RESEARCH ARTICLE

Response of methanotrophic activity and community structure to temperature changes in a diffusive CH_4/O_2 counter gradient in an unsaturated porous medium

Karina Urmann, Anna Lazzaro, Isabella Gandolfi, Martin H. Schroth & Josef Zeyer

ETH Zurich, Institute of Biogeochemistry and Pollutant Dynamics, Zurich, Switzerland

Correspondence: Martin H. Schroth, ETH Zurich, Institute of Biogeochemistry and Pollutant Dynamics, Universitaetstr. 16, CH-8092 Zurich, Switzerland. Tel.: +41 44 633 6039; fax: +41 44 633 1122; e-mail: martin.schroth@env.ethz.ch

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Abstract

Microbial methane oxidation is a key process in the global methane cycle. In the context of global warming, it is important to understand the responses of the methane-oxidizing microbial community to temperature changes in terms of community structure and activity. We studied microbial methane oxidation in a laboratory-column system in which a diffusive CH_4/O_2 counter gradient was maintained in an unsaturated porous medium at temperatures between 4 and 20 °C. Methane oxidation was highly efficient at all temperatures, as on average $99 \pm 0.5\%$ of methane supplied to the system was oxidized. The methanotrophic community that established in the model system after initial inoculation appeared to be able to adapt quickly to different temperatures, as methane emissions remained low even after the system was subjected to abrupt temperature changes. FISH showed that Type I as well as Type II methanotrophs were probably responsible for the observed activity in the column system, with a dominance of Type I methanotrophs. Cloning and sequencing suggested that Type I methanotrophs were represented by the genus *Methylobacter* while Type II were represented by *Methylocystis*. The results suggest that in an unsaturated system with diffusive substrate supply, direct effects of temperature on apparent methanotrophic activity and community may be of minor importance. However, this remains to be verified in the field.

Introduction

Methane is the second most important greenhouse gas after carbon dioxide (Forster *et al.*, 2007). It is microbially produced in a number of ecosystems such as rice fields, peat bogs and landfills under anaerobic conditions (Reeburgh, 2003), and is subsequently transported from the methanogenic zone to the atmosphere. However, before CH_4 reaches the atmosphere, it can be oxidized to CO_2 under aerobic conditions (Hanson & Hanson, 1996), which reduces CH_4 emissions by over 50% (Reeburgh, 2003). Furthermore, CH_4 is taken up from the atmosphere into oxic soils, which can provide a sink for atmospheric CH_4 (Reeburgh, 2003). The CH_4 oxidation reaction can be described by the following stoichiometry:

$$CH_4 + (2-x)O_2 \rightarrow (1-x)CO_2 + xCH_2O + (2-x)H_2O \quad (1)$$

where x is the fraction of carbon that is assimilated into biomass (CH_2O). The reaction is mediated by methanotrophic bacteria that use CH_4 as their main source of carbon and energy (Hanson & Hanson, 1996). Traditionally, methanotrophs have been divided into two major groups, Type I with the subgroup X, and Type II, belonging to the *Gammaproteobacteria* and *Alphaproteobacteria*, respectively (Hanson & Hanson, 1996). The two types are distinguished by typical enzymes, assimilation pathways, internal membrane structures and predominant phospholipid fatty acids (Wise *et al.*, 1999). Despite recent discovery of species that do not fit into this traditional division (Dedysh *et al.*, 2000), the concept remains useful for categorizing commonly found methanotrophs and for comparison with most of the existing literature.

Because of the crucial role of methanotrophic activity in the reduction of CH_4 emissions, an improved understanding of the temperature-dependence of CH_4 oxidation and

methanotrophic community structure is important to predict effects of a changing climate on the CH_4 cycle. Temperature affects enzymatic activity and temperature variations may lead to changes in the structure of the methanotrophic community (Borjesson *et al.*, 2004; Mohanty *et al.*, 2007). Active Type I and Type II methanotrophs have been found over a wide range of temperatures (Nozhevnikova *et al.*, 2001; Trotsenko & Khmelenina, 2005). However, it is still not clear how the two types of methanotrophs may differ in their responses to temperature fluctuations. For example, in a landfill-cover soil, Type I methanotrophs dominated in incubations at low temperatures (Borjesson *et al.*, 2004), whereas in thawed permafrost soils at 0–18 °C the distribution of Type I and II methanotrophs did not correlate with temperature (Liebner & Wagner, 2007).

In contrast to community structure, the temperature dependence of CH_4 oxidation activity in ecosystems with elevated CH_4 concentrations, such as peat bogs or landfills, has frequently been investigated in well-mixed batch incubations under CH_4-saturated conditions (e.g. Nedwell & Watson, 1995; Borjesson *et al.*, 2004). These experiments provide information on the direct effect of temperature on cell-specific, i.e. enzymatic, activity and possibly growth (Nozhevnikova *et al.*, 2001). Results are usually described as Q_{10}-values, i.e. the factor by which the reaction rate changes with a 10 °C change in temperature. Usually, Q_{10}-values for CH_4 oxidation in batch experiments under CH_4-saturated conditions are around 3 (Segers, 1998) with some higher values of 3–4 reported for landfill-cover soils (Borjesson *et al.*, 2004). However, CH_4 oxidation under field conditions may not just be enzymatically controlled, especially in unsaturated systems. Rather, a range of direct and indirect factors such as substrate concentration, which may increase with temperature, and water content, which may decrease with temperature, can be responsible for the observed response of a process to temperature in the field (Davidson *et al.*, 2006). Even temperature effects inverse to the effects expected for enzymatically controlled processes have been observed due to the interaction of different factors (Ding *et al.*, 2004). However, it is often challenging to separate the effect of different factors on CH_4 oxidation in the field (e.g. Roslev & King, 1996).

