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ORIGINAL ARTICLE

One millimetre makes the difference: high-resolution analysis of methane-oxidizing bacteria and their specific activity at the oxic–anoxic interface in a flooded paddy soil

Andreas Reim, Claudia Lüke, Sascha Krause[1], Jennifer Pratscher[2] and Peter Frenzel
Max Planck Institute for Terrestrial Microbiology, Department of Biogeochemistry, Marburg, Germany

Aerobic methane-oxidizing bacteria (MOB) use a restricted substrate range, yet >30 species-equivalent operational taxonomical units (OTUs) are found in one paddy soil. How these OTUs physically share their microhabitat is unknown. Here we highly resolved the vertical distribution of MOB and their activity. Using microcosms and cryosectioning, we sub-sampled the top 3-mm of a water-saturated soil at near *in situ* conditions in 100-μm steps. We assessed the community structure and activity using the particulate methane monooxygenase gene *pmoA* as a functional and phylogenetic marker by terminal restriction fragment length polymorphism (t-RFLP), a *pmoA*-specific diagnostic microarray, and cloning and sequencing. *pmoA* genes and transcripts were quantified using competitive reverse transcriptase PCR combined with t-RFLP. Only a subset of the methanotroph community was active. Oxygen microprofiles showed that 89% of total respiration was confined to a 0.67-mm-thick zone immediately above the oxic–anoxic interface, most probably driven by methane oxidation. In this zone, a *Methylobacter*-affiliated OTU was highly active with up to 18 *pmoA* transcripts per cell and seemed to be adapted to oxygen and methane concentrations in the micromolar range. Analysis of transcripts with a *pmoA*-specific microarray found a *Methylosarcina*-affiliated OTU associated with the surface zone. High oxygen but only nanomolar methane concentrations at the surface suggested an adaptation of this OTU to oligotrophic conditions. No transcripts of type II methanotrophs (*Methylosinus, Methylocystis*) were found, which indicated that this group was represented by resting stages only. Hence, different OTUs within a single guild shared the same microenvironment and exploited different niches.
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Introduction

Methane is, next to water vapor and carbon dioxide, the most important greenhouse gas (Intergovernmental Panel on Climate Change, 2007), with natural wetlands and irrigated rice fields together emitting about one-third of the total (Conrad, 2009). Their contribution would be even higher without the activity of aerobic methane-oxidizing bacteria (MOB), which act as a biofilter, mitigating emissions to the atmosphere (Reeburgh *et al.*, 1993). MOB use methane as the sole source of carbon and energy, provided oxygen is available (Trotsenko and Murrell, 2008). Owing to this dual dependency, they thrive at oxic–anoxic interfaces, where both substrates are supplied (Brune *et al.*, 2000). In flooded soils and sediments, these interfaces are located at the soil surface and in the rhizosphere of macrophytes when present (Bosse and Frenzel, 1997; Bodelier *et al.*, 2006). Rhizospheric MOB in both paddy fields and natural wetlands have been often studied (Calhoun and King, 1997; Eller and Frenzel, 2001; Sorrell *et al.*, 2002; Shrestha *et al.*, 2008; Vishwakarma *et al.*, 2009), but work at the soil surface has been mainly focused on process measurements (Conrad and Rothfuss, 1991; Frenzel *et al.*, 1992; Bosse *et al.*, 1993). The soil surface is characterized by sharp counter-gradients of oxidized and reduced species. Where these gradients overlap,

Correspondence: P Frenzel, Max-Planck Institute for Terrestrial Microbiology, Department of Biogeochemistry, Karl-von-Frisch-Str. 10, Marburg D-35043, Germany.
E-mail: frenzel@mpi-marburg.mpg.de
[1]Current address: Netherlands Institute of Ecology (NIOO-KNAW), Department of Microbial Ecology, 6708 AB Wageningen, The Netherlands.
[2]Current address: The University of Warwick, Coventry CV4 7AL, UK.
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⩾90% of potentially emitted methane is oxidized (Frenzel *et al.*, 1990; Conrad and Rothfuss, 1991).

MOB can be divided into two major groups, type I and type II, being equivalent to the families *Methylococcaceae* (γ-proteobacteria), and *Methylocystaceae* and *Bejerinckiaceae* (α-proteobacteria), respectively. The key enzyme of all MOB is methane monooxygenase (MMO), and the overwhelming majority of cultivated MOB possess a membrane-bound MMO (particulate MMO). Only the genera *Methylocella* and *Methyloferula* lack this enzyme and instead have a soluble MMO (sMMO) (Dedysh *et al.*, 2000; Dedysh, 2009; Vorobev *et al.*, 2011). The *pmoA* gene, which encodes the β-subunit of particulate MMO, is an excellent functional marker for studying MOB in most environments (McDonald and Murrell, 1997; Dumont and Murrell, 2005; McDonald *et al.*, 2008). Its phylogeny reflects very well that of the 16S rRNA gene (Kolb *et al.*, 2003; Degelmann *et al.*, 2010). Type I MOB can be further divided into type Ia (for example, *Methylomonas*, *Methylobacter*, *Methylosarcina* and *Methylomicrobium*), and type Ib (for example, *Methylococcus* and *Methylocaldum*). Recently, MOB belonging to the phylum Verrucomicrobia have been isolated, but these seem to be restricted to extreme environments (Dunfield *et al.*, 2007; Pol *et al.*, 2007; Op den Camp *et al.*, 2009).

Sanger- and pyro-sequencing have provided a large database of high-quality *pmoA* sequences (Lüke *et al.*, 2010; Lüke and Frenzel, 2011). Based on *pmoA* phylogeny, type II MOB form a coherent cluster well represented by cultivated strains. Many distinct groups lacking cultivated representatives have been allocated to type I MOB. Numerous sequences are located at an intermediate position between the *pmoA* gene of MOB and the *amoA* gene of ammonia oxidizers. The substrate of the enzymes encoded by these sequences remains uncertain, with the exception of a few proven methane (Stoecker *et al.*, 2006; Dunfield *et al.*, 2007) and alkane oxidizers (Sayavedra-Soto *et al.*, 2011; Coleman *et al.*, 2012).

