Erschienen in: FEMS Microbiology Ecology ; 92 (2016), 8. - fiw123
https://dx.doi.org/10.1093/femsec/fiw123

Methane release from sediment seeps to the atmosphere is counteracted by highly active Methylococcaceae in the water column of deep oligotrophic Lake Constance

Maren Bornemann[1], Ingeborg Bussmann[2], Lucas Tichy[1], Jörg Deutzmann[1,3], Bernhard Schink[1] and Michael Pester[1,*]

[1]Department of Biology, University of Konstanz, Universitätsstrasse 10, 78457 Konstanz, Germany, [2]Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Meeresstation Helgoland, Kurpromenade 201, D-27498 Helgoland, Germany and [3]Department of Civil and Environmental Engineering, Stanford University, 318 Campus Drive, Stanford, CA 94305, USA

*__Corresponding author:__ Department of Biology, University of Konstanz, D-78457 Konstanz, Germany. Tel: +49-7531-883282; Fax: +49-7531-884047; E-mail: Michael.Pester@uni-konstanz.de

One sentence summary: Pelagic Methylococcaceae are highly active in scavenging methane released from sediment seeps of a deep pre-alpine lake, Lake Constance.

ABSTRACT

Methane emissions from freshwater environments contribute substantially to global warming but are under strong control of aerobic methane-oxidizing bacteria. Recently discovered methane seeps (pockmarks) in freshwater lake sediments have the potential to bypass this control by their strong outgassing activity. Whether this is counteracted by pelagic methanotrophs is not well understood yet. We used a ^{3}H-CH_4-radiotracer technique and *pmoA*-based molecular approaches to assess the activity, abundance and community structure of pelagic methanotrophs above active pockmarks in deep oligotrophic Lake Constance. Above profundal pockmarks, methane oxidation rates (up to 458 nmol CH_4 l^{-1} d^{-1}) exceeded those of the surrounding water column by two orders of magnitude and coincided with maximum methanotroph abundances of 0.6% of the microbial community. Phylogenetic analysis indicated a dominance of members of the Methylococcaceae in the water column of both, pockmark and reference sites, with most of the retrieved sequences being associated with a water-column specific clade. Communities at pockmark and reference locations also differed in parts, which was likely caused by entrainment of sediment-hosted methanotrophs at pockmark sites. Our results show that the release of seep-derived methane to the atmosphere is counteracted by a distinct methanotrophic community with a pronounced activity throughout bottom waters.

Keywords: pockmark; methane seep; Lake Constance; water column; aerobic methanotrophic bacteria; methane oxidation activity

INTRODUCTION

Methane is an important greenhouse gas and contributes substantially to radiative forcing, being second only to carbon dioxide among the long-lived greenhouse gases (Wuebbles and Hayhoe 2002; Ciais *et al.* 2013; Kirschke *et al.* 2013). Lakes and other freshwater systems are currently estimated to release about 40–103 Tg CH_4 yr^{-1}, making them an important natural source to the global methane budget (Bastviken *et al.* 2011; Ciais *et al.* 2013). Methane released from lakes is mainly produced by methanogenic archaea in anoxic sediments (Zehnder and Stumm 1988; Conrad 2009), although methane production has also been reported to occur in oxic water columns (Schmidt and Conrad 1993; Hofmann 2013; Bogard *et al.* 2014; Tang *et al.* 2014). While diffusing through surface sediments and water columns, a large proportion of the previously formed methane is oxidized to CO_2 by aerobic methanotrophic bacteria (e.g. Frenzel, Thebrath and Conrad 1990; Bastviken, Ejlertsson and Tranvik 2002; Deutzmann, Wörner and Schink 2011). These organisms are crucial for the transfer of methane-derived carbon and energy to higher trophic levels of aquatic food webs while reducing additional greenhouse gas fluxes to the atmosphere (Bastviken *et al.* 2003; Jones and Grey 2011; Sanseverino *et al.* 2012). Phylogenetically, they belong to the Alphaproteobacteria (type II methanotrophs; families Methylocystaceae and Beijerinckiaceae), Gammaproteobacteria (type I methanotrophs; families Methylococcaceae, Methylothermaceae and Crenotrichaceae) or Verrucomicrobia (type III methanotrophs; family Methylacidiphilaceae), with the latter being so far restricted to acidic habitats (Knief 2015). With exceptions among the Beijerinckiaceae, all aerobic methanotrophs can activate methane via a membrane-bound (particulate) methane monooxygenase (pMMO). Therefore, the *pmoA* gene, which encodes the α-subunit of pMMO, became the most commonly used functional marker gene to assess the diversity and abundance of methanotrophs in the environment (Knief 2015).

Pockmarks are crater-like depressions in the lake floor that result from gas or fluid emissions from subsurface sediments (Hovland and Judd 1988; Hovland, Gardner and Judd 2002). While pockmarks in marine environments have been investigated in numerous studies, reports from freshwater systems are still scarce (Pickrill 1993; Manley *et al.* 2004; Duck and Herbert 2006; Reusch *et al.* 2015). In Lake Constance, several hundred pockmarks were recently discovered near the entrance of the main tributary, the alpine River Rhine (Wessels *et al.* 2010; Bussmann *et al.* 2011). About 40% of them were found to be actively seeping methane of biogenic origin (Bussmann *et al.* 2013). Previous studies identified pockmark sediments as environmental niches sustaining distinct communities of aerobic methane-oxidizing bacteria with exceptionally high activity and abundance (Deutzmann, Wörner and Schink 2011). Studies conducted at non-seep areas in Lake Constance indicated that sediment-hosted aerobic methanotrophs are an important biofilter for methane ascending from the sediment into the water column (Frenzel, Thebrath and Conrad 1990). In addition, two recent studies established that denitrifying anaerobic methanotrophs represent the second major methane sink in benthic environments of Lake Constance (Deutzmann and Schink 2011; Deutzmann *et al.* 2014).

At pockmark sites where the direct release of methane bubbles into the water column bypasses sediment-associated methanotrophic activity, isotopic signals suggest that water column-associated methanotrophs play an important role in mitigating methane emissions (Bussmann *et al.* 2013). Therefore, the focus of our study was to investigate methanotrophic communities in the water column above methane-emitting seeps using radiotracer techniques and molecular approaches. Our objectives were (i) to determine whether methane oxidation activities in the water column are stimulated by plumes of methane (ii) to identify potential differences in methanotrophic abundances between seep and reference water columns and (iii) to assess how the water column-associated methanotrophic community composition compares between pockmark and reference sites.

METHODS

Study area

Lake Constance is an oligotrophic lake consisting of a large, deep basin (Upper Lake Constance) and a smaller, shallow basin (Lower Lake Constance). In most years, the mixing regime appears to be monomictic with a complete turnover of the water column in winter. Even during summer stratification, the entire water body of Upper Lake Constance exhibits elevated oxygen concentrations never falling below 6 mg l^{-1} close to the sediment surface (IGKB 2014). The alpine river Rhine represents the main tributary of the lake with 60%–65% of the total water inflow (Wessels *et al.* 2010).

Sampling procedure

Water column and sediment samples were taken on two cruises in spring (03 March 2012) and fall (17 October 2012) in the southeastern part of Upper Lake Constance near the entrance of the Alpine Rhine, where pockmarks had been located and mapped before (Wessels *et al.* 2010). Sampling was conducted with the help of a remotely operated vehicle that was operated from a research vessel. Samples were collected at profundal (ca. 80 m water depth at 47°31.38′N and 9°35.89′E) and littoral pockmark sites (ca. 9 m water depth at 47°29.97′N and 9°35.71′E). Deep (ca. 150 m at 47°32.82′N and 9°34.22′E) and shallow reference samples (ca. 10 m water depth at 47°30.82′N and 9°36.59′E) were taken outside the pockmark area.

Water sampling above profundal pockmarks and reference sites was performed using 5-l Niskin bottles attached to a rosette sampler, which was equipped with a Seabird Conductivity-Temperature-Depth probe. The sampling device was located within the methane plume of the profundal pockmark with the help of a horizontal scanning sonar (Kongsberg MS1000), which was deployed with a tripod at the lake floor as described previously (Wessels *et al.* 2010). Water column samples of littoral pockmarks were collected by scuba diving with Niskin bottles being positioned within the pockmark bubble stream just before closing. Samples were taken at different water depths: near the surface (above the thermocline), from intermediate water layers and close to the lake floor.