Laboratory-column systems provide the opportunity to study CH_4 oxidation under controlled conditions, while mimicking important physical parameters, such as substrate transport, of a field situation. Methane oxidation at different temperatures has been investigated in a laboratory column with high advective influx of CH_4 mimicking a landfill-cover soil (Kettunen *et al.*, 2006). Conversely, we recently developed a laboratory-column system with a diffusive counter gradient of CH_4 and O_2 (Urmann *et al.*, 2007) mimicking the unsaturated zone above a contaminated aquifer. Similar conditions, but with varying physical parameters such as water content and porosity, can be found for example in peat bogs or wetlands. The system consists of a vertical column with unsaturated sand inoculated with a methanotrophic enrichment culture. In a first study, the response of CH_4 oxidation activity to changes in substrate-concentration gradients was investigated. We observed high CH_4 oxidation activity, which responded quickly to changing gradients while following apparent first-order kinetics with respect to CH_4 gas-phase concentrations.

The aim of the present work was to study the effect of temperature on CH_4 oxidation activity and methanotrophic community structure in a CH_4/O_2 counter-gradient system. To this end, a laboratory column containing sand inoculated with a methanotrophic enrichment culture was operated under unsaturated conditions for a total of 273 days at temperatures ranging from 4 to 20 °C.

Materials and methods

Enrichment of methanotrophic culture

A methanotrophic culture was enriched from groundwater from a methanogenic, petroleum hydrocarbon-contaminated aquifer in Studen, Canton Bern, Switzerland, and from the sediment above the aquifer, where methanotrophic activity has previously been detected (Urmann *et al.*, 2005, 2008). Temperatures previously observed in the groundwater ranged from 9 to 17 °C (Bolliger *et al.*, 2000). Cultures were initially enriched from water and sediment in nitrate minimum-salts medium (NMS) diluted 2 : 10 [0.2 NMS: NMS as described in Bowman (2006) except that ferric ammonium EDTA was replaced by chelated iron solution (2 mL L^{-1} medium of 0.1 g ferric (III) ammonium citrate, 0.2 g EDTA, sodium salt, 0.3 mL concentrated HCl in 100 mL deionized water)]. Enrichments were kept at 20 °C in gas-tight serum bottles that were placed on a shaker at 110 r.p.m. for 17–19 days. No growth was observed in cultures at 12 or 4 °C during the same time period. The inoculum was subsequently grown from water and sediment cultures transferred together into fresh 0.2 NMS at 28 °C for 6 days (initial headspace concentrations for all cultures: CH_4, 90 mL L^{-1}; CO_2, 10 mL L^{-1}; O_2, 190 mL L^{-1}). Here and throughout this paper, we present gas-phase concentrations in units of mL L^{-1}; note that 1 mL L^{-1} is equivalent to 0.1% by volume. For inoculation, 1650 mL of culture was combined with 2500 mL diluted NMS (2 : 10 with demineralized water).

Column experiments

The response of the methanotrophic enrichment culture to different temperatures was investigated in a 80-cm-high laboratory column with a diffusive CH_4/O_2 counter gradient (Fig. 1). The column was operated as described previously

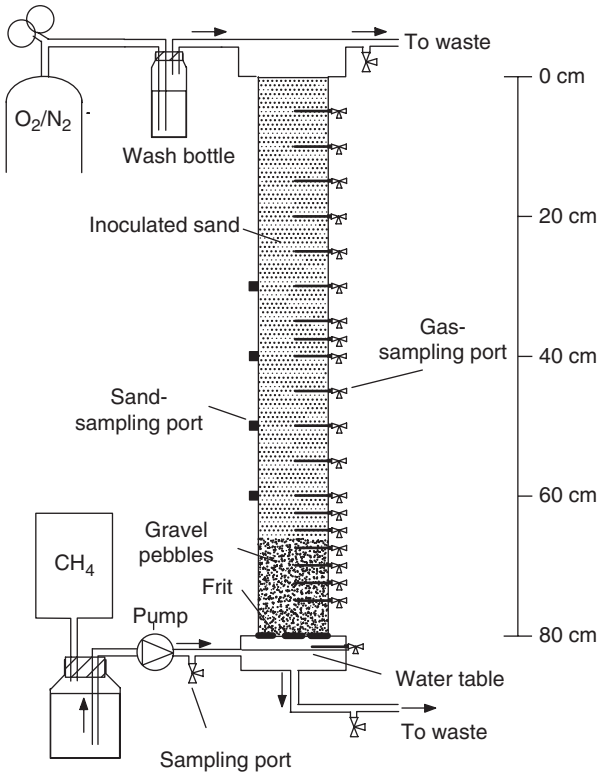

Fig. 1. Sketch of the diffusive CH_4/O_2 counter-gradient column containing unsaturated sand and gravel pebbles inoculated with a methanotrophic enrichment culture.

(Urmann *et al.*, 2007), but placed in a climate chamber to control temperature. Briefly, CH_4 was supplied to the column by pumping water, which was saturated with CH_4 at the respective temperature and kept under a CH_4 headspace, through a chamber at the bottom of the column. Methane partitioned into the headspace of the chamber and diffused upwards into the column. At the top of the column, a chamber was flushed with a gas mixture of 4 mL L^{-1} O_2 in N_2 at an average flow rate of 50 mL min^{-1} allowing O_2 to diffuse downwards into the column. In addition to the 18 gas sampling ports used in the previous experiment, three additional ports were added in the lower part of the column, and four 1-cm-diameter sampling ports were installed at 30, 40, 50 and 60 cm depth to allow collection of sand for molecular analysis (Fig. 1). The column was packed from 80- to 66-cm depth with gravel pebbles (3–5.6-mm diameter, porosity 0.38, commercial quality) to facilitate drainage, and from 66- to 0-cm depth with coarse sand (0.7–1.2-mm diameter, porosity 0.43, commercial quality). Initially, the column was operated under abiotic conditions (with dry sand) to test gas tightness and CH_4 supply. A linear profile (similar to the one shown in Urmann *et al.*, 2007) indicated gas tightness of the system. In order to achieve nearly constant CH_4 supply at all temperatures, CH_4 saturation was lowered from 100% to 85% at 4 and 12 °C by flushing the water with a mixture of 85% CH_4 and 15% N_2 instead of 100% CH_4. However, during the experiment, constant CH_4 supply was not fully achieved, as CH_4 fluxes were higher at 20 °C than at 4 and 12 °C (see Results). In a second step, the sand was inoculated with a methanotrophic enrichment culture, prepared as described above. This inoculum was pumped into the column up to 10 cm below the sand surface, left overnight and then drained. Subsequently, the column was operated in four phases (I–IV) for a total of 273 days at different temperatures (Table 1) to investigate (1) microbial CH_4 oxidation in the counter gradient and (2) changes in the methanotrophic community composition.