More than 30 operational taxonomical units (OTUs) corresponding to the species level have been found in a single paddy soil (Lüke *et al.*, 2010). This raises the question whether and how their niches are separated, and whether all these individual OTUs really contribute to overall methane oxidation. However, not all of these 30 OTUs need to be active at the same time as methanotrophs form resting stages (Whittenbury *et al.*, 1970a, b). Indeed, the development and activity of methanotroph communities has been suggested to vary depending on methane flow (Krause *et al.*, 2012), nitrogen supply (Rudd *et al.*, 1976; Graham *et al.*, 1993; Bodelier *et al.*, 2000a, b; Noll *et al.*, 2008), disturbance (Ho *et al.*, 2011) and grazing (Murase and Frenzel, 2008). At the macroscale, MOB community structure within a paddy field varies randomly, probably as ploughing prevents the development of explicit spatial patterns (Krause *et al.*, 2009). Rice roots select for specific communities, thereby favouring the growth of certain OTUs (Lüke *et al.*, 2011). Another aspect of spatial organization, however, has not yet been addressed — the community structure at the microscale. We focused on the soil surface and hypothesized that activity would be highest right at the oxic–anoxic interface, which would potentially separate MOB according to substrate availability, for example, high methane/low oxygen in deeper layers and low methane/high oxygen in shallower layers.

The study of gradient organisms requires a physical model that mimics naturally occurring gradients. With MOB, these are primarily the counter-gradients of oxygen and methane (Gilbert and Frenzel, 1998). We constructed microcosms that allow incubation of the top 3-mm of a water-saturated soil at near *in situ* conditions (Murase and Frenzel, 2007). When methane was supplied from below and air was supplied from above, a functioning methanotrophic community developed within a few days, oxidizing virtually all the methane that otherwise would have passed through this soil layer. We used cryosectioning (Murase *et al.*, 2006) to subsample the soil from top to bottom in 100-μm steps. Focusing on *pmoA* as a functional and phylogenetic marker, we analyzed genes and transcripts along this depth profile, using *pmoA* transcripts as a proxy for species-specific activity. The community structure was assessed by terminal restriction fragment length polymorphism (t-RFLP), by a *pmoA*-specific diagnostic microarray (Bodrossy *et al.*, 2003), and by cloning and sequencing. Oxygen microprofiles were used to model the depth-dependent oxygen consumption rate (Berg *et al.*, 1998). Rates were correlated to copy numbers of *pmoA* genes and transcripts. Transcripts were quantified using a combination of competitive reverse transcriptase PCR (RT-PCR) and t-RFLP.

In addition to *pmoA*, we used *mmoX* encoding for a subunit of the sMMO to search for genes and transcripts of aerobic MOB that lack a *pmoA*. Three different primer sets were applied to DNA and RNA extracted from microcosms incubated for 2, 4 and 6 weeks, respectively. While this design allows to cover potential successional changes, the chosen primer sets are expected to target a wide range of *mmoX* diversity.

Materials and methods

Soil microcosm incubation and sampling

The construction and setup of the microcosms have been described previously (Murase and Frenzel, 2007). Briefly, 14 g dry rice field soil from Vercelli (Italy) was saturated with 7 ml demineralized water and incubated on a polytetrafluoroethylene membrane, which divided the microcosm into an upper and a lower compartment. The upper compartment

contained oxygen at atmospheric concentrations, while the lower chamber was connected to an external reservoir (volume 1 l) with nitrogen gas supplemented with methane (15%).

We set up four microscosms (1–4). During incubation, methane and oxygen concentrations were monitored by gas chromatography. Methane was added regularly to the reservoir keeping concentration stable (s.e. 0.16% CH_4, $n = 18$). The microcosms were incubated in the dark for 14 days at 25 °C. Present and active methanotroph populations show some succession, but most changes occur from 25 days onwards (Krause *et al.*, 2010). Prior to sampling, vertical oxygen profiles were determined using an oxygen microelectrode (OX50, Unisense, Aarhus, Denmark). The microcosms were then shock frozen in liquid nitrogen and stored at −80 °C until further analysis. For sub-sampling, the frozen soil was attached to a pre-cooled stage with Tissue-Tek OCT Compound (Sakura Finetek, Staufen, Germany). A cryotome (cryostat HM500M, MICROM, Walldorf, Germany) was used to prepare subsamples 100 μm thick (30 subsamples per microcosm). The subsamples were stored in 500 μl RNAlater-ICE (Ambion, Austin, TX, USA) at −20 °C for subsequent nucleic acid extraction.

Nucleic acid extraction

DNA and RNA were extracted following the protocol of Lueders *et al.* (2004) with minor modifications (Krause *et al.*, 2010). RNA was prepared by digestion of 1 mg total nucleic acid with RQ1 RNase-free DNase (Promega, Madison, WI, USA) and subsequent purification using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. *pmoA* transcripts were enriched using the mRNA-only Prokaryotic mRNA Isolation Kit (Epicentre Biotechnologies, Madison, WI, USA) and again purified with the RNeasy Mini Kit (Qiagen).

