RESEARCH ARTICLE

Structure and function of methanotrophic communities in a landfill-cover soil

Ruth Henneberger[1], Claudia Lüke[2], Lona Mosberger[1] & Martin H. Schroth[1]

[1]Institute of Biogeochemistry and Pollutant Dynamics, ETH Zurich, Zurich, Switzerland; and [2]Max Planck Institute for Terrestrial Microbiology, Marburg, Germany

Correspondence: Martin H. Schroth, Institute of Biogeochemistry and Pollutant Dynamics, ETH Zurich, Universitätstr. 16, CH-8092 Zurich, Switzerland. Tel.: +41 44 6336039; fax: +41 44 6331122; e-mail: martin.schroth@env.ethz.ch

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Abstract

In landfill-cover soils, aerobic methane-oxidizing bacteria (MOB) convert CH_4 to CO_2, mitigating emissions of the greenhouse gas CH_4 to the atmosphere. We investigated overall MOB community structure and assessed spatial differences in MOB diversity, abundance and activity in a Swiss landfill-cover soil. Molecular cloning, terminal restriction-fragment length polymorphism (T-RFLP) and quantitative PCR of *pmoA* genes were applied to soil collected from 16 locations at three different depths to study MOB community structure, diversity and abundance; MOB activity was measured in the field using gas push-pull tests. The MOB community was highly diverse but dominated by Type Ia MOB, with novel *pmoA* sequences present. Type II MOB were detected mainly in deeper soil with lower nutrient and higher CH_4 concentrations. Substantial differences in MOB community structure were observed between one high- and one low-activity location. MOB abundance was highly variable across the site [4.0×10^4 to 1.1×10^7 (g soil dry weight)$^{-1}$]. Potential CH_4 oxidation rates were high [1.8–58.2 mmol CH_4 (L soil air)$^{-1}$ day^{-1}] but showed significant lateral variation and were positively correlated with mean CH_4 concentrations ($P < 0.01$), MOB abundance ($P < 0.05$) and MOB diversity (weak correlation, $P < 0.17$). Our findings indicate that *Methylosarcina* and closely related MOB are key players and that MOB abundance and community structure are driving factors in CH_4 oxidation at this landfill.

Introduction

Landfills are important anthropogenic sources of the potent greenhouse gas methane (CH_4), with an estimated global release between 35 and 69 Tg CH_4 per year (Denman *et al.*, 2007). Production of CH_4 in anoxic zones of landfill bodies is far greater, but 10–100% of total CH_4 is converted to CO_2 by aerobic methane-oxidizing bacteria (hereafter referred to as MOB) in oxic layers of cover soils before release into the atmosphere (Reeburgh, 1996; Spokas *et al.*, 2006). MOB mainly belong to the Proteobacteria and have been divided into two major groups: Type I (γ-Proteobacteria) and Type II (α-Proteobacteria), differing not only in phylogenetic affiliation, but also in biochemical and physiological properties such as carbon assimilation pathways, internal membrane structures and predominant phospholipid fatty acids (Hanson & Hanson, 1996; Murrell, 2010). The MOB have a unique metabolism and utilize CH_4 as sole energy and carbon source. The initial step of this metabolic pathway, i.e. the aerobic oxidation of CH_4 to methanol, is catalysed by the enzyme methane monooxygenase (MMO). This enzyme exists in two forms: the membrane-bound particulate (pMMO) and the soluble (sMMO) form. Particulate MMO is present in all known MOB (except the genera *Methylocella* and *Methyloferula*; Dedysh *et al.*, 2005; Vorobev *et al.*, 2011) and is often used for the detection and identification of MOB, as the phylogeny of the *pmoA* gene (coding for the β-subunit of the pMMO) is congruent with the 16S rRNA phylogeny (Kolb *et al.*, 2003).

As a result of the importance of MOB in mitigating CH_4 emissions, their distribution, diversity and abundance have been thoroughly investigated. It has been shown that

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Type I and Type II MOB are ubiquitous but can also inhabit different niches (Murrell, 2010). In addition, community structures often change with varying environmental factors such as CH_4 and O_2 concentrations, nitrogen availability or pH. For example, nutrient-rich, well-aerated soils are frequently dominated by Type Ia MOB (Amaral *et al.*, 1995; Henckel *et al.*, 2000), but under nitrogen-limited conditions, Type II MOB and some *Methylomonas* species have been reported to be dominant, as these organisms are known to be capable of nitrogen fixation (Auman *et al.*, 2001; Bodelier, 2011). Type II MOB were also more abundant in slightly acidic landfill-cover soils (Wise *et al.*, 1999; Cébron *et al.*, 2007), while both, Type I and Type II MOB, have been detected in similar abundance in other landfill environments (Bodrossy *et al.*, 2003; Uz *et al.*, 2003; Crossman *et al.*, 2004; Lin *et al.*, 2009).

MOB in landfill environments have been studied not only for their diversity but also for inherent CH_4 oxidation capacity (e.g. Chen *et al.*, 2003; Boerjesson *et al.*, 2004; Gebert *et al.*, 2009). MOB activity has commonly been investigated in laboratory-based incubation studies, providing important information on CH_4 oxidation under controlled environmental conditions. Nevertheless, sampling procedures and sample preparation may disturb natural communities, and incubation schemes may differ from field settings. Thus, rates of CH_4 oxidation obtained from laboratory experiments may not reflect *in situ* conditions and may potentially hamper extrapolation of these data to the field (Madsen, 1998). In addition, CH_4 oxidation is sensitive to many factors including volumetric water content and temperature as well as pH and nutrient availability, leading to substantial spatial and temporal heterogeneity at large ecosystem scales (e.g. Scheutz *et al.*, 2009).

A limited number of studies have quantified CH_4 oxidation activity directly in the field, using methods including vertical CH_4 gas concentration profiles (e.g. Born *et al.*, 1990; Jones & Nedwell, 1993; Damgaard *et al.*, 1998) or stable C-isotope analysis of CH_4 (Liptay *et al.*, 1998; Chanton *et al.*, 2008). An alternative method for the quantification of CH_4 oxidation in the field is the gas push-pull test (GPPT; Urmann *et al.*, 2005), during which a gas mixture containing reactive gases (CH_4, O_2) and at least one nonreactive (tracer) gas (e.g. Ar) is injected into the soil at a location of interest. Thereafter, the soil-gas-diluted mixture is extracted from the same location and sampled periodically. While the gas mixture is in the soil, reactive gases can be consumed by MOB, and CH_4 oxidation is quantified from reactant- and tracer-gas concentrations measured during extraction (Schroth & Istok, 2006). So far, this method has been applied in soil above a contaminated aquifer (Urmann *et al.*, 2005, 2008), in a peat bog (Urmann *et al.*, 2007), and in landfill-cover soils (Gómez *et al.*, 2009; Streese-Kleeberg *et al.*, 2011). While few studies have analysed spatial differences in MOB distribution in landfill-cover soils (Kumaresan *et al.*, 2009; Lin *et al.*, 2009), these studies lack lateral resolution of CH_4 oxidation activity. Furthermore, a link between *in situ* (field-scale) activity and MOB diversity has not yet been established.

