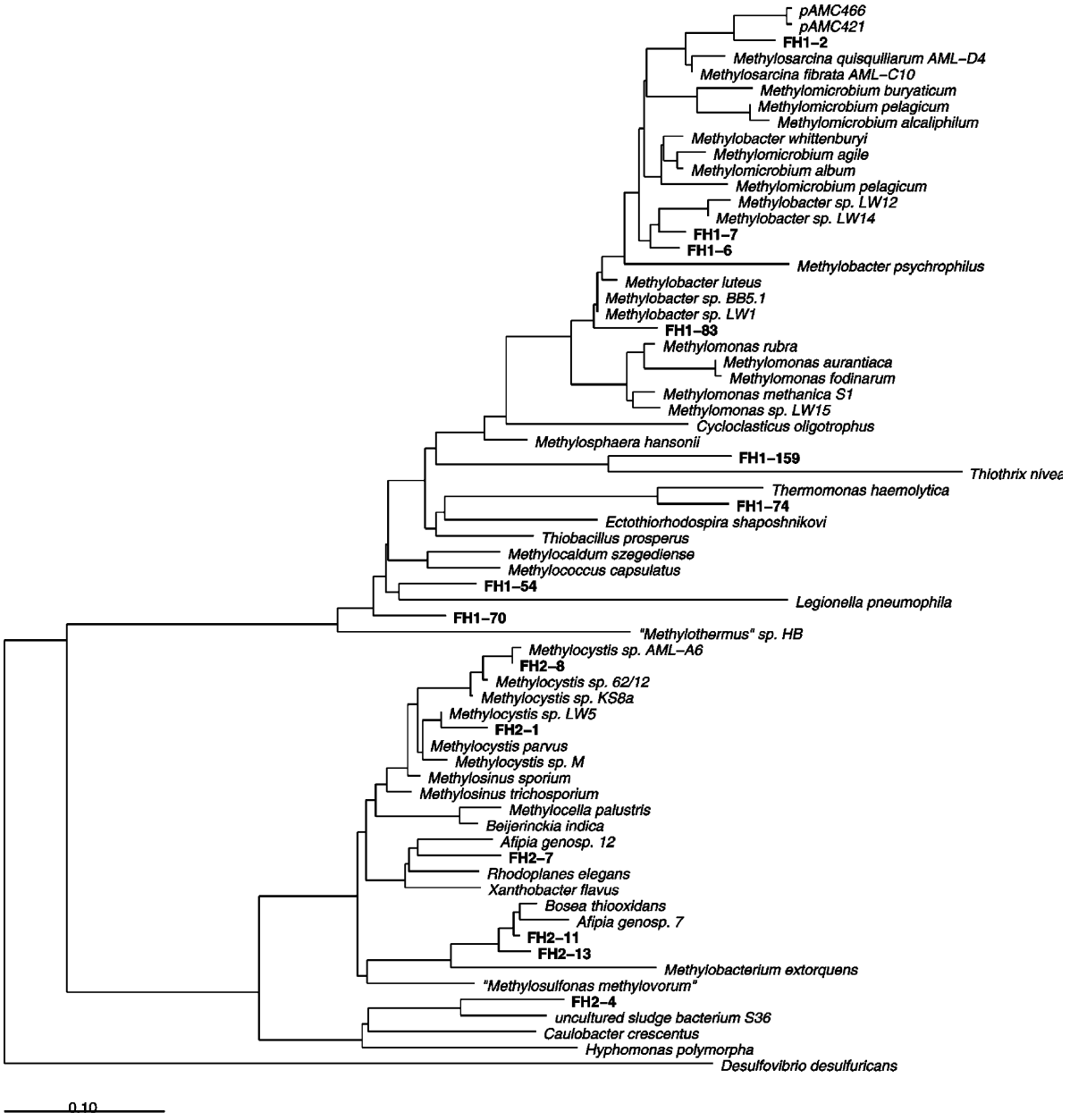

Fig. 3. Nearest neighbor tree indicating the phylogenetic affiliation of type I and type II methanotroph 16S rRNA gene clone sequences retrieved from landfill soil. The bar indicates the estimated number of base changes per nucleotide sequence position.

