Gammaproteobacterial methanotrophs dominate methanotrophy in aerobic and anaerobic layers of boreal lake waters

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ABSTRACT: Small oxygen-stratified humic lakes of the boreal zone are important sources of methane to the atmosphere. Although stable isotope profiling has indicated that a substantial part of methane is already oxidized in the anaerobic water layers in these lakes, the contributions of aerobic and anaerobic methanotrophs in the process are unknown. We used next-generation sequencing of mcrA and 16S rRNA genes to characterize the microbial communities in the water columns of 2 boreal lakes in Finland, Lake Alinen-Mustajärvi and Lake Mekkojärvi, and complemented this with a shotgun metagenomic analysis from Alinen-Mustajärvi and an analysis of pmoA genes and 16S rRNA, mcrA, and pmoA transcripts from Mekkojärvi. Furthermore, we tested the effect of various electron acceptors and light on methane oxidation (13C-CH4 labeling) in incubations of water samples collected from the lakes. Aerobic gammaproteobacterial methanotrophs (order Methylococcales) exclusively dominated the methanotrophic community both above and below the oxycline in the lakes. A novel lineage within Methylococcales, Candidatus Methyloumidiphilus alinensis, defined here for the first time, dominated in Alinen-Mustajärvi, while methanotrophs belonging to Methylobacter were more abundant in Mekkojärvi. Light enhanced methane oxidation in the anoxic water layer, while alternative electron acceptors (SO4^{2-}, Fe^{3+}, Mn^{4+}, and anthraquinone-2,6-disulfonate), except for NO3^{-}, suppressed the process. Our results suggest that oxygenic photosynthesis potentially fuels methanotrophy below the aerobic water layers in methane-rich boreal lakes. Furthermore, incubation results, together with the detection of denitrification genes from metagenome-assembled genomes of gammaproteobacterial methanotrophs, imply that boreal lake methanotrophs may couple methane oxidation with NOx reduction in hypoxic conditions.

KEY WORDS: Methanotroph · Methane oxidation · Boreal lake · Water column · Shotgun metagenomics · 16S rRNA · mcrA · pmoA

INTRODUCTION

The concentration of atmospheric methane (CH4), a critical greenhouse gas, has increased substantially since industrialization, with current total emissions in the order of 500 to 600 Tg yr^{-1} (Kirschke et al. 2013). Roughly 50% of these emissions stem from natural sources (Kirschke et al. 2013), mostly produced by archaea in methanogenesis, the final step in the anaerobic degradation of organic matter.
(Conrad 1999). Although lakes occupy only 3.7% of the global non-glaciated land area (Verpoorter et al. 2014), their CH₄ emissions are estimated to be as high as 6 to 24% of the total natural CH₄ release (Bastviken et al. 2004, 2011). The numerous lakes and ponds in the northern areas (north of 50° N) with annual CH₄ emissions of ~16.5 Tg (6 to 7% of natural release) are especially significant components of the global CH₄ budget (Wik et al. 2016). Thus, knowledge about CH₄ cycling in lakes, especially in northern areas, is essential to better constrain its global input and will ultimately aid in predicting climate change.