Profundal pockmark sediment cores and all reference sediment samples outside the pockmark area were obtained with a gravity multicorer. A horizontal scanning sonar was used to ensure sampling of seep sediment in close vicinity to the gas ebullition site. Littoral pockmark sediment was sampled by scuba diving, allowing most accurate sampling close to gas emanation sites. All obtained samples were taken to the laboratory as undisturbed sediment cores and stored at 4°C until further processing (within 3 days of collection).

Water column, methane concentrations and oxidation rates

Right after sampling, collected water was transferred from each Niskin bottle into duplicate 120-ml glass serum bottles. Bottles were flushed extensively with sample water, filled completely, closed with butyl stoppers and sealed with aluminum crimps. Excess water could escape via a needle in the stopper. Samples were poisoned with 0.3 ml of 5 M NaOH to avoid further methane oxidation. Headspace methane concentrations were analyzed as described in detail by Bussmann *et al.* (2013).

To determine methane oxidation rates (MOX), water samples were transferred aboard the research vessel to 120-ml glass serum bottles that were sealed gas-tight. Rate measurements were performed following a radiotracer technique using tritiated methane (American Radiolabeled Chemicals, St. Louis, MO, USA, 740 GBq $mmol^{-1}$). The tracer (5×10^5 Bq in 0.1 ml gas volume) was added to triplicate water samples and one killed control. Samples were incubated in the dark in a refrigerator near *in situ* temperatures (3°C –7°C). After ~24 h the incubation was stopped by adding 0.3 ml of 5 M NaOH. Following incubation, the radioactivity of the total amount of added tracer (^3H-$CH_4 + {}^3H$-H_2O) and of produced tritiated water (^3H-H_2O) was determined with a liquid scintillation counter (Tri-Carb 2910 TR, Perkin Elmer, Waltham, MA, USA) as described by Bussmann *et al.* (2015). Thereafter, the first order rate constant k' and MOX were calculated as follows:

$$k' = \left[{}^3H - H_2O / \left({}^3H - CH_4 + {}^3H - H_2O\right)\right] / t \quad (1)$$

$$MOX = k' \times [CH_4] \quad (2)$$

with the incubation time (t) and the ambient methane concentration $[CH_4]$ in nmol l^{-1}. The first order rate constant k' equals the fractional turnover rate, which can be used to compare the relative activity of different water samples (Heintz, Mau and Valentine 2012). A minor proportion of the tritiated water results typically from tracer contamination instead of methane oxidation (Bussmann *et al.* 2015), which however did not exceed 1.47% on average in our killed controls. The detection limit of the method applied was a MOX of 0.176 nmol CH_4 l^{-1} d^{-1}. The complete data set was deposited at www.pangaea.de (doi:10.1594/PANGAEA.854468).

DNA extraction

For total DNA extraction from water samples, 2–3 l of lake water were pre-filtered through a 70-μm and a 30-μm mesh to remove large detritus particles and eukaryotic organisms. Following pre-filtration, samples were subsequently filtered through a 5.0-μm and a 0.2-μm pore-size syringe filter (Minisart HF, Sartorius, Göttingen, Germany) using a peristaltic pump. After filtration, 1 ml of Tris-EDTA buffer (10 mM Tris/HCl pH 8.0, 10 mM EDTA) was injected into the filters. DNA extraction was performed as previously described by Kesberg and Schleheck (2013). In brief, filters were subjected to ultrasound for 30 s (35 kHz, peak-output 480 W) followed by backflushing with lysis solutions containing lysozyme, proteinase K and SDS. The DNA in the flow-through was purified and concentrated with DNA-binding magnetic beads (Dynabeads MyOne SILANE, Life Technologies, Carlsbad, CA, USA). DNA extraction from sediment samples proceeded right after transport to the laboratory. The uppermost 3 cm of undisturbed sediment cores were homogenized and total DNA extracted with the NucleoSpin soil kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions.

PCR amplification

Partial *pmoA* genes from sediment samples were amplified using the *pmoA* primer pair pmoA189f-mb661r to cover maximum *pmoA* diversity without co-amplification of closely related ammonia monooxygenase *amoA* genes (Costello and Lidstrom 1999). Amplification of partial *pmoA* genes derived from water samples was performed with the primer set wcpmoA189f-wcmb661r, which is a modified version of the pmoA189f-mb661r primer pair and was initially designed to amplify *pmoA* phylotypes from marine pelagic environments (Tavormina, Ussler and Orphan 2008). Compared to the conventional primer pair pmoA189f-mb661r used for the sediment samples, this primer pair provided a substantial enhancement in product yield from water column samples. The PCR reaction mixture consisted of 0.5 μl of Phire Hot Start II DNA Polymerase (Thermo Fisher Scientific, Ulm, Germany), 1× Phire Reaction Buffer, 200 μM of each dNTP, 1 μM of each primer, 10 ng of BSA (bovine serum albumin) and 3 μl of DNA template in a final volume of 25 μl. Initial denaturation at 98°C for 30 s was followed by 40 cycles of denaturation at 98°C for 15 s, annealing at 56.5°C for 45 s and elongation at 72°C for 1 min. The final elongation step was at 72°C for 10 min. Following amplification, all PCR products were purified using the DNA Clean and Concentrator kit (Zymo Research, Freiburg, Germany). Successful amplification was confirmed by gel electrophoresis on a 1.5% (w/v) agarose gel. PCR product concentrations were determined and purity-checked with a NanoDrop Spectrophotometer (Thermo Fisher Scientific).

qPCR analysis

Quantitative real time PCR (qPCR) analysis of *pmoA* gene abundances was performed with the wcpmoA189f-wcmb661r primer pair according to a previously described method (Deutzmann, Wörner and Schink 2011), with minor modifications. PCR reactions contained 10 ng of template DNA, 10 μl of SsoFast Evagreen Supermix with Low ROX (Bio-Rad, Munich, Germany) and 50 nM of each primer in a final volume of 20 μl. A total of 10 ng of BSA was added to the samples to prevent inhibition of amplification. Samples were analyzed in biological triplicates. Each biological replicate was analyzed in five technical parallels on an ABI 7500 fast machine (Applied Biosystems, Foster City, CA, USA). A two-step PCR protocol was used with an initial denaturation at 98°C for 2 min, followed by 40 cycles at 98°C for 6 s and 60°C for 30 s for annealing, elongation and data acquisition. Melting curves were obtained at 60°C–95°C at a 0.5°C-step heating rate and correlated well between environmental samples and *pmoA* standards. Calibration standards were obtained via 10-fold serial dilutions of a plasmid (PCR2.1, Invitrogen, Carlsbad, CA, USA) containing a single copy of the *pmoA* gene (HQ383803), with concentrations ranging from 10^1 to 10^7 copies per PCR reaction. Dilution series were included in five parallels in every run to determine the calibration curves and qPCR efficiencies (ranging between 86% and 95%). Partial inhibition of the qPCR assays was tested via 10-fold serial dilutions of an environmental sample resulting in no obvious signs of inhibition. To reduce the complexity of the data set, results obtained from the 0.2-μm and 5.0-μm fractions were combined in the final data analysis.

T-RFLP analysis

For terminal restriction fragment length polymorphism (T-RFLP) analysis, both the conventional reverse primer mb661r as well as the modified primer wcmb661r were labeled with 6-carboxyfluorescein. Samples were analyzed in biological

triplicates. A total of 200 ng of amplification product was digested by mixing with 0.5 U of the restriction endonuclease *Msp*I (recognition site C/CGG, Thermo Fisher Scientific). Digestions were carried out in a total volume of 20 μl for 3 h at 37°C. After heat inactivation of the enzyme at 80°C for 20 min, 10 ng of the digested samples was mixed with an internal DNA fragment length standard (0.5 μl diluted in 10 μl of water, GeneScan 500 ROX, Applied Biosystems). The standard contained 16 different 6-carboxy-X-rhodamine labeled fragments ranging in length from 35 to 500 nucleotides. Samples were denatured at 94°C for 5 min and immediately placed on ice. T-RFs (terminal restriction fragments) were size-separated by capillary electrophoresis on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). After electrophoresis, the sizes of the T-RF patterns ranging between 40 and 508 bp were determined by comparison with the internal standard using the Peak Scanner Software v1.0 (Applied Biosystems). Only T-RFs with peak areas of at least 50 fluorescence units were considered for analysis. Output files containing peak height and area were converted to binned T-RF tables using a R binning script (Ramette 2009). Relative fluorescence intensities of the peaks were calculated with this script by dividing individual peak areas by the total peak area for the respective samples. Again, to reduce the complexity of the data set, results obtained from the 0.2-μm and 5.0-μm fractions were combined in the final data analysis.