3.4. Dot blot hybridisation

Oligonucleotide probes targeting specifically type I and type II methanotrophs were used to estimate the abundance of the 16S rRNA from methanotrophs. A dilutions series of reference strains *Methylomonas methanica* S1 and *Methylosinus trichosporium* OB3b (type I and type II methanotrophs, respectively)

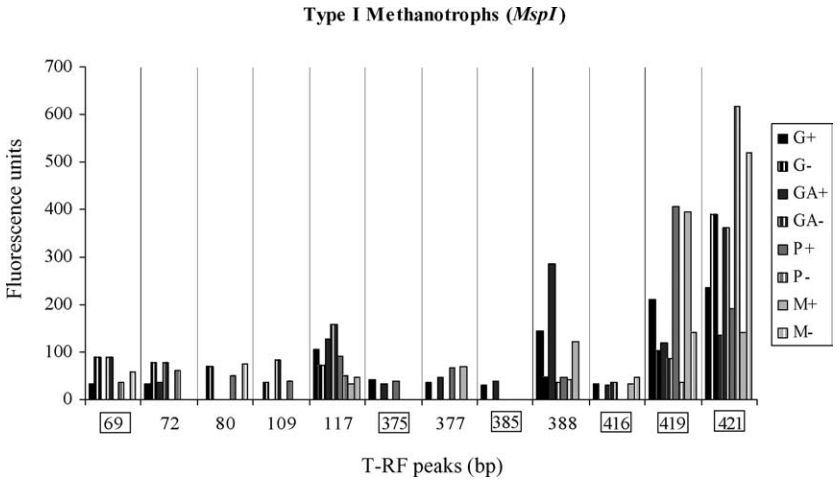

Fig. 4. Representative sample T-RFLP profiles (*Msp*I) of type I methanotrophs of rhizosphere samples treated with (+) and without (−) LFG. T-RF peaks highlighted by boxes were represented in the clone library of *Miscanthus* (M2+ and M2−) by clones with corresponding predicted T-RF sizes.

hybridised with Gm633 and Gm705 (type I specific), and Am445 and Am976 (type II specific) probes gave clear signals with intensities correlating with concentration (data not shown), down to 25 ng. Nevertheless, hybridisation to 1000 ng rhizosphere sample rRNA with all four oligonucleotide probes exhibited hardly detectable signal. The same rRNA dot blots yielded strong signals for all samples analysed when hybridised with universal 16S rRNA probe (Eub338).

4. Discussion

4.1. T-RFLP as a method to study methanotroph communities

In this study, the applicability of T-RFLP analysis based on the 16S rRNA genes specifically amplified from methanotrophs was validated by analysis of 16S rDNA clone libraries. The community profiling method was then applied for the study of methano-

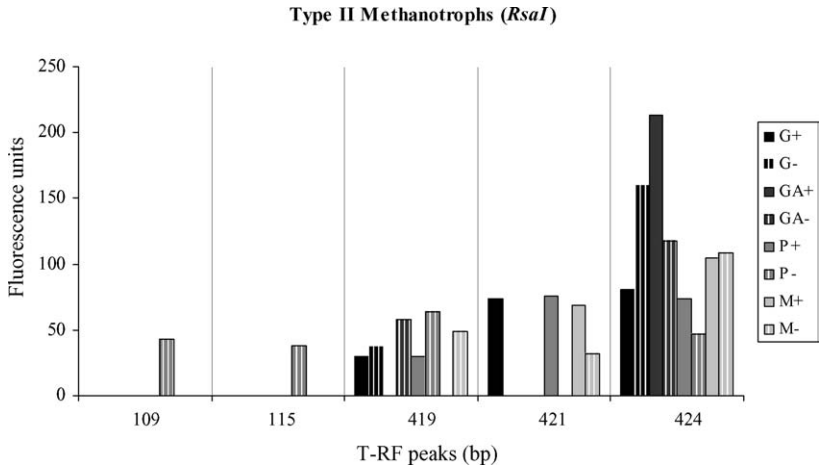

Fig. 5. Representative sample T-RFLP profiles (*Rsa*I) of type II methanotrophs of rhizosphere samples treated with (+) and without (−) LFG.