Therefore, the objectives of this study were (1) to assess the overall methanotrophic community structure in the cover soil of a Swiss landfill, (2) to identify lateral and vertical differences in diversity and abundance and (3) to investigate potential patterns between diversity, abundance and activity of the MOB communities present at the study site. These objectives were addressed in a field campaign during summer 2010 by collecting soil samples from three different depths at 16 spatially distinct locations across a significant area of the landfill cover. DNA-based microbial-ecology techniques [terminal restriction-fragment length polymorphism (T-RFLP), molecular cloning and quantitative PCR of *pmoA* genes present] were used in conjunction with GPPTs at each location to determine MOB diversity, abundance and *in situ* CH_4 oxidation rates. This work is an extension of a pilot investigation that focused on the methodology for the quantification of CH_4 oxidation at this specific field site (Gómez *et al.*, 2009).

Materials and methods

Study site and sample collection

All sampling and field-based studies were performed at the Lindenstock landfill [Liestal (BL), Switzerland], previously described in detail elsewhere (Gómez *et al.*, 2009). Sixteen locations were sampled in June/July 2010, covering a large area of the landfill cover, with three locations situated on the central plateau (locations C1, C2 and C3; Fig. 1) and seven locations positioned along the slopes in each cardinal direction (locations EM and EB towards east–northeast, S towards south–southeast, WM and WB towards west, and NM and NB towards north). The final six locations were placed at a right angle with location C1 as apex (Fig. 1, insert). Of those locations, three were situated ~ 30, 115 and 1000 cm north of C1 (C1_30N, C1_115N, C1_1000N) and the remaining three were ~ 50, 150 and 1000 cm east of C1 (C1_50E, C1_150E, C1_1000E). Exact positions were determined using the global positioning system and converted into Swiss Grid geographical coordinates.

Soil cores were collected using a HUMAX hollow-stem auger system (80 and 35 mm inner diameter; Martin Burch AG, Rothenburg, Switzerland). At each location, we drilled to a depth of ~ 105–110 cm, and core samples

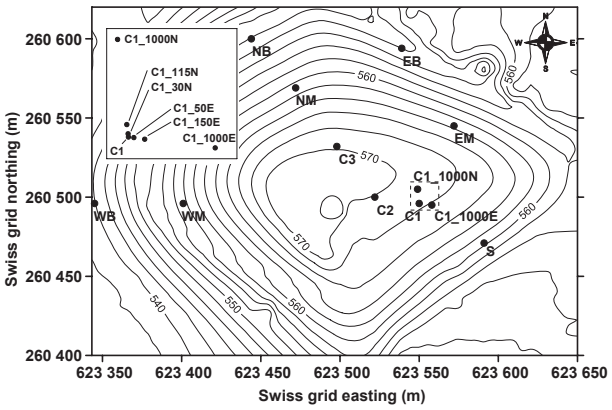

Fig. 1. Contour plot of the Lindenstock landfill showing elevation (in m a.s.l.) and sampling locations. The area near location C1 (dashed square) is shown in detail in the figure insert. Elevation data courtesy of Oester Messtechnik, Thun, Switzerland.

were collected in plastic sleeves from 5 to 15, 45 to 55 and 95 to 105 cm depths (hereafter termed 'location'_10, 'location'_50 and 'location'_100). Core samples were stored on ice for several hours and subsampled immediately upon return to the laboratory. Tools for subsampling were treated with 5% sodium hypochlorite for several hours, rinsed with sterile water and baked at 180 °C for 12 h. Portions of each subsample were stored at −80 °C (DNA extraction) and −20 °C until further processing.

Chemical and physical soil properties

Soil pH was determined in 0.01 M $CaCl_2$ soil extracts (3 g of soil in 30 mL of 0.01 M $CaCl_2$ was mixed on overhead shaker overnight; pH of supernatant was determined after centrifugation at 4500 ***g*** for 10 min). For total phosphorous (TP), 4 g of dried, milled soil was mixed with 0.9 g of Hoechst wax C (Reactolab SA, Servien, Switzerland), pressed into pellets (Specac Press; Portmann Instruments AG, Biel Benken, Switzerland) and analysed by X-ray fluorescence (XRF; Spectro-X-Lab 2000, Spectro, Kleve, Germany). Total carbon (TC) and total nitrogen (TN) were analysed by Dumas combustion with a detection limit of 0.03% N/w and 0.02% C/w, based on a 40-mg sample (NC 1500, CE Instruments, Wigan, UK). Sulphate, nitrate and phosphate concentrations were measured by ion chromatography (DX-1000; Dionex, Sunnyvale, CA) after KCl extraction (3 g of soil in 30 mL of 1 M KCl was mixed on overhead shaker overnight; supernatant was analysed after centrifugation at 4500 ***g*** for 10 min). The same extract was also used to determine ammonia concentrations colorimetrically as described by Sims *et al.* (1995).

Soil temperature was recorded throughout the field campaign in 3-h intervals using Thermochron iButton dataloggers (DS1921G#F50; Maxim, Sunnyvale) installed at two different locations (near locations C1 and EB) and at five different depths each (soil surface and depths of 2, 10, 50 and 100 cm). Volumetric water content was measured by time-domain reflectometry (TDR; TDR100; Campbell Scientific, Lougborough, UK) using pairs of brass rods (15 mm i.d.) of 30 cm, 70 cm and 110-cm length that were permanently installed near all sampling locations except locations close to C1 (only one set installed for all locations shown in Fig. 1 insert). Particle-size distribution was analysed by laser diffraction (LS 13320; Beckman Coulter Inc., Miami, FL) in soil suspensions (3 g soil < 2 mm suspended in 30 mL of 10% (w/v) sodium hexametaphosphate).

Soil gas sampling and analyses

Teflon tubes (2 mm i.d.) were installed to 10, 50 and 100 cm depth after removal of the soil cores and prior to refilling the boreholes. The lower end of each tube was embedded in approx. 10 cm of sand (Quarzsand 0.7–1.2 mm; Carlo Bernasconi AG, Zürich, Switzerland). Void spaces between the three sampling depths and the final 5 cm to the soil surface were filled with commercially available Bentonite (Fatto, Migros, Switzerland). Tubes were protected from soil-particle clogging by covering the tips with steel wool; upper ends were fitted with three-way valves to allow extraction of gas samples using syringes fitted with a luer-lock valve. Composition of soil gas collected at 10, 50 and 100 cm depths at all 16 locations was analysed monthly over the course of 1 year following installation. Tubes and syringes were flushed with 20–30 mL of soil gas prior to collecting 20 mL of gas and injecting 15 mL into N_2-flushed glass vials (19.7 mL) with butyl rubber stoppers. Pressure of the vials before and after sample addition was measured with a manometer (Keller AG, Winterthur, Switzerland), and the pressure difference was used to calculate the dilution of the soil gas. Gas concentrations were measured by gas chromatography (Trace GC Ultra; Thermo Electron Corporation, Rodano, Italy). The N_2 carrier gas flow velocity was 30 mL min^{-1} (30 kPa), the flame ionization detector (FID) hydrogen was set at 35 kPa and the synthetic air flow at 350 kPa. For CH_4, a FID and 2 m Porapak N 100/120 mesh column (1/16″ o.d., 1 mm i.d.) were used; column and detector temperatures were 30 and 250 °C, respectively. Concentration of O_2 and CO_2 was measured using a thermal conductivity detector (TCD), using a HayeSep D column (100/120) with column and detector temperatures of 85 and 250 °C, respectively.