Water content in the column for 5- to 10-cm intervals was determined gravimetrically at the end of the experiment. The volumetric water content of the sand increased from 0.013 to 0.020 between 5 and 35 cm depth with an average of 0.016 cm^3 cm^{-3} sand. Between 40 and 65 cm, the water content of the sand increased from 0.028 to 0.071 and was on average 0.048 cm^3 cm^{-3} sand. Finally, in the gravel layer, volumetric water content was relatively constant with an average of 0.054 cm^3 cm^{-3} gravel. Volumetric water contents at the top and bottom of the column, measured once during the experiment by briefly opening the top and bottom of the column, was similar to water contents measured at the end of the experiment, indicating that the water content remained fairly constant throughout the experiment.

Sampling and analytical methods

Methane concentration profiles in the column were measured seven to 14 times and profiles of CO_2 and O_2 three to four times per phase (Table 1). Methane sampling and analysis were performed as described previously (Urmann *et al.*, 2007). Methane was measured by GC on a Trace GC Ultra (Thermo Electron, Rodano, Italy) at 35 °C with a HayeSep N column and a flame ionization detector. Samples (1 mL) for CO_2/O_2 analysis were taken from gas-sampling ports of the column (Fig. 1) with a gas-tight glass syringe after flushing three times with 1 mL sample gas and measured directly by GC on a Trace GC Ultra (Thermo Electron) at 85 °C with a Hayesep-D column and a thermal conductivity detector. With this system, the detection limit for O_2 was around 0.5 mL L^{-1}.

Concentration profile analysis

Gas fluxes, J (μmol h^{-1}), into and out of the column were calculated from measured CH_4, CO_2 or O_2 data using Fick's first law of diffusion (Urmann *et al.*, 2007):

$$J = -D_{\text{eff}} \frac{\mathrm{d}c}{\mathrm{d}z} A \qquad (2)$$

Table 1. Column operational parameters, sampling frequency, CH_4 fluxes, oxidation rates and total cell counts

Experimental phase	Phase I	Phase II	Phase III	Phase IV
Duration (days)	115	100	29	29
T (°C)	12	4	12	20
Sampling frequency*				
CH_4	9	14	9	7
CO_2/O_2	4	4	3	3
DNA/FISH	1	1	1	1
Fluxes and rates (μmol CH_4 h^{-1} ± 1 SD)				
J_{in}†	28.7 ± 3.4 (n = 9)	26.7 ± 4.8 (n = 14)	28.3 ± 5.0 (n = 9)	37.1 ± 6.5 (n = 7)
J_{out}†	0.28 ± 0.12 (n = 9)	0.23 ± 0.08 (n = 14)	0.19 ± 0.16 (n = 9)	0.57 ± 0.33 (n = 7)
r‡	28.4 ± 3.3 (n = 9)	26.5 ± 4.8 (n = 14)	28.2 ± 4.9 (n = 9)	36.5 ± 6.3 (n = 7)
Efficiency of CH_4 removal (% ± 1 SD)				
ε§	99.0 ± 0.3 (n = 9)	99.2 ± 0.3 (n = 14)	99.4 ± 0.4 (n = 9)	98.5 ± 0.6 (n = 7)
Total cell counts (DAPI-stained cells × 10^6 g^{-1} dry wt sand ± 1 SE, n = 8)				
	3.66 ± 0.32	2.54 ± 0.41	2.67 ± 0.46	2.38 ± 0.67

*The number of sampling occasions for CH_4, CO_2/O_2 and molecular analysis is given for each phase.

†J_{in} is the average CH_4 flux into the column; J_{out} the average CH_4 flux out of the column (equals emissions). All fluxes were computed assuming steady-state conditions.

‡r is the average total CH_4 oxidation rate in the column, calculated as $J_{in} - J_{out}$.

§CH_4 removal efficiency calculated as $(r/J_{in}) \times 100$.

The gas-phase concentration gradients with depth, dc/dz, were obtained by regression from the linear parts of the profiles. The flux was converted to μmol h^{-1} using the cross-sectional area, A, of the column (93.3 cm^2).

Average effective diffusion coefficients (D_{eff}) in the sand between 5 and 35 cm, 40 and 65 cm and in the gravel were computed from gas-diffusion coefficients in air D_a adjusted to the actual temperature T (in K) [D_a at $T_0 = 273$ K: 0.1952 cm^2 s^{-1} (CH_4) (Massman, 1998), 0.138 cm^2 s^{-1} (CO_2) and 0.178 cm^2 s^{-1} (O_2) (Perry & Green, 1997)], measured porosity (ϕ), and soil-air content (θ_{air}) derived from porosity and the average measured soil-water content for the respective depth interval, using the Millington & Quirk (1961) equation:

$$D_{eff} = \frac{\theta_{air}^{10/3}}{\phi^2} D_a(273\,\mathrm{K}) \left(\frac{T}{T_0}\right)^{1.81} \quad (3)$$

Methane and CO_2 fluxes in the bottom part of the column were calculated from D_{eff} in the gravel and the profiles measured in the gravel. To calculate CH_4, CO_2 and O_2 fluxes in the top part of the column, D_{eff} down to 35 cm and linear profiles to a maximum depth of 35 cm were used.