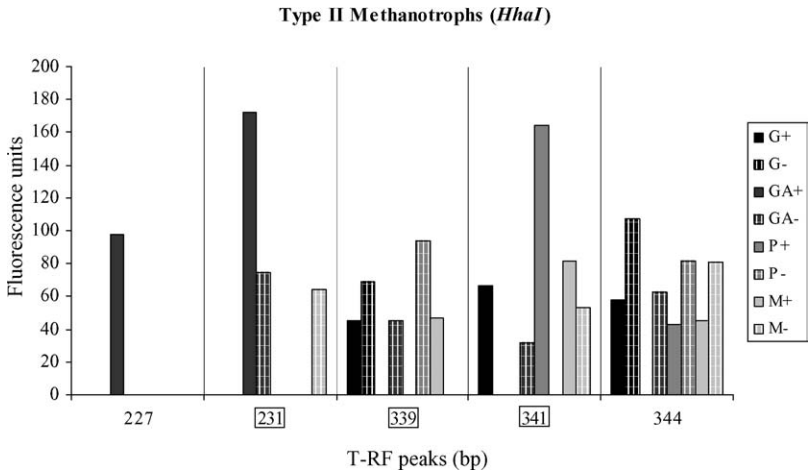

Fig. 6. Representative sample T-RFLP profiles (*Hha*I) of type II methanotrophs of rhizosphere samples treated with (+) and without (−) LFG. T-RF peaks highlighted by boxes were represented in the clone library of grass–alfalfa mixture with LFG supply (GA+) by clones with corresponding predicted T-RF sizes.

troph communities in the rhizosphere of different plants used for the vegetation of landfill site simulating lysimeters. 16S rRNA-based T-RFLP combined with methanotroph specific primers was demonstrated to detect changes and differences in methanotroph community structures. For validation, three samples were selected and analysed by RFLP and sequence analysis of 16S rRNA clone libraries. Generally, good agreement was found between results using these two approaches. There were only a few clones in the clone libraries where no corresponding T-RF peaks could be detected in the T-RFLP profiles. These can be attributed most likely to the low number of clones analysed, resulting in a non-statistical representation of the methanotroph diversity in the clone libraries (especially for the type II analysis). Some T-RFs could not be assigned to known methanotroph sequences. We assume that these fragments represent either ''pseudo T-RFs'' (false T-RFs) (Egert and Friedrich, 2003) caused by secondary structure formation during PCR resulting in partly single-stranded 16S rRNA gene amplicons and consequently partial digestion products and false positive T-RFs. Alternatively, unspecific amplification of 16S rRNA genes may have occurred.

The major limitation of the approach is the lack of coverage for the more recently described genera *Methylosphaera*, *Methylocaldum*, ''*Methylothermus*'', *Methylocapsa* and *Methylocella*, which could be overcome by new primer design. Thus, the complete diversity and richness of the methanotroph community is not covered and only the phylotypes that were efficiently amplified by the ''specific'' primer sets are represented in the profiles. Despite this shortcoming T-RFLP analyses were shown to be a reliable method to be used for the fingerprinting of methanotroph communities in landfill site cover soil.

4.2. Methanotroph communities in landfill simulating lysimeters

Various vegetation strategies were tested regarding their effect on the methane oxidation capacity of landfill sites. Plants were considered to play a beneficial role as the roots increase oxygen concentrations even in deeper layers of the soil and provide niches to be colonized by aerobic bacteria. As most methanotrophs are strictly aerobes and require oxygen for efficient methane oxidation, oxygen availability in landfill sites is a major limitation to the growth and activity of methane oxidising bacteria. Furthermore, the rhizosphere is a hot-spot of microbial activity as root exudates provide a broad range of nutrients supporting microbial growth. This study showed that the dominant methanotrophs colonizing the rhizospheres of various plants tested belonged to the genera