Cloning, sequencing and phylogenetic analysis

Clone libraries were generated from samples obtained in spring and fall from water columns above the profundal pockmark (40, 70 and 80 m water depth), the profundal reference site (145 m water depth) and the littoral pockmark (8 m water depth). Prior to cloning, blunt-end *pmoA* PCR products were generated as described above and subjected to an additional elongation step at 72°C for 10 min using 0.5 U of a recombinant *Taq* polymerase and 200 μM dATP in 1× reaction buffer to add A-overhangs. For cloning, the Topo TA cloning kit (Invitrogen) was used according to the manufacturer's instructions. Randomly selected clones were subjected to sequencing of the partial *pmoA* gene insert using the vector-specific M13 primer set and PCR conditions as provided in the manufacturer's instructions. Quality checks of the obtained sequences were conducted with FinchTV v1.5.0 (Perkin Elmer).

Sequence data were analyzed with the ARB software package (Ludwig *et al*. 2004). New *pmoA* sequences were added to a *pmoA* ARB database using the Fast Aligner tool implemented in the software. If necessary, alignments were corrected manually. Representative *pmoA* sequences obtained previously on Lake Constance habitats were included in the phylogenetic analysis (Pester *et al*. 2004; Bussmann, Rahalkar and Schink 2006; Rahalkar and Schink 2007; Rahalkar, Bussmann and Schink 2007; Deutzmann, Wörner and Schink 2011, 15 unpublished sediment sequences). Screening for chimeric sequences was performed by partial treeing as described previously by Pester *et al*. (2010). Sequences were grouped into operational taxonomic units (OTUs) based on a *pmoA* sequence identity threshold of 90% using the Mothur software package v1.33.3 (Schloss *et al*. 2009). Phylogenetic trees were re-constructed from distance matrices using the method of Fitch and Margoliash (1967) included in the PHYLIP software package (Felsenstein 1989). Bootstrapping was performed with the PHYLIP parsimony tool (100 replicates). Clone sequences were subjected to *in silico* T-RFLP analysis using the TRiFLe software (Junier, Junier and Witzel 2008) and assigned to experimentally determined T-RFs using a range of ±1 bp. Nucleotide sequences obtained in this study have been deposited at the National Center for Biotechnology Information under accession numbers KU140435 to KU140596.

Statistical analysis

The Shannon–Wiener (H′) index of diversity was calculated based on the relative fluorescence intensity values of the T-RFLP profiles using the BioDiversity Pro software package (McAleece *et al*. 1997). Multivariate statistical analyses were performed using the R software v3.1.3 (R-Core-Team 2015). The devtools package (Wickham and Chang 2015) and the ggbiplot package (Vu 2011) were used for creating principal component analysis (PCA) plots. To determine the number of ubiquitous and site-specific T-RFs, Venn diagrams were drawn from presence–absence data using Venny v2.0 (Oliveros 2007).

RESULTS

Distribution of methane and its oxidation in the profundal water column

The vertical distribution of methane was measured in the highly stratified water column (Fig. S1A, Supporting Information) at profundal pockmark and non-seep reference sites in fall. Methane concentrations in surface (5–10 m water depth) and intermediate (40–60 m water depth) water layers above seeps ranged on average from 40 to 125 nM and increased to a maximum of 1442 nM ∼10 m above the lake floor (70–80 m water depth) (Fig. 1A). In comparison, highest methane concentrations in the reference water column were detected near the water surface and did not exceed 53 nM. In spring, we observed a similar distribution pattern at pockmark and non-seep locations in the less stratified water column (Figs S1B and S2A, Supporting Information).

Vertical profiles of methane oxidation activities in the water column followed those of methane concentrations. Oxidation activities in surface and intermediate layers of the seep-related water column ranged from 3 to 63 nmol CH_4 l^{-1} d^{-1}, and maximum oxidation rates of 458 nmol CH_4 l^{-1} d^{-1} were observed in deeper layers ∼10 m above the pockmark. Below this depth in closer vicinity to ebullition sites, oxidation rates declined again to values in the range of 298 and 330 nmol CH_4 l^{-1} d^{-1} (Fig. 1B). Compared to pockmark sites, methanotrophic activities were considerably lower in surrounding reference water columns and did not exceed 31 nmol CH_4 l^{-1} d^{-1} in surface waters and 1.7 nmol CH_4 l^{-1} d^{-1} in bottom waters (Fig. 1B). In spring, methanotrophic activities showed similar trends at seep and reference sites, although activities were found to be consistently lower (Fig. S2B, Supporting Information).

As the calculations of methane oxidation activities are linearly dependent on methane concentrations, we also used the fractional turnover rates (or fraction of used tracer) to characterize the methanotrophic activity. For pockmark water columns, we detected high turnover rates of 0.027 h^{-1} in surface water layers, which decreased in midwater layers to 0.002–0.006 h^{-1} and increased again to a maximum of 0.019 h^{-1} in bottom water layers above pockmarks. Reference sites showed a different distribution of turnover rates throughout the water column. In subsurface waters, turnover rates were similar to pockmark sites with a maximum of 0.025 h^{-1}, showed a large variance in midwaters but decreased substantially in bottom waters to a maximum of 0.006 h^{-1}. In spring, turnover rates were low (<0.010 h^{-1}) in the upper and midwater column for both, pockmark and

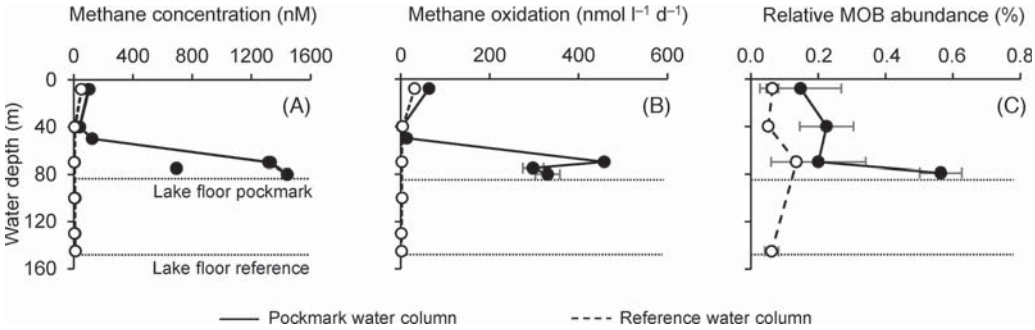

Figure 1. Methanotrophic activity and abundance as determined for profundal pockmark and reference stations in fall. (**A**) Vertical profiles of methane concentrations as determined and shown in duplicates (duplicates most often overlap). The outlier at 75-m depth in the pockmark water column was only determined once and therefore not included into the trendline. (**B**) MOX from $^{3}H\text{-}CH_4$ turnover measurements as determined in triplicates (mean ±st. dev. is given). (**C**) Relative abundance of aerobic methane-oxidizing bacteria (MOB) as determined in triplicates by *pmoA*-based qPCR (mean ±st. dev. is given).

reference sites. As in fall, turnover rates increased towards bottom waters at pockmark (max. 0.017 h^{-1}) but not at non-seep sites (max. 0.008 h^{-1}) (Fig. S3, Supporting Information).

Abundance profiles of pelagic methanotrophs

To gain deeper insights into the vertical distribution of aerobic methanotrophs in the profundal zone, we selected the fall samples for qPCR analysis targeting the *pmoA* gene. The relative abundance of methanotrophs was estimated by calculating the ratio of *pmoA* copy numbers to total microbial genome copies as inferred from the total DNA amount used for qPCR and assuming that a methanotrophic cell contains two *pmoA* copies (Kolb *et al.* 2003), on average a bacterial species has a genome size of 4 Mbp (Islas *et al.* 2004) (equivalent to 4.3×10^{-6} ng double-stranded DNA), and most of the extracted DNA is from living microorganisms. At profundal pockmark sites, abundances of methanotrophic populations were found to be distributed quite unevenly throughout the water column. Abundances turned out to be lowest in surface water layers where methanotrophs contributed on average 0.1% of the total microbial community. They increased with depth and were found to be most abundant in bottom water layers close to methane bubble release, accounting for up to 0.6% of the total microbial community. Methanotrophic populations recovered from profundal reference samples were detected at considerably lower abundances as compared to pockmark-related sites. They first increased with depth, reaching a maximum relative abundance of 0.1% at intermediate water layers. At deeper water layers, abundances decreased again and were similar to those obtained from surface layers (Fig. 1C). This analysis was extended also to littoral water columns above pockmark and non-seep sites in fall. Methanotrophs contributed an extraordinary high percentage to the total microbial community in water samples taken above littoral pockmarks, ranging on average from 3.8% to 7.1%. In this habitat, numbers of methanotrophic bacteria slightly increased at greater water depth proximal to methane venting. Compared to pockmark sites, substantially lower abundances were observed in shallow water columns without seep influence, where maximum values were restricted to 0.04% (Fig. S4, Supporting Information).