Total CH_4 oxidation rate, r (μmol h^{-1}), in the column was calculated by a mass balance approach similar to Chaplin *et al.* (2002) by subtracting the flux out of the top of the column, J_{out}, from the flux into the column, J_{in}:

$$r = J_{in} - J_{out} \quad (4)$$

Finally, the efficiency of CH_4 removal in the column, ε (%), was computed as:

$$\varepsilon = \frac{r}{J_{in}} \times 100 \quad (5)$$

Total production of CO_2 was computed in a similar fashion by adding together the fluxes out of the top and bottom of the column. Oxygen consumption equalled the O_2 flux into the column as all O_2 was consumed in the system. To estimate an apparent first-order rate constant k from for example CH_4 profiles, an analytical solution for a one-dimensional steady-state diffusion–consumption model (Reeburgh *et al.*, 1997) was fitted to the average data for the days when all gases were measured using the CH_4 concentration at the bottom of the active zone (see Results) and, as an approximation, a concentration of zero at the top of the column as boundary concentrations. For these computations, the effective diffusion coefficient [Eqn. (3)] was calculated using the average water content in the active zone (sand between 40 and 65 cm).

Analysis of methanotrophic cultures

At the end of each phase, four sand samples (*c.* 0.4 g each) were collected with a sterile spatula from sampling ports located at the side of the column at 30, 40, 50 and 60 cm (Fig. 1). For FISH, each sand sample (*c.* 0.2 g) and 1–2 mL of the inoculum were fixed at room temperature for 3 h in 1 mL of 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS). The samples were washed twice by centrifugation for 5 min at 13 000 ***g***, and resuspension of the pellet in

1 mL PBS. Finally, the cells were resuspended in 100 μL ethanol : PBS (50 : 50 v/v) and stored at −20 °C until analysis.

For terminal-restriction fragment length polymorphism (T-RFLP) profiling, the other half of each sample (*c.* 0.2 g) was stored in 0.5 mL DNA extraction buffer [50 mM NaCl, 50 mM Tris-HCl (pH 7.6), 50 mM EDTA, 5% sodium dodecyl sulphate; all reagents provided by Sigma-Aldrich, Buchs, Switzerland] and 0.3 g glass beads (0.10–0.11-mm diameter) at −80 °C.

DNA extraction and T-RFLP profiling of the *pmoA* gene

DNA from different depths of the sand pack and from the inoculum was extracted according to a modified version of the protocol of Noll *et al.* (2005). DNA quantification was performed with Nanodrop (Thermo Fisher Scientific, Wilmington, DE).

For PCR, 30–100 ng of DNA was amplified in a master mix containing 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 μM dNTPs, 1 mg mL^{-1} bovine serum albumin and 2 U *Taq* polymerase (Invitrogen, Carlsbad, CA) and 0.2 μM of each *pmoA*-specific primers 189F (GGNGACTGGGACTTCTGG; Holm *et al.*, 1992), labelled with FAM, and 661Rev (CCGGMGCAACGTCYTTACC; Burrows *et al.*, 1984). A touchdown PCR was applied, with an initial step at 94 °C for 3 min, followed by 30 cycles with denaturation at 94 °C for 2 min, 30 s annealing from 62 to 56 °C, followed by extension at 72 °C for 1 min. The PCR amplification was ended by a final step at 72 °C for 4 min. PCR was carried out in a BioRad Icycler thermal cycler (BioRad, Reinach, Switzerland).

The PCR product was digested with an equal volume of digestion mix, consisting of 10 U per reaction of a selected restriction enzyme in 1% Y^+ Tango buffer (Fermentas, Burlington, ON, Canada) for 3 h at 37 °C. A first digestion was performed with the enzyme MspI (Fermentas) as it is considered the most appropriate to analyse the diversity of the methanotrophic community on the *pmoA* gene sequences (Horz *et al.*, 2001). To better resolve the variability of the *pmoA* gene within the Type I methanotrophs, a parallel digestion was performed with the enzyme HaeIII (Fermentas).

Three microlitres of digestion products was added to 10.5 μL of HIDI Formamide (Applied Biosystems, Foster city, CA) and 0.5 μL of MapMarker1000_ROX DNA fragment length standard (Bioventures, Murfreesboro, TN). The samples were denatured for 2 min at 95 °C and immediately transferred to ice. Electrophoresis was performed for 60 min at 60 °C with an ABI 3130XL genetic analyser (Applied Biosystems), using POP7 as the running polymer. The lengths of the fluorescently labelled T-RFs were determined using GENEMAPPER software (v.3.7, Applied Biosystems). Each operational taxonomic unit (OTU) was represented by a peak of a certain size ± 1 bp. As it was not possible to obtain replicate samples from the sand column, T-RFLP was used as a descriptive tool to visualize the general composition of the methanotrophic community.

Cloning and sequencing of the *pmoA* gene

To identify the main methanotrophic groups in the sand column, a PCR product, derived from amplification of the sample from 60-cm depth at the end of phase I with the *pmoA*-specific primers A189F (unlabelled) and 661Rev, was purified with a PCR-cleanup-microspin column (Qiagen) and cloned into the TOPO vector (Invitrogen) according to the manufacturer's instructions. Transformants were screened with colony PCR in 25 μL of a PCR mix under the same conditions as described above, using 25 cycles. The positive PCR products were further purified with PCR clean-up centrifuge plates (Millipore, Billericay, MA). One hundred to 150 ng of purified PCR product was then sequenced using the BigDye v.1.1 terminator sequencing kit (Applied Biosystems) containing 1.6 pmol of sequencing vector-specific primer M13F (CACGACGTTCTAAAAC GACGGC), according to the manufacturer's instructions. Reactions were purified with sequencing reaction clean-up plates (Millipore) to remove excess dye, and then run with an ABI3130XL sequencer using POP7 as running polymer (Applied Biosystems) and running the samples for 60 min at 50 °C.

