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ORIGINAL ARTICLE

Microbial minorities modulate methane consumption through niche partitioning

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Microbes catalyze all major geochemical cycles on earth. However, the role of microbial traits and community composition in biogeochemical cycles is still poorly understood mainly due to the inability to assess the community members that are actually performing biogeochemical conversions in complex environmental samples. Here we applied a polyphasic approach to assess the role of microbial community composition in modulating methane emission from a riparian floodplain. We show that the dynamics and intensity of methane consumption in riparian wetlands coincide with relative abundance and activity of specific subgroups of methane-oxidizing bacteria (MOB), which can be considered as a minor component of the microbial community in this ecosystem. Microarray-based community composition analyses demonstrated linear relationships of MOB diversity parameters and *in vitro* methane consumption. Incubations using intact cores in combination with stable isotope labeling of lipids and proteins corroborated the correlative evidence from *in vitro* incubations demonstrating γ-proteobacterial MOB subgroups to be responsible for methane oxidation. The results obtained within the riparian flooding gradient collectively demonstrate that niche partitioning of MOB within a community comprised of a very limited amount of active species modulates methane consumption and emission from this wetland. The implications of the results obtained for biodiversity–ecosystem functioning are discussed with special reference to the role of spatial and temporal heterogeneity and functional redundancy.
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Introduction

Biodiversity is generally regarded as a stabilizing parameter in the sustainable functioning of our ecosystems (Cardinale *et al.,* 2006, 2012). The current loss of biodiversity (Mora *et al.,* 2011) has evoked numerous studies focusing on biodiversity and ecosystem functioning (BEF) relationships in eukaryotic communities (Hillebrand and Matthiessen, 2009; Loreau, 2010; Cardinale *et al.,* 2012). However, microorganisms (i.e., bacteria, archaea, protozoa, fungi and algae), comprising a major part of the total biomass of organisms inhabiting earth (Whitman *et al.,* 1998) and representing the largest source of biodiversity (Gans *et al.,* 2005), are absent in the ongoing BEF debates and conservation policy, despite various pleas to do so (Cockell and Jones, 2009; Griffith, 2012). Moreover, microorganisms play critical roles in natural biogeochemical processes (Falkowski *et al.,* 2008) as well as in engineered or managed systems like wastewater treatment plants, agricultural fields and industrial bioreactors providing many services to our societies. A number of gaps in the knowledge of environmental microbial communities are fundamental to the absence of microbes on the global biodiversity conservation agenda (Bodelier, 2011), the inability to link microbial species to the processes they catalyze as well as the assumed high functional redundancy in microbial communities being the most crucial ones (Bodelier, 2011). These

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issues may be the reason why links between microbial diversity and ecosystem processes have been observed only for processes carried out by narrow groups of microbes carrying out a specific process (e.g., chitin and cellulose degradation) (Wohl *et al.,* 2004; Peter *et al.,* 2011) in diversity manipulation or artificial community experiments. The often-observed saturating species–function curves in these experiments, interpreted as functional redundancy, may be caused by including species not actively contributing to function. For microbial communities in soils, it has been demonstrated that up to 80% of all species present are inactive or in a dormant state (Lennon and Jones, 2011). Another explanation may be a lack of niches for species to be active due to the absence of spatial and temporal environmental heterogeneity in highly reductionist microbial BEF experiments. In fact, the lack of environmental heterogeneity and thus of suitable niches has been put forward as one of the crucial points to address in future BEF experiments in ecology in general (Cardinale *et al.,* 2012).

The best available approach to link microbial species to substrate consumption (i.e., analog for microbial behavior) in complex environmental settings is the tracking of isotopic labels into compounds of taxonomic relevance (e.g., polar lipid-derived fatty acids (PLFAs), RNA, DNA and proteins). In that respect, aerobic methane-oxidizing bacteria (MOB) are a group of environmentally highly relevant microbes that form an ideal model system for performing microbial BEF experiments. MOB have a very restricted substrate range generating their energy and providing in their biomass carbon largely by the utilization of CH_4 (Semrau *et al.,* 2010; Stein *et al.*, 2012). Next to this, the special C1 metabolism distinguishes MOB from the activity of other bacteria, which in combination with the possession of unique PLFAs (Bodelier *et al.,* 2009) facilitates the use of stable isotope labeling approaches (Boschker *et al.,* 1998; Bodelier *et al.*, 2012) to capture the active MOB in the environment. The molecular detection of MOB is facilitated by the use of a functional marker gene (particulate methane monooxygenase (*pmoA*)) that is strongly linked to the phylogeny of aerobic proteobacterial MOB (Holmes *et al.,* 1995; Kolb *et al.,* 2003; Lüke and Frenzel, 2011). The phylogeny of the methane-oxidizing guild has been changed substantially in the last decade. The classical canonical assignment of MOB subgroups (i.e., types I, X and II), which was based on physiology, biochemistry, morphology and phylogeny, does not reflect the current situation any longer considering the discovery of facultative methane oxidizers (Dedysh *et al.,* 2005; Belova *et al.,* 2011) (Semrau *et al.,* 2011), Verrucomicrobial methanotrophs (Dunfield *et al.,* 2007; Pol *et al.,* 2007; Op den Camp *et al.,* 2009) and 'aerobic methanotrophs' generating their own oxygen by nitrite reduction (Ettwig *et al.,* 2010) belonging to the NC10 phylum. Hence, currently it is more useful to refer to MOB subgroups based on phylogenetic assignments exclusively (Op den Camp *et al.,* 2009). The well-studied γ- and α-proteobacterial MOB harbor the families of the *Methylococcaceae* (γ-*Proteobacteria*), the *Methylocystaceae* and *Beijerinckiaceae* both belonging to the α-*Proteobacteria.* Within these families, a large number of classified genera and species have been described that, on the basis of phylogenetic assignment, are referred to as types Ia (*Methylomonas, Methylomicrobium, Methylobacter, Methylosarcina, Methylosoma, Methylovulum, Methylosphaera, Crenothrix* and *Clonothrix*), Ib (*Methylococcus, Methylocaldum, Methylogaea, Methylohalobius* and *Methylothermus*) and II (*Methylocystis, Methylosinus, Methylocella, Methylocapsa* and *Methyloferula*) (Lüke and Frenzel, 2011; Stein *et al.*, 2012). The application of molecular tools (McDonald *et al.,* 2008) based on the *pmoA* gene in combination with stable isotope probing of PLFA (SIP-PLFA) and/or DNA/RNA has rendered type Ia, Ib and II MOB as the best-studied group of environmental microbes, even allowing for assigning of life-history strategies to this group of bacteria (Ho *et al.,* 2013).

Aerobic MOB constitute the largest methane sink in terrestrial habitats, especially thriving at oxic–anoxic interfaces in wetlands (e.g., rice paddies, peat lands and floodplains) and surfaces of lake sediments, thereby directly contributing to the regulation of earth's climate through modulation of atmospheric methane concentrations (Conrad, 2007; Semrau *et al.,* 2010). Methane emission estimates from wetlands are characterized by high uncertainty (Bloom *et al.,* 2010). Recent variation in atmospheric methane concentrations, including the resumed increase from 2007 onwards, has been linked to changes in wetlands (e.g., in surface area, fertilizer use in rice cultivation and microbial processes) (Bousquet *et al.,* 2006; Kai *et al.,* 2011; Spahni *et al.,* 2011). Especially, wet mineral soils (e.g., riparian floodplains), which can serve as a sink as well as a source of methane depending on the hydrological conditions, are proposed to contribute to the 2007 increase (Spahni *et al.,* 2011). The question is to what extent the diversity of MOB communities and the traits of the species therein modulate methane consumption, thereby contributing to variability in methane emission from wetland habitats.

In this study, we investigate the response of aerobic MOB in an irregularly flooded wetland and assess the role of diversity of MOB communities in the modulation of methane consumption in these soils. A combination of molecular biological tools (McDonald *et al.,* 2008) and stable isotope labeling of lipids (Boschker *et al.,* 1998; Neufeld *et al.,* 2007) and proteins (Seifert *et al.,* 2012) is applied to exploit the characteristics of aerobic MOB to serve as a model group in microbial BEF studies in the most optimal way. More specifically, we want to investigate the role of available niches on the diversity–functioning relationship by assessing the

active MOB community members in a spatial and temporal investigation carried out in a riparian floodplain that is characterized by a natural gradient of hydrological conditions providing a variable amount of niches for MOB.

Materials and methods

Sampling site

The sampling site was located at Ewijkse Waard (51°88′ N, 5°73′ E), a riparian floodplain along the river Rhine in the Netherlands. The soil properties have been described previously in detail (Kemnitz *et al.,* 2004; Steenbergh *et al.,* 2010). The sampling site is located on the embankment of a small oxbow lake that is connected to the river on one side but is separated from the river by an outflow barrier, ensuring that the oxbow lake does not run dry completely. This outflow barrier results in slow retreat of water from the sampling plot after flooding, which results in a gradient of flooding days within the plot. The highest part is flooded up to 2 weeks per year, whereas the lowest part can be flooded up to 150 days per year. The flooding occurs very irregularly when the water level of the Rhine reaches a threshold level upon which the oxbow lake fills up with water (Supplementary Figure S1). A 10 × 10 m plot was established situated on a slope on the embankment of a small oxbow lake, resulting in an elevation difference of 1.1 m between the lowest and highest point of the plot (Figure 1) (Wang *et al.,* 2012).

Soil methane emission

Methane emissions were measured in the field in May, July and October 2007 in vented closed flux chambers using a photoacoustic infrared gas analyzer with a multisampler (Bruel and Kjaer, Naerum, Denmark). The flux chambers had an inner diameter of 15.2 cm and a height of 24.2 cm and were attached to preinstalled frames to minimize disturbance of the soil during measurement. Within each flux chamber, at least five gas samples were taken over a measurement period of 1 h.