CH₄ emissions from natural ecosystems are largely regulated by aerobic oxidation by methane oxidizing bacteria (MOB), utilizing O₂ as an electron acceptor (EA) (Hanson & Hanson 1996), or through anaerobic oxidation of methane (AOM) by anaerobic methanotrophic archaea (ANME archaea), utilizing alternative inorganic (NO₃⁻, SO₄²⁻, Mn⁴⁺ or Fe³⁺) or organic EAs (e.g. humic acids) (Beal et al. 2009, Knittel & Boetius 2009, Haroon et al. 2013, Ettwig et al. 2016, Scheller et al. 2016). In addition, bacteria of the phylum NC10 may gain oxygen for the oxidation of CH₄ in anaerobic conditions using the nitric oxide dismutase enzyme (Ettwig et al. 2010). Some methanogens also oxidize small amounts of CH₄ without external EAs during trace methane oxidation due to enzymatic backflux (Moran et al. 2005, Timmers et al. 2017). While AOM coupled with SO₄²⁻ reduction by ANME archaea is an efficient CH₄ sink in oceanic sediments and waters (Knittel & Boetius 2009), a variety of EAs, i.e. SO₄²⁻, Fe³⁺, and NO₃⁻/NO₂⁻, have been shown to be important drivers of the AOM process in freshwater sediments (Sivan et al. 2011, Deutzmann et al. 2014, á Nordi & Thamdrup 2014, Timmers et al. 2016). However, recent geochemical and microbiological evidence from water columns of oxygen-stratified lakes (i.e. lakes with a temporary or permanently anoxic hypolimnion) of the temperate zone strongly suggests that aerobic MOBs dominate CH₄ oxidation in both oxic and anoxic water layers (Biderre-Petit et al. 2011, Blees et al. 2014, Milucka et al. 2015, Oswald et al. 2015, 2016a,b). Aerobic MOBs were also recently seen to dominate anaerobic CH₄ oxidation in sub-arctic and temperate lake sediments (Bar-Or et al. 2017, Martinez-Cruz et al. 2017). Under oxygen limitation, MOBs may efficiently use the limited O₂ to activate CH₄ and are suggested to further support their metabolism by fermentation (Kalyuzhnaya et al. 2013) or by anaerobic respiration using alternative EAs, i.e. NO₃⁻, NO₂⁻ and Fe and Mn oxides (Kits et al. 2015a,b, Oswald et al. 2016b). Recently, it has been suggested that in situ oxygen production by photosynthetic algae (Milucka et al. 2015) or episodic oxygen introduction, events from the surface waters (Blees et al. 2014) could fuel MOBs in the anoxic waters. However, indirect evidence from lake sediments suggests that MOBs could also drive AOM independently of any external O₂ source (Bar-Or et al. 2017, Martinez-Cruz et al. 2017).

A large number of small, shallow, brown-water lakes characterize the arctic and boreal regions (Kortelainen 1993, Downing et al. 2006). During summer, many of these lakes are steeply stratified with respect to temperature and chemical properties (including oxygen) (Salonen et al. 1984). Similar to lakes in the temperate zone, CH₄ accumulates in the anoxic hypolimnion (Houser et al. 2003, Kankaala et al. 2007), and CH₄ oxidation taking place in the water column acts as an efficient CH₄ sink (Kankaala et al. 2006, Peura et al. 2012). In fact, isotopic profiling shows that a substantial part of CH₄ oxidation already takes place in the anoxic water phase (Peura et al. 2012, Nykänen et al. 2014). However, clone library analyses of the mcrA gene coding for archaeal methyl co-enzyme M reductase (Milferstedt et al. 2010, Youngblut et al. 2014) and a recent shotgun metagenomic analysis (Peura et al. 2015), although with modest sequencing depth, did not detect any AOM organisms in the anoxic waters of humic lakes. Furthermore, analyses targeting bacterial biomarkers have shown that MOBs constitute a significant part of the bacterial community in the anoxic waters of boreal lakes, overlapping with the strictly anaerobic Chlorobium (Taipale et al. 2009, Peura et al. 2012, Garcia et al. 2013, Schiff et al. 2017). Yet, the contributions of aerobic CH₄ oxidation and AOM in the water columns of boreal lakes remain unresolved.

We studied the contribution of aerobic CH₄ oxidation and AOM in water columns of 2 boreal oxygen-stratified lakes by geochemical profiling and by conducting water sample incubations amended with ¹³C-labeled CH₄ and various EAs. CH₄-oxidizing microbial communities were studied by next-generation sequencing (NGS) of pmoA (coding for particulate methane monooxygenase Subunit a of aerobic MOBs), mcrA, and 16S rRNA genes and their RNA transcripts, and by shotgun metagenomics. We hypothesized that aerobic MOBs dominate the methanotrophic community as well as CH₄ oxidation below the oxycline (oxic–anoxic interface) of water column of these boreal, CH₄-rich lakes.
MATERIALS AND METHODS