Complementary DNA (cDNA) synthesis and pmoA amplification

cDNA was synthesized and *pmoA* was amplified using the One-step Access RT-PCR System (Promega) with the forward primer A189f (5′-GGNGACTG GGACTTCTGG-3′) (Holmes *et al.*, 1995) and the reverse primer mb661r (5′-CCGGMGCAACGTCYTT ACC-3′) (Costello and Lidstrom, 1999). For t-RFLP, the forward primer was FAM labeled. For microarray analysis, the reverse primer contained the T7 promoter site (Bodrossy *et al.*, 2003). A total of 1 μl purified template RNA was mixed with 5 μl AMV/Tfl 5 × reaction buffer (Promega), 0.01 mg bovine serum albumin (Roche, Basel, Switzerland), 2.5 nmol of each dNTP (Promega), 8 pmol of each primer, 25 nmol $MgSO_4$ (Promega), 5% (v/v) DMSO, 20 U RNasin Plus (Promega), 2.5 U Tfl DNA polymerase (Promega), 2.5 U AMV reverse transcriptase (Promega) and molecular-grade water (Sigma-Aldrich, Munich, Germany) in a total volume of 25 μl. Reactions without AMV reverse transcriptase were used to check for DNA contamination. The first strand of cDNA was synthesized at 45 °C in 45 min, followed by 2 min at 94 °C to inactivate the AMV reverse transcriptase. The template was amplified in 35 cycles (30 s at 94 °C, 1 min at 55 °C, 1 min at 68 °C, final elongation 7 min at 68 °C). PCR products were checked on a 1% agarose gel and extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen). Genomic copies of the *pmoA* gene were amplified following the same protocol, but without the initial cDNA synthesis step.

Cloning and sequencing

Cloning and sequencing was done as described before (Lüke *et al.*, 2010).

t-RFLP analysis

The purified PCR product (100 ng) was digested with FastDigest MspI enzyme (Fermentas, St Leon-Rot, Germany) at 37 °C for 6 min. Digested samples were purified with Post-Reaction Clean-Up Spin Columns (Sigma-Aldrich) according to the manufacturer's instructions. A total of 2 μl of each purified sample was mixed with 11 μl Hi-Di Formamide (Applied Biosystems, Foster City, CA, USA) and 0.2 μl of an internal DNA fragment length standard (MapMarker 1000, 50–1000 bp, x-rhodamine, Eurogentec, Ougree, Belgium) and denatured for 2 min at 94 °C. The terminal restriction fragments (tRFs) were separated and detected with capillary electrophoresis and an automatic sequencer (3130 Genetic Analyzer, Applied Biosystems; 30 min at 15 kV and 9 μA). The tRF patterns were analyzed with GeneMapper Version 4.0 (Applied Biosystems).

Microarray analysis

In vitro transcription, fragmentation, hybridization, scanning and data analysis were performed as described elsewhere (Stralis-Pavese *et al.*, 2004; Stralis-Pavese *et al.*, 2011).

Competitive t-RFLP

A competitive PCR (cPCR) assay (Han and Semrau, 2004) was adapted to quantify copy numbers of the *pmoA* gene and its transcripts. The assay was optimized for the most abundant and active OTU. A vector-born copy of an environmental *Methylobacter*-related *pmoA* gene (accession number) was used for standard preparation. Primers were A189f_T7 (5′-TAATACGACTCACTATAGGGGGNGA CTGGGACTTCTGG-3′) and Inner-rev-661 (5′-CCG GMGCAACGTCYTTACCACTCAGGAGTACCAGTTC TT-3′). Concentrations of DNA and RNA standards were determined using RiboGreen and PicoGreen, respectively (Molecular Probes Inc., Eugene, OR, USA).

For each sample, a minimum of three PCR or RT-PCR amplifications was performed as described above. Each reaction contained equal amounts of the environmental template, but varying standard concentrations. After amplification, PCR products were processed and analyzed by t-RFLP. We regressed the logarithms of standard-to-sample ratios to the logarithms of standard added; the amount of standard at the equivalence point equaled the unknown copy number (Freeman *et al.*, 1999). For further details and an example see Supplement 1.

Statistical analysis
t-RFLP data were analyzed as described before (Krause *et al.*, 2010). Briefly, the tRFs were binned to OTUs based on an *in silico* analysis of about 500 sequences from field and greenhouse experiments of Vercelli soil (Lüke *et al.*, 2010). tRF profiles were standardized (Dunbar *et al.*, 2001) and expressed as fractions. For microarray analysis, signals were standardized (i) against the mean of the overall array intensities (Lüke *et al.*, 2011) and (ii) against an experimentally determined reference value for positive detection (Bodrossy *et al.*, 2003). Statistical analysis and graphics were done in R (R Development Core Team, 2011). Analysis of similarity and non-metric multidimensional scaling (using the function *metaMDS*) were done with the *vegan* package, version 2.1-0 (Oksanen *et al.*, 2011).

Soil microcosms for mmoX analyses
To evaluate the potential role of sMMO, we used another 8 microscosms that were installed and incubated as described above. Two microcosms were killed after 2 weeks, and three microcosms each after 4 and 6 weeks, respectively. Soil was sampled in 0.5 g aliquots, shock frozen in liquid nitrogen, and stored at $-80\,^{\circ}C$ till further analysis. DNA and RNA were simultaneously extracted and prepared as described above. RNA samples were reverse transcribed with random hexamer primers and SuperScript III reverse transcriptase (Invitrogen, Darmstadt, Germany). Amplification of *mmoX* gene and transcript sequences was done using (i) primer set mmoX206f/886r (Hutchens *et al.*, 2004), (ii) primer set mmoXf92/r1430 (Islam *et al.*, 2008), and (iii) primer set mmoXLF/LR (Rahman *et al.*, 2011). Primer set mmoX206f/886r covers a fairly wide range of *mmoX* diversity, whereas mmoXf92/r1430 includes verrrucomicrobial sequences. Primer set mmoXLF/LR is specific for *Methylocella.* To check for cDNA quality, we amplified both rRNA and *pmoA* with primer sets 8F/1392R (Amann *et al.*, 1995) and A189f/682r (Holmes *et al.*, 1995), respectively. We got products from all samples. Amplicons generated with mmoX206f/886r from DNA extracted from microcosms after 2 and 6 weeks of incubation were cloned and sequenced as described above. Sequencing was carried out by GATC (GATC Biotech AG, Konstanz, Germany). Phylogenetic trees were constructed from sequence data using the ARB software package (Ludwig *et al.*, 2004).