Sample collection for in vitro experiments

In September 2006 and May, July and October 2007, five soil cores (7 cm in diameter and 40 cm in length), evenly distributed over the diagonal of the sampling plot, were collected (Figure 1) and transported to the laboratory for further analyses the next day. The soil cores are subsectioned into three layers (0–2, 7–9 and 27–29 cm) and sieved (2 mm) and mixed before further processing. The choice for these layers was based on pilot experiments where the most active methane-oxidizing horizons were determined using methane oxidation assays as described below (data not shown).

Soil physicochemistry

For the samples collected in September 2006, a range of soil physico chemical parameters were measured (i.e., moisture content, water-filled pore space (WFPS), pH (water and KCl), organic matter, total N, total P, granulometric composition, available cations (NH_4^+, Mg^{2+}, Ca^{2+}) and anions by $CaCl_2$ extraction). These parameters were analyzed as described earlier (Bodelier *et al.*, 2012). In the other sampling months, only moisture, WFPS, pH (water and KCl), organic matter and mineral N (water and 2 M KCl, i.e., NH_4^+ and NO_3^-) availability were measured.

Methane oxidation activity and kinetics

Methane oxidation activity was executed in soil slurries as described by Steenbergh *et al.* (2010) using a headspace methane concentration of 10 000 p.p.m.v. (parts per million volume). This concentration proved to be saturating the methane-oxidizing enzymes in the floodplain samples (data not shown). In short, for each sample, 5 g of the sieved and mixed soil was suspended in 10 ml

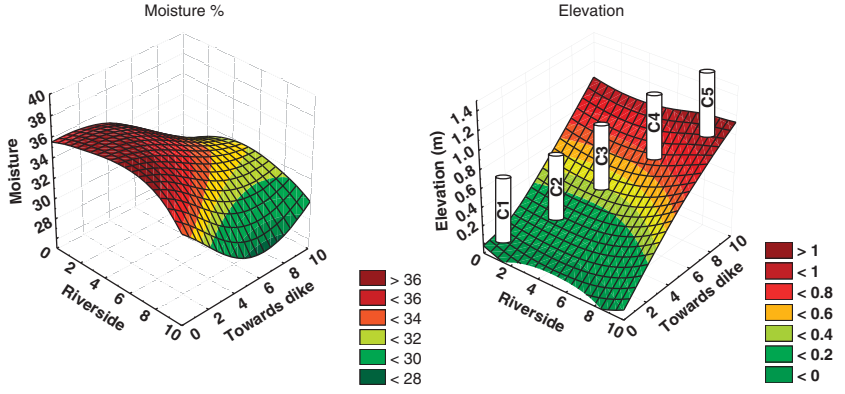

Figure 1 Information on sampling design and moisture content in the experimental plot. Gravimetric water content (left panel) as function of the location in the plot. The right-hand panel depicts the elevation differences with the 10 × 10 m experimental plot. The cylinders (C1–C5) depict the spatial arrangement of the cores taken monthly. Samples for moisture content were taken in November 2006 in 73 samples collected within the plot.

of Milli-Q water (Milli-Q Reagent Water System, Millipore, Billerica, MA, USA) in a 150-ml flask capped with a rubber stopper. A portion of 1.4 ml pure CH_4 was added to obtain a CH_4-mixing ratio of approximately 10 000 p.p.m.v. The CH_4 concentration in the headspace was monitored during the PMO assays by gas chromatography flame ionization detector (GC-FID) analysis (HP 5890 Gas Chromatograph, Hewlett-Packard, Wilmington, DE, USA). To assess methane oxidation kinetics of the methane-consuming communities in the floodplain samples, identical slurry incubations as above were carried out using seven different initial methane concentrations (100, 300, 600, 1000, 5000, 10 000 and 20 000 p.p.m.v.). Methane consumption rates were calculated based on headspace samples taken during 24 h of incubation. Apparent K_m and V_{max} were determined using Monod kinetics calculated by applying nonlinear fitting routine included in Sigmaplot 12.3 (Sigmaplot, Systat Software Inc., San Jose, CA, USA). Specific methane oxidation affinity was calculated as V_{max}/K_m.

Methane production activity
Potential methane production rates were determined as described earlier (Bodelier *et al.,* 2000) following methane production in anaerobic slurry incubations using 10 g of fresh soil in 20 ml of Milli-Q water. Headspace methane measurements in all laboratory assays were measured by GC-FID analyses (HP 5890 Gas Chromatograph, Hewlett-Packard).

DNA and RNA extraction
DNA was extracted as described by Pan *et al.* (2010), based on the FastDNA spin kit for soil (MP Biomedicals LLC, Solon, OH, USA). RNA was extracted as described by Steenbergh *et al.* (2010). RNA extraction was executed for the samples collected in 2007 only. Quality and quantity of DNA and RNA were determined spectrophotometrically using the NanoDrop ND-1000 (Nanodrop Technology, Wilmington, DE, USA).

PCR amplification and reverse transcription
Amplicons of the particulate methane monooxygenase gene (*pmoA*) were produced by a two-step PCR protocol using the primer pairs A189–A682 followed by A189–Mb661 following the conditions as described by Pan *et al.* (2010). mRNA was reverse transcribed and cDNA was produced as described by Steenbergh *et al.* (2010) using the Super-Script III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA).

DNA- and mRNA-based microarray analyses
Diversity analyses of the methanotrophic community were executed using a diagnostic microarray approach based on the *pmoA* gene as described earlier (Pan *et al.,* 2010). For every sample, three independent PCRs were carried out and pooled prior to the *in vitro* transcription step in the microarray procedure. The normalized signal intensities derived from the scanned microarrays of only the non-redundant probes were used to infer diversity parameters (i.e., Shannon index (log e based), evenness) using the 'Diverse Routine' implemented in the Primer-E software (Plymouth Marine Laboratory, Plymouth, UK).

Quantitative PCR analyses of methanotrophic bacteria
Gene copy numbers of three subgroups of methanotrophic bacteria (types Ia, Ib and II) (Supplementary Figure S3) were determined using quantitative PCR (qPCR) of the *pmoA* gene as described earlier (Pan *et al.,* 2010).

Clone library construction and phylogenetic analyses
Clones were generated from PCR products using a seminested approach (primers A189–A682 followed by A189–mb661) (clones assigned WP1AN, WP1BN). Cloning was performed using the TAs cloning kit (Invitrogen), using a mix of three replicate PCR products for ligation. Of both libraries (WP1AN and WP1BN), 75 clones carrying the insert were randomly selected for sequencing. Clones were sequenced using an ABI 3130 capillary sequencer (Applied Biosystems, Foster city, CA, USA). Clone sequences were compared with the sequences available in public databases, using the BLAST software from the National Center for Biotechnology Information (available at http://www.ncbi.nlm.nih.gov/BLAST/), to determine their phylogenetically closest relatives. *pmoA* sequences have been submitted in the GenBank database under accession numbers KF256139–KF256263.

Intact cores: 'in situ' methane consumption
In July 2009, triplicate soil cores were collected (1.8 cm in diameter and 5 cm in length) at the same five elevation levels within the 10 × 10 m plot as were sampled for the '*in vitro*' experiments. The cores were transported to the laboratory and incubated intact in the same bottles as used for slurry incubations (see the Methane oxidation activity and kinetics section). In these static incubations, methane was added amounting to 10 000 p.p.m.v. headspace concentration. Methane decrease in the headspace was monitored by GC-FID as described by Steenbergh *et al.* (2010) to determined actual methane consumption by intact cores. For SIP-PLFAs and stable isotope probing of proteins (protein-SIP) (see below), three times 10 000 p.p.m.v. (i.e., 4.2 ml) methane was consumed before stopping incubation. In case of SIP-PLFA, 30 µl 99% ^{13}C-CH_4 (Campro Scientific BV, Veenendaal, The Netherlands) was

added to increase the signature of the methane-C to approximately 1400‰. In case of protein-SIP, 70% of the methane in the headspace (7000 p.p.m.v.) was ^{13}C-CH_4. For both SIP-PLFA and protein-SIP, five unlabeled control cores were analyzed. Soil cores were freeze dried before extraction of lipids and proteins.

SIP-PLFA

PLFAs were extracted and analyzed as described by Mohanty *et al.* (2006) using 4 g of freeze-dried soil with a Bligh and Dyer extraction procedure. Compound-specific gas chromatography isotope ratio mass spectrometry (MS) was applied as described by Mohanty *et al.* (2006) to determine ^{13}C incorporation into individual PLFA. Labeling profiles of soil samples were compared with those of a PLFA database (Mohanty *et al.,* 2006; Bodelier *et al.,* 2009) to determine the active methanotrophic species in these samples.

Protein-SIP

Protein Extraction Protocol and In-Solution Digestion. Two protein extraction protocols were used. First, 2.5 g of soil sample was used for the protocol described by Benndorf *et al.* (2007), which uses an NaOH extraction step followed by a phenol extraction and an ammonium acetate precipitation. The extracted proteins from the water and inter phase as well as from the phenol phase were operated separately in the following steps. The proteins were washed with an acetone and a subsequent ethanol step and suspended in Tris buffer. An in-solution digestion procedure was applied to all protein fractions. Either all or at least 30 μg of the protein extracts were incubated with 8 M urea, reduced with DTT and alkylated with iodoacetamide. Trypsin was added in a ratio of 1:100, and the digest was incubated overnight at 37 °C. The peptide mixture was desalted using C_{18}-Zip Tips columns. A second protocol using an SDS buffer extraction was processed (Chourey *et al.,* 2010). In this protocol, 5 g of soil was resuspended in a 2% SDS buffer and boiled for 20 min. The proteins in the supernatant were precipitated with 20% TCA overnight. After washing the protein pellet with acetone, the proteins were denatured using guanidine-HCl + DTT. The ammonium bicarbonate-buffered protein extract was then directly used for an in-solution digestion with trypsin overnight. The peptide mixture was desalted using C_{18}-Zip Tips columns.