Gas push–pull tests

The GPPTs were conducted with slight modifications from the procedure described previously (Urmann *et al.*, 2005; Gómez *et al.*, 2009). Briefly, ~ 20–22 L of gas mixture containing 0.8×10^5 μL L^{-1} CH_4, 1.7×10^5 μL L^{-1} O_2 and 2.5×10^5 μL L^{-1} each helium (He), neon (Ne) and argon (Ar) was injected into the soil at 50 cm depth over a period of 30 min at flow rates of 0.57–0.67 L min^{-1}. For extraction, the flow rate was reduced to ~ 0.5 L min^{-1}, and 15 L of gas mixture diluted with soil gas was extracted over a period of 34 min. Two background samples of soil gas were collected prior to injection. Three samples were collected during injection, and the extracted gas mixture was sampled at 2-min intervals.

All samples were analysed using a Trace GC Ultra gas chromatograph with TCD and a capillary Molsieve 5A column (Varian, Palo Alto, CA; 50 m × 0.53 mm i.d., 50 μm) at 30 °C with a micropacked ShinCarbon ST precolumn (Restec, Bellefonte, PA) at 50 °C; H_2 was used as carrier gas (Urmann *et al.*, 2007). Kinetic parameters of CH_4 oxidation (apparent first-order rate constants k, potential CH_4 oxidation rates) were subsequently estimated from GPPT extraction data as described previously (Urmann *et al.*, 2008; Gómez *et al.*, 2009).

DNA extraction

Total DNA from the different subsamples was extracted in triplicate by bead beating using approx. 0.5 g of freeze-dried soil. The method described by Henckel *et al.* (1999) was applied with the following modifications: Soil was resuspended in 750 μL of sodium phosphate buffer and 250 μL of sodium dodecyl sulphate solution. After centrifugation for 10 min at 13 000 ***g***, the supernatant was collected and the soil pellet re-extracted with 600 μL of sodium phosphate buffer and 200 μL of sodium dodecyl sulphate solution. Purification and precipitation was carried out as described by Lueders *et al.* (2004). Nucleic acid pellets were finally resuspended in 50 μL of H_2O, and extracts from soil triplicates were pooled and stored at –20 °C. Recovery of nucleic acids was confirmed by gel electrophoresis on a 1% agarose gel, and concentrations were determined and purity was checked using a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE).

PCR of *pmoA* gene

pmoA genes were amplified by using 0.2 mM of the primers A189f (Holmes *et al.*, 1995) and mb661r (Costello & Lidstrom, 1999), 1× MasterAmpF PCR premix (Epicentre® Biotechnologies, Madison, WI), 0.5 U of *Taq* polymerase (Invitrogen, Carlsbad, CA) and 1 μL of template DNA in a 25-μL total reaction volume under the following cycling conditions: 94 °C for 5-min initial denaturation, 11 cycles of touchdown reaction which consisted of 94 °C for 60 s and an annealing step of 62–52 °C for 60 s (start at 62 °C and decrease by 1 °C per cycle) and 72 °C for 60 s. This was followed by 24 cycles with annealing at 52 °C for 60 s and a 10-min final extension at 72 °C. Template DNA was diluted routinely in H_2O (1 : 25–1 : 200) to test for PCR inhibitors that might have been coextracted from the soil. For each DNA template, the dilution that exhibited the highest yields of PCR product (5 μL was analysed on 1% agarose gel) was used for further analysis. Gene copy numbers were calculated g^{-1} dry weight (d.w.) soil.

T-RFLP analysis

PCR products for T-RFLP analysis were generated using the conditions specified above with the FAM (6-carboxyfluorescein)-labelled primer A189f. PCR products were digested for 3–4 h at 37 °C with 2.5 U of *Msp*I restriction enzyme (Fermentas, St. Leon-Roth, Germany) in 25 μL reaction volumes and purified using a PCR purification kit (Fermentas). One to three microlitres of digested and purified product was mixed with 10 μL of HIDI Formamide and 0.1 μL of MapMarker 1000_ROX (Bioventures, Murfreesboro, TN), denatured for 2 min at 95 °C and placed on ice immediately. Fragments were analysed by electrophoresis for 60 min at 60 °C with an ABI 3130Xl genetic analyser (Applied Biosystems, Foster City, CA), using POP7 as a running polymer. Lengths of the fluorescently labelled terminal restriction fragments (T-RF) were determined using the GENEMAPPER software package (v.3.7; Applied Biosystems) and validated by T-RFLP analysis of selected *pmoA* clones. T-RFs were binned to operational taxonomic units based on *in silico* analysis of the clone library sequences complimented with publicly available *pmoA* sequences. After binning, a data set was generated consisting of T-RF sizes in base pairs and peak heights in fluorescence units. The statistical analysis was performed using the R software environment for statistical computing and graphics (R Development Core Team, 2009). The T-RFLP data were standardized according to Dunbar *et al.* (2000), and the heat map was created using *heatmap2* provided by the *gplots* package (v. 2.7.4; Venables, 2009). The constrained correspondence analysis was performed using *caa* within the *vegan* package (v. 1.15-4; Oksanen *et al.*, 2009). Diversity indices are based on peak heights in fluorescence units and calculated as 1 − Simpson's index D (*BiodiversityPro* software package, http://www.sams.ac.uk/; accessed September 2011). Diversity indices range from 0 (no diversity) to 1 (infinite diversity).

Quantitative PCR

Copy numbers of the *pmoA* genes present in the DNA extracts were determined by quantitative PCR on an ABI 7300 (Applied Biosystems) using 1× Kapa Sybr® Fast Universal qPCR Mix (Kapa Biosystems, Woburn), 0.2 μM of primers A189f and mb661r and 1 μL of template DNA (dilution 1 : 25–1 : 150) in a 20-μL reaction volume. The thermal profile consisted of an initial denaturation (3 min at 95 °C), 10 touchdown cycles of 95 °C for 15 s, 62–53 °C (–1 °C per cycle) for 30 s and 72 °C for 30 s, followed by 30 cycles of 95 °C for 15 s, 52 °C for 30 s, 72 °C for 30 s and an additional step at 85 °C for 30 s for fluorescent data acquisition. Melting curve analysis was performed during a final 95 °C cycle for 15 s, 60 °C for 60 s, 95 °C for 30 s and 60 °C for 15 s. Purified DNA from *Methylococcus capsulatus* (strain Bath; courtesy of Prof. Svenning, University of Tromsø, Norway) was quantified with SYBR Green I as described by Matsui *et al.* (2004) and serially diluted for use as a standard. This dilution series was included in duplicate for every run to determine the calibration curve, which was plotted as Ct values as a function of log-transformed copy numbers. Samples were analysed in triplicate, and a total of three runs were performed to include all samples. Efficiencies calculated from the slopes of the calibration curves ranged between 94.3% and 98.8% (r^2 = 0.9984–0.9988).