The sequences were analysed with SEQUENCING ANALYSIS software v.2.5 (Applied Biosystems) and aligned with BIOEDIT software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequences were matched with GenBank sequences using the BLASTN of the National Centre for Biotechnology Information (Benson *et al.*, 2005).

FISH with methanotroph-specific probes

For FISH, the samples were first shaken horizontally on a microcentrifuge tube vortex adapter (MoBio, Carlsbad, CA) for 15 min at minimum speed. Ten microlitres of the slurry was transferred to a clean microcentrifuge tube, to which 10 μL of a 60% Hystodenz (Sigma-Aldrich) was added. This step has been recommended to efficiently separate cells from sediment particles (Caracciolo *et al.*, 2005). Following centrifugation at 13 000 ***g*** for 90 min, the supernatant (containing the cells) was carefully transferred onto wells on ethanol-rinsed eight-well glass slides, and dried at 30 °C. Cells were permeabilized with the application of 10 μL of 1% lysozyme solution (Sigma-Aldrich) onto the wells and incubated at 46 °C for 1 h. The slides were briefly rinsed with autoclaved deionized water, and dried at 30 °C. Hybridization with Cyt-labelled Type I and Type II

methanotroph-specific probes MA445 (CTTATCCAGG TACCGTCATTATCGTCC) and MG705 (CTGGTGTTCCT TCAGATC) (Gulledge *et al.*, 2001) was conducted at 46 °C for 3 h in the dark (Dedysh *et al.*, 2000). In parallel, for 4′-6-diamidino-2-phenylindole (DAPI) counterstaining, 10 μL of a DAPI solution (25 μg μL^{-1}) was pipetted over each well and left in the dark for 15 min. The slides were rinsed with water for 1 min, dried and mounted with Citifluor antifadent (Citifluor Ltd, Leicester, UK). Analysis was performed immediately with a Leica DM6000B fluorescence microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Leica 350FX camera for fluorescence microscopy.

Results

Gas-concentration profiles

Initially, the column was maintained at 12 °C. Following inoculation, the observed CH_4 profile was curved over the entire depth of the column (data not shown), indicating that CH_4 oxidation was initially occurring along the whole profile. After 2 weeks, a distinct zone, where CH_4 oxidation activity was concentrated, had developed, but only after an additional 4 weeks was the system fully equilibrated and a steady state reached, as indicated by a stable CH_4 profile (Fig. 2). During the entire experiment with temperatures between 4 and 20 °C, observed CH_4, CO_2 and O_2 profiles remained similar despite some variability within each phase and between phases, with deviations especially in phase IV. Averages of three to four CH_4, CO_2 and O_2 profiles (Fig. 2), obtained on different days of each phase, were used for further analysis.

In all four phases, CH_4 profiles were linear in the lower part of the column. Transition from linear to nonlinear profiles, i.e. a decline in the gradient d*c*/d*z*, indicated CH_4 oxidation. Around 15-cm (phase I–III) to 20-cm (phase IV) depth, above the transition point, CH_4 concentrations dropped to low values (0.02–0.04 mL L^{-1}). This 15–20-cm-deep zone will be designated the 'active zone' throughout this paper. A decrease in the curvature of the CH_4 profiles, i.e. a decline in the gradient change, indicated a decrease of CH_4 oxidation from the bottom towards the top of this zone. In the upper and lower part of the column, CO_2 profiles were also linear, indicating no or low CO_2 production in these regions (Fig. 2). In phases I–III, the maximum CO_2 concentrations coincided with the transition point in CH_4 profiles, confirming that maximum activity occurred at this depth. This depth will be designated the 'depth of maximum activity' throughout this paper. The depth of maximum activity was around 60 cm on days when CO_2 and O_2 were measured in all phases (Fig. 2), but fluctuated

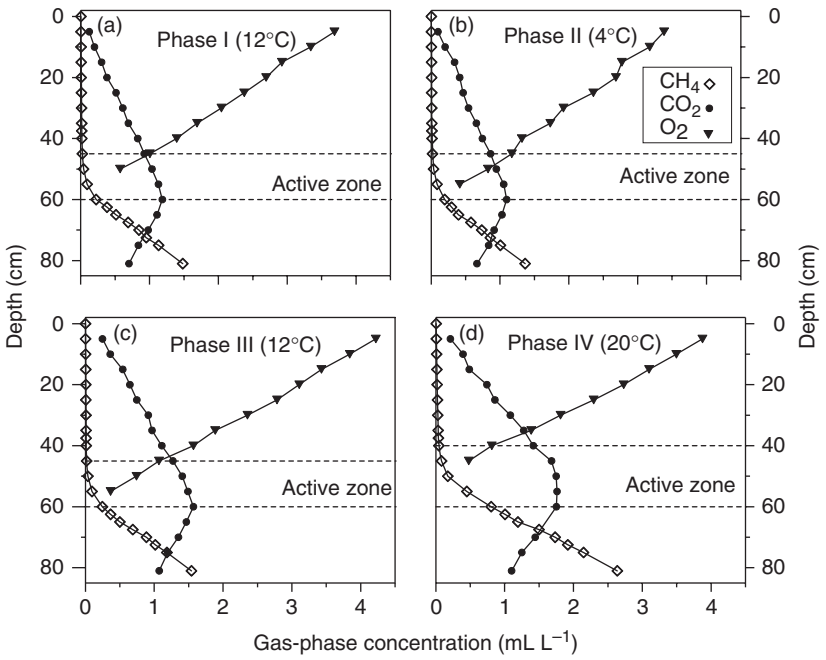

Fig. 2. Measured CH_4, CO_2 and O_2 profiles in the column in phases I–IV with temperatures of 12, 4, 12 and 20 °C. The average of three to four profiles measured on different days is shown for each phase. Relative SD (n = 3–4) in the slopes of the profiles, and therefore variability of gas fluxes, for each phase ranged from 10% to 16% (CH_4), 8% to 30% (CO_2) and 2% to 11% (O_2). Variability in the slopes of all measured CH_4 profiles is given in Table 1. The 'active zone' is the zone where at least 95% of CH_4 oxidation occurred.

between 60 and 65 cm on other days (data not shown). Only in phase IV, similar CO_2 concentrations occurred in the entire active zone and CH_4 profiles indicated little activity at the bottom of the active zone, suggesting that activity was more spread out. In all phases, O_2 concentration profiles were linear in the upper part of the column and dropped below the detection limit of the analytical method within the active zone.