Study lakes and sampling

The study lakes—Lake Mekkojärvi (61° 13′ N, 25° 8′ E) (area 0.004 km², max. depth 4 m, dissolved organic carbon [DOC] concentration ~30 mg C l⁻¹), and Lake Alinen-Mustajärvi (61° 12′ N, 25° 06′ E) (area 0.007 km², max depth 6.5 m, DOC ~10 mg C l⁻¹)—are small humic headwater lakes located in southern Finland. The lakes are usually ice-free from early May to mid-November and spring meromictic, i.e. the whole water column turns over in autumn but only partially in spring. Before the autumn overturn, the lakes are steeply stratified with respect to temperature and oxygen. For example, the oxycline was at 1 and 2 m depths in Mekkojärvi and Alinen-Mustajärvi, respectively, during summer stratification in 2009 (Karhunen et al. 2013). Photosynthetically active radiation (PAR), during a bright summer day, decreases from 107.5 to 0.1 µmol photons m⁻² s⁻¹ between 1.5 and 5.5 m depth in Alinen-Mustajärvi; while in Mekkojärvi, it decreases from 96.4 to 0.5 µmol photons m⁻² s⁻¹ between 0.5 and 1.5 m depth (surface PAR is 1400 µmol photons m⁻² s⁻¹) (Karhunen et al. 2013). Thus, the potential zone for oxygenic photosynthesis, i.e. where PAR exceeds ~0.1 µmol photons m⁻² s⁻¹ (Gibson 1985, Brand et al. 2016), can extend well below the oxycline, to ~2 m in Mekkojärvi and ~5.5 m in Alinen-Mustajärvi. Accordingly, there was chlorophyll a below the oxycline in both study lakes in July 2009 (~3 µg l⁻¹ at 2.5 m in Mekkojärvi, and ~10 µg l⁻¹ at 5.5 m in Alinen-Mustajärvi; Karhunen et al. 2013). The lakes were sampled at their deepest points on 9 September 2013 for Alinen-Mustajärvi and 1 September 2014 for Mekkojärvi. Vertical O₂ and temperature profiles were measured using a ŶSI model 55 dissolved oxygen instrument (Yellow Springs Instruments). The water for the analysis of vertical variation in microbial communities (via DNA- and RNA-based amplicon sequencing) and background variables were collected using a Limnos water sampler. The background variables included oxidation–reduction potential (ORP), pH, concentrations of CH₄, CO₂ and sulfide, and ¹³C/¹²C of dissolved inorganic carbon (DIC) for both lakes. In addition data was collected on total dissolved Fe and Mn for Mekkojärvi, and on ¹³C/¹²C of CH₄ and concentrations of inorganic nutrients (NO₃⁻+NO₂⁻, NH₄⁺, PO₄³⁻, SO₄²⁻), total N, total P, DOC and particulate organic carbon (POC) for Alinen-Mustajärvi. For CH₄ oxidation experiments, water was collected from the epilimnion (1.2 m), meta- (1.6 m), and hypolimnion (2.8 m) in Mekkojärvi and at the depth with the lowest estimated PAR suitable for oxygenic photosynthesis (5.5 m) in Alinen-Mustajärvi. Furthermore, an additional sampling for shotgun metagenomic analyses of vertical variation in microbial communities in Alinen-Mustajärvi water column was conducted on 23 September 2013. See Supplement 1 at www.int-res.com/articles/suppl/a081p257_supp.pdf for a more detailed description of the sampling.

In vitro determination of potential CH₄ oxidation

To test the effects of EAs on the anaerobic CH₄ oxidation of Mekkojärvi, the collected samples (epilimnion: n = 3; metalimnion: n = 9; hypolimnion: n = 9) were divided into the treatments reported in Table 1. Each treatment included 2 replicates with ¹³C-labeled CH₄ and 1 replicate with ¹²C-labeled CH₄. Incubations took 21 d. The bottles were positioned upside down, partially submerged in water to prevent air exposure of the caps, and gently shaken once a week during the incubation. The sampling for ¹³C-content of DIC, concentrations of CH₄ and CO₂, as well as DNA and RNA, was done once, on the last day of incubations.