Mass Spectrometric Analyses. Peptides were reconstituted in 0.1% formic acid and concentrated on a trapping column (nanoAcquity UPLC column, C_{18}, 180 μm × 2 cm, 5 μm, Waters, Eschborn, Germany) with water containing 0.1% formic acid at flow rates of 15 μl min^{-1}. The complex peptide mixture was fractionated using a nanoAcquity UPLC (Waters) equipped with a C_{18} column (75 μm × 250 mm, 1.7 μm). Chromatography was performed at a flow rate of 0.3 μl min^{-1} by using 0.1% formic acid in solvents A (100% water) and B (100% acetonitrile), with peptides eluted over 147 min (samples phenol extraction) or 90 min (samples SDS extraction) with a 8–40% solvent B gradient using a nano-HPLC system (nanoAcquity, Waters) coupled to an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Schwerte, Germany). Continuous scanning of eluted peptide ions was carried out between 300 and 2000 m z^{-1} (samples phenol extraction) or 400 and 1400 m z^{-1} (samples SDS extraction), automatically switching to an MS/MS CID mode on ions exceeding an intensity of 2000 (samples phenol extraction) or 3000 (samples SDS extraction).

MS raw data were processed for database search using Thermo Proteome Discoverer software (version 1.2.0.208, Thermo Fisher Scientific). Search was performed by tandem MS ion search algorithms from the Mascot house server (version 2.3.01) (Perkins *et al.,* 1999). The following parameters were selected: taxonomy ID 403 (*Methylococcaceae*), 206350 (*Methylophilales*), 31993 (*Methylocystaceae*), 120652 (*Methylocella*), 184923 (*Methylocapsa*) 82115 (*Rhizobiaceae*) and 74201 (*Verrucomicrobia*) of NCBI nr (National Center for Biotechnology Information, Rockville Pike, Bethesda, MD, USA; state time 6 January 2013) as criteria for taxonomy, tryptic cleavage, maximum two missed cleavage sites. A peptide tolerance threshold of ±10 p.p.m. and an MS/MS tolerance threshold of ±0.8 Da were chosen. Carbamidomethylation at cysteines was given as static and oxidation of methionines as variable modification. Peptides were considered to be identified by Mascot when a false-positive probability <0.05 (probability-based ion scores threshold >40) was achieved. The level of ^{13}C incorporation was calculated as described by Taubert *et al.* (2011) using the Excel spreadsheet (Taubert *et al.,* 2011).

Results and discussion

The methane flux measurements from the studied floodplain were highly variable, with negative (methane uptake) as well as positive (methane emission) values well within the normal range for this type of ecosystem (Supplementary Figure S2). Methane consumption (initial rates as well as V_{max}) displayed a distinct distribution with highest rates in between the low (prolonged wet) and the high (predominantly dry) ends of the flooding gradient (Figures 2a and b). This is the opposite trend compared with the emitted methane flux (Supplementary Figure S2), suggesting that the MOB community is capable of scavenging all methane produced at the intermediate elevation levels.

MOB genera and species, belonging to the lineage of the *Proteobacteria* (α- and γ- *Proteobacteria*

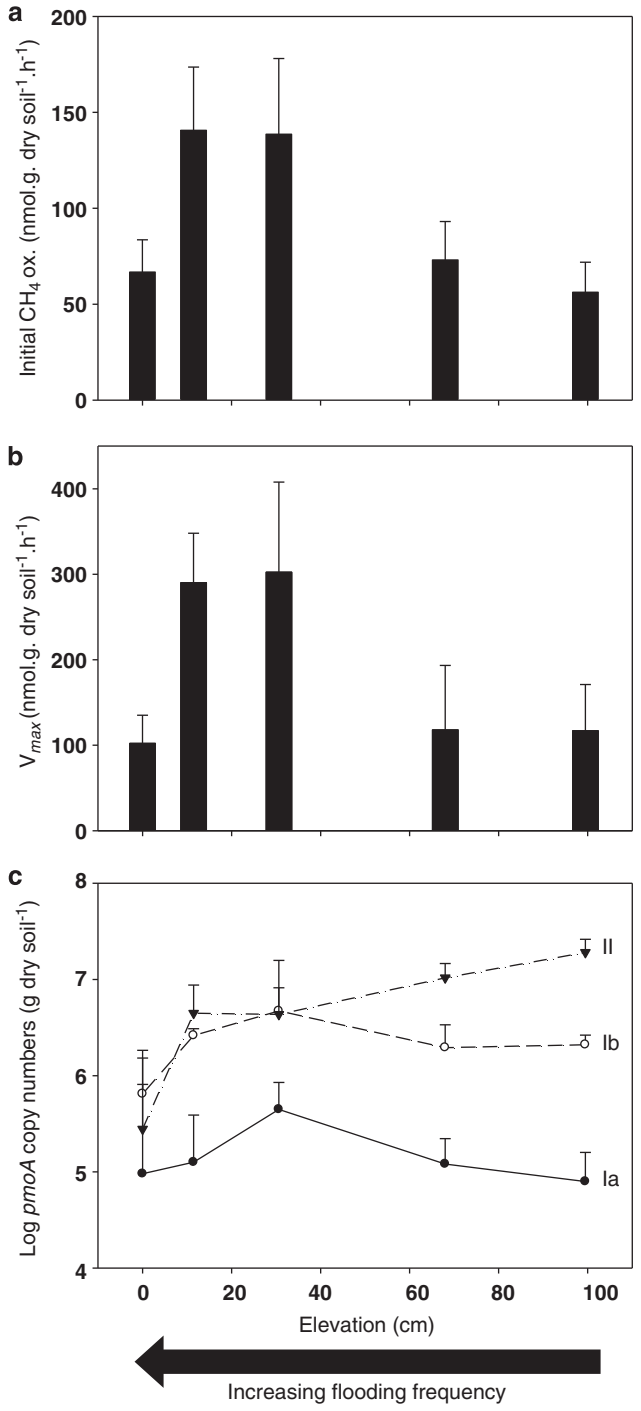

Figure 2 (**a**) *In vitro* methane consumption, (**b**) maximum methane oxidation capacity (V_{max}) and (**c**) *pmoA* gene copy numbers of type Ia (closed circles), type Ib (open circles) and type II (closed triangles) MOB in the surface 0–2 cm of soil collected at different elevations in a riparian floodplain. Values are seasonal averages of four sampling months plus standard deviation.

subdivision), are divided in type Ia, Ib and II subgroups (see also Supplementary Figure S3) based on *pmoA* phylogeny, which is largely congruent with the phylogeny based on 16S rRNA (Lüke and Frenzel, 2011). qPCR assays targeting these subgroups individually in the 0–2 cm layer of the soil revealed clear differentiated distributions, with type II increasing in number with increasing elevation with respect to the lowest point of the plot (Figure 2c). In contrast, numbers of types Ia and Ib reached highest values in the mid-elevation levels, reflecting the overall pattern of methane consumption (Figure 2). Initial methane consumption activity as well as specific affinity (i.e., specific affinity for methane defined as V_{max}/K_m) was highest at elevations of 0.1–0.3 m corresponding to moisture content of the soil between 35% and 40% (Supplementary Figure S4), indicating the niche boundaries for highest methane consumption in this soil. qPCR results showed that type II MOB are numerically more abundant than type Ia at all elevations and than type Ib at elevations >0.3 m (Figure 2c). Clone libraries obtained from samples from both ends of the flooding gradient confirmed the dominance of type II (Supplementary Figure S3).The orders of magnitude difference between types II and Ia in the floodplain in this study was in line with parallel studies on the same site (Steenbergh *et al.,* 2010; Wang *et al.,* 2012), confirming the observed relative abundances.

MOB community composition (i.e., Shannon diversity index) as determined by *pmoA*-based microarray analyses was positively correlated with methane consumption when considering the data obtained from all sampling months (Figure 3a). One has to consider that the Shannon index is applied using microarray probes as 'species' analogs. In reality, however, the highest resolving probes still cover multiple species and sequence types. Hence, the Shannon index in this study reflects presence and relative abundance of taxonomical units and cannot be used to compare diversity with other prokaryotic or eukaryotic communities. The observed correlation, however, only occurred in the upper soil layer (0–2 cm) (Figure 3a) and not in the lower layers (7–9 and 27–29 cm) (Figures 3c and e). The short-term *in vitro* methane oxidation assays are an analog for the potential active community at the time of sampling (Steenbergh *et al.,* 2010). The congruence between abundance of specific MOB subgroups, community composition and activity in the 0–2 cm layer, exclusively, suggests that these subgroups contribute predominantly to methane oxidation in the soil itself.

The observed correlation between diversity index and methane consumption was determined by the evenness of the MOB community and, hence, changes in relative abundance of the active species in line with change in activity (Figure 3). Richness was not related with methane oxidation activity (linear regression: $R^2 = -0.02$, $F = 0.58$, $P < 0.46$). Other activity components were also positively correlated with diversity index. Specific affinity was correlated with Shannon index on DNA basis and V_{max} on RNA basis (Supplementary Table S1). Dynamics of signals of individual microarray probes