Methylobacter and *Methylocystis.* In the presence of methane, the rhizospheres showed a higher abundance of the genus *Methylobacter* belonging to type I methanotrophs as opposed to control plants. In the latter treatments generally a lower abundance of methanotrophs was detected. This finding indicates that the growth of methanotrophs, in particular that of *Methylobacter* was induced by the presence of LFG in this environment. In general, type I methanotrophs showed a higher diversity represented by a number of different T-RFs, which were found in all LFG-treated samples. Methane had also a significant effect on population structure of type II methanotrophs, and particularly in lysimeters planted with a mixture of grass–alfalfa and poplar, methanotrophs were induced. Analysis of T-RFLP by both individual cleavages demonstrated high abundance of type II methanotrophs belonging to the species *Methylocystis parvus* in these lysimeters. This result was confirmed by clone library sequence analysis as well. *Methylocystis parvus* has a ubiquitous distribution in the environment. It has been detected in a wide variety of environments including landfill (Wise et al., 1999), sediments (Bussmann et al., 2004) and on the rhizosphere of flooded rice (Rotthauwe et al., 1997). In general little is known about methanotroph diversity in landfill cover soils. A recent analysis (Uz et al., 2003) of 16S rRNA gene libraries from landfill samples 3 m below the surface detected type I methanotrophs *Methylobacter* and type II methanotrophs *Methylocystis* and *Methylosinus* despite the relatively deep sampling. In previous work on landfill site simulating lysimeters with the same plant covers (Stralis-Pavese et al., 2004), methanotrophs community was analysed throughout the lysimeter using *pmoA* as phylogenetic marker and diagnostic microarrays. In this work, GA+ lysimeters harboured a major *Methylocystis* population throughout the depth profile, including the top layer. Furthermore, no *Methylosinus* population has been found in our previous study. Type Ib methanotroph *Methylocaldum* was the dominant population using *pmoA* primers (T3c-*pmoA*189 and T7c-mb661). This result did not link with our T-RFLP analysis due to the lack of coverage for this genus by the 16S rRNA primers pairs. In contrast, only a small population of type Ia *Methylobacter* has been found in the upper layer of the fumigated control in this work. Other works (Costello and Lidstrom, 1999; Horz et al., 2001), have similarly concluded that 16S rRNA based and *pmoA* based T-RFLP methods yield similar methanotroph community profile results, both approaches being suitable for such work, complementing each other. 16S rRNA (Mb1007r, Mc1005r, Mm835 and Type2b) and the *pmoA* (*pmoA-mb*661) primers sets were applied for studying natural population of methanotroph community, identifying a broad, similar range of diversity of these genes in the sediment of Lake Washington (Costello and Lidstrom, 1999) and on the roots of submerged rice plants (Horz et al., 2001) showed a large variety of type I, including the genus *Methyloccocus* and type II methanotrophs. Dot blot hybridisation did not allow quantitative assessment of methanotrophs. The relative abundance of type I and type II methanotrophs was below the threshold of the method (found to be slightly below 2%) in the environments analysed. As in most lysimeters, the vast majority if not all of the methane was oxidised, it is most likely that methanotrophs were methane limited, especially in the top layer analysed in the current work.

5. Conclusions

Both type I and type II methanotrophs were affected by the type of plant cover, but LFG had a more pronounced effect on type I populations showing higher abundance/diversity by samples fumigated with LFG than those without fumigation. In addition, we can speculate that some type I methanotrophs (*Methylobacter*) appear to be best adapted to growth in the presence of landfill gas in the upper layer (oxic zone) of the plant rhizosphere. In correlation with the results of gas profiles, all planted lysimeters supplied with LFG oxidised most of the methane supplied at a rate of 35 g methane m^{-2} day^{-1} or higher, while bare soil lysimeters (BS+) displayed methane oxidation rate of 25 g methane m^{-2} day^{-1}. The best performance was shown in lysimeters with a grass and alfalfa mixture (GA+), where a high relative abundance of *Methylocystis* was found. The second highest methane oxidation was found in *Miscanthus* (M+) and poplar (P+) lysimeters while grass alone (G+) exerted a significantly less positive effect. Bare soil had the lowest level of methane oxidation

capacity. Our result showed that LFG had a positive effect on the population structure of methannotrophic bacteria. However, a clear correlation between methane oxidation capacity and the methanotroph communities associated with the different plant covers was difficult to deduce. The relative abundance of methanotrophs in the top layers analysed was found to be below 2%, in accordance to the relatively low methane concentrations in these top layers and the increased availability of oxygen, the latter potentially giving rise to a diverse aerobic bacterial community.

T-RFLP technique is dependent on efficient unbiased DNA extraction and PCR amplification of a target gene. The non-complete coverage of the known diversity of methanotrophs by the existing PCR primer sets and the limited phylogenetic resolution of the T-RFLP method determine the limitation of the approach. Nevertheless, in this study, T-RFLP profiles combined with clone library sequence analyses demonstrated reliable results and proved to be suitable for a rapid molecular fingerprinting of methanotroph communities in landfill cover soils.

The results of this study indicate a general trend of the dynamics of the methanotrophic population structures in the rhizospheres of planted lysimeters.

In general, 16S rRNA gene based T-RFLP analysis of methanotrophic community profiles is suitable for indicating general trends in spatial or temporal changes or differences. T-RF peaks cannot usually be translated, however, to certain genera or species of methanotrophs, for this analysis the community profiling has to be combined with sequence analysis of clone libraries. The approach could be improved by the design of new PCR primers specific to so far uncovered genera of methanotrophs and by more methanotrophic 16S rDNA sequences becoming known.

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