Comparison of pockmark and reference site related methanotrophic communities

T-RFLP analysis of amplified *pmoA* fragments was used as a rapid and reproducible method for comparing methanotrophic communities in the water column above profundal and littoral pockmarks as well as surrounding reference sites. In fall, a total of 39 T-RFs were detected of which 18 (46%) were shared among all sample locations. We detected the considerable number of nine T-RFs (23%) that occurred exclusively at seep-related sites, with only one of these T-RFs each being exclusively detected above profundal or littoral pockmarks, respectively. In comparison, only one T-RF (3%) appeared to be restricted to non-seep environments (Fig. 2B).

Multivariate statistical analysis was performed to test for discernable community differentiation among sampling locations. PCA biplots of the first two principal components clearly showed that methanotrophic populations at pockmark and non-seep sites clustered separately from each other. Furthermore, littoral samples were separated from profundal ones. Differing relative fluorescence intensities of a 502-bp T-RF had the largest impact on clustering of T-RFLP profiles (Fig. 2A). Compared to pockmark samples, this T-RF was found to be more abundant throughout littoral and profundal water columns without seep influence. Besides this *pmoA* amplicon, T-RFs with a length of 53, 102, 128, 72, 63, 40 and 96 bp accounted each for at least 5% of the variance explained by one of the first two dimensions (listed in decreasing order of explained variance). Among them, T-RFs of 102, 72 and 40 bp exhibited considerably higher abundances at littoral and profundal pockmarks relative to corresponding non-seep sites (Fig. S5, Supporting Information). In addition, differentiation of methanotrophic communities among sites was assessed by calculating Shannon–Wiener (H′) diversity indices based on the observed T-RFs. Seep-associated water columns were found to possess a consistently higher methanotrophic diversity ($H' = 1.17–1.29$) than those located off seeps ($H' = 0.87–1.03$) (Table S1, Supporting Information).

T-RFLP profiles of water column-associated methanotrophic populations were also compared to profiles of sediment-affiliated methanotrophs, both for littoral and profundal sites. In fall, a total of 46 T-RFs were observed of which 13 T-RFs (28%) were shared among water columns and sediments at pockmark sites. A total of 22 T-RFs (48%) occurred exclusively in seep-related water columns, whereas only 8 T-RFs (17%) were restricted to pockmark sediments (Fig. 3B). In accordance with these results, PCA biplots showed that water column-related and sediment-hosted methanotrophs formed distinct clusters (Fig. 3A). However, comparability of water column and sediment samples is partially limited due to different DNA extraction and *pmoA* amplification procedures.

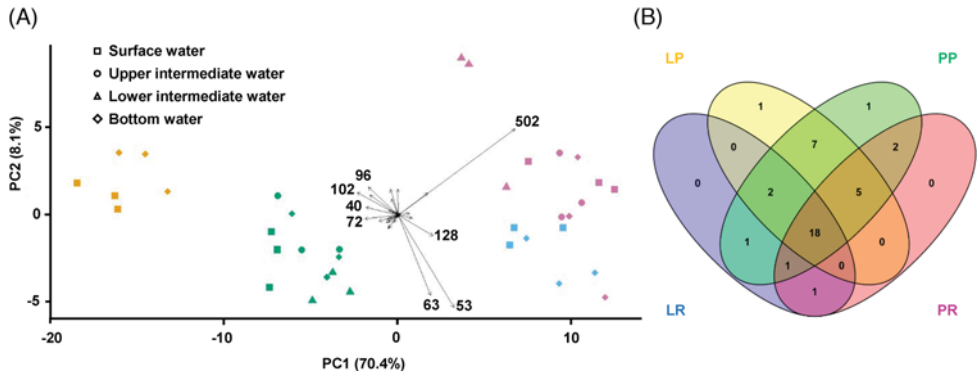

Figure 2. Beta-diversity analysis of aerobic methanotrophs in water columns overlying profundal and littoral pockmark and reference sites in fall. (**A**) PCA biplot of *pmoA*-based T-RFLP profiles. The first two principal components are shown, explaining 78.5% of the total variance. Arrows represent individual T-RFs. The length and direction of the arrows indicate the contribution of the variables to the principal components of the biplot. T-RFs with a relative contribution of ≥5% to either one of the principal components are labeled with their respective length. Different colors indicate different sampling locations: littoral pockmark (yellow), profundal pockmark (green), littoral reference (blue) and profundal reference (red). (**B**) Venn diagram illustrating the distribution of T-RFs between depth-integrated pockmark and reference water samples at littoral and profundal sampling sites. Diagrams are based on presence or absence of T-RFs. LP: littoral pockmark, PP: profundal pockmark, LR: littoral reference, PR: profundal reference.

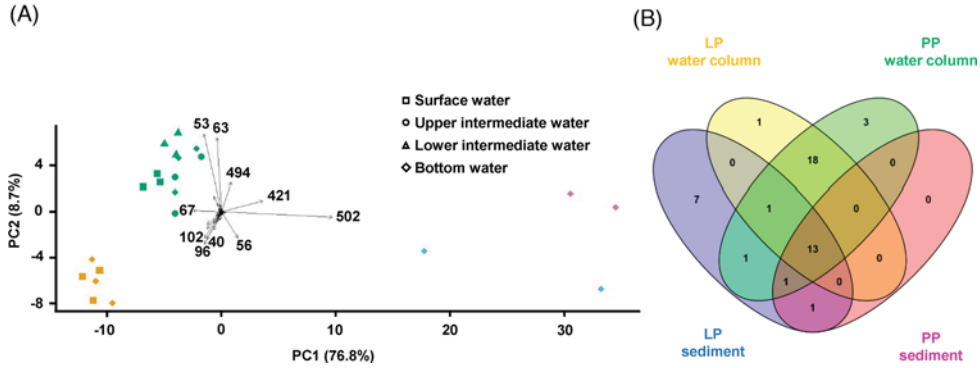

Figure 3. Beta-diversity analysis of aerobic methanotrophs residing in sediments and water columns of littoral and profundal pockmarks in fall. (**A**) PCA biplot of *pmoA*-based T-RFLP profiles obtained from the water column and sediment-hosted methanotrophic communities at littoral and profundal pockmark locations. The first two principal components are displayed, accounting for 85.5% of the total variance. Arrows represent individual T-RFs. The length and direction of the arrows indicate the contribution of the variables to the principal components of the biplot. Arrows with a relative contribution of ≥5% to either one of the principal components are labeled with the corresponding T-RF length. Colors denote sampling locations: littoral pockmark water column (yellow), profundal pockmark water column (green), littoral pockmark sediment (blue) and profundal pockmark sediment (red). (**B**) Venn diagram depicting the distribution of T-RFs between water column and sediment samples of littoral and profundal pockmarks. LP: littoral pockmark, PP: profundal pockmark.

Phylogenetic identity of pelagic methanotrophs

To assess the phylogenetic relatedness of water column-affiliated and sediment-hosted methanotrophic bacteria at pockmark and non-seep sites, *pmoA* clone libraries were generated from lake water sampled above pockmark and reference sites in fall and spring (Table S2, Supporting Information). Sequences of 147 clones were analyzed together with 228 sediment-affiliated sequences obtained previously from pockmark and non-seep locations in Lake Constance (Pester *et al.* 2004; Bussmann, Rahalkar and Schink 2006; Rahalkar and Schink 2007; Rahalkar, Bussmann and Schink 2007; Rahalkar *et al.* 2009; Deutzmann, Wörner and Schink 2011, 15 unpublished sequences). Using a 90%-sequence identity threshold to distinguish among different methanotrophic bacteria at the approximate species-level (Heyer, Galchenko and Dunfield 2002; Lüke *et al.* 2010), a total of 69 OTUs were observed to reside at different sites in Lake Constance. Reconstruction of a phylogenetic tree revealed a diverse community of Methylococcaceae among these OTUs (Fig. 4), which clustered into the so-called type Ia methanotrophs (Knief 2015). The largest proportion of water column-derived *pmoA* phylotypes formed a clade that was distinct from sediment-hosted methanotrophs (maximum of 84.3% *pmoA* nucleotide identity) and *Methylovulum miyakonense* as closest cultured relative (maximum of 86.2% *pmoA* nucleotide identity). Phylotypes affiliated with this water column-specific clade were recovered from waters above profundal and littoral pockmarks as well as from profundal reference sites (Fig. 4; Table S2, Supporting Information). Phylotypes closely related to *Methylobacter* species constituted another large fraction of our and previous clone libraries. These OTUs were obtained from pockmark and non-seep sediments but were only detected in the water column above pockmarks so far. Only a small proportion of the retrieved sequences could be affiliated to Methylococcaceae representatives of the so-called type Ib methanotrophic bacteria. Members grouping within this cluster were recovered from sediments and seep-associated water columns and were distantly related to cultivated representatives of the genera *Methylococcus* and *Methylocaldum* (62.1%–85.3% *pmoA* nucleotide identity). Methanotrophic bacteria belonging to the Alphaproteobacteria (type II) were not detected in the analyzed water column samples and were restricted with four OTUs to non-seep sediments (Fig. 4).