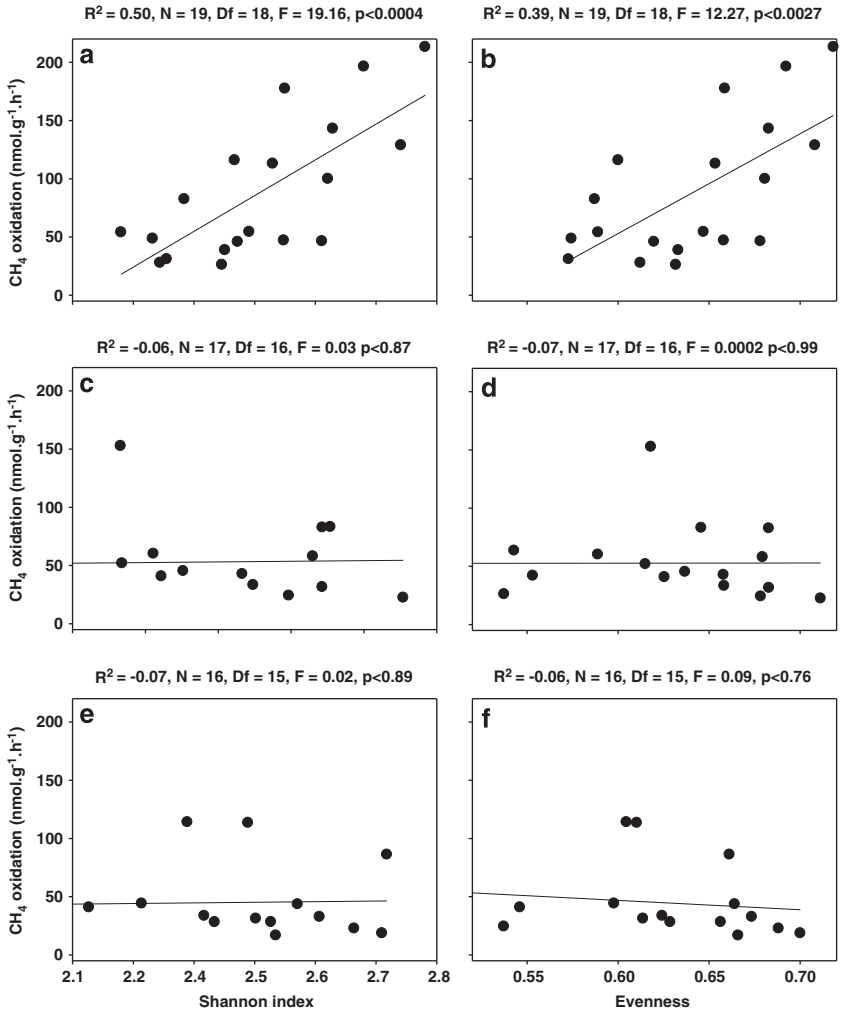

Figure 3 Relationship between *in vitro* methane consumption and diversity components (Shannon index and evenness) of the methane-oxidizing community in floodplain soil samples collected in September 2006 and May, July and October 2007. Diversity of the MOB community was assessed using a *pmoA*-based microarray. Data points represent the results from five soil cores taken per month, which were subsectioned in (**a** and **b**) 0–2 cm, (**c** and **d**) 7–9 cm and (**e** and **f**) 27–29 cm. Equations above panels are linear regression results (d.f. = degrees of freedom, F = ANOVA F-value; *P* = significance level of the regression).

revealed that only type Ia- and Ib-related general and species-specific probes correlated to methane measures of methane-oxidizing activity (i.e., initial consumption, V_{max} and specific affinity), whereas type II probes did not (Supplementary Figure S5 and Supplementary Table S1), suggesting that changes in types Ia and Ib to be responsible for the change in evenness of the community. qPCR data also demonstrated strong correlations between abundance of type Ib and both initial methane oxidation and V_{max}, whereas numbers of type Ia are correlated with specific affinity (Supplementary Table S1). A principal component biplot of the most relevant components analyzed in this study also clearly demonstrates the exclusive association of type I MOB with all relevant methane consumption parameters, whereas the variation found in type II MOB-related parameters, with *Methylocystis* species in particular, correlates with the elevation (Supplementary Figure S6). The relationship between mRNA transcript numbers, which can be used as a proxy for enzymatic activity (Freitag *et al.,* 2010), also contributes to the line of correlative evidence for type I MOB as being the active species in this floodplain (Supplementary Figure S7).

What has to be kept in mind with respect to the results described above is the limitations of the *pmoA*-based methods we have used in this study. The genera containing only soluble methane monooxygenase (i.e., *Methylocella* and *Methyloferula*) as well as the MOB belonging to the *Verrucomicrobia* and the NC10 cluster cannot be detected. More recent described genera (*Methylovulum*, *Methylogae*, *Methylosoma*, *Crenothrix* and *Clonothrix*) are not included in the version of the microarray used. The genus *Methylocapsa* is not detected by the type II qPCR assay but is present on the microarray. Although this genus was detected by micro array probes, the relative abundance did not correlate with activity components but only with elevation,

similar to other type II MOB (data not shown). Hence, we cannot exclude that the observed methane consumption is carried out partly by non-detected MOB. However, this does not alter the relationships between abundance, community composition and activity of the genera we could reliably detect.

To gather more direct evidence for the active species and to exclude the possibility that the correlations between *in vitro* methane oxidation and type Ia and Ib MOB, as observed above, are the result of a negative bias against type II MOB caused by the slurry and shaking procedure of the *in vitro* assays, we used an SIP approach on intact soil cores. These cores were collected in the field in July 2009 and incubated intact without shaking in an atmosphere spiked with ^{13}C-CH$_4$, which can be traced in taxonomically relevant compounds (e.g., PLFAs and proteins). The intact-core methane consumption pattern revealed higher methane consumption in the lower parts of the flooding gradient, without a distinct optimum (Supplementary Figure S8). This could be related to the long period without flooding preceding the sampling (Supplementary Figure S1). Stable isotope labeling of PLFAs clearly showed that the majority of the label ends up in C16 PLFA, which is known to be mainly associated with type I MOB (Bodelier *et al.,* 2009) (Figure 4). Using multivariate ordination techniques, the labeling pattern, as displayed in Figure 4, can be compared with PLFA profiles of cultivated species (Mohanty *et al.,* 2006; Bodelier *et al.*, 2012), which clearly shows that a combination of both type Ia- and Ib-related MOB is actually consuming the methane in these cores (Figure 5). The labeling of the MOB-specific PLFA (i.e., not present in other microbes; C16:1ω5t and C16:1ω8c) is direct evidence for the methane consumption by representatives of the genera *Methylomonas/Methylomicrobium/Methylobacter/Methylosarcina* (type Ia) (Bodelier *et al.,* 2009), which were also linked to methane consumption *in vitro* by microarray analyses (Supplementary Figures S5 and S6 and Supplementary Table S1). The PLFA C16:1ω8c is also present in two type II strains, isolated from acidic habitats (Dedysh *et al.,* 2007); however, significant incorporation by these species would be mirrored by an increase of labeling of the PLFA C18:1ω8c, which they also posses in large amount. In our samples, this did not occur, strengthening the conclusion that type Ia MOB are responsible for incorporation of label in C16:1ω8c.

However, PLFA analysis also gives evidence for methane consumption by *Methylocaldum* species (type Ib) based on a high label signal in C16:0 and C16:1ω7c relative to the aforementioned PLFAs. These type Ib MOB were detected by cloning, but *Methylocaldum*-specific microarray probe signals did not correlate with methane consumption. Considering the fact that the used probes cover the clone sequences detected, this suggests the presence of yet uncultivated MOB with a similar PLFA as

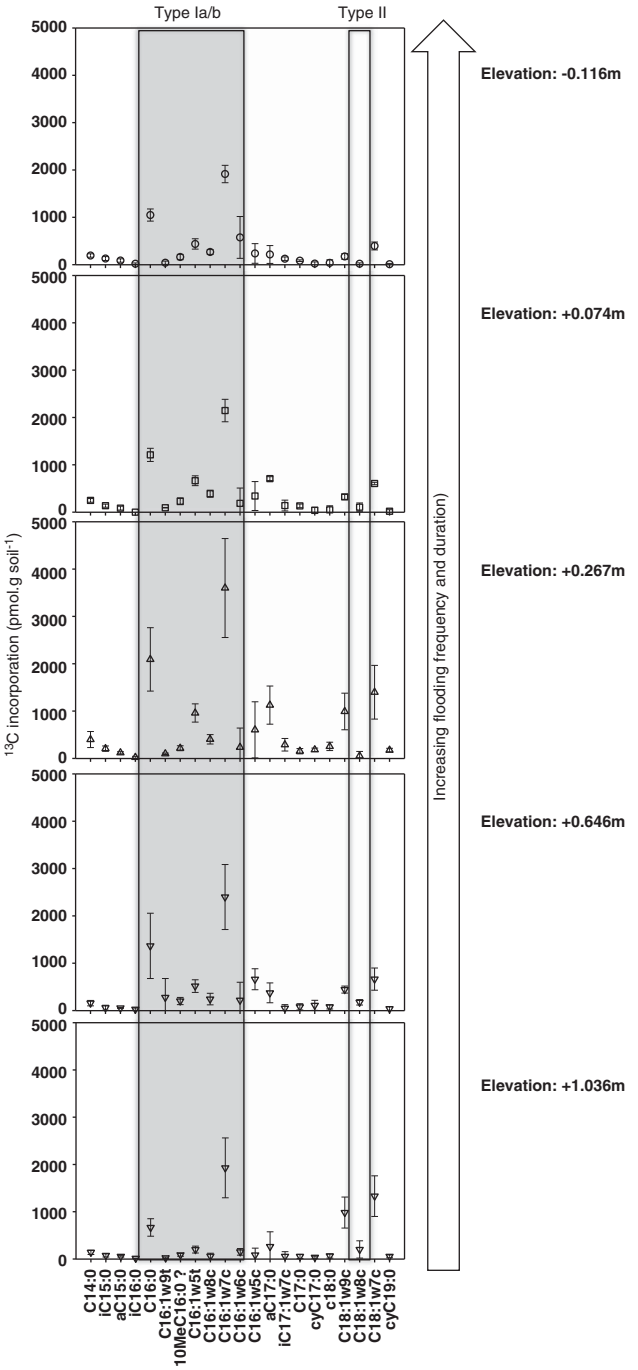

Figure 4 Incorporation of ^{13}C-CH$_4$ using SIP-PLFAs in intact soil cores collected at different elevations within a riparian floodplain. Intact cores were incubated with ^{13}CH$_4$ to label PLFA associated to MOB communities. Each data point is the average of three soil cores per elevation level. Error bars indicate standard deviation. The grey-shaded area indicates PLFA associated to type I MOB, whereas the open box depicts PLFA indicative for type II MOB.