For the incubations in Alinen-Mustajärvi, water was concentrated 20-fold, using tangential flow filtration. Anaerobic pre-incubation (dark, 7°C, ~6.5 mo), in gas-tight bottles amended with either ¹³C-CH₄ (6 bottles), isotopically natural CH₄ (3 bottles), or nothing (3 bottles), preceded the actual EA and light experiments of Alinen-Mustajärvi samples (Table 1). The samples for the temporal monitoring of CH₄-concentration were taken 14 times, while those for ¹³C-DIC and sulfide were taken 5 and 2 times, respectively, from the bottles amended with ¹³C-CH₄ or normal CH₄, during the 6.5 mo pre-incubation. One further sampling of CH₄ and ¹³C-DIC was also performed thereafter from the pre-incubation bottles, after a total of 9 mo of incubation. Originally, the pre-incubation phase was done for DNA- and RNA-stable isotope probing (SIP) experiments. However, SIP failed due to insufficient nucleic acid extraction efficiency, which was tested from 3 freeze-dried samples (1 with isotopically natural CH₄ and 2 with ¹³C-CH₄) sacrificed after 6 d of incubation and from 2 ml subsamples collected after 5.5 mo of pre-incubation through septa and pelleted using centrifugation (20 000 × g for 8 min). However, the pelleted samples taken after 5.5 mo of pre-incubation (thus, 1 mo before the onset of the actual EA and light experiments) were used to
analyze the change in the bacterial community structure during the pre-incubation period.

The subsamples (altogether 63 vials), taken from one of the pre-incubation bottles that had been amended with isotopically natural CH$_4$, were used in the actual experiments, which tested the effects of various EAs and light on the CH$_4$ oxidation of Alinen-Mustajärvi hypolimnion samples. The vials were degassed (made anoxic) before the onset of the experiment. The 9 experimental treatments reported in Table 1 each included 5 and 2 replicate vials with $^{13}$C-labeled and isotopically natural CH$_4$, respectively. The incubations lasted for 134 d, except for the O$_2$ treatment, which lasted for 27 d. PAR, measured using a LI-185B Quantum/Radiometer/Photometer with Quantum Q sensor (both LI-COR), was adjusted to ~0.3 µmol photons m$^{-2}$ s$^{-1}$ at the surface of the incubation bottles in both light treatments to represent the lowest PAR thresholds previously reported for oxygenic photosynthesis, i.e. 0.09 to 0.34 µmol photons m$^{-2}$ s$^{-1}$ (Gibson 1985, Brand et al. 2016) (Table 1). A red light was chosen since it penetrates furthest in brown-water lakes (Kirk 1983) and, thus, may best represent the light conditions in deep layers. Sampling for $^{13}$C-content of CO$_2$ was done 4 times during the incubation period. To avoid O$_2$ contamination of the samples, the incubations and injections (using He-flushed syringes and needles) were always done submerged in water. See Supplement 1 for a detailed description of experiments in both study lakes.

The added EA concentrations in experiments of both study lakes were either similar to or lower than those in previous AOM studies of aquatic and wetland environments (Beal et al. 2009, Blazewicz et al. 2012). However, they were higher than in situ concentrations to ensure the detection of EA effects on CH$_4$ oxidation.

### Concentration and stable isotope analyses

The analysis of dissolved sulfide, SO$_4^{2-}$, nutrients, DOC, POC, Fe, and Mn is described in Supplement 1. Concentrations of CH$_4$ and CO$_2$ in the water column of both lakes, as well as in EA experiments of Mekkojärvi, were measured using a gas chromatograph (GC), as described in Ojala et al. (2011). CH$_4$ during the pre-incubation period of Alinen-Mustajärvi samples was measured using a Perkin Elmer Clarus 500 GC with a flame-ionization detector (FID). The $^{13}$C/$^{12}$C of CH$_4$ was measured using Isoprime 100 isotope ratio mass spectrometer (IRMS) coupled with a trace gas pre-concentrator, while the
\(^{13}\text{C}/^{12}\text{C}\) of DIC and CO\(_2\) was analyzed either with the same device (Mekkojärvi samples) or with a Thermo Finnigan GasBench II connected to an XP Advantage IRMS (Alinen-Mustajärvi samples), using the same in-house carbon standard (CaCO\(_3\)). Isotope results were expressed as \(^{8}\text{C}\) values for water column data and as excess concentration of \(^{13}\text{C}\)-CO\(_2\) or \(^{13}\text{C}\)-DIC for incubations (i.e. the concentration of \(^{13}\text{C}\) produced solely from the added \(^{13}\text{C}\)-CH\(_4\)) according to Supplement 1.