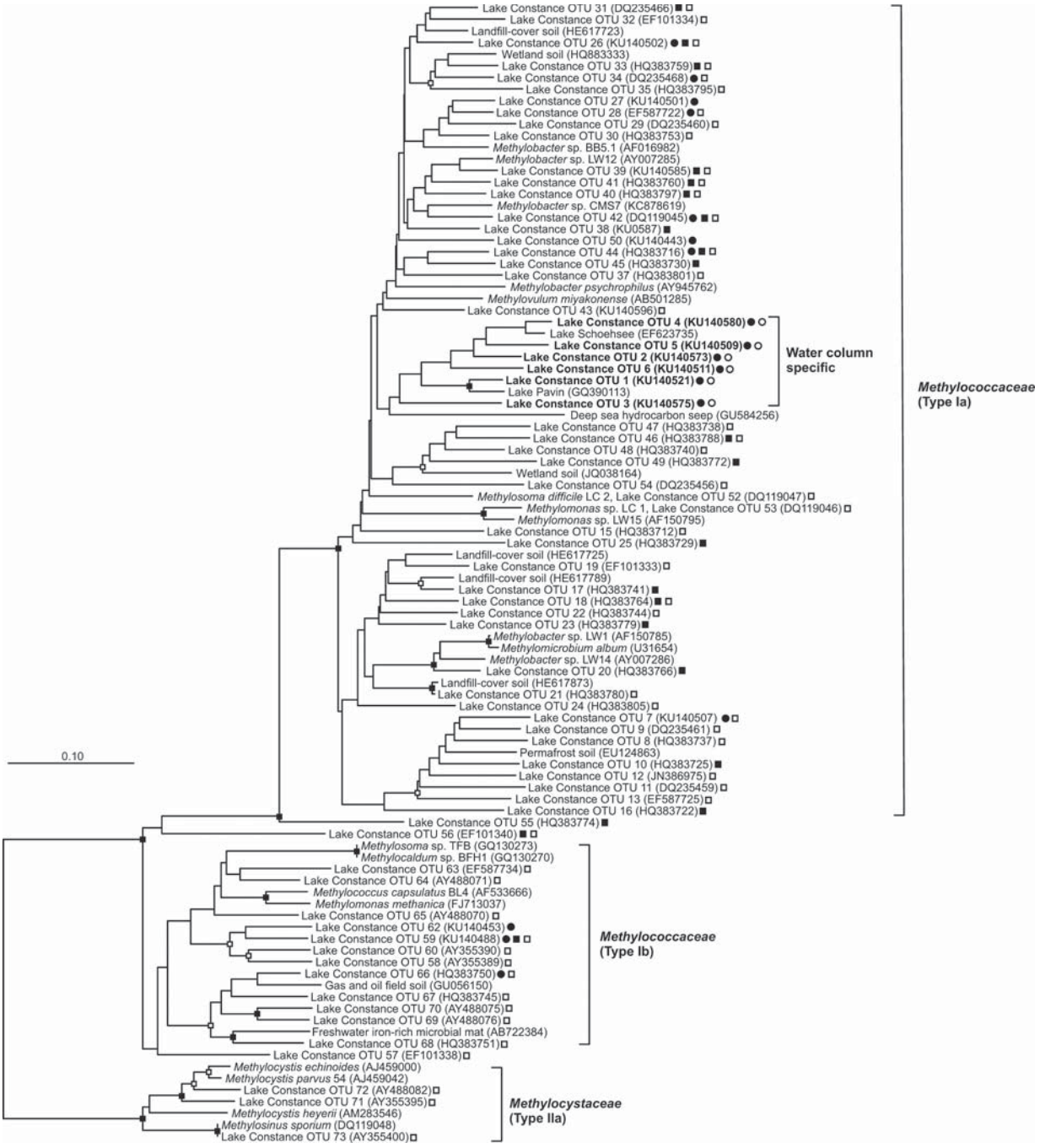

Figure 4. Phylogenetic tree based on the Fitch–Margoliash algorithm illustrating the phylogenetic relationship of *pmoA* fragments retrieved from littoral and profundal pockmark (filled circles) and reference site (open circles) water columns as well as from pockmark (filled squares) and non-seep (open squares) sediments obtained in this and previous studies on Lake Constance (LC). Approximate species-level OTUs clustered at 90% nucleotide sequence identity are displayed by their representing sequence (the respective NCBI accession number is displayed in brackets). OTUs belonging to the water-column specific cluster are displayed in bold. OTUs 52 and 53 are represented by pure cultures obtained from Lake Constance. The number of water-column-derived clone sequences contained in each OTU is given in Table S2, Supporting Information. Open and filled squares along the branches of the phylogenetic tree indicate lineages with ≥70% and ≥90% maximum parsimony bootstrap support, respectively. The scale bar represents 10% estimated sequence divergence.

Dominance of Methylococcacea representatives belonging to type Ia methanotrophs was corroborated by comparison of *in silico* T-RFs of obtained *pmoA* clones and the actual T-RFs of the analyzed samples. Sequences with the *Msp*I recognition site at 502 bp were found to constitute a substantial part of all T-RFLP-analyzed samples (Figs S5 and S6, Supporting Information) and could clearly be assigned to this group (Table S2, Supporting Information). The same was true for the T-RF at 72 bp. *In silico* digestion of cloned *pmoA* fragments further showed that T-RFs of 53, 64 and 77 bp were related to Methylococcacea representatives belonging to type Ib methanotrops (Table S2, Supporting Information). Despite our sequencing effort and meta-analysis of

existing *pmoA* clones from Lake Constance, the bulk of T-RFs observed in the environmental samples could not be assigned to a specific cloned *pmoA* sequence.

DISCUSSION

Pockmarks sustain a highly active methanotrophic community

Pockmarks are just starting to be discovered in freshwater lakes (Pickrill 1993; Manley *et al*. 2004; Duck and Herbert 2006; Wessels *et al*. 2010; Reusch *et al*. 2015) but their influence on the biogeochemistry and ecology of the overlying pelagic zone has not yet been explored in detail. Lake Constance, which is an important and well-studied model system for lakes, is characterized by hundreds of pockmarks near the entrance of the main tributary, the Alpine River Rhine (Wessels *et al*. 2010; Bussmann *et al*. 2011), with roughly 40% of them actively seeping methane of biogenic origin (Bussmann *et al*. 2013). We observed methane concentrations that were up to two orders of magnitude higher directly above seep locations as compared to reference sites, indicating that pockmarks represent an important source of methane in the water column of Lake Constance near the Alpine Rhine estuary (Fig. 1A and S2A, Supporting Information). Above pockmarks, methane concentrations steeply decreased in intermediate and surface water layers to concentrations similar to reference sites. Such distribution patterns were congruent with those measured previously above profundal pockmarks in Lake Constance (Bussmann *et al*. 2013). Ascending methane bubbles with an initial diameter of 5 mm are predicted to dissolve almost completely in the hypolimnion of Lake Constance, resulting in hardly any methane input into epilimnetic water layers (Bussmann *et al*. 2013). This is also supported by our data and has been described in studies on methane seeps and methane gas hydrates in marine environments (Valentine *et al*. 2001; Durisch-Kaiser *et al*. 2005). At reference stations, methane concentrations were highest in surface waters and thus could not result from methane escaping methanogenic sediments. Methane peaks in surface waters have been repeatedly observed in pelagic environments of Lake Constance and other lakes (Schmidt and Conrad 1993; Hofmann, Federwisch and Peeters 2010; Grossart *et al*. 2011; Hofmann 2013; Bogard *et al*. 2014; Tang *et al*. 2014; Blees *et al*. 2015). The source of this epilimnetic methane is still a matter of discussion but lateral transport from nearshore littoral zones (Schmidt and Conrad 1993; Hofmann, Federwisch and Peeters 2010) and methane production in anoxic microenvironments such as detrital particles and digestive tracts of zooplankton (Schmidt and Conrad 1993; Schulz *et al*. 2001; Blees *et al*. 2015) have been suggested for pre-alpine lakes like Lake Constance. In analogy to alternative pathways of methane formation proposed for marine environments, aerobic decomposition of methylated compounds could also contribute to methane accumulation in near-surface water layers (Karl *et al*. 2008; Damm *et al*. 2010; Metcalf *et al*. 2012).