Cloning and sequencing and phylogenetic analysis

Amplified *pmoA* genes were purified by gel extraction (Fermentas, St. Leon-Roth, Germany) and cloned using the TA Cloning kit (Invitrogen) and blue–white screening. Randomly selected clones were subjected to sequencing of the *pmoA* gene insert, using a vector-specific primer (sequencing performed by GATC Biotech, Konstanz, Germany). Identity of *pmoA* gene sequences was confirmed by database searches using nucleotide BLAST (http://www.ncbi.nml.nih.gov/BLAST/). Phylogenetic analysis of the gene and deduced amino acid sequences was carried out using the ARB program package (Ludwig *et al.*, 2004). Tree construction was performed based on 122 amino acid positions using the Neighbor-joining algorithm implemented in ARB. *pmoA* sequences obtained in this study were deposited at the EMBL nucleotide sequence database under accession numbers HE613037–HE613041 , and HE617679 – HE617968.

Results

Chemical and physical soil properties

The majority of Lindenstock cover soil samples exhibited a near-neutral pH of 7.3–7.9 (except sample C1_150E_100 with pH 9.8; Supporting Information, Table S1). TC, TN, nitrate and ammonia concentrations were similar at the different locations and generally decreased with depth, with few exceptions. Sulphate concentrations showed a reverse trend with depth, or differences between the different depths were relatively minor. TP varied from 0.05% to 0.13% d.w., with an average of 0.08 ± 0.02% d.w. (Table S1). However, phosphate concentrations in the extracts were below the detection limit of the extraction method used here [0.8 μM (L soil extract)$^{-1}$], notwithstanding sample S_10 with a phosphate concentration of 3.68 μg (g soil)$^{-1}$.

Soil texture was heterogeneous; samples contained pebbles, rocks, boulders and construction material. The < 2-mm fraction of the different samples showed high silt content (40.3–57.0% d.w.), and the soil could mostly be characterised as gravely loam, silt loam or loam (USDA nomenclature). Total porosity has been estimated to be ~ 0.49 during a previous study, for a location in close proximity to C1 (Gómez *et al.*, 2009). TDR measurements showed the lowest yearly mean volumetric water content for the 30–70-cm-depth interval at location S (0.16), while location NB had the highest yearly mean value (0.41; Table 1). Locations C1 and NM (specifically addressed below) had yearly means of 0.24 ± 0.09 and 0.27 ± 0.04, respectively, with individual measurements ranging from 0.06 to 0.40 for C1 and 0.18 to 0.33 for NM.

Overall MOB community structure

MOB presence was confirmed by PCR amplification of the *pmoA* genes for each individual sample. To assess the overall MOB community structure, recombinant libraries were constructed from composite DNA samples (100 ng of DNA for each individual sample). A total of 142 high-quality sequences were obtained and used for phylogenetic placement. The majority of sequences (85%) were placed within the Type Ia MOB, with *Methylosarcina*-like (44% of all sequences; Fig. 2) and the related aquifer cluster (15% of the sequences) being the most abundant groups. Approximately 7% of all clone sequences grouped within the type Ib MOB, whereas only 6% clustered within the genus *Methylocystis*, the only group of Type II MOB detected in this study. The remaining 2% of sequences grouped within the Upland Soil Cluster-γ as well as within the Deep Sea Cluster-5 (for a definition of lineages see Lueke & Frenzel, 2011). This latter cluster was also referred to as OPU-1 (Tavormina *et al.*, 2010) and comprised sequences so far exclusively obtained from marine environments.

Several novel sequences were also present that could not clearly be affiliated to known *pmoA* clusters (Fig. 2). In particular, clone LL_F11 showed only 76% nucleotide

Table 1. Abundance, diversity and activity of MOB at 16 different locations distributed across the Lindenstock landfill-cover soil

Sample ID	MOB abundance *pmoA* copy no. ± SD × 10^4 (g soil d.w.)$^{-1}$ ‡	Diversity index*	MOB activity		CH_4 concentration in soil gas			Vol. water content†
		Based on T-RFLP (–)	First-order rate const. k ± SD (h^{-1})	Potential CH_4 oxid. rate ± SD [mmol CH_4 (L soil air)$^{-1}$ day^{-1}]	Yearly mean (μL L^{-1})	Min (μL L^{-1})	Max (μL L^{-1})	Yearly mean (–)
C1_10	1110 ± 96	0.70			7	1	52	
C1_50	110 ± 9.6	0.73	4.69 ± 0.16	58.2 ± 12.9	44	< 1	626	0.20
C1_100	190 ± 22	0.77			103	< 1	699	
C1_30N_10	540 ± 126	0.68			3	1	7	
C1_30N_50	210 ± 27	0.77	4.62 ± 0.37	57.6 ± 5.5	306	< 1	4380	n.a.
C1_30N_100	35 ± 6.5	0.81			2180	< 1	3.3×10^4	
C1_115N_10	860 ± 110	0.71			9	1	97	
C1_115N_50	3320 ± 430	0.77	2.61 ± 0.07	35.6 ± 4.0	396	< 1	5770	n.a.
C1_115N_100	210 ± 37	0.26			2.1×10^4	1	1.1×10^5	
C1_1000N_10	21 ± 4.0	0.73			86	1	1160	
C1_1000N_50	550 ± 26	0.66	2.30 ± 0.07	32.9 ± 3.8	2	< 1	5	n.a.
C1_1000N_100	730 ± 81	0.69			550	< 1	7640	
C1_50E_10	370 ± 14	0.70			12	1	139	
C1_50E_50	190 ± 58	0.60	2.41 ± 0.15	32.1 ± 2.0	3	< 1	34	n.a.
C1_50E_100	73 ± 17	0.64			191	< 1	2540	
C1_150E_10	460 ± 44	0.65			20	2	215	
C1_150E_50	430 ± 35	0.61	1.86 ± 0.23	26.4 ± 6.8	1	< 1	6	n.a.
C1_150E_100	89 ± 13	0.66			2	< 1	13	
C1_1000E_10	52 ± 3.2	0.27			166	2	1470	
C1_1000E_50	41 ± 5.8	0.65	0.21 ± 0.02	5.7 ± 0.5	201	< 1	1620	n.a.
C1_1000E_100	71 ± 21	0.78			16§	< 1	81	
C2_10	15 ± 1.8	0.77			2	1	4	
C2_50	b.d.¶	0.60	0.26 ± 0.03	6.1 ± 0.7	20	1	200	0.32
C3_10	200 ± 52	0.56			4	2	19	
C3_50	b.d.	0.69	0.35 ± 0.07	6.3 ± 2.9	98§	1	572	0.25
Em_10	180 ± 26	0.78			100	1	1120	
Em_50	340 ± 23	0.67	0.52 ± 0.02	14.4 ± 0.5	2.5×10^4§	1	1.9×10^5	0.34
Em_100	120 ± 26	0.81			2.2×10^4§	1	1.6×10^5	
Eb_10	37 ± 8.6	0.47			47	2	563	
Eb_50	31 ± 12	0.81	0.27 ± 0.04	7.4 ± 1.1	113	1	1510	0.31
Eb_100	38 ± 5.0	0.81			219§	1	1210	
S_10	9.9 ± 3.7	0.47			6§	2	23	
S_50	16 ± 2.8	0.62	0.21 ± 0.03	6.4 ± 0.9	4	< 1	25	0.16
S_100	64 ± 15	0.56			59	< 1	739	
Wm_10	5.5 ± 1.1	0.49			152	1	2060	
Wm_50	8.1 ± 1.5	0.67	0.15 ± 0.02	4.1 ± 0.4	4	< 1	37	0.29
Wm_100	16 ± 2.6	0.69			127	< 1	1510	
Wb_10	8.9 ± 0.8	0.27			3	2	4	
Wb_50	5.2 ± 0.3	0.68	0.20 ± 0.03	5.5 ± 0.7	26§	1	273	0.34
Wb_100	4.5 ± 0.7	0.56			50§	1	449	
Nm_10	19 ± 8.3	0.43			4	2	14	
Nm_50	28 ± 6.5	0.61	0.16 ± 0.01	4.4 ± 0.3	1	< 1	3	0.27
Nm_100	46 ± 4.6	0.79			2	< 1	10	
Nb_10	13 ± 4.6	0.64			12	2	120	
Nb_50	26 ± 4.6	0.68	0.06 ± 0.01	1.8 ± 0.3	8§	1	72	0.41
Nb_100	4.0 ± 2.9	0.72			5530§	18	3.6×10^4	