Fluxes and activity

Methane flux into the column, obtained from CH_4 profiles during steady state, ranged from 18 to 39 μmol h^{-1} in all phases (Fig. 3a). In phases I–III average fluxes into the column were similar while in phase IV the average flux was around 30% higher (Table 1) with one exceptionally high value of 48 μmol h^{-1} on day 260 (Fig. 3a). Emission fluxes usually ranged from 0.04 to 0.59 μmol CH_4 h^{-1} (Fig. 3b, note the different scale of the *y*-axis) and remained within the same range on the days after temperature was changed. The highest emission flux (1.3 μmol h^{-1}) occurred within phase IV on day 260, i.e. the day of the highest influx (Fig. 3a), and corresponded to a maximum relative emission of 2.5%. The average total CH_4 oxidation rate, *r*, in the column increased significantly in phase IV compared with phases I–III (Table 1), indicating a positive correlation between *r* and temperature (Friedman test, $P = 0.048$). Conversely, the efficiency of CH_4 removal, ε, ranged from 97.5% to 99.8% with little variation within each phase and between phases. Over 95% of oxidation occurred within the active zone and only an average of 1% of CH_4 entering the column was emitted at the top of the column.

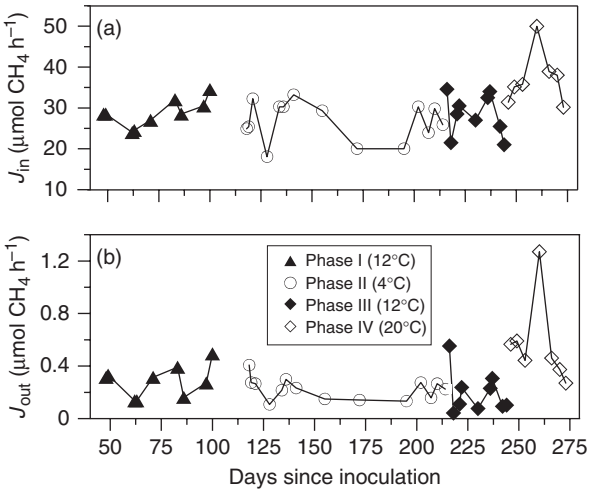

Fig. 3. (a) Total CH_4 flux (J_{in}) into the column. (b) Total CH_4 emission flux (J_{out}) out of the column. Note the different scale of the *y*-axes. Only data from 48 days (since inoculation) onward are shown, as no data analysis was previously performed because the system was not yet fully equilibrated.

Apparent first-order rate constants *k* for CH_4 oxidation were estimated from profiles using a simple one-dimensional diffusion–consumption model (Reeburgh *et al.*, 1997). The estimated *k* values ranged from 9 to 14 h^{-1}, referred to CH_4 concentrations in soil air. Comparing CH_4 oxidation with CO_2 production obtained from CO_2 profiles, an assimilation rate can be estimated based on the assumption that all CO_2 originates from CH_4. On average, $27 \pm 10\%$ ($n = 14$, 1 SD) of CH_4 was assimilated in all phases with no apparent trend throughout the experiment. Calculating carbon assimilation from CH_4 and O_2 consumption yielded a similar assimilation rate of $32 \pm 24\%$ ($n = 14$, 1 SD), but with higher variations probably due to lower reliability of O_2 data. As only one instead of two O_2 molecules is required for one CH_4 assimilated, the obtained average assimilation rates translate into a stochiometric ratio of $CH_4 : O_2$ of *c.* 1 : 1.7.

DAPI cell counts and FISH analysis of active methanotrophic populations

Total cell number (DAPI-stained cells) in the sand pack after inoculation [$(2.33 \pm 0.54) \times 10^5$ cells g^{-1} dry wt sand] was estimated from cell numbers in the inoculum and water content in the column at 60 cm. During phase I (12 °C), total cell number in the active zone of the sand column increased in comparison with cell numbers after inoculation by a factor of 10, indicating growth (Table 1). In the subsequent phases, the cell number remained fairly stable.

The percentage of DAPI-stained cells hybridizing with methanotroph-specific probes was generally higher for Type I than Type II, with a pronounced increase in Type II towards the end of the experiment (Fig. 4).

Taxonomic assignment of *pmoA* sequences

The T-RFLP profiles of the sand samples in the active zone (at 60-cm depth) were highly similar throughout the experimental phases, sharing similar patterns. The enzymatic digestion of the *pmoA* amplicon with MspI produced four detectable T-RFs (77, 245, 440, and 485 bp) and an undigested fragment. Digestion with HaeIII allowed us to further distinguish populations of Type I methanotrophs (three detectable T-RFs of 333, 348 and 350 bp). In contrast, this enzyme was not suitable for analysis of Type II methanotrophs, as it produced T-RFs for Type II that were too small to be detected (< 50 bp).