Methane that dissolves from emanating bubbles can either be diluted with methane-depleted surrounding water or can be oxidized by methanotrophic bacteria (Bussmann *et al*. 2013). In the deep hypolimnion close to gas ebullition sites, elevated methane concentrations apparently stimulated methanotrophic activities, resulting in MOX of up to 458 nmol CH_4 l^{-1} d^{-1}. In comparison, at intermediate and surface water depths, the rates were only 3–63 nmol CH_4 l^{-1} d^{-1} and were comparable to values obtained at non-seep reference sites. Our data confirm earlier results derived from depth profiles of methane isotopic carbon signatures, which indicated that around 90% of the pockmark-derived methane is oxidized within the deep hypolimnion (Bussmann *et al*. 2013). Thus, methane availability appears to largely determine the activity of methanotrophic bacteria, as has been repeatedly observed in studies on lake water columns and on marine pelagic environments adjacent to methane seeps or methane gas hydrates (Valentine *et al*. 2001; Durisch-Kaiser *et al*. 2005; Sundh, Bastviken and Tranvik 2005; Kankaala *et al*. 2006; Zakharenko *et al*. 2015). The positive correlation between methane concentrations and MOX should be typically double-checked since activity calculations depend linearly on methane concentrations (see Material and Methods). Therefore, we additionally followed the fractional turnover rate of methane, which is an activity measure independent of ambient concentrations (Heintz, Mau and Valentine 2012; Osudar *et al*. 2015). Fractional turnover rates overlapped for pockmark- and reference-related surface and intermediate water layers, whereas bottom waters above seep-derived methane plumes exhibited 2–3 times higher fractional turnover rates as compared to reference sites. Thus, both methane oxidation and fractional turnover rates indicate that the water column-associated methanotrophic community is stimulated by methane emissions from profundal pockmarks and is able to maintain a high level of activity throughout near-bottom waters.

Pelagic methanotrophs are most abundant in waters directly overlying pockmarks

We performed *pmoA*-based qPCR analysis to assess the distribution of methanotrophs in the water column above profundal and littoral pockmark and reference sites. During our fall survey of the profundal zone, bottom water layers close to gas-emitting sites harbored increased numbers of methane-oxidizing bacteria, which decreased in intermediate and surface water layers to abundances observed at reference sites (Fig. 1C). This increased relative abundance in the deep hypolimnion above profundal pockmarks coincided with elevated methane concentrations and oxidation activities (Fig. 1). Thus at these locations, rates of methane oxidation seem to be controlled by methanotrophic population size. This is supported by a general positive relationship between methane supply, oxidation capacities and methanotrophic abundances as being observed also for other lakes (Ross *et al*. 1997; Sundh, Bastviken and Tranvik 2005; Oswald *et al*. 2015).

The relative abundances of methanotrophs above littoral seeps were even one to two orders of magnitude higher than those obtained at littoral reference sites (Fig. S4, Supporting Information). These results coincide with higher abundances of sediment-hosted methanotrophs at littoral pockmarks as compared to reference sites (Deutzmann, Wörner and Schink 2011). Above littoral pockmarks, methane-oxidizing bacteria were found to be quite evenly distributed throughout the water column and increased only slightly in close vicinity to methane plumes. In contrast to profundal pockmarks, methane released from littoral pockmark sediments has been described to pass the water column with nearly no dissolution and oxidation (Bussmann *et al*. 2013). A similar observation has been made in Lake Baikal, reporting escape of methane from shallow sources into the atmosphere (Schmid *et al*. 2007). As DNA-based qPCR analysis does not reflect microbial activity, we could not distinguish between active methanotrophs and resting stages or spores. Inactive methanotrophic bacteria have been shown to constitute a substantial part of the methanotrophic community in pockmark and non-seep sediments of Lake Constance (Rahalkar *et al*.

2009; Deutzmann, Wörner and Schink 2011). Thus, enhanced abundances of methanotrophs above shallow pockmarks might not primarily depend on increased methane concentration but could, at least in parts, result from release of sediment-hosted methanotrophs to the overlying water column by gas ebullition. Irrespective of the reason of enhanced methanotroph abundances above profundal and littoral pockmarks, their increased biomass in the water column might represent a crucial link for re-entry of methane-derived carbon and energy into the pelagic food web that would otherwise be emitted as additional greenhouse gas to the atmosphere (Sundh, Bastviken and Tranvik 2005; Jones and Grey 2011).

Members of the Methylococcaceae dominate the methanotrophic community in the pelagic zone of Lake Constance

The major part of the methane-oxidizing community was shared between pockmark and reference water columns, as indicated by *pmoA*-based T-RFLP analysis (Fig. 2B). However, we also detected a considerable number of methanotrophs (ca. 20% of T-RFs) whose presence was apparently supported by local pockmark-specific conditions. Surprisingly, we detected a stable community composition of methanotrophs throughout the entire water column above deep seep locations, which was apparently not coupled to the greater availability of methane in close proximity to profundal gas emanation sites (Fig. 2A). Here, other parameters such as oxygen concentrations, nitrogen availability or trace metal concentrations may have been the major factors shaping the methanotrophic community as has been suggested previously for marine waters overlying seep and reference sites (Durisch-Kaiser *et al.* 2005). We also detected a partial overlap of water column-associated and sediment-affiliated methanotrophic communities at littoral and profundal pockmarks (Fig. 3). While the majority of sediment-affiliated methanotrophs could be detected also in the water column, the opposite was not true. This type of overlap is not surprising as sediment re-suspension at gas emanation sites likely results in a benthic-pelagic exchange of methanotrophs. Such a (methane) bubble-related transport of benthic methanotrophs into the overlying water column has been also suggested for the marine environment (Schmale *et al.* 2015).

The phylogenetic composition of pelagic methanotrophs was dominated by a diverse community of gammaproteobacterial Methylococcaceae (members of type I methanotrophic bacteria), whereas alphaproteobacterial methanotrophs (type II) were not detected. A predominance of gammaproteobacterial (type I) over alphaproteobacterial (type II) methanotrophs could have been caused by a partial discrimination of the reverse primer mb661r against *pmoA* variants within the Alphaproteobacteria (Knief 2015). However, a dominance of methanotrophic Gammaproteobacteria has previously been reported for pockmark and non-seep sediments in Lake Constance (Pester *et al.* 2004; Rahalkar and Schink 2007; Deutzmann, Wörner and Schink 2011) and from various other studies on methanotrophic communities in benthic and pelagic freshwater environments (Ross *et al.* 1997; Costello *et al.* 2002; Carini *et al.* 2005; Nercessian *et al.* 2005; Sundh, Bastviken and Tranvik 2005; Biderre-Petit *et al.* 2011; Grossart *et al.* 2011; Oswald *et al.* 2015). We did not check for methanotrophs within the Beijerinckiaceae that contain only a soluble (cytoplasmic) methane monooxygenase but not the particulate (membrane-bound) enzyme targeted by the *pmoA* gene. However, a recent review on methanotrophs revealed that members of the Beijerinckiaceae have been detected preferentially in various soil environments rather than in freshwater habitats (Knief 2015). Therefore, our results indicate that members of the Methylococcaceae might play a key role in methane oxidation in Lake Constance, regardless of habitat-dependent selection pressures due to changes in methane and oxygen concentrations or nutrient availability.

The majority of water column-derived *pmoA* phylotypes formed a clade that was distinct from sediment-affiliated methanotrophs and only distantly related (≤86% nucleotide sequence identity) to methanotrophs of the genera *Methylovulum* and *Methylobacter* (type Ia methanotrophs, Fig. 4). Phylotypes affiliated with this clade were retrieved from both pockmark and reference sites, indicating a widespread distribution of these yet uncultivated bacteria in the water column of Lake Constance. Another considerable proportion of the retrieved sequences clustered into groups that were more closely related to *Methylobacter* species (also type Ia). Sediment-associated phylotypes of this cluster were recovered from pockmark and non-seep locations, whereas water column-associated ones solely occurred in pockmark surroundings. Again, this indicates entrainment of sediment-hosted methanotrophs into the water column by sediment re-suspension at gas emanation sites (Schmale *et al.* 2015).