In addition, CH_4 concentrations in soil gas and volumetric water contents (selected locations) are shown.

Sample locations are shown in Fig. 1. For each sample ID, the last designation indicates sampling depth (cm).

*Diversity index computed as (1 − Simpson's index D).

†Volumetric water content computed for 30–70-cm-depth interval.

‡Actual values equal reported values times the indicated factor.

§Sampling of soil gas impeded on several occasions owing to the soil being apparently water-saturated.

¶Below detection.

sequence identity to known *pmoA* sequences. The remaining novel sequences either branched within the Type Ib MOB in close proximity to two different Rice Paddy Clusters (RPC) or were phylogenetically related to *Methylomonas*-like Type Ia (Lueke & Frenzel, 2011; Group 1 in Fig. 2). The rarefaction curve for the composite DNA samples approached a plateau (data not shown) and a clone-coverage value of 96.5% was calculated, indicating that a sufficient number of clones was analysed.

Based on the differences observed for potential CH_4 oxidation rates at 50 cm depth (see below), MOB community structure was analysed in detail for two individual locations: C1 with high and NM with low potential CH_4 oxidation rate. Recombinant *pmoA* libraries were constructed from samples C1_50 and NM_50, and 79 and 75 high-quality sequences (clone-coverage values 97.5% and 97.3%) were obtained, respectively. Both libraries were clearly dominated by Type Ia MOB (94% for C1 and 97% for NM; Fig. 3). However, the dominant group in the C1 library was *Methylosarcina*-like Type Ia (61%), while the NM library contained 80% RPC-2 sequences, constituting only 1% of the C1 library. So far, the RPC-2 cluster contained sequences almost exclusively obtained from rice paddy studies. Type II MOB were absent in the NM library, and one sequence was placed within the *pxmA* in the AOB-/*Crenothrix*-related group (Lueke & Frenzel, 2011).

Spatial differences in MOB diversity and abundance

To compare spatial (vertical and lateral) differences in MOB diversity, T-RFLP profiles were generated for each

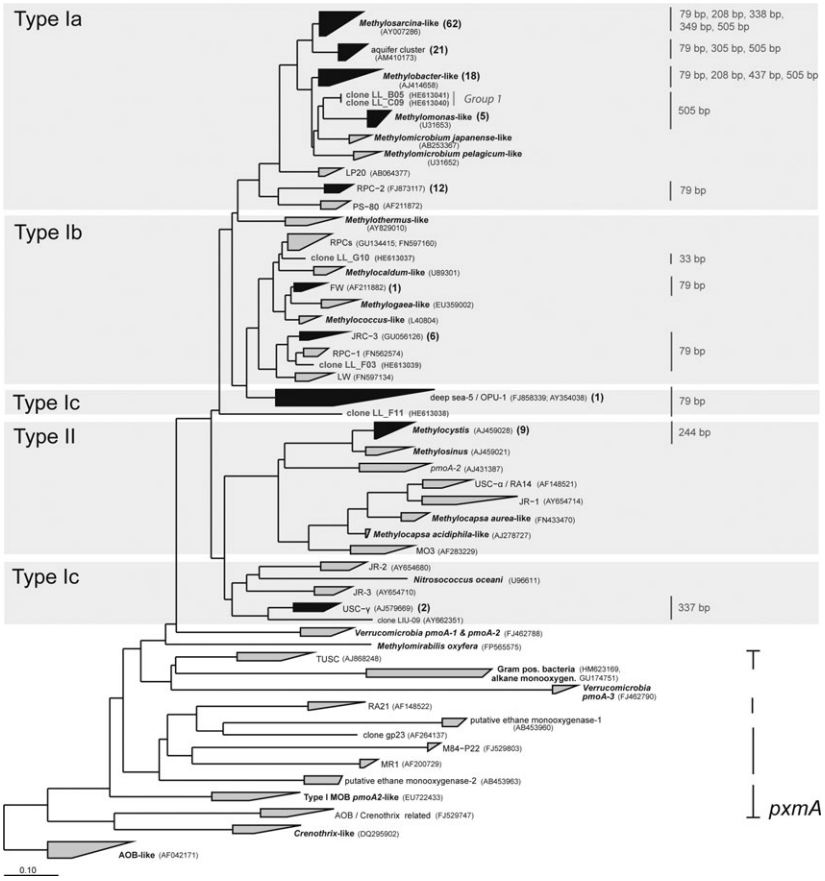

Fig. 2. Neighbor-joining tree showing the phylogenetic relationship of partial *pmoA* sequences based on 122 deduced amino acid positions. Clusters containing sequences obtained from the Lindenstock landfill clone library are depicted in black, and single clone sequences are shown in grey. The number of clone sequences grouping in the respective cluster is given in parentheses. Major *pmoA* lineages were defined and named according to Lueke & Frenzel (2011). The *Methanosarcina*-like lineage was divided into two clusters, *Methylosarcina*-like and aquifer cluster. Genbank accession numbers of representative isolates or environmental clone sequences for each cluster are given in parentheses. *pxmA* is used for sequences clustering between the *pmoA* gene and the homologous *amoA* gene of ammonia oxidizers, for which the substrate specificity of the corresponding protein is still unknown. Numbers depicted on the right side of the tree show the T-RF lengths of the corresponding landfill *pmoA* sequences. The scale bar represents 0.1 changes per amino acid position.