T-RFLP analysis with two different enzymes permitted assignment of a putative taxonomic affiliation to the dominant T-RFs in the profile through *in silico* comparison with *pmoA* sequences in the NCBI database (Fig. 5). In agreement with Horz *et al.* (2001), the database search showed that the

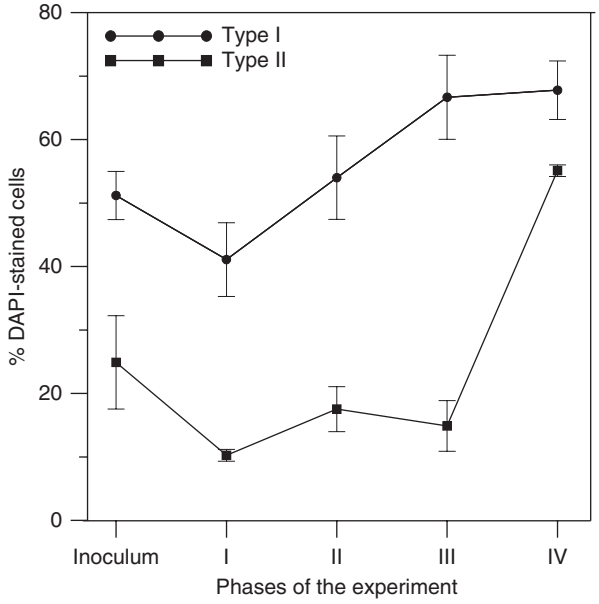

Fig. 4. Percentage values of cells hybridizing with the methanotrophic probes GM705 and AM445 in relation to total cell number detectable by DAPI staining in the original inoculum and in all phases (I–IV) of the experiment. Bars represent SE of cell counting, which was performed in eight separate wells per sample (for each data point, $n = 8$).

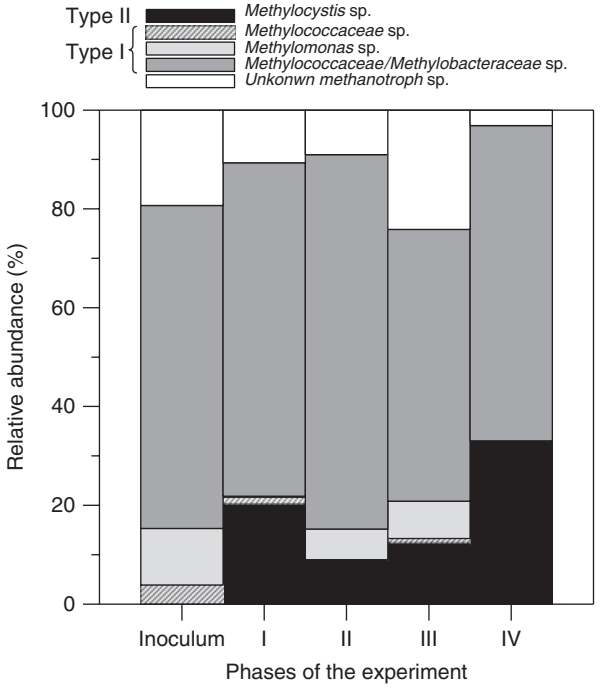

Fig. 5. MspI-generated methanotrophic patterns in the active zone of the sand pack (at 60-cm depth) based on T-RFLP analysis of the *pmoA* gene. Each T-RF is represented by the taxonomic assignment obtained through cloning of the *pmoA* PCR product and *in silico* comparison with database *pmoA* sequences.

MspI-derived T-RF 77 can be assigned to the class *Methylococcaceae*, MspI-derived T-RF 440 to the genus *Methylomonas*, and the undigested PCR product to the genera *Methylococcaceae* or *Methylobacteraceae*. All the *pmoA* sequences of the mentioned Type I groups have a restriction site for HaeIII at 350 bp, which was detected in the T-RFLP analysis. The MspI-derived T-RF 245 could instead be referred to Type II methanotrophs such as *Methylocystis*, while the MspI-derived T-RF 485 and the HaeIII-derived T-RFs 333 and 348 found in the T-RFLP profiles could not be referred to any known methanotrophic group.

The sequences obtained from the cloned *pmoA* PCR gave two positive BLAST matches in the NCBI database. Forty-one sequences were highly similar (98%) to the *pmoA* gene of an uncultured bacterium clone (accession number EF212347) and of a *Methylobacter* sp. (accession number DQ400895). One sequence showed instead 99% similarity to the *pmoA* gene of a Type II methanotroph (*Methylocystis* sp., accession number AJ584611).

Discussion

Methanotrophic activity

In the column system presented here, CH_4 emissions were equally low at temperatures between 4 and 20 °C and did not markedly increase after changes in temperature with the exception of the beginning of the experiment, when the active, methanotrophic zone established. Similarly, the efficiency of CH_4 removal, ε, remained close to 100% throughout the experiment (Table 1). Consequently, the methanotrophic community in the column, originally enriched from a petroleum-contaminated site, maintained its function as an effective biofilter for CH_4 independent of temperature. On the other hand, in absolute terms, the total oxidation rate, r, was higher at 20 °C than at 4 and 12 °C. This effect, however, was largely due to a change in the system's boundary conditions, as the influx of CH_4 into the column was significantly higher at 20 °C than at 4 and 12 °C (Table 1). Thus, the observed increase in r in phase IV may be attributed to an increase in substrate availability rather than a direct effect of temperature on enzymatic activity.

Furthermore, no major differences were observed in the distribution of activity in the column between the different temperatures apart from the apparently wider active zone in phase IV (20 °C), which may have been due to the higher influx of CH_4. In phase IV, an interruption in CH_4 supply due to technical difficulties for 3 days before measurements and/or potential nutrient limitations due to the long duration of the experiment may have slowed down growth or activation of methanotrophs in response to the higher CH_4 fluxes. However, this did not impact the overall function of the column system as a biofilter.