Unfortunately, we were not able to relate all obtained T-RFs to methanotrophic phylotypes despite our sequencing effort. Nevertheless, both the T-RFLP analysis and the meta-analysis of *pmoA* clones obtained from pelagic and benthic pockmark and reference sites indicated that the methanotrophic community in waters overlying pockmarks are shaped by water-associated methanotrophs as well as sediment-affiliated methanotrophs that enter the water column by gas ebullition (Schmale *et al.* 2015).

CONCLUSIONS

In methane-seeping pockmark areas of freshwater lakes, the flux of methane exceeds the capacity of sediment-hosted methanotrophs to counteract release of this potent greenhouse gas. In this study, we show for the first time that in a deep pre-alpine lake pockmark-overlying waters harbor a methanotrophic community that is shaped by pelagic and seep-associated methanotrophs and displays a pronounced metabolic activity coupled to an increased population size. This water column-associated community is dominated by members of the Methylococcaceae and acts as an effective biological filter in the deep hypolimnion, precluding the release of most of seep-associated methane to the atmosphere.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

ACKNOWLEDGEMENTS

We are grateful to Martin Wessels, to the scientific diving groups of the Alfred Wegener Institute and the University Konstanz, and to the crew and captains of R/V Kormoran for enabling sampling. We also thank Martin Wessels and Michal Tomczyk for providing a digital elevation model illustration of pockmarks on the lake floor of Lake Constance. We additionally thank Claudia Lüke for providing a detailed *pmoA* ARB database and Benjamin Frommeyer, Manuel Hornung and Svenja Mayer for help with sampling, qPCR and T-RFLP analyses. Further thanks go to K.-W. Klings for excellent technical assistance in methane analysis and David Schleheck and Anna Kesberg for the introduction to the used DNA extraction procedure.

FUNDING

Funding was provided by the German Research Foundation (SCHI 180/14-1 to Bernhard Schink, BU 996/5-1 to Ingeborg Bussmann) and the Zukunftskolleg of the University of Konstanz (to Michael Pester). The authors declare no conflict of interest.

Conflict of interest. None declared.

REFERENCES

Bastviken D, Ejlertsson J, Sundh I *et al*. Methane as a source of carbon and energy for lake pelagic food webs. *Ecology* 2003;**84**:969–81.

Bastviken D, Ejlertsson J, Tranvik L. Measurement of methane oxidation in lakes: a comparison of methods. *Environ Sci Technol* 2002;**36**:3354–61.

Bastviken D, Tranvik LJ, Downing JA *et al*. Freshwater methane emissions offset the continental carbon sink. *Science* 2011;**331**:50.

Biderre-Petit C, Jézéquel D, Dugat-Bony E *et al*. Identification of microbial communities involved in the methane cycle of a freshwater meromictic lake. *FEMS Microbiol Ecol* 2011;**77**:533–45.

Blees J, Niemann H, Erne M *et al*. Spatial variations in surface water methane super-saturation and emission in Lake Lugano, southern Switzerland. *Aquat Sci* 2015;**77**:535–45.

Bogard MJ, del Giorgio PA, Boutet L *et al*. Oxic water column methanogenesis as a major component of aquatic CH_4 fluxes. *Nat Commun* 2014;**5**, DOI: 10.1038/ncomms6350.

Bussmann I, Damm E, Schlüter M *et al*. Fate of methane bubbles released by pockmarks in Lake Constance. *Biogeochemistry* 2013;**112**:613–23.

Bussmann I, Matousu A, Osudar R *et al*. Assessment of the radio ^3H-CH_4 tracer technique to measure aerobic methane oxidation in the water column. *Limnol Oceanogr-Meth* 2015;**13**:312–27.

Bussmann I, Rahalkar M, Schink B. Cultivation of methanotrophic bacteria in opposing gradients of methane and oxygen. *FEMS Microbiol Ecol* 2006;**56**:331–44.

Bussmann I, Schlömer S, Schlüter M *et al*. Active pockmarks in a large lake (Lake Constance, Germany): effects on methane distribution and turnover in the sediment. *Limnol Oceanogr* 2011;**56**:379–93.

Carini S, Bano N, LeCleir G *et al*. Aerobic methane oxidation and methanotroph community composition during seasonal stratification in Mono Lake, California (USA). *Environ Microbiol* 2005;**7**:1127–38.

Ciais P, Sabine C, Bala G *et al*. Carbon and other biogeochemical cycles. In: Stocker TF, Qin D, Plattner G-K (eds). *Climate Change 2013 – The Physical Science Basis: Working Group I Contribution to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge: Cambridge University Press, 2013, 465–571.

Conrad R. The global methane cycle: recent advances in understanding the microbial processes involved. *Environ Microbiol Rep* 2009;**1**:285–92.

Costello AM, Auman AJ, Macalady JL *et al*. Estimation of methanotroph abundance in a freshwater lake sediment. *Environ Microbiol* 2002;**4**:443–50.

Costello AM, Lidstrom ME. Molecular characterization of functional and phylogenetic genes from natural populations of methanotrophs in lake sediments. *Appl Environ Microb* 1999;**65**:5066–74.

Damm E, Helmke E, Thoms S *et al*. Methane production in aerobic oligotrophic surface water in the central Arctic Ocean. *Biogeosciences* 2010;**7**:1099–108.

Deutzmann JS, Schink B. Anaerobic Oxidation of methane in sediments of Lake Constance, an oligotrophic freshwater lake. *Appl Environ Microb* 2011;**77**:4429–36.

Deutzmann JS, Stief P, Brandes J *et al*. Anaerobic methane oxidation coupled to denitrification is the dominant methane sink in a deep lake. *P Natl Acad Sci USA* 2014;**111**:18273–8.

Deutzmann JS, Wörner S, Schink B. Activity and diversity of methanotrophic bacteria at methane seeps in eastern Lake Constance sediments. *Appl Environ Microb* 2011;**77**:2573–81.

Duck RW, Herbert RA. High-resolution shallow seismic identification of gas escape features in the sediments of Loch Tay, Scotland: tectonic and microbiological associations. *Sedimentology* 2006;**53**:481–93.

Durisch-Kaiser E, Klauser L, Wehrli B *et al*. Evidence of intense archaeal and bacterial methanotrophic activity in the Black Sea water column. *Appl Environ Microb* 2005;**71**:8099–106.

Felsenstein J. PHYLIP-phylogeny inference package. *Cladistics* 1989;**5**:164–6.

Fitch WM, Margoliash E. Construction of phylogenetic trees. *Science* 1967;**155**:279–84.

Frenzel P, Thebrath B, Conrad R. Oxidation of methane in the oxic surface layer of a deep lake sediment (Lake Constance). *FEMS Microbiol Ecol* 1990;**6**:149–58.

Grossart H-P, Frindte K, Dziallas C *et al*. Microbial methane production in oxygenated water column of an oligotrophic lake. *P Natl Acad Sci USA* 2011;**108**:19657–61.

Heintz MB, Mau S, Valentine DL. Physical control on methanotrophic potential in waters of the Santa Monica Basin, Southern California. *Limnol Oceanogr* 2012;**57**:420–32.

Heyer J, Galchenko VF, Dunfield PF. Molecular phylogeny of type II methane-oxidizing bacteria isolated from various environments. *Microbiology* 2002;**148**:2831–46.

Hofmann H. Spatiotemporal distribution patterns of dissolved methane in lakes: how accurate are the current estimations of the diffusive flux path? *Geophys Res Lett* 2013;**40**:2779–84.

Hofmann H, Federwisch L, Peeters F. Wave-induced release of methane: littoral zones as source of methane in lakes. *Limnol Oceanogr* 2010;**55**:1990–2000.

Hovland M, Gardner JV, Judd AG. The significance of pockmarks to understanding fluid flow processes and geohazards. *Geofluids* 2002;**2**:127–36.

Hovland M, Judd AG. *Seabed Pockmarks and Seepages: Impact on Geology, Biology, and the Marine Environment*. London: Graham and Trotman, 1988.

IGKB (ed.). *Jahresbericht der Internationalen Gewässerschutzkommission für den Bodensee: Limnologischer Zustand des Bodensees Nr. 40 (2012–2013)*, Vol. 40. Langenargen, Germany: Internationale Gewässerschutzkomission für den Bodensee, 2014, 112.