Sequences
pmoA sequence data have been submitted to EMBL under accession numbers HE805099–HE805112. *mmoX* sequence data have been submitted to GenBank under accession numbers JQ889714–JQ889792.

Results

Methane oxidation and t-RFLP analysis
We followed methane concentrations over time in both the lower and upper compartments of the microcosms. The lower compartment with the methane source simulated the methanogenic soil layer. The upper compartment, or headspace, was replenished with air every 2 days. Initially, in all microcosms, up to 3% methane accumulated in the headspace, but after 5 days of incubation, accumulation ceased resulting in an average headspace concentration of 228 ppm_v CH_4 which indicated the presence of an active and efficient methanotrophic community.

After 2 weeks of incubation, the oxic–anoxic interface in all microcosms stabilized between 1.5 and 1.7 mm (Figure 1a). After 14 days, the soil of four microcosms was shock frozen with liquid nitrogen, removed intact, mounted in a cryotome and sub-sampled in 100-μm layers by sectioning. A total of 30 subsamples per microcosm were analyzed (total $n = 120$).

For an initial overview, we analyzed the genes and transcripts by *pmoA* t-RFLP. We assigned OTUs based on an *in silico* analysis of 500 *pmoA* clone sequences plus another 3500 sequences retrieved by pyrosequencing (Lüke *et al.*, 2011; Lüke and Frenzel, 2011). The assignments were supported by 15 sequences generated from cloned mRNA (another 80 clones were derived from rRNA, see below). Figure 1 shows a synopsis of the average oxygen microprofile and the DNA and mRNA-based t-RFLP profiles from microcosm 1. The DNA-based pattern was diverse, with dominating fragments identified as type Ia (*Methylobacter*) and Ib, and as type II (*Methylocystis* and *Methylosinus*). The type-II-specific fragment was dominant below 2.2 mm depth, whereas fragments assigned to type Ib MOB had their highest relative abundance around the oxic–anoxic interface between 1.0 and 2.0 mm depth (Figure 1b). This increasing dominance of type-I-specific fragments was even more pronounced in the *pmoA* transcripts (Figure 1c). *Methylobacter*-related tRFs dominated around the oxic–anoxic interface, but also in the upper 0.5 mm. Based on t-RFLP, type II MOB did not transcribe the *pmoA* gene. This was consistent with cloning and sequencing of *pmoA*

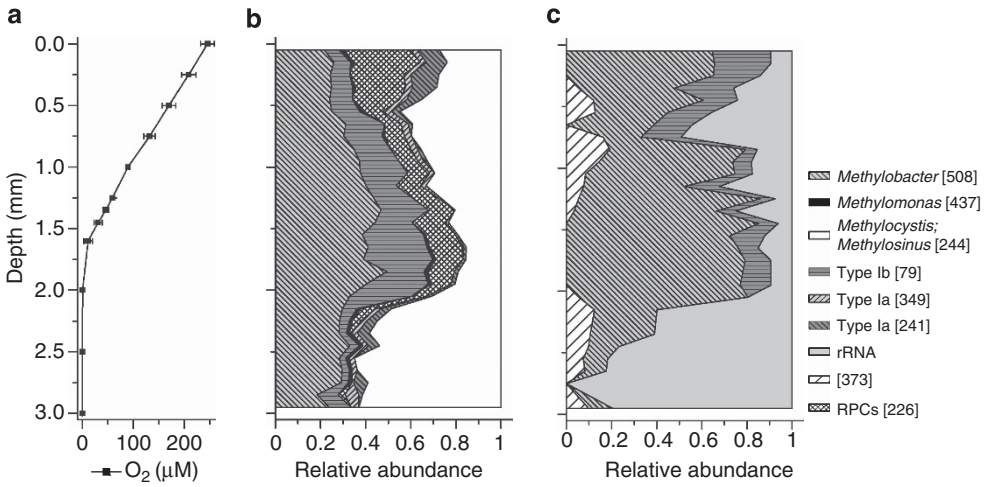

Figure 1 Vertical profiles of oxygen concentration and of tRFs derived from *pmoA* genes and their transcripts. Data are from microcosm 1. (**a**) Average oxygen profile (± s.e., $n = 4$) measured with a microelectrode. Relative abundance of *pmoA* tRFs derived from (**b**) DNA and (**c**) RNA. The phylogenetic affiliation of tRFs is given together with their size in brackets. rRNA: combined relative abundances of different tRFs derived from unspecific reverse and amplification of 16S and 23S rRNA sequences; RPCs: rice paddy clusters (Lüke *et al.*, 2010).

transcripts: type-II-specific sequences were lacking. A considerable fraction of tRFs was not derived from *pmoA* transcripts but rather from rRNA, as observed previously with *pmoA* RT-PCR (Krause *et al.*, 2010). These false-positive tRFs became most obvious below the oxic–anoxic interface, which suggested an extremely low number of target molecules in the anoxic soil.

The *pmoA* gene diversity along the depth profiles was consistent between all four microcosms. On average, the dominating methanotroph groups (type II and type Ia) showed an alternating pattern with a predominance of type Ia around the oxic–anoxic interface (Figure 2). Also the transcript analysis was consistent with the results described for microcosm 1: a lack of type II, a pronounced dominance of type Ia around the oxic–anoxic interface, and a high fraction of false-positive products at depths where no aerobic methane oxidation was expected.