Despite somewhat lower CH_4 fluxes into the column and consequently lower CH_4 oxidation (r) in this experiment, CH_4, CO_2 and O_2 profiles were generally comparable with previous experiments conducted under similar conditions but at room temperature (23–26 °C) (Urmann *et al.*, 2007). In accordance with similar profiles, CH_4 oxidation followed apparent first-order kinetics in both studies, albeit with a lower first-order rate constant in this study due to the lower CH_4 influx. In contrast to previous experiments, we were able to measure O_2 profiles in this study. Within the active zone, values dropped below the detection limit, indicating low O_2 concentrations at the depth of maximum activity as observed previously in simulations of O_2 profiles (Urmann *et al.*, 2007).

The fact that we did not observe a temperature dependence of CH_4 removal efficiency ε in the column indicates that CH_4 oxidation is mainly controlled by physical processes, i.e. gas-phase diffusion and transport of CH_4 to the cells, rather than the enzymatic reaction. The latter is highly dependent on temperature, while physical processes show a comparatively low temperature dependence, which in this experiment is already accounted for in flux calculations. Control of CH_4 oxidation by physical processes is in agreement with observed apparent first-order kinetics with respect to CH_4 in the gas phase. Similarly, in previous studies on CH_4 uptake by oxic soils following first-order kinetics, temperature effects were usually small and were mainly attributed to gas-phase diffusion with enzymatic activity playing a secondary role (King & Adamsen, 1992; Smith *et al.*, 2003). In contrast, in soils with high diffusivity, an increase of CH_4 oxidation with temperature suggested a larger influence of enzymatic activity on CH_4 uptake (Mosier *et al.*, 1996). In comparison with these studies, CH_4 oxidation activity in our column system was several orders of magnitude higher and was higher than activity observed for example in peat bogs (Whalen & Reeburgh, 2000; Pearce & Clymo, 2001). In a column study of CH_4 oxidation in landfill-cover soils with even higher, advective CH_4 fluxes, some dependence of activity on temperature between 4 and 23 °C was observed (Kettunen *et al.*, 2006). However, the drop in activity with temperature was less than in concomitant batch incubations at CH_4 saturation. This confirms that CH_4 oxidation in field situations, even with high activity and high CH_4 fluxes, is not necessarily controlled only enzymatically, and physical characteristics may also play an important role. Therefore, Q_{10}-values obtained from batch incubations may not adequately represent temperature dependence of CH_4 oxidation in the field.

In our experiment, it appeared that apart from physical properties, one crucial factor for the functioning of the system as a biofilter for CH_4 was the initial establishment, i.e. growth, of the methanotrophic community. Methanotrophic biomass and conditions for growth, such as substrate and nutrient availability, are an important factor determining methanotrophic activity (Bender & Conrad, 1994; King, 1997; Willison *et al.*, 1997), and can also play a role in the complex response of methanotrophic activity to temperature changes in the field (Davidson *et al.*, 2006).

Methanotrophic community structure

Molecular analysis of sand from the column permitted us to link the observed CH_4 oxidation activity with the corresponding methanotrophic community. Total cell numbers suggest that initially growth occurred to establish activity in the column. Subsequently, assimilation rates were in the range of 25–35%. Such values fall within the wide range from 2% to 73% previously reported for methanotrophs (references in Whalen *et al.*, 1990), but similar total cell numbers suggested that the community reached a steady state during phase I with no further significant net growth occurring at later stages of the experiment.

Positive FISH signals for both Type I and Type II methanotrophs in the inoculum and in the column throughout the experiment suggested that both groups were responsible for the observed activity in the column. Type I species, which appear to be represented by the genus *Methylobacter*, were the dominant active methanotrophic species throughout the experiment. The percentage of active Type II species (putative *Methylocystis* sp.) increased significantly during the last phase of the experiment, at 20 °C. A similar increase was not seen for the Type I methanotrophs. Such growth or activation of cells of Type II methanotrophs would suggest that they favour higher temperatures, as was also shown by Mohanty *et al.* (2007) for Type II methanotrophs of rice soils incubated at temperatures > 15 °C. However, at the end of the experiment there could have also been a reduction of available nutrients in the column. Therefore, Type II methanotrophs might have had a competitive advantage over Type I methanotrophs. This effect would be in agreement with previous findings that Type II methanotrophs outcompeted Type I methanotrophs under nutrient-limited conditions (Graham *et al.*, 1993).

The T-RFLP profiles from the active zone of the column shared a similar pattern in the temperature range from 4 to 20 °C. An *in silico* assignment of taxonomic affiliations to specific T-RFs can have uncertainties due to the comparison with sequence information derived from other experimental systems. However, the analysis of sequence data derived from cloning of the *pmoA* gene confirmed the taxonomic affiliations of two major T-RFs and the presence of both Type I and Type II methanotrophs in the sand pack. Based on these data, T-RF 245 clearly corresponds to a species related to *Methylocystis* (Type II), while the undigested fragment is strongly related to a *Methylobacter* species (Type I). The lack of replicate sequences related to T-RF 245 may

indicate experimental difficulties of cloning the *pmoA* gene of the Type II methanotrophs (Horz *et al*., 2001).

Conclusions

In a laboratory-column system, it was possible to assess successfully the response of methanotrophic activity and community structure to changing temperature under physical conditions that resemble a field situation. Methane oxidation removed CH_4 from the system effectively at all temperatures. The observed methane oxidation rates were probably maintained by both Type I and Type II members of a relatively stable methanotrophic community able to adapt to temperatures between 4 and 20 °C. Even though results cannot be directly extrapolated and the enriched methanotrophic community probably only represents a portion of the community present at the field site from which the inoculum was obtained, methanotrophic communities may also be able to maintain their activity at a wide temperature range in the field without major shifts in community composition. The fact that activity in our system appeared to be primarily controlled by physical parameters rather than by enzymatic activity underlines that care should be taken when applying simple relationships between reaction rate and temperature observed in laboratory-batch incubations to a field situation. As in our model system, substrate transport, among other possible factors, may be more important than enzymatic activity in the field, leading to a negligible apparent temperature dependence of observed activity.

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