Methylocaldum species. The non-generic type Ib-related probe (501-286, Supplementary Figure S5 and Supplementary Table S1) that correlates most significantly with methane oxidation activity is in fact a probe targeting a cluster of uncultivated type 1b-related sequences that are most closely

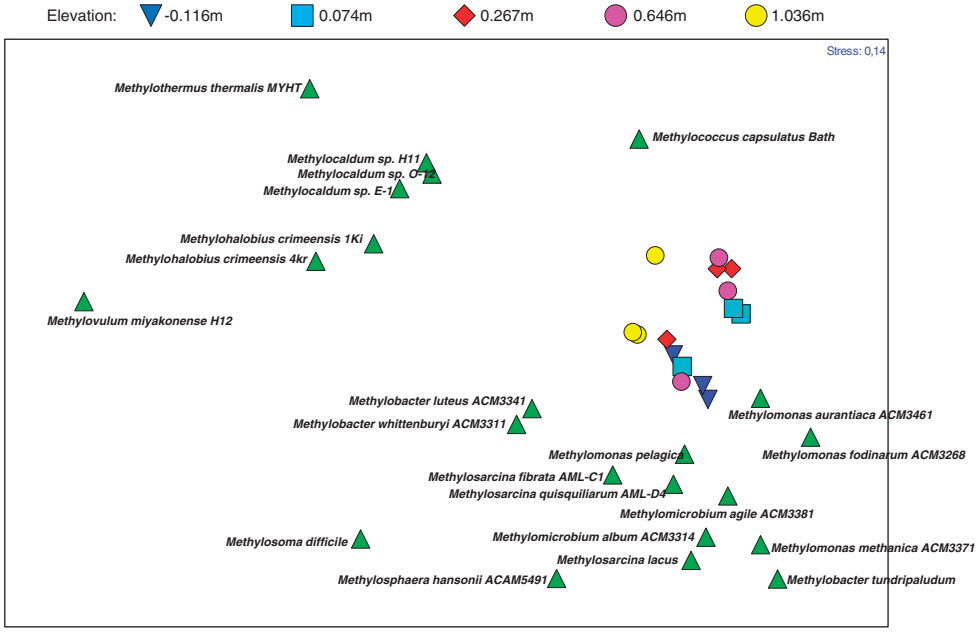

Figure 5 Multivariate statistical analyses of SIP $^{13}CH_4$-PLFA profiles of the MOB community (type I only) in intact soil retrieved from a riparian floodplain in July 2009. The inputs of the multidimensional scaling (MDS) analyses were PLFA profiles of MOB cultures (expressed as percentage of total PLFA content) and of SIP profiles of soil samples (expressed as percentage of ^{13}C incorporated in separate PLFAs of the total PLFA ^{13}C uptake). The two-dimensional distances between samples in the MDS graph show the relative similarity between samples. Stress value of the MDS plot was 0.14. For the MDS analyses, the profiles were transformed [$\log(x+1)$] before the Bray–Curtis similarity matrix was established.

related to *Methylococcus capsulatus* (Supplementary Figure S5).

The type II MOB detected by cloning as well as by microarray were all *Methylocystis*-related species that contain the MOB-specific PLFA C18:1ω8c as a major component (Bodelier *et al.,* 2009). Label incorporation into this PLFA was insignificant compared with the total incorporation, which also did not allow for comparison of labeling profiles with known cultures as has been performed for type I (Figure 5). However, the displayed incorporation and distribution over the flooding gradient was similar to type II qPCR numbers and microarray probe signals. Type II label incorporation was higher towards higher elevations, whereas incorporation in the specific type I PLFA showed an optimum (Figure 6), similar to the optimum in methane consumption and dynamics of numbers of types Ia and Ib as determined by *in vitro* assays.

Similar to the analysis of stable isotope-labeled PLFAs, ^{13}C-labeled proteins can be used to identify the organisms that have assimilated the labeled substrate (Jehmlich *et al.,* 2010). For this study, protein-SIP was used for the first time on MOB communities. The shift in mass spectra resulting from the incorporation of ^{13}C into proteins can be assessed by MS (Supplementary Figure S9) and used to calculate label incorporation by comparison with the spectra of the corresponding unlabeled peptides, which have to be identified in a control sample in parallel.

Protein identification was focused towards members of α-, β- and γ-*Proteobacteria* and

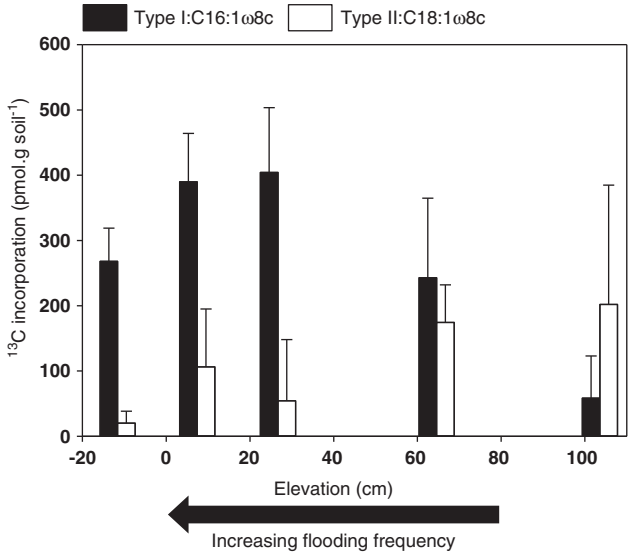

Figure 6 ^{13}C-CH_4 incorporation into specific PLFA for type I and II MOB in intact soil cores, retrieved from a riparian floodplain in July 2009. Bars represent the average of three replicate cores, with error bars showing standard deviation.

Verrucomicrobia as they include representatives of MOB. The protein hits were mainly affiliated to *Methylobacter tundripaludum* SV96, *Verrucomicrobium spinosum*, *Methylacidiphilum infernorum* V4, *Rhizobium* spp. and *Sinorhizobium* spp. (Supplementary Tables S2–S6). Although several genome sequences of MOB (*Methylomonas methanica* MC09 (Boden *et al.,* 2011), *Methylomicrobium album*

strain BG8, *Methylococcus capsulatus* strain Bath and Texas (Ward *et al.,* 2004; Kleiveland *et al.,* 2012), *Methylocystis* strain Rockwell (Stein *et al.,* 2011), *Methylocystis* strain SC2 (Dam *et al.,* 2012), *Methylocystis parvus* OBBP (del Cerro *et al.,* 2012), *Methylosinus trichosporium* OB3b (Stein *et al.,* 2010)) were deposited in the consulted NCBI nr database, almost no other labeled peptide sequences affiliated to MOB were identified besides peptides of *Methylobacter tundripaludum* SV96. In addition, most of the identified peptide sequences had to be excluded according to the stringent Mascot scoring parameters, which may be related to the soil matrix. Protein extraction from soil is still a great challenge for environmental proteomics. Several protocols are available (Benndorf *et al.,* 2009; Chourey *et al.,* 2010; Keiblinger *et al.,* 2012) and two were also tested here, but none improved the number of identified and validated peptides in a sufficient manner. Nevertheless, the prominent identified protein was PmoB. The preferred detection of PmoB may be caused by the localization in the cytosol compared with PmoA, which is membrane bound and hard to solubilize during protein extraction. In a recent study (Paszczynski *et al.,* 2011), the dominance of peptides belonging to PmoB of *Methylosinus trichosporium* OB3b was also described by the analyses of groundwater samples from a site contaminated with chlorinated ethenes. Peptides of other subunits were either not identified or identified in minor numbers.

The calculation of stable isotope incorporation in the proteins clearly indicated methane consumption by type Ia-related species (Table 1). Label incorporation was not detected at the highest elevation, whereas at intermediate elevations (0.20 m), proteins (PmoB, guanosine triphosphate-binding protein, methanol dehydrogenase) associated with the type Ia MOB *Methylobacter tundripaludum* SV96 could be detected (Table 1). This points to highest amounts of labeled proteins in the intermediate samples, corroborating the results found with PLFA and molecular analyses. No ^{13}C incorporation was found in proteins of both type Ib and II MOB and of *Verrucomicrobia* (Supplementary Tables S2–S6). Either none of the corresponding masses were found in the mass spectra of the control samples, or just the unlabeled (i.e., ^{12}C) masses were detected.

The evaluation of the mass spectra during the calculation of the relative isotope abundance (RIA) showed two ^{13}C incorporation features, a high incorporation of about 90% ^{13}C and a medium

Table 1 RIA of proteins showing ^{13}C incorporation into peptides

Peptide		*Charge*	*^{12}C ($m z^{-1}$)*	*Maximum peptide score*	*High incorporation*		*Medium incorporation*	
					^{13}C ($m z^{-1}$)	*RIA (%)*	*^{13}C ($m z^{-1}$)*	*RIA (%)*
Core 1: −0.116 m								
	gi307826358	Methane monooxygenase/ammonia monooxygenase, subunit B (*Methylobacter tundripaludum* SV96) MW = 45.3 kDa, max. protein score = 699.07						
LGEFNTAGVR		2	532.280	85	553.852	93.2 ± 0.1	545.324	62.4 ± 1.5
LADLIYDPDSR		2	639.322	68	665.911	93.6 ± 0.2	655.878	62.0 ± 0.3
Core 2: 0.074 m								
	gi307826358	Methane monooxygenase/ammonia monooxygenase, subunit B (*Methylobacter tundripaludum* SV96) MW = 45.3 kDa, max. protein score = 684.14						
LGEFNTAGVR		2	532.280	85	553.861	91.2 ± 0.7	546.327	61.2
LADLIYDPDSR		2	639.322	68	665.410	92.3 ± 0.9	656.881	61.9 ± 0.7
TVQVTASDAAWEVYR		2	848.423	62	884.040	93.5	ND	ND
QGSWIGGQLVPR		2	649.354	58	ND	ND	ND	ND
FAGLLFFFDEAGNR		2	802.400	53	ND	ND	ND	ND
	gi381151332	PQQ-dependent dehydrogenase, methanol/ethanol family (*Methylomicrobium album* BG8) MW = 66.6 kDa, max. protein score = 89.29						
IFLQQSDTVLTALDAK		2	881.985	41	919.110	93.1	ND	ND
Core 3: 0.267 m								
	gi307826358	Methane monooxygenase/ammonia monooxygenase, subunit B (*Methylobacter tundripaludum* SV96) MW = 45.3 kDa, max. protein score = 735.09						
LGEFNTAGVR		2	532.280	86	553.852	92 ± 0.1	546.829	62.8 ± 0.1
LADLIYDPDSR		2	639.322	67	665.409	92.7 ± 0.4	657.383	62.6 ± 0.1
TVQVTASDAAWEVYR		2	848.421	62	884.040	93.3	873.003	64.6
QGSWIGGQLVPR		2	649.355	57	675.943	90.9	667.916	61.2
Core 4: 0.646 m								
	gi307826358	Methane monooxygenase/ammonia monooxygenase, subunit B (*Methylobacter tundripaludum* SV96) MW = 45.3 kDa, max. protein score = 217.17						
LGEFNTAGVR		2	532.280	83	553.351	90.4 ± 0.3	ND	ND
LADLIYDPDSR		2	639.322	67	664.908	90.9	658.386	63.5
QGSWIGGQLVPR		2	649.355	45	675.943	90.2	ND	ND