Quantification of transcripts by competitive t-RFLP

Neither the *pmoA* microarray nor the classic t-RFLP (Horz *et al.*, 2001) allow quantification *sensu stricto,* even if they are often regarded as semi-quantitative. To quantify copy numbers of the *pmoA* gene and its transcripts, we combined cPCR or RT-PCR with t-RFLP, respectively. We focused on a fragment affiliated to *Methylobacter* that showed high relative abundances at the oxic–anoxic interface (tRF 508, Figure 1b). This OTU reached copy numbers up to 3.73×10^8 *pmoA* genes per gram fresh weight of soil, and a maximum of 9.5 transcripts per genomic *pmoA* copy at the oxic–anoxic interface (Figure 3). Based on this distribution and further supported by microarray data (Figure 4, see below), we defined three depth zones: surface zone, oxic–anoxic interface and anoxic zone (Table 1). The average number

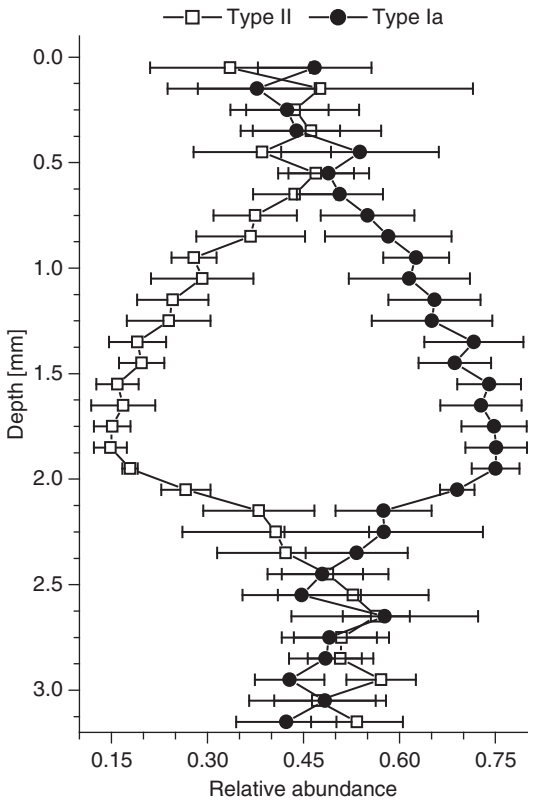

Figure 2 Vertical differentiation of the DNA-based population structure in all four microcosms. Mean relative abundances of type II and type Ia MOB (± s.e., $n = 4$). Type II corresponds to tRF 244; type Ia is the sum of all other fragments. Unlike RT-PCR, the DNA-based analysis did not suffer from unspecific amplification of rRNA genes.

of *pmoA* transcripts in the highly active oxic–anoxic interface were one order of magnitude higher than in the surface and anoxic zones (Table 1). The highest

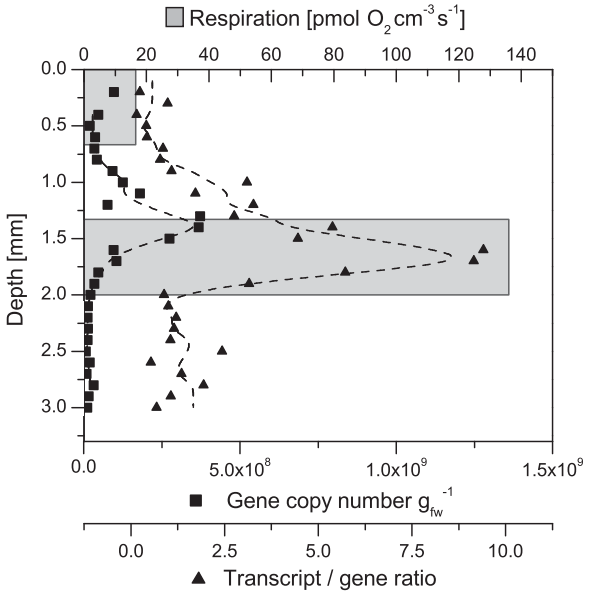

Figure 3 Competitive t-RFLP analysis of a *Methylobacter*-related fragment (tRF 508). Copy numbers of the *pmoA* gene and the *pmoA* RNA/DNA ratio are shown with Sawitzky–Golay smoothers ($n=5$). The oxygen consumption rate was calculated from the microprofile shown in Figure 1a using Berg's program PROFILE ver. 1.0 (Berg *et al.*, 1998), considering the top 2 mm.

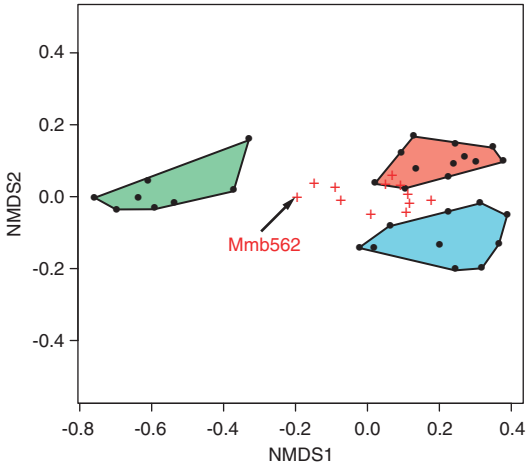

Figure 4 Non-metric multidimensional scaling (NMDS; Bray–Curtis dissimilarities; stress = 0.0689; $R^2=0.997$; linear fit, $R^2=0.989$) of transcript patterns derived from hybridization to a *pmoA* microarray; average values from four microcosms. Layers are shown as black symbols. The green, red and blue hulls visualize the three different zones (surface, 0–0.8 mm; oxic–anoxic interface, 0.8–2 mm and anoxic, 2–3 mm), respectively. The differences between the zones are highly significant (analysis of similarity, Bray–Curtis dissimilarity, $P<0.001$). Red symbols mark the different probes. Probe O_Mmb562, which separated the surface zone from the other two zones, is specific for *Methylosarcina*. The original data from each microcosm are documented in Supplement 3, and probes considered for the ordination together with the rationale for selecting them are given in Supplement 2.

transcript:gene copy ratios coincided with the area of highest oxygen consumption, as calculated from the oxygen microprofiles (Figure 3).