The accumulation of excess \(^{13}\text{C}\)-CO\(_2\) or \(^{13}\text{C}\)-DIC was converted into production rates (nmol l\(^{-1}\) d\(^{-1}\)). This was done as a simple end-point calculation for Mekkojärvi samples, assuming negligible concentration of excess \(^{13}\text{C}\)-DIC at the start of incubations. For Alinen-Mustajärvi, CH\(_4\) oxidation was considered to take place only in treatments that showed linear accumulation of \(^{13}\text{C}\)-CO\(_2\) in time through all the 4 time (sampling) points (linear regression, \(p < 0.05\)), while CH\(_4\) oxidation was regarded negligible for other treatments. The production rates of \(^{13}\text{C}\)-CO\(_2\) in Alinen-Mustajärvi samples were then calculated using the end-point approach, but for 3 time periods, covering the whole incubation period: (1) 0–6 d (treatment with CH\(_4\) + O\(_2\)) or 0–21 d (other treatments), (2) 6–9 d (treatment with CH\(_4\) + O\(_2\)) or 21–71 d (other treatments), and (3) 9–27 d (treatment with CH\(_4\) + O\(_2\)) or 71–134 d (other treatments).

**DNA- and RNA-based amplicon sequencing analyses**

The DNA and RNA of water column and EA experiment samples from Mekkojärvi were extracted from filters using the PowerWater RNA Isolation Kit (MO BIO Laboratories) according to the manufacturer’s instructions. For Alinen-Mustajärvi, DNA was extracted from 1.2 to 4.5 mg of freeze-dried water column biomass, using the PowerSoil DNA Isolation Kit (MO BIO). In addition, a phenol-chloroform and bead-beating protocol was used to extract DNA from the pelleted sample collected from the pre-incubation bottle of Alinen-Mustajärvi 1 mo before the water in the bottle was subjected to the EA and light experiments (Griffiths et al. 2000).

Bacterial communities were studied by using NGS of the bacterial 16S rRNA gene and 16S rRNA amplicons. Potential and active methanogenic/methanotrophic archaea were studied by using NGS of mcrA amplicons from DNA and mRNA, while methanotrophic bacteria were studied by targeting pmoA. Primers, PCR, reverse-transcriptase PCR (RT-PCR), preparation of NGS libraries, and the sequencing (Ion Torrent™ Personal Genome Machine) are described in detail in Supplement 1.

Mothur (Schloss et al. 2009) was used in all subsequent sequence analyses, unless reported otherwise. Barcodes and primer sequences, as well as low-quality sequences (containing ≥1 mismatch in primer or barcode sequences, ambiguous nucleotides, homopolymers longer than 8 nucleotides, and not fulfilling the quality parameters qwindowaverage = 20 and qwindowsize = 10) were removed. FrameBot (from the FunGene website, http://fungene.cme.msu.edu/FunGenePipeline) (Fish et al. 2013, Wang et al. 2013) was used to correct frameshift errors in mcrA and pmoA reads.

Bacterial 16S rRNA gene sequences were aligned using Silva reference alignment (Release 119), while pmoA and mcrA were aligned using reference alignments retrieved from FunGene (http://fungene.cme.msu.edu/index.spr). Chimeric sequences, identified using Uchime (Edgar et al. 2011), were removed from each library, and a preclustering algorithm (Huse et al. 2010) was used to reduce the effect of sequencing errors. 16S rRNA sequences were assigned taxonomies with a naïve Bayesian classifier (bootstrap cutoff value 75%) (Wang et al. 2007), using the Silva database (Release 128), and sequences classified as archaea, chloroplast, mitochondria, and eukaryota were removed. Taxonomic classification of the functional genes took place similarly but with recently constructed databases for mcrA (Rissanen et al. 2017) and pmoA (Dumont et al. 2014).

Sequences were divided into operational taxonomic units (