Abbreviations: 12C $m z^{-1}$, mass to charge values for the monoisotopic peak; ^{13}C $m z^{-1}$, mass to charge values for the most intense peak with heavy isotope incorporation; MW, molecular weight; ND, mass not detected in the spectra; PQQ, pyrroloquinoline-quinone; RIA, relative isotope abundance. Two types of ^{13}C incorporation (high and medium) are detected in the proteins of the methylotrophic species.

incorporation of about 62% ^{13}C. Both belonged to the same peptide sequence. This occurrence may point to either *de novo* enzyme synthesis of existing cells, growth of novel cells or differences in enzyme turnover between cells. Another explanation would be that there is still internal production of unlabeled methane within the cores, resulting in methanotrophs consuming unlabeled methane in the inside of the core and labeled methane at the outer parts, resulting in a mix of differently labeled proteins in the final mixed sample used for extraction.

Theoretically, some type Ib and all type II MOB can incorporate carbon from CO_2 next to methane (Jahnke *et al.,* 1999; Templeton *et al.,* 2006). Very recently, an elegant study using a stable isotope-labeling approach in cultures of *Methylosinus trichosporium* OB3b demonstrated that even up to 60% of the biomass carbon can be derived from CO_2 (Yang *et al.,* 2013). Basically, this could also have led to the fact that both types are not detected in protein-SIP and to low incorporation in PLFA by type II. When assuming that 60% of the methane is respired to CO_2, the ratio of labeled CO_2 to unlabeled atmospheric CO_2 in our incubations will be 32 (12 600/390 p.p.m.v.), which makes it highly unlikely that the observed results have been influenced by this mechanism.

Very recently, it has been shown that Verrucomicrobial methanotrophs are autotrophs and do not utilize methane but CO_2 for generating cell carbon (Khadem *et al.,* 2011; Sharp *et al.,* 2012). Hence, possible activity of these MOB would be missed in our incubations, but as explained above, labeled CO_2 is produced during incubation. However, the amount produced in combination with the incubation time may not have been sufficient for effective labeling of Verrucomicrobial proteins. Therefore, we cannot exclude activity of these MOB normally found only in geothermal environments (Sharp *et al.,* 2012). If the presence of Verrucomicrobial MOB proteins detected in our floodplain soil will be confirmed by molecular detection techniques as recently developed (Sharp *et al.,* 2012), then this would be the first evidence of the presence of these methanotrophs in non-extreme habitats. Future work will reveal whether this indeed is the case.

Synthesis

By using a polyphasic approach combining biogeochemical, molecular biological methods and stable isotope labeling, we were able to connect microbial methane oxidation with the active species in the environment. Collectively, the data obtained give support for a linear relationship between biodiversity components and microbial functioning in natural complex soil ecosystems. The investigated natural flooding gradient provided a combination of environmental drivers (e.g., methane, oxygen and nitrogen) leading to proliferation of subgroups of MOB (i.e., type Ia/Ib), resulting in highest methane consumption coinciding with highest community evenness. The congruence between community evenness and function is probably observed in this study because we assessed the species actually responsible for the measured function in a range of conditions allowing them to be active. Thereby, this study highlights three important aspects of BEF research in general and of microbial BEF studies more specifically:

(i) *Spatial and temporal environmental heterogeneity* giving rise to variability in the number of niches has been neglected so far in BEF studies (Hillebrand and Matthiessen, 2009; Caliman *et al.,* 2010). In comprehensive meta-analyses on BEF studies of the past decades, the incorporation of temporal and spatial scales, representing environmental heterogeneity and the number of niches, in BEF studies is designated as one of the most important issues to address in future studies (Cardinale *et al.,* 2012). Our study incorporated both temporal and spatial scales, which led to a linear relationship between community composition and function, which seems in contrast with the often-saturating species–function curves in artificial community experiments. However, what occurs in our study is that the number of active species, which we are obviously detecting, is increasing with time and environmental variability. The distribution of MOB species and methane consumption in the studied environmental gradient can be represented conceptually in a three-dimensional space (Figure 7), where the relationship between function and biodiversity (i.e., richness, evenness) is determined by the number of niches (defined as the Hutchinson's 'ecological niche'; i.e., a multidimensional space where the dimensions are environmental conditions and the resources that define the requirements of an individual or a species to practice 'its' way of life) present allowing species to be active and contribute to function. Many BEF studies do not include environmental heterogeneity (i.e., variation in niches), leading to a high number of niche-less (i.e., niche–species mismatch as shown in Figure 7) microbial species that remain inactive and therefore do not contribute to function. This is represented by the solid line in Figure 7, which can be misinterpreted as functional redundancy. The results obtained in our study are more similar to the dashed line as displayed in Figure 7, where the environmental gradient dictates the number of niches available for the large natural diversity of microbial species to emerge from seed banks and become active and contribute to function. Environmental historical contingencies can determine the size of the species pool present in the seed banks and the species emerging. For MOB in rice fields, it has already been shown that similar environmental conditions over thousands of years lead to a rather stable seed bank and to a selection in the species emerging from the seed bank under conditions of methane and oxygen availability (Ho *et al.,* 2011).

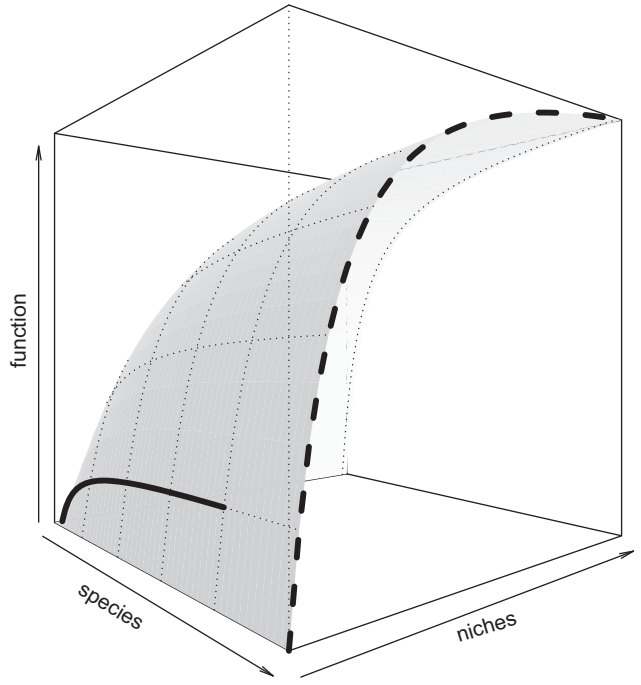

Figure 7 Graphical representation of the three dimensions involved in BEF relationships. The curve of the species–function relationship will depend on the number of niches present, allowing species to be active and contribute to function. Many BEF studies do not include environmental heterogeneity (i.e., variation in niches), leading to a high number of niche-less microbial species that remain inactive and therefore do not contribute to function. This is represented by the solid line, which can be misinterpreted as functional redundancy. The results obtained in our study are more similar to the dashed line; the environmental gradient dictates the number of niches available for the large natural diversity of microbial species to become active and contribute to function.

The rice cultivation over centuries has led to a restricted number of niches and also to a selection of type I MOB emerging from the seed bank. Why type II MOB are abundant in many wetland soils, also in our floodplain, but rarely are detected as dominating active MOB is still a very open question (Ho *et al.,* 2013). Following the logic of Figure 7, no suitable niches are available in our system or alternatively we fail to detect their contribution to methane consumption for methodological reasons.

More supporting evidence for the importance of habitat heterogeneity for the BEF has recently been generated in highly controlled stream biofilms, where algal species richness positively correlated to nitrate assimilation under variable flow regimes leading to heterogeneous habitat conditions by which the number of niches could be varied experimentally (Cardinale, 2011). Hence, BEF studies in artificial as well as manipulated natural ecosystems using a fixed number of niches will give an incomplete and even biased picture of BEF relationships in natural ecosystems, especially when results are used for future predictions of ecosystem functioning.

(ii) *Functional redundancy in microbial communities* as explanation of saturating species–function curves may very well be an invalid concept because the biodiversity axis consists of many species that do not contribute to the measured function, leading to the solid line shown in Figure 7. Next to this, the species that do contribute to function can often not be assigned. Microbial BEF studies use DNA-based molecular diversity assessment techniques bearing no diagnostic means to determine which of the species is contributing to the observed function (Bell *et al.,* 2009). Positive BEF relationships are most often observed with narrow functions (e.g., methane oxidation (Levine *et al.,* 2011), polymer degradation (Wohl *et al.,* 2004; Peter *et al.,* 2011) or pesticide degradation (Monard *et al.,* 2011)), where species are brought together to determine which all contribute to the specific function in the respective experimental setup. Hence, BEF studies in microbial ecosystems where species that are actively contributing to ecosystem functioning cannot be pinpointed will give biased results, often concluding functional redundancy to be occurring which actually is either a lack of niche heterogeneity or cumulative counting of 'seed bank' species that are not actively contributing to the measured function.