Microarray analysis of pmoA transcripts

We analyzed the transcript patterns of the four microcosms using non-metric multidimensional scaling (Figure 4). Each of the three depth zones formed a distinct cluster with significant differences to each other (analysis of similarity, Bray–Curtis dissimilarity, $P<0.001$). Probes used for ordination and a few others yielding obvious spatial patterns are listed in Supplement 1.

The original microarray data from all four microcosms (1–4) provided additional information (Supplement 2). Hybridization signals for type-II-specific probes were observed mostly in the oxic–anoxic interface, but the signals were faint. The only type II probe that gave a stronger signal (P_MM_MsT343) is unspecific, that is, it binds also to many type Ib sequences. This signal coincided with that of the general probes for type Ib (Ib453 and Ib559).

The general probes for type Ia (O_Ia193, O_Ia575) gave strong signals not only at the oxic–anoxic interface but also in the anoxic zone. Furthermore, probe Mb271 gave signals in all three depth zones, but most signals in microcosms 1 and 2 were in the oxic–anoxic interface. In three out of the four microcosms, the upper 0.4 mm was characterized by a strong signal of probe O_Mmb562, which is indicative for *Methylosarcina*. Also the largely redundant probes Mmb303 and Mmb304 (Supplement 2) gave consistently high signals, which suggested that the activity of *Methylosarcina* was indeed high in the surface zone (Supplement 3, microcosms 2–4).

To evaluate the potential role of sMMO, we sampled two to three replicate microcosms after 2, 4 and 6 weeks, respectively. All *mmoX* sequences retrieved could be affiliated with type II MOB of the genera *Methylosinus* and *Methylocystis* (Supplement 4). No transcripts were found, whereas a *Methycella*-specific assay even failed to produce products from DNA suggesting that this genus is missing (Supplement 4).

Discussion

Previous experiments with gradient microcosms have already shown that focusing on the active layer allows processes and interactions to be analyzed in unparalleled detail (Murase and Frenzel, 2007; Krause *et al.*, 2010). While only 3 mm thick, the soil layer in the microcosm was considerably thinner and allowed a stronger focus on the organisms of interest than in many other experiments (Dumont *et al.*, 2011, Siljanen *et al.*, 2011). Even working at a resolution of 1 cm dilutes the active layer with the microbial seed bank in the bulk soil and limits interpretability, regardless if major soil compartments are sampled separately (Eller *et al.*, 2005). Dividing the soil further into 100-μm-thick layers brought an unprecedented resolution that was sufficient not only to analyze the

Table 1 Average number of *pmoA* gene copies and transcripts in the three different depth zones defined in Figure 4

Depth zone (mm)	pmoA *copies*			pmoA *transcripts*		
		P-*value*			P-*value*	
	(10^7 g^{-1})	*Anoxic–oxic interface*	*Surface*	*(10^7 g^{-1})*	*Anoxic–oxic interface*	*Surface*
Surface (0–0.8)	4.6	<0.05	n.a.	1.3	<0.01	n.a.
Anoxic–oxic interface (0.8–2)	16.1	n.a.	<0.05	44.9	n.a.	<0.01
Anoxic (2–3)	1.5	<0.01	<0.005	2.8	<0.001	0.3

Abbreviation: n.a., not applicable.
P-values are for *t*-tests comparing averages per depth zone.

vertical position of different OTUs but also physiological differences within one OTU. While applied here to a laboratory system, this technique can also be adapted to retrieve real-time snapshots from sediment surfaces using freeze cores (Macumber *et al.*, 2011).

Using the *pmoA* gene as a functional and phylogenetic marker for MOB, we analyzed genes and transcripts at the sub-mm scale along a depth profile from the soil surface down into the anoxic zone. As predicted from oxygen microprofiles, methanotrophic activity was indeed located at the oxic–anoxic interface. Using *pmoA* transcripts as a proxy for species-specific activity, we correlated the transcript-to-gene ratio of specific methanotroph taxa to methane-driven respiration.

Transcripts of *mmoX*, a gene encoding for a subunit of sMMO found as a second monooxygenase in some MOB, could not be detected, even if the gene was found (Supplement 4). MOB of the genus *Methylocella* lack *pmoA*, but has *mmoX* (Dedysh, 2009). This genus was previously thought to be acidophilic, but could recently be detected in circum-neutral environments, too (Rahman *et al.*, 2011). However, it was undetectable in the paddy soil under study (Supplement 4). Hence, *pmoA* is a reliable functional and phylogenetic marker for this microcosm experiment.

Concentration profiles and activity

At the oxic–anoxic interface, oxygen is not only used for the direct mineralization of organic matter but also for the re-oxidation of end products from anaerobic processes (Brune *et al.*, 2000). In freshwater environments, methane is most important and may be the dominant oxidation substrate at the interface. Our microcosms were designed to model this situation, but can in principle be re-configured to focus on other redox processes or to study the interaction between different competing processes.

The 16 oxygen microprofiles measured (4 per microcosm) showed the same characteristics: a near-linear decrease in oxygen concentration down to a depth of about 1.3 mm, followed by a pronounced curvature before the concentration reached zero at about 1.8 mm depth (Figure 1a). The concomitant methane concentrations at the oxic–anoxic interface were most probably in the lower micromolar range (Gilbert and Frenzel, 1998). The methane sensors built for previous work (Rothfuss *et al.*, 1994; Gilbert and Frenzel 1998) do not achieve the sub-mm resolution required for this experiment. While further miniaturization is feasible, the detection limit would decrease proportionally, making a sensitive analysis impossible. Membrane-inlet mass spectrometry (Lloyd *et al.*, 1986; Lloyd *et al.*, 1996; Beckmann *et al.*, 2004) suffers from the same constraints, and the development of a microscale biosensor for methane (Damgaard and Revsbech, 1997) has been discontinued. Hence, interpretation has to rely on oxygen microprofiles alone.