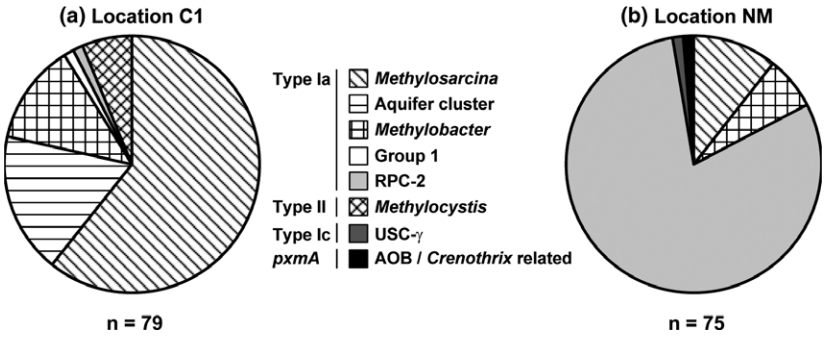

Fig. 3. Relative distribution of *pmoA* clone sequences obtained from two selected locations at 50 cm depth within the Lindenstock landfill-cover soil. The sequences were phylogenetically grouped according to the clusters shown in Fig. 2. Location C1 (a) was characterized by a high potential CH_4 oxidation rate [58.2 mmol CH_4 (L soil air)$^{-1}$ day^{-1}], whereas location (b) NM exhibited a low potential oxidation rate [4.4 mmol CH_4 (L soil air)$^{-1}$ day^{-1}].

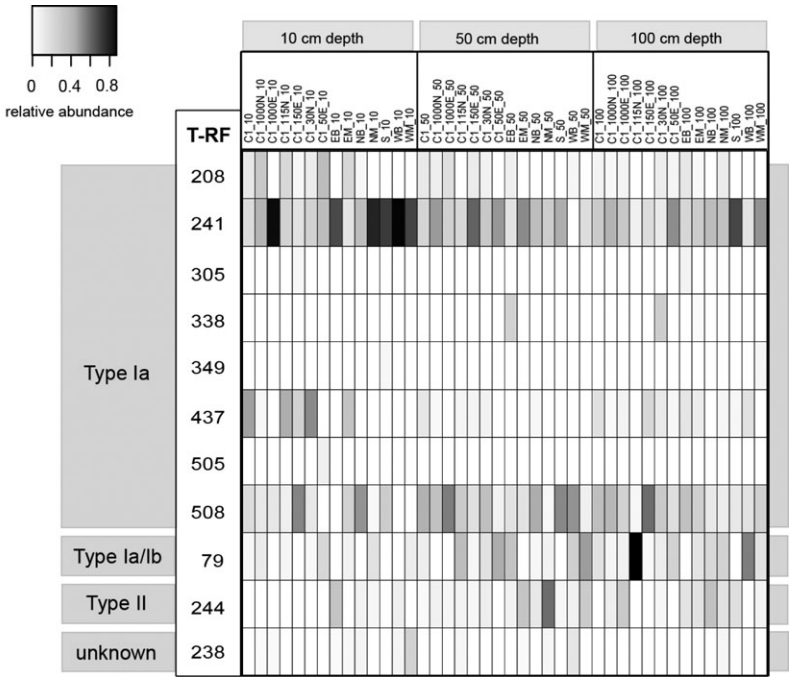

Fig. 4. Relative abundances of standardized T-RFLP data obtained from different locations within the Lindenstock landfill-cover soil. Samples were collected at three different depths (last designation of sample ID). T-RFs are shown according to *in silico* analysis of landfill clone sequences (see Fig. 2).

individual sample. Eleven distinct T-RFs were identified, and all but one could be assigned to specific groups of MOB by *in silico* and *in vitro* digestion of recombinant *pmoA* genes (Fig. 4). In accordance with clone libraries, T-RFLP profiles of individual samples were also dominated by T-RFs that correspond to Type Ia MOB. In particular, T-RFs 241 and 508 were highly abundant in most samples, while T-RF 241 was most abundant in the 10-cm-depth samples (Fig. 4). T-RF 208 (*Methylosarcina*- or *Methylobacter*-like; Fig. 2) was present in many samples but appeared to be also more abundant in samples taken at 10 cm depth. In contrast, T-RFs 79 (Type Ia and Ib) and 244 (Type II) showed higher abundances in samples collected from 50 and 100 cm depth. T-RFs 437 (Type Ia) and 238 (unknown) were also present in many samples, and in particular T-RF 437 was highly abundant in several 10-cm samples collected around location C1. The remaining T-RFs were detected in very few samples. Constrained correspondence analysis showed a highly significant ($P < 0.005$) separation of top soil (10 cm depth) and bottom soil (50 + 100 cm depth) communities (data not shown). This separation could be

explained by different relative abundances of the T-RFs 79, 244 and 508 (high abundance in bottom soil) and 241 (high abundance in top soil), respectively. Locations C2 and C3 were excluded from this analysis, as no 100-cm samples could be collected at these sites owing to difficulties encountered with drilling deeper than 60 cm.

Correlation of diversity indices (based on T-RFLP analysis) with soil depth was not significant, but several locations showed the highest diversity at 100 cm depth (Table 1). In similar fashion, MOB abundance (i.e. *pmoA* copy numbers) did not show a correlation with soil depth. Copy numbers were highly variable in the different samples and ranged from 4.0×10^4 (sample NB_100) to 3.3×10^7 (g soil d. w.)$^{-1}$ (sample C1_115N_50; Table 1). At some locations, differences of up to one order of magnitude were observed between different depths (e.g. C1 with 1.1×10^7 g^{-1} at 10 cm and 1.1×10^6 g^{-1} at 50 cm depth), while other locations showed only slight variation with depth (e.g. EB with 3.7×10^5 g^{-1}, 3.1×10^5 g^{-1} and 3.8×10^5 g^{-1} at 10, 50 and 100 cm depth, respectively).