Islas S, Becerra A, Luisi PL *et al*. Comparative genomics and the gene complement of a minimal cell. *Origins Life Evol B* 2004;**34**:243–56.

Jones RI, Grey J. Biogenic methane in freshwater food webs. *Freshwater Biol* 2011;**56**:213–29.

Junier P, Junier T, Witzel K-P. TRiFLe, a program for in silico terminal restriction fragment length polymorphism analysis with user-defined sequence sets. *Appl Environ Microb* 2008;**74**:6452–6.

Kankaala P, Huotari J, Peltomaa E *et al*. Methanotrophic activity in relation to methane efflux and total heterotrophic

bacterial production in a stratified, humic, boreal lake. *Limnol Oceanogr* 2006;**51**:1195–204.
Karl DM, Beversdorf L, Bjorkman KM *et al*. Aerobic production of methane in the sea. *Nat Geosci* 2008;**1**:473–8.
Kesberg AI, Schleheck D. Improved protocol for recovery of bacterial DNA from water filters: sonication and backflushing of commercial syringe filters. *J Microbiol Meth* 2013;**93**:55–7.
Kirschke S, Bousquet P, Ciais P *et al*. Three decades of global methane sources and sinks. *Nat Geosci* 2013;**6**:813–23.
Knief C. Diversity and habitat preferences of cultivated and uncultivated aerobic methanotrophic bacteria evaluated based on pmoA as molecular marker. *Front Microbiol* 2015;**6**: 1346.
Kolb S, Knief C, Stubner S *et al*. Quantitative detection of methanotrophs in soil by novel pmoA-targeted real-time PCR assays. *Appl Environ Microb* 2003;**69**:2423–9.
Ludwig W, Strunk O, Westram R *et al*. ARB: a software environment for sequence data. *Nucleic Acids Res* 2004;**32**:1363–71.
Lüke C, Krause S, Cavigiolo S *et al*. Biogeography of wetland rice methanotrophs. *Environ Microbiol* 2010;**12**:862–72.
Manley PL, Manley TO, Watzin MC *et al*. Lakebed pockmarks in Burlington Bay, Lake Champlain: I. Hydrodynamics and implications of origin. In: Manley PL, Manley TO, Mihuc TB (eds). *Lake Champlain: Partnerships and Research in the New Millennium*. New York: Springer, 2004, 299–329.
McAleece N, Lambshead J, Paterson G *et al*. *Biodiversity Pro (version 2)*. London: The Natural History Museum, Oban, UK: The Scottish association of Marine Science, 1997.
Metcalf WW, Griffin BM, Cicchillo RM *et al*. Synthesis of methylphosphonic acid by marine microbes: a source for methane in the aerobic ocean. *Science* 2012;**337**:1104–7.
Nercessian O, Noyes E, Kalyuzhnaya MG *et al*. Bacterial populations active in metabolism of C1 compounds in the sediment of Lake Washington, a freshwater lake. *Appl Environ Microb* 2005;**71**:6885–99.
Oliveros JC. *VENNY. An Interactive Tool for Comparing Lists With Venn Diagrams*. 2007. http://bioinfogp.cnb.csic.es/tools/venny/.
Osudar R, Matoušů A, Alawi M *et al*. Environmental factors affecting methane distribution and bacterial methane oxidation in the German Bight (North Sea). *Estuar Coast Shelf S* 2015;**160**:10–21.
Oswald K, Milucka J, Brand A *et al*. Light-dependent aerobic methane oxidation reduces methane emissions from seasonally stratified lakes. *PLoS One* 2015;**10**:e0132574.
Pester M, Bittner N, Pinsurang D *et al*. A 'rare biosphere' microorganism contributes to sulfate reduction in a peatland. *ISME J* 2010;**4**:1591–602.
Pester M, Friedrich MW, Schink B *et al*. *pmoA*-based analysis of methanotrophs in a littoral lake sediment reveals a diverse and stable community in a dynamic environment. *Appl Environ Microb* 2004;**70**:3138–42.
Pickrill RA. Shallow seismic stratigraphy and pockmarks of a hydrothermally influenced lake, Lake Rotoiti, New Zealand. *Sedimentology* 1993;**40**:813–28.
Rahalkar M, Bussmann I, Schink B. *Methylosoma difficile* gen. nov., sp. nov., a novel methanotroph enriched by gradient cultivation from littoral sediment of Lake Constance. *Int J Syst Evol Microbiol* 2007;**57**:1073–80.
Rahalkar M, Deutzmann J, Schink B *et al*. Abundance and activity of methanotrophic bacteria in littoral and profundal sediments of Lake Constance (Germany). *Appl Environ Microb* 2009;**75**:119–26.
Rahalkar M, Schink B. Comparison of aerobic methanotrophic communities in littoral and profundal sediments of Lake Constance by a molecular approach. *Appl Environ Microb* 2007;**73**:4389–94.
R-Core-Team. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing, 2015.
Ramette A. Quantitative community fingerprinting methods for estimating the abundance of operational taxonomic units in natural microbial communities. *Appl Environ Microb* 2009;**75**:2495–505.
Reusch A, Loher M, Bouffard D *et al*. Giant lacustrine pockmarks with subaqueous groundwater discharge and subsurface sediment mobilization. *Geophys Res Lett* 2015;**42**:3465–73.
Ross JL, Boon PI, Ford P *et al*. Detection and quantification with 16S rRNA probes of planktonic methylotrophic bacteria in a floodplain lake. *Microbial Ecol* 1997;**34**:97–108.
Sanseverino AM, Bastviken D, Sundh I *et al*. Methane carbon supports aquatic food webs to the fish level. *PLoS One* 2012;**7**:e42723.
Schloss PD, Westcott SL, Ryabin T *et al*. Introducing mothur: open source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microb* 2009;**75**:7537–41.
Schmale O, Leifer I, Deimling JSv *et al*. Bubble transport mechanism: indications for a gas bubble-mediated inoculation of benthic methanotrophs into the water column. *Cont Shelf Res* 2015;**103**:70–8.
Schmid M, Batist MD, Granin NG *et al*. Sources and sinks of methane in Lake Baikal: a synthesis of measurements and modeling. *Limnol Oceanogr* 2007;**52**:1824–37.
Schmidt U, Conrad R. Hydrogen, carbon monoxide, and methane dynamics in Lake Constance. *Limnol Oceanogr* 1993;**38**:1214–26.
Schulz M, Faber E, Hollerbach A *et al*. The methane cycle in the epilimnion of Lake Constance. *Arch Hydrobiol* 2001;**151**:157–76.
Sundh I, Bastviken D, Tranvik LJ. Abundance, activity, and community structure of pelagic methane-oxidizing bacteria in temperate lakes. *Appl Environ Microb* 2005;**71**:6746–52.
Tang KW, McGinnis DF, Frindte K *et al*. Paradox reconsidered: methane oversaturation in well-oxygenated lake waters. *Limnol Oceanogr* 2014;**59**:275–84.
Tavormina PL, Ussler W, Orphan VJ. Planktonic and sediment-associated aerobic methanotrophs in two seep systems along the North American margin. *Appl Environ Microb* 2008;**74**:3985–95.
Valentine DL, Blanton DC, Reeburgh WS *et al*. Water column methane oxidation adjacent to an area of active hydrate dissociation, Eel river Basin. *Geochim Cosmochim Ac* 2001;**65**:2633–40.
Vu VQ. *ggbiplot: a ggplot2 based biplot*. R package version 055, 2011, https://github.com/vqv/ggbiplot.
Wessels M, Bussmann I, Schloemer S *et al*. Distribution, morphology, and formation of pockmarks in Lake Constance, Germany. *Limnol Oceanogr* 2010;**55**:2623–33.
Wickham H, Chang W. *devtools: tools to make developing R code easier*. R package version 1, 2015, https://github.com/hadley/devtools.
Wuebbles DJ, Hayhoe K. Atmospheric methane and global change. *Earth-Sci Rev* 2002;**57**:177–210.
Zakharenko AS, Pimenov NV, Ivanova VG *et al*. Detection of methane in the water column at gas and oil seep sites in central and southern Lake Baikal. *Microbiology* 2015;**84**:90–7.
Zehnder AJB, Stumm W. Geochemistry and biogeochemistry of anaerobic habitats. In: Zehnder AJB (ed.). *Biology of Anaerobic Microorganisms*. New York: Wiley, 1988, 1–38.