We calculated oxygen consumption using Berg's program PROFILE ver. 1.0 (Berg *et al.*, 1998) and a sediment diffusion coefficient measured in this paddy soil (Rothfuss and Conrad, 1994; Noll *et al.*, 2005). The algorithm calculates the zone-specific respiration rate using Fick's second law (Figure 3). Assuming constant porosity, the respiration rate at the surface was modest, followed by an area where no significant respiration took place. High values contributing 89% of the total oxygen respiration were calculated for the zone above the interface (Figure 3). Assuming a stoichiometry of $CH_4{:}O_2 = 1{:}2$, the total respiration of 36.6 nmol $O_2\,cm^{-2}\,h^{-1}$ corresponded to a methane oxidation rate of 18.3 nmol $CH_4\,cm^{-2}\,h^{-1}$.

t-RFLP patterns and quantification

In community profiling studies of MOB, t-RFLP analysis targeting the *pmoA* gene has a long tradition (Horz *et al.*, 2001; Hoffmann *et al.*, 2002; Mohanty *et al.*, 2007). The results, however, depend on the choice of primer sets. Compared with the 682r reverse primer (Holmes *et al.*, 1995), the reverse primer mb661r (Costello and Lidstrom, 1999; Bourne *et al.*, 2001) covers methanotroph diversity, but not the homologous *amoA* gene encoding for a subunit of ammonium monooxygenase. Furthermore, primer mb661r seems to be superior for resolving type I diversity (Bourne *et al.*, 2001; Lüke *et al.*, 2010).

When we compared DNA- and RNA-based community profiles, we found striking differences between the extant (DNA-based) and active (RNA-based) populations (Figure 1). The extant population changed consistently with depth in all four microcosms studied (Figure 2). Virtually no transcripts of type II MOB were found, but a *Methylobacter*-like OTU (tRF 508) was most prominent around the oxic–anoxic interface (Figures 1b and c). Taking transcription as a proxy for activity, this dominance suggested a corresponding localization of *Methylobacter*-specific methane oxidation. This conclusion, however, depends largely on the high fraction of unspecific tRFs derived from ribosomal RNA. While this large fraction of false-positive tRFs suggested an extremely low content of *pmoA* mRNA compared with rRNA, it is only a tentative measure. We therefore adapted a cPCR assay (Han and Semrau, 2004) and used it to quantify the most prominent *Methylobacter*-like OTU (tRF 508; Figure 3).

cPCR has rarely been applied in microbial ecology (Han and Semrau, 2004), but it has certain advantages, in particular if combined with t-RFLP. t-RFLP alone gives only relative values. Provided adequate standards can be constructed, t-RFLP can easily be modified to retrieve truly quantitative data. In addition, RT-cPCR helps in overcoming the variability inherent to the RT step (Freeman *et al.*, 1999). In the particular case of *pmoA*, RT-cPCR helped in circumventing the problem with unspecific RT-PCR products; the latter had restriction sites that were different from those of the OTU of interest. Some limitations of t-RFLP still apply, for example, a limited phylogenetic resolution and the co-occurrence of particular tRFs in more than one phylotype. Hence, a decent sequence database is a must. However, cPCR combined with t-RFLP has the major advantage that not only quantification but also other tasks such as community analysis (t-RFLP, microarray) and sequencing can be based on the same assays, whereas covering different MOB types by qPCR requires different primer sets (Kolb *et al.*, 2003).

In the anoxic zone below 2 mm, the *pmoA* copy number was low and may correspond to the initial numbers present in the dry soil when the experiment was started. The slightly higher copy number in the top zone suggested that growth had taken place there at least for some time (Table 1). The maximum transcript:gene ratio coincided with the highest respiration rate, but was localized slightly deeper in the soil than the maximum *pmoA* copy number (Figure 3). If we consider two *pmoCAB* operons per cell (Semrau *et al.*, 1995), the *Methylobacter*-affiliated OTU had up to 18 transcripts per cell (Figure 3). The half-life of *pmoA* mRNA is unknown. The half-life of other mRNAs may be as short as 30 s, but could also be much longer depending on the environment and the growth state (Arraiano *et al.*, 2010; Steglich *et al.*, 2010). However, we are confident that we preserved the *in situ* mRNA content by shock freezing the soil with liquid nitrogen when still in the microcosm.

The soil surface in the microcosms was characterized by high oxygen (Figure 1) and low methane concentrations. Before we sampled the soil for molecular analysis, 318 ppm_v methane had accumulated in the headspace, which corresponds to 400 nM methane in the pore water near the soil surface. Hence, MOB living in the top layers of the microcosms were exposed to this or a slightly higher methane concentration immediately before shock freezing. However, the *Methylobacter*-affiliated OTU had less than one transcript per cell (Table 1), which suggested that this situation was rather unfavorable. In the anoxic zone, however, the *Methylobacter*-affiliated OTU had nearly four transcripts per cell (Table 1). Our microcosm design aimed at producing a planar system varying only with depth. Microelectrode measurements have a high spatial resolution (<0.1 mm, corresponding to ca. 0.53 nl), whereas the molecular data refer to the entire area of the microcosm (28.3 cm^2, 0.28 ml per layer). However, the 16 oxygen profiles were so reproducible that we can rule out spatial heterogeneities. Hence, factors other than locally deviating oxygen penetration depth must be responsible for the high transcript:gene ratio below the oxic–anoxic interface. The nitrite-reducing methanotroph, '*Candidatus* Methylomirabilis oxyfera' is able to generate O_2 from NO via dismutase (Ettwig *et al.*, 2010; Strous, 2011). However, we have no indication that *Methylobacter* possesses this trait, and nitrite was not detectable in another microcosm experiment with the same soil (Krause *et al.*, 2010). Recently developed microelectrodes (Revsbech *et al.*, 2009; Revsbech *et al.*, 2011) have demonstrated nanomolar oxygen concentrations in areas that have been considered anoxic so far. However, the design of our microcosms includes a trap to remove any oxygen that might have diffused into the lower compartment (Murase and Frenzel, 2007). Hence, oxygen may have been present in trace amounts in the methane-rich 'anoxic' zone below the oxic–anoxic interface, but diffusive transport of oxygen to MOB must have been negligible, if it occurred at all. Therefore, the mRNA:DNA ratio points to a differentiated physiological status of the cells depending on depth and a surprisingly high number of transcripts in the anoxic zone.