However, a lateral trend was notable when comparing different locations at 50 cm depth. Diversity indices showed a weak positive correlation with *pmoA* copy numbers ($r = +0.34$), meaning that higher MOB diversity was observed in several samples with higher MOB abundance (Fig. 5a and b), but the correlation was not significant ($P < 0.2$). Abundance of MOB was clearly highest around location C1, while lowest abundance was observed along the west slope (WB, WM) and at C2 and C3 (copy numbers below the quantification limit, $< 1 \times 10^4$ g^{-1}, for

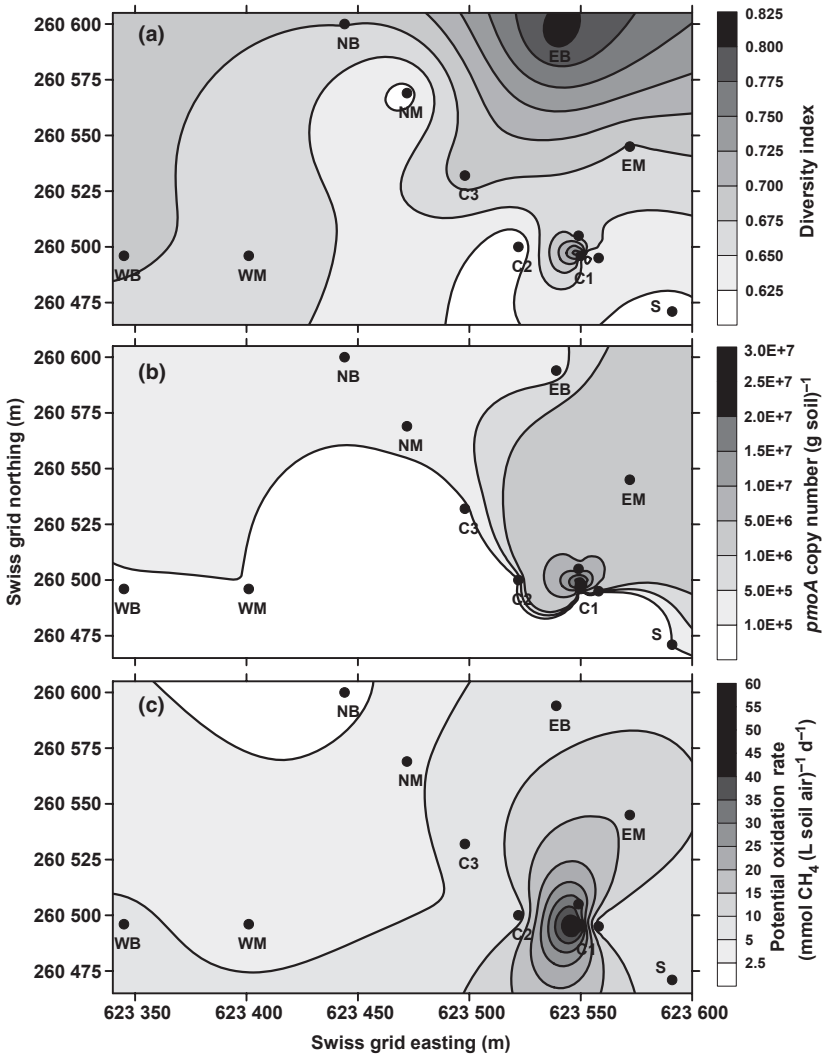

Fig. 5. Contour plots showing MOB (a) diversity index (1 − Simpson's index D), (b) abundance (*pmoA* copy number) and (c) activity (potential CH_4 oxidation rate) across the landfill-cover soil at 50-cm depth based on measurements at 16 sampling locations. To obtain contours, a standard kriging procedure was employed for data interpolation.

C2_50 and C3_50). Some nonspecific amplification was noted in many samples during quantitative PCR. These nonspecific amplicons showed slightly lower melting temperatures compared to the *pmoA* amplicons and were excluded from quantification by adjusting the detection temperature to 85 °C.

Methanotrophic activity

GPPTs at all 16 locations were performed within a 4-week period during July and August 2010. Soil temperature at 50 cm depth ranged from 17 to 19 °C during this period, and volumetric water content measured in early August ranged from 0.12 to 0.38 and was somewhat lower than yearly averages (Table 1), except for EM and NB sites, which appeared very moist (volumetric water content 0.52 and 0.56, respectively). Background concentrations of CH_4 in soil gas measured just prior to GPPTs were low compared to injection concentrations, ranging from 1 to 700 μL L^{-1}, and no increase in relative concentrations of CH_4 during extraction (as noted in Gómez *et al.*, 2009) was observed.

Apparent first-order rate constants (k), characterising indigenous CH_4 oxidation for variable, substrate (CH_4)-limited conditions, differed substantially between individual locations and ranged from 0.06 h^{-1} at NM to 4.69 h^{-1} at C1 (Table 1). For direct comparison of MOB activity, potential CH_4 oxidation rates were calculated for a CH_4 concentration of 3.0×10^4 μL L^{-1}, i.e. for a CH_4 concentration within the range of those attained during all GPPTs. A 'hot spot' of activity was observed on the central plateau around C1, with rates ranging from 26.4 (C1_150E) to 58.2 mmol CH_4 (L soil air)$^{-1}$ day^{-1} (C1; Fig. 5c). Rates clearly decreased at locations C2 and C3 and along the slopes of the landfill. Interestingly, CH_4 oxidation rates for three locations within a similar distance to C1 showed a high variation: 32.9 mmol CH_4 (L soil air)$^{-1}$ day^{-1} at C1_1000N in contrast to 5.7 and 6.1 mmol CH_4 (L soil air)$^{-1}$ day^{-1} at C1_1000E and C2, respectively. Lateral distribution of potential CH_4 oxidation rates across the landfill was notably correlated with MOB abundance (Fig. 5b and c). In fact, oxidation rates and *pmoA* copy numbers in 50-cm-depth samples were significantly positively correlated ($r = +0.49$, $P < 0.05$). Excluding a single outlier (C1_115N_50), this correlation became highly significant ($r = +0.72$, $P < 0.01$). A weak positive correlation of CH_4 oxidation rates and diversity was observed ($r = +0.36$, $P < 0.17$), with several high-activity locations showing also high diversity indices at 50 cm depth (Fig. 5a and c). In contrast, at locations NM, C2 and S, low CH_4 oxidation rates as well as low diversity were detected.

A positive correlation ($r = +0.62$, $P < 0.01$) was also found to exist between potential CH_4 oxidation rates and the mean CH_4 concentration measured in soil gas at 50 cm depth over the course of 1 year (outlier EM excluded from calculations). Strong variation in CH_4 soil gas concentrations was observed between the different locations (Table 1). Highest CH_4 concentrations were detected at location EM in March 2011, with values of 1.9×10^5 μL (L soil air)$^{-1}$ at EM_50 and 1.6×10^5 μL (L soil air)$^{-1}$ at EM_100. Moreover, high CH_4 concentrations [up to 1.3×10^5 μL (L soil air)$^{-1}$] were detected at this location during eight subsequent sampling dates. In contrast, CH_4 concentrations measured at NM did not exceed 14 μL (L soil air)$^{-1}$, and the yearly mean at this specific location was around 2 μL (L soil air)$^{-1}$. Oxygen concentrations in soil gas decreased with depth and showed no significant difference between the different locations (not shown). Nevertheless, even at a depth of 100 cm, the soil was still oxic, with a yearly mean oxygen concentration of $8.9 \pm 2.3 \times 10^4$ μL O_2 (L soil air)$^{-1}$. However, gas sampling from 100 cm, in some cases also 50 cm depth, was impeded one to several times at several locations between October and March due to the soil being apparently water-saturated (Table 1).