(iii) *Globally highly important processes are regulated by minor components of microbial communities*. The active methane-oxidizing species (types Ia and Ib) in the studied wetland constitute a numerically subordinate group within the functional guild of methanotrophs, which is already a minority on itself. When assuming the total amount of bacteria to be approximately 1.10^9 cells per gram of dry soil (Wessen *et al.,* 2010; Ho *et al.,* 2011; Daniell *et al.,* 2012; Kuramae *et al.,* 2012), which is even the lower limit of the range typically observed, then type Ia and Ib MOB constitute 0.001–0.05% and 0.08–0.5% of the total community, respectively. The individual species carrying out the observed activity that are collectively targeted by the qPCR assays constitute an even lower percentage of the community. Type Ia MOB related to the ones found in our study have been demonstrated to be responsible for methane consumption in many important methane-emitting habitats such as rice paddies (Bodelier *et al.,* 2000), arctic wetlands (Graef *et al.,* 2011), landfills (Chen *et al.,* 2007), lake sediments (Dumont *et al.,* 2011) and floodplains (Bodelier *et al.*, 2012), which has been ascribed to their specific traits enabling them to be very responsive to the periodic availability of methane and other nutrients (Steenbergh *et al.,* 2010; Bodelier *et al.*, 2012). Recent studies assigning species-specific contributions to important biogeochemical cycles using stable isotopes also indicated a disproportionate role of single rare microbial species to globally important processes (Musat *et al.,* 2008; Pester *et al.,* 2010; Peter *et al.,* 2011), strongly suggesting that the traits of the organisms involved will be fundamental to the variability and dynamics in the biogeo-

chemical process catalyzed. Recently, a strong link between diversity of microbes consuming atmospheric methane and soil methane uptake was demonstrated (Levine *et al.,* 2011) for high-affinity methanotrophs in upland soils. In combination with our results on low-affinity MOB in wetland habitats, it can be concluded that microbial diversity and microbial traits are important regulating factors in the global methane budget. The relevance of microbial minorities and their respective niches for globally important ecosystem functions calls for consideration of conservation policy with respect to microbial diversity, especially in light of climate change and other anthropogenic disturbances.

Acknowledgements

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References

Bell T, Gessner MO, Griffiths RI, McLaren J, Morin PJ, van der Heijden M *et al.* (2009). Microbial biodiversity and ecosystem functioning under controlled conditions and in the wild. In: Naeem SB, Bunker DE, Hector A, Loreau M, Perrings C (eds) *Biodiversity, Ecosystem Functioning, and Human Wellbeing: An Ecological and Economic Perspective*. Oxford University Press: Oxford, pp 121–133.

Belova SE, Baani M, Suzina NE, Bodelier PLE, Liesack W, Dedysh SN. (2011). Acetate utilization as a survival strategy of peat-inhabiting *Methylocystis* spp. *Environ Microbiol Rep* **3**: 36–46.

Benndorf D, Balcke GU, Harms H, von Bergen M. (2007). Functional metaproteome analysis of protein extracts from contaminated soil and groundwater. *ISME J* **1**: 224–234.

Benndorf D, Vogt C, Jehmlich N, Schmidt Y, Thomas H, Woffendin G *et al.* (2009). Improving protein extraction and separation methods for investigating the metaproteome of anaerobic benzene communities within sediments. *Biodegradation* **20**: 737–750.

Bloom AA, Palmer PI, Fraser A, Reay DS, Frankenberg C. (2010). Large-scale controls of methanogenesis inferred from methane and gravity spaceborne data. *Science* **327**: 322–325.

Bodelier PLE, Hahn AP, Arth IR, Frenzel P. (2000). Effects of ammonium-based fertilisation on microbial processes involved in methane emission from soils planted with rice. *Biogeochemistry* **51**: 225–257.

Bodelier PLE. (2011). Toward understanding, managing, and protecting microbial ecosystems. *Front Microbiol* **2**: 80.

Bodelier PLE, Bar-Gilissen MJB, Meima-Franke M, Hordijk K. (2012). Structural and functional response of methane-consuming microbial communities to different flooding regimes in riparian soils. *Ecol Evol* **2**: 106–127.

Bodelier PLE, Bar-Gillisen MJB, Hordijk K, Sinninghe Damsté JSS, Rijpstra WIC, Geenevasen JAJ *et al.* (2009). A reanalysis of phospholipid fatty acids as ecological biomarkers for methanotrophic bacteria. *ISME J* **3**: 606–616.

Boden R, Cunliffe M, Scanlan J, Moussard H, Kits KD, Klotz MG *et al.* (2011). Complete genome sequence of the aerobic marine methanotroph methylomonas methanica MC09. *J Bacteriol* **193**: 7001–7002.

Boschker HTS, Nold SC, Wellsbury P, Bos D, de Graaf W, Pel R *et al.* (1998). Direct linking of microbial populations to specific biogeochemical processes by C-13-labelling of biomarkers. *Nature* **392**: 801–805.

Bousquet P, Ciais P, Miller JB, Dlugokencky EJ, Hauglustaine DA, Prigent C *et al.* (2006). Contribution of anthropogenic and natural sources to atmospheric methane variability. *Nature* **443**: 439–443.

Caliman A, Pires AF, Esteves FA, Bozelli RL, Farjalla VF. (2010). The prominence of and biases in biodiversity and ecosystem functioning research. *Biodivers Conserv* **19**: 651–664.

Cardinale BJ, Srivastava DS, Duffy JE, Wright JP, Downing AL, Sankaran M *et al.* (2006). Effects of biodiversity on the functioning of trophic groups and ecosystems. *Nature* **443**: 989–992.

Cardinale BJ. (2011). Biodiversity improves water quality through niche partitioning. *Nature* **472**: 86–89.

Cardinale BJ, Duffy JE, Gonzalez A, Hooper DU, Perrings C, Venail P *et al.* (2012). Biodiversity loss and its impact on humanity. *Nature* **486**: 59–67.

Chen Y, Dumont MG, Cebron A, Murrell JC. (2007). Identification of active methanotrophs in a landfill cover soil through detection of expression of 16S rRNA and functional genes. *Environ Microbiol* **9**: 2855–2869.

Chourey K, Jansson J, VerBerkmoes N, Shah M, Chavarria KL, Tom LM *et al.* (2010). Direct cellular lysis/protein extraction protocol for soil metaproteomics. *J Proteome Res* **9**: 6615–6622.

Cockell CS, Jones HL. (2009). Advancing the case for microbial conservation. *Oryx* **43**: 520–526.

Conrad R. (2007). Microbial ecology of methanogens and methanotrophs. *Adv Agron* **96**: 1–63.

Dam B, Dam S, Kube M, Reinhardt R, Liesack W. (2012). Complete genome sequence of *Methylocystis* sp strain SC2, an aerobic methanotroph with high-affinity methane oxidation potential. *J Bacteriol* **194**: 6008–6009.

Daniell TJ, Davidson J, Alexander CJ, Caul S, Roberts DM. (2012). Improved real-time PCR estimation of gene copy number in soil extracts using an artificial reference. *J Microbiol Methods* **91**: 38–44.

Dedysh SN, Knief C, Dunfield PF. (2005). *Methylocella* species are facultatively methanotrophic. *J Bacteriol* **187**: 4665–4670.

Dedysh SN, Belova SE, Bodelier PLE, Smirnova KV, Khmelenina VN, Chidthaisong A *et al.* (2007). *Methylocystis heyeri* sp nov., a novel type II methanotrophic bacterium possessing 'signature' fatty acids of type I methanotrophs. *Int J Syst Evol Microbiol* **57**: 472–479.

del Cerro C, Garcia JM, Rojas A, Tortajada M, Ramon D, Galan B *et al.* (2012). Genome sequence of the methanotrophic poly-beta-hydroxybutyrate producer *Methylocystis parvus* OBBP. *J Bacteriol* **194**: 5709–5710.

Dumont MG, Pommerenke B, Casper P, Conrad R. (2011). DNA-, rRNA- and mRNA-based stable isotope probing of aerobic methanotrophs in lake sediment. *Environ Microbiol* **13**: 1153–1167.

Dunfield PF, Yuryev A, Senin P, Smirnova AV, Stott MB, Hou SB *et al.* (2007). Methane oxidation by an extremely acidophilic bacterium of the phylum Verrucomicrobia. *Nature* **450**: 879–882.

Ettwig KF, Butler MK, Le Paslier D, Pelletier E, Mangenot S, Kuypers MMM *et al.* (2010). Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature* **464**: 543–548.

Falkowski PG, Fenchel T, Delong EF. (2008). The microbial engines that drive Earth's biogeochemical cycles. *Science* **320**: 1034–1039.

Freitag TE, Toet S, Ineson P, Prosser JI. (2010). Links between methane flux and transcriptional activities of methanogens and methane oxidizers in a blanket peat bog. *FEMS Microbiol Ecol* **73**: 157–165.

Gans J, Wolinsky M, Dunbar J. (2005). Computational improvements reveal great bacterial diversity and high metal toxicity in soil. *Science* **309**: 1387–1390.

Graef C, Hestnes AG, Svenning MM, Frenzel P. (2011). The active methanotrophic community in a wetland from the High Arctic. *Environ Microbiol Rep* **3**: 466–472.

Griffith GW. (2012). Do we need a global strategy for microbial conservation? *Trends Ecol Evol* **27**: 1–2.

Hillebrand H, Matthiessen B. (2009). Biodiversity in a complex world: consolidation and progress in functional biodiversity research. *Ecol Lett* **12**: 1405–1419.

Ho A, Lüke C, Cao ZH, Frenzel P. (2011). Ageing well: methane oxidation and methane oxidizing bacteria along a chronosequence of 2000 years. *Environ Microbiol Rep* **3**: 738–743.

Ho A, Kerckhof FM, Lüke C, Reim A, Krause SMB, Boon N *et al.* (2013). Conceptualizing functional traits and ecological characteristics of methane-oxidizing bacteria as life strategies. *Environ Microbiol Rep* **5**: 335–345.