Microarray analyses

Although the microarray analysis leads to essentially the same conclusions as t-RFLP analysis, the higher phylogenetic resolution of the microarrays may allow differentiation down to the species level (Stralis-Pavese *et al.*, 2011). First developed for DNA-based analyses, the microarray has been successfully applied to study *pmoA* transcripts (Bodrossy *et al.*, 2006; Chen *et al.*, 2007; Krause *et al.*, 2010). Here we used it to refine the transcript

analysis (Figure 4, Supplement 3). The microarray design follows a multiple-probes approach. This has significant advantages and may help in detecting groups not yet covered by a specific probe by another, more conserved and general probe. A certain degree of redundancy also helps to exclude false-positive hybridization signals, but may introduce a bias in ordination analyses if a particular phylotype hybridizes with more than one probe. Hence, we used only mutually exclusive probes with a well-defined phylogenetic coverage for ordination analysis. These probes and a few others showing obvious spatial patterns are listed in Supplement 2. The ordination (non-metric multidimensional scaling, Figure 4) gave a clear separation between the surface, oxic–anoxic interface and bottom zones.

The only signal that could be associated with type II MOB was most probably false positive, as the respective probe hybridizes also with different rice paddy clusters (Lüke *et al.*, 2010) belonging to type I (Supplement 3); the general probes for type II gave no signal. Type II MOB have been described as more abundant than type I but as contributing to methane oxidation mainly under high-methane concentrations, whereas type I may thrive if the methane source strength decreases (Henckel *et al.*, 2000). This, however, may be a misconception: even at a high source strength, that is, high-methane production rates, methanotrophic activity shapes counter-gradients, resulting in a steady state with concomitantly low oxygen and methane concentrations at the oxic–anoxic interface. Thus, not methane concentration but rather the energy flow through a population may be the decisive factor (Krause *et al.*, 2012). Type II MOB may occasionally become active in such a situation, but the controlling factor(s) are unknown (Krause *et al.*, 2010). However, extinguishing 97.5% of all microbiota stimulates the exponential growth of type II MOB, which eventually become by far the dominant group (Ho *et al.*, 2011). Hence, thanks to their robust resting stages (Whittenbury *et al.*, 1970a, b), type II MOB may apply a sit-and-wait strategy, taking advantage of changing situations that may be a catastrophe for others.

Type I MOB may have benefited from the rather constant conditions in the microcosms. Probes indicative for *Methylobacter* gave positive signals in the oxic–anoxic interface and the anoxic zone (Supplement 3), but much less so in the surface zone. This corresponds to the quantification with cPCR/t-RFLP (Figure 3), which revealed a higher mRNA:DNA ratio in the anoxic zone than in the surface zone. The role of type II MOB and *Methylobacter* is surprisingly similar to that found in a seasonal study on an alpine meadow (Abell *et al.*, 2009), in which type II MOB remained largely unaffected by season and environment but nevertheless represented the dominant MOB. *Methylobacter*-related MOB, however, were found to be responsible for the majority of methane oxidation.

Probes indicative for *Methylosarcina* gave significant signals in the surface zone (Supplement 3), which suggested activity at high oxygen concentrations (245–132 μM) and low methane concentrations (ca. 400 nM). This is much higher than the atmospheric methane concentration (1.8 ppm_v, corresponding to 2.3 nM), but cultures of and sequences affiliated to *Methylosarcina* have so far only been retrieved from high-methane environments, such as lake sediments, rice paddies and landfills (Wise *et al.*, 2001; Kalyuzhnaya *et al.*, 2005; Henneberger *et al.*, 2011; Lüke and Frenzel 2011). Apparent K_m constants in environments with high source strength are usually $>1\,\mu M\,CH_4$ (Conrad, 1996), but some cultivated MOB, in particular *Methylocystis* strains, may grow for an extended period at 120 nM CH_4 and less (Knief and Dunfield, 2005). For *Methylosarcina*-like MOB, however, activity at low methane concentrations has not yet been reported.

Conclusions

As shown before, only a restricted subset of a diverse methanotroph community was active, and most activity was confined to a zone 0.67-mm thick. However, our experiment showed how different OTUs within a single guild can share the same microenvironment, thereby exploiting different niches. We hypothesized that activity would be highest immediately at the oxic–anoxic interface separating MOB according to substrate availability. This was indeed the case, with a *Methylobacter*-affiliated OTU and dominating overall methane oxidation located at the oxic–anoxic interface. This OTU seems to be well adapted to the oxic–anoxic interface, where oxygen and, presumably, methane concentrations are in the micromolar range. This OTU was not active at the surface with its high oxygen but only nanomolar methane concentrations. In contrast, transcripts of a *Methylosarcina*-affiliated OTU were associated with this surface layer, which suggested an adaptation to oligotrophic conditions.

Conflict of interest

The authors declare no conflict of interest.

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