Discussion

MOB community structure

The cover soil of the Lindenstock landfill harbours a highly diverse and active MOB community. All known clades of MOB were represented (except the thermo-acidophilic Verrucomicrobia; reviewed in Op den Camp *et al.*, 2009) as well as novel sequences identified in the clone libraries of the composite DNA samples, but members of the genus *Methylosarcina* were the most dominant. Two of the three characterised species, *Methylosarcina fibrata* and *Methylosarcina quisquilarium*, have been isolated from a landfill site (Wise *et al.*, 2001), and this genus has been reported to be abundant in other landfill-cover soils (e.g. Chen *et al.*, 2007; Héry *et al.*, 2008). Their ability to form cysts and produce capsules or diffuse slime layers might enhance survival under changing environmental conditions. The aquifer cluster of MOB, related to *Methylosarcina*, was also highly abundant in the Lindenstock samples and has been previously detected in other landfill soil samples (e.g. NCBI accession number GQ857592). Assuming that a 7% amino acid distance of the *pmoA* genes reflects the species level (Degelmann *et al.*, 2010), this cluster with 10–13% amino acid distance to *Methylosarcina* might reflect a novel genus of MOB with similar traits and habitats as *Methylosarcina*. The dominance of these two groups indicates that they might be well-adapted key players in CH_4 oxidation in the Lindenstock cover soil and potentially in other landfill environments with high CH_4 fluxes.

Novel *pmoA* sequences with up to 24% nucleotide sequence distance to known *pmoA* sequences indicate the presence of previously unknown species or possibly genera. It is also remarkable that one sequence was placed within Deep Sea Cluster-5, which hitherto has been exclusively reported from marine environments (e.g. Nercessian *et al.*, 2005). The detection of these unusual and novel sequences in the limited data set comprised by clone libraries highlights the importance for further investigation in this highly diverse environment, potentially harbouring novel organisms. Even though the pMMO-specific primer set selected for this study (A189f/mb661r) is known to result in high coverage of MOB diversity (McDonald *et al.*, 2008), use of other primers (e.g. A682r and A650r) might result in the discovery of additional novel sequences. Organisms that only contain sMMO have so far only been isolated from acidic environments (Dedysh *et al.*, 2005; Vorobev *et al.*, 2011). As the Lindenstock soils are neutral to slightly alkaline, sMMO-specific primers have not been employed in this study but may be included in a future investigation.

Unlike other diversity studies on landfills where both, Type I and Type II MOB, were highly abundant (e.g. Cébron *et al.*, 2007; Gebert *et al.*, 2009; Lin *et al.*, 2009), the Lindenstock cover soil was clearly dominated by Type Ia MOB, while Type II MOB constituted only a minor component of the clone libraries analysed. Type I MOB have been reported to outcompete Type II MOB in high-nutrient, high-oxygen environments (e.g. Henckel *et al.*, 2000; Stralis-Pavese *et al.*, 2004), conditions also found at our study site. Dominance of Type I MOB is also partially reflected in the T-RFLP profiles. T-RFs assigned to Type Ia MOB were highly abundant in all samples. On the other hand, the only T-RF representing Type II MOB (T-RF 244) was more abundant in samples collected from 50 and 100 cm depth than in the 10-cm samples. Oxygen and nutrient concentrations were reduced in the deeper soil layers, and substantial fluctuation in volumetric water content was observed (to the extent that the soil occasionally appeared water-saturated). The ability to form cysts as resting stages may provide *Methylocystis* with an advantage in environments where growth is periodically restricted, e.g. by the lack of oxygen under water-saturated conditions (Hanson & Hanson, 1996).

Methanotrophic activity

Landfill-cover soils have been reported to show significantly higher CH_4 oxidation capacities compared to other environments (Boerjesson *et al.*, 1998; Gebert *et al.*, 2009; Streese-Kleeberg *et al.*, 2011). This is also true for the cover soil of the Lindenstock landfill, where the highest potential CH_4 oxidation rates observed during this study were roughly 2–30 times higher than those measured at other sites with nearby CH_4 sources, such as above a methanogenic aquifer (Urmann *et al.*, 2005) or in a peat bog (Urmann *et al.*, 2007). Nevertheless, the highest CH_4 oxidation rates determined for July/August 2010 were below most rates observed during a campaign that was conducted at this location in 2007 (Gómez *et al.*, 2009). As a positive correlation between oxidation rates and mean CH_4 concentration was detected in our study, this decrease in activity might be due to a decline in CH_4 production within the landfill body. Further long-term studies are required to confirm the potential decline in CH_4 production and MOB activity at this site.

Spatial differences in activity, abundance and diversity

Significant differences in MOB activity were observed among different locations within the cover soil. Lateral comparison showed a positive correlation between potential CH_4 oxidation rates and *pmoA* copy numbers, with a 'hot spot' of MOB activity and abundance located in the central plateau area. These lateral differences could be correlated with differences in yearly mean CH_4 soil gas concentrations. A more rigorous geostatistical analysis, however, was not possible due to the limited number of sampling locations across the site.

In contrast to CH_4 concentrations, TC, TN, ammonia or nitrate in the soil did not appear to influence lateral differences in MOB abundance or activity. In particular, TN did not seem to be a limiting factor for methanotrophic activity at our site. This is in contrast to the findings by Gebert *et al.* (2009), who reported a positive correlation between TN and CH_4 oxidation rates. However, while lowest TN concentrations measured in both studies were highly similar, MOB community structures were quite different between the two sites.

The lateral differences in diversity indices observed in our study could not be correlated with soil depth or any soil property analysed. Nevertheless, a weak positive correlation was observed between activity and diversity, indicating that high-diversity, more resilient and functionally stable communities might be present at the active sites, rather than lower-diversity, highly resistant MOB communities (Girvan *et al.*, 2005). Unfortunately, T-RFLP resolution was too coarse to determine the differences in diversity to a greater detail, as several T-RFs could be assigned to different clusters of MOB. As a consequence, calculated diversity indices may not reflect the true diversity at the site and further analysis, e.g. by diagnostic microarray, will be required.

Clone libraries from two sites with opposing activity and diversity index showed significant differences in

community structure, supporting the hypothesis that activity may not be driven exclusively by MOB abundance. The two dominant groups (*Methylosarcina* and RPC-2) both show T-RFs of 79 base pairs, masking such diversity differences in the T-RFLP profiles and distorting statistical analyses. Nevertheless, our findings indicate that *Methylosarcina* and closely related MOB are key players and that CH_4 soil gas concentration is a driving factor in CH_4 oxidation at the Lindenstock landfill, ultimately affecting both MOB abundance and diversity. Further work (e.g. analysis of *pmoA* mRNA or stable isotope probing) will attempt to correlate the findings of this diversity study with the identity of specific, active members of the MOB community.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Chemical properties of cover-soil samples collected at different depths from 16 locations distributed across the Lindenstock landfill.

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