Holmes AJ, Costello A, Lidstrom ME, Murrell JC. (1995). Evidence that particulate methane monooxygenase and ammonia monooxygenase may be evolutionary related. *FEMS Microbiol Lett* **132**: 203–208.

Jahnke LL, Summons RE, Hope JM, des Marais DJ. (1999). Carbon isotopic fractionation in lipids from methanotrophic bacteria II: the effects of physiology and environmental parameters on the biosynthesis and isotopic signatures of biomarkers. *Geochim Cosmochim Acta* **63**: 79–93.

Jehmlich N, Schmidt F, Taubert M, Seifert J, Bastida F, von Bergen M *et al.* (2010). Protein-based stable isotope probing. *Nat Protoc* **5**: 1957–1966.

Kai FM, Tyler SC, Randerson JT, Blake DR. (2011). Reduced methane growth rate explained by decreased Northern Hemisphere microbial sources. *Nature* **476**: 194–197.

Keiblinger KM, Wilhartitz IC, Schneider T, Roschitzki B, Schmid E, Eberl L *et al.* (2012). Soil metaproteomics — comparative evaluation of protein extraction protocols. *Soil Biol Biochem* **54**: 14–24.

Kemnitz D, Chin KJ, Bodelier P, Conrad R. (2004). Community analysis of methanogenic archaea within a riparian flooding gradient. *Environ Microbiol* **6**: 449–461.

Khadem AF, Pol A, Wieczorek A, Mohammadi SS, Francoijs K-J, Stunnenberg HG *et al.* (2011). Autotrophic methanotrophy in Verrucomicrobia: *Methylacidiphilum fumariolicum* SolV uses the Calvin–Benson–Bassham cycle for carbon dioxide fixation. *J Bacteriol* **193**: 4438–4446.

Kleiveland CR, Hult LTO, Kuczkowska K, Jacobsen M, Lea T, Pope PB. (2012). Draft genome sequence of the methane-oxidizing bacterium *Methylococcus capsulatus* (Texas). *J Bacteriol* **194**: 6626–6626.

Kolb S, Knief C, Stubner S, Conrad R. (2003). Quantitative detection of methanotrophs in soil by novel pmoA-targeted real-time PCR assays. *Appl Environ Microbiol* **69**: 2423–2429.

Kuramae EE, Yergeau E, Wong LC, Pijl AS, van Veen JA, Kowalchuk GA. (2012). Soil characteristics more strongly influence soil bacterial communities than land-use type. *FEMS Microbiol Ecol* **79**: 12–24.

Lennon JT, Jones SE. (2011). Microbial seed banks: the ecological and evolutionary implications of dormancy. *Nat Rev Microbiol* **9**: 119–130.

Levine UY, Teal TK, Robertson GP, Schmidt TM. (2011). Agriculture's impact on microbial diversity and associated fluxes of carbon dioxide and methane. *ISME J* **5**: 1683–1691.

Loreau M. (2010). Linking biodiversity and ecosystems: towards a unifying ecological theory. *Philos Trans R Soc Lond B Biol Sci* **365**: 49–60.

Lüke C, Frenzel P. (2011). Potential of *pmoA* amplicon pyrosequencing for methanotroph diversity studies. *Appl Environ Microbiol* **77**: 6305–6309.

McDonald IR, Bodrossy L, Chen Y, Murrell JC. (2008). Molecular ecology techniques for the study of aerobic methanotrophs. *Appl Environ Microbiol* **74**: 1305–1315.

Mohanty SR, Bodelier PLE, Floris V, Conrad R. (2006). Differential effects of nitrogenous fertilizers on methane-consuming microbes in rice field and forest soils. *Appl Environ Microbiol* **72**: 1346–1354.

Monard C, Vandenkoornhuyse P, Le Bot B, Binet F. (2011). Relationship between bacterial diversity and function under biotic control: the soil pesticide degraders as a case study. *ISME J* **5**: 1048–1056.

Mora C, Tittensor DP, Adl S, Simpson AGB, Worm B. (2011). How many species are there on earth and in the ocean? *PLoS Biol* **9**: e1001127.

Musat N, Halm H, Winterholler B, Hoppe P, Peduzzi S, Hillion F *et al.* (2008). A single-cell view on the ecophysiology of anaerobic phototrophic bacteria. *Proc Natl Acad Sci USA* **105**: 17861–17866.

Neufeld JD, Wagner M, Murrell JC. (2007). Who eats what, where and when? Isotope-labelling experiments are coming of age. *ISME J* **1**: 103–110.

Op den Camp HJM, Islam T, Stott MB, Harhangi HR, Hynes A, Schouten S *et al.* (2009). Environmental, genomic and taxonomic perspectives on methanotrophic Verrucomicrobia. *Environ Microbiol Rep* **1**: 293–306.

Pan Y, Bodrossy L, Frenzel P, Hestnes A, Krause SBM, Lüke C *et al.* (2010). Impacts of inter- and intralaboratory variations on the reproducibility of microbial community analyses. *Appl Environ Microbiol* **76**: 7451–7458.

Paszczynski AJ, Paidisetti R, Johnson AK, Crawford RL, Colwell FS, Green T *et al.* (2011). Proteomic and targeted qPCR analyses of subsurface microbial communities for presence of methane monooxygenase. *Biodegradation* **22**: 1045–1059.

Perkins DN, Pappin DJC, Creasy DM, Cottrell JS. (1999). Probability-based protein identification by searching

sequence databases using mass spectrometry data. *Electrophoresis* **20**: 3551–3567.
Pester M, Bittner N, Deevong P, Wagner M, Loy A. (2010). A 'rare biosphere' microorganism contributes to sulfate reduction in a peatland. *ISME J* **4**: 1591–1602.
Peter H, Beier S, Bertilsson S, Lindstrom ES, Langenheder S, Tranvik LJ. (2011). Function-specific response to depletion of microbial diversity. *ISME J* **5**: 351–361.
Pol A, Heijmans K, Harhangi HR, Tedesco D, Jetten MSM, den Camp H. (2007). Methanotrophy below pH1 by a new Verrucomicrobia species. *Nature* **450**: 874–878.
Seifert J, Taubert M, Jehmlich N, Schmidt F, Volker U, Vogt C *et al.* (2012). Protein-based stable isotope probing (protein-SIP) in functional metaproteomics. *Mass Spectrom Rev* **31**: 683–697.
Semrau JD, DiSpirito AA, Yoon S. (2010). Methanotrophs and copper. *Fems Microbiol Rev* **34**: 496–531.
Semrau JD, DiSpirito AA, Vuilleumier S. (2011). Facultative methanotrophy: false leads, true results, and suggestions for future research. *FEMS Microbiol Lett* **323**: 1–12.
Sharp CE, Stott MB, Dunfield PF. (2012). Detection of autotrophic verrucomicrobial methanotrophs in a geothermal environment using stable isotope probing. *Front Microbiol* **3**: 303–303.
Spahni R, Wania R, Neef L, van Weele M, Pison I, Bousquet P *et al.* (2011). Constraining global methane emissions and uptake by ecosystems. *Biogeosciences* **8**: 1643–1665.
Steenbergh AK, Meima MM, Kamst M, Bodelier PLE. (2010). Biphasic kinetics of a methanotrophic community is a combination of growth and increased activity per cell. *FEMS Microbiol Ecol* **71**: 12–22.
Stein LY, Yoon S, Semrau JD, DiSpirito AA, Crombie A, Murrell JC *et al.* (2010). Genome sequence of the obligate methanotroph *Methylosinus trichosporium* strain OB3b. *J Bacteriol* **192**: 6497–6498.
Stein LY, Bringel F, DiSpirito AA, Han S, Jetten MSM, Kalyuzhnaya MG *et al.* (2011). Genome sequence of the methanotrophic Alphaproteobacterium *Methylocystis* sp strain Rockwell (ATCC 49242). *J Bacteriol* **193**: 2668–2669.
Stein LY, Roy R, Dunfield PF. (2012). *Aerobic Methanotrophy and Nitrification: Processes and Connections*. eLS. John Wilet & Sons Ltd.: Chichester.
Taubert M, Jehmlich N, Vogt C, Richnow HH, Schmidt F, von Bergen M *et al.* (2011). Time resolved protein-based stable isotope probing (protein-SIP) analysis allows quantification of induced proteins in substrate shift experiments. *Proteomics* **11**: 2265–2274.
Templeton AS, Chu KH, Alvarez-Cohen L, Conrad ME. (2006). Variable carbon isotope fractionation expressed by aerobic CH_4-oxidizing bacteria. *Geochim Cosmochim Acta* **70**: 1739–1752.
Wang J, Krause S, Muyzer G, Meima-Franke M, Laanbroek HJ, Bodelier PLE. (2012). Spatial patterns of iron- and methane-oxidizing bacterial communities in an irregularly flooded, riparian wetland. *Front Microbiol* **3**: 64–64.
Ward N, Larsen O, Sakwa J, Bruseth L, Khouri H, Durkin AS *et al.* (2004). Genomic insights into methanotrophy: the complete genome sequence of *Methylococcus capsulatus* (Bath). *PLoS Biol* **2**: 1616–1628.
Wessen E, Hallin S, Philippot L. (2010). Differential responses of bacterial and archaeal groups at high taxonomical ranks to soil management. *Soil Biol Biochem* **42**: 1759–1765.
Whitman WB, Coleman DC, Wiebe WJ. (1998). Prokaryotes: the unseen majority. *Proc Natl Acad Sci USA* **95**: 6578–6583.
Wohl DL, Arora S, Gladstone JR. (2004). Functional redundancy supports biodiversity and ecosystem function in a closed and constant environment. *Ecology* **85**: 1534–1540.
Yang S, Matsen JB, Konopka M, Green-Saxena A, Clubb J, Sadilek M *et al.* (2013). Global molecular analyses of methane metabolism in methanotrophic Alphaproteobacterium, *Methylosinus trichosporium* OB3b. Part II. Metabolomics and ^{13}C-labeling study. *Front Microbiol* **4**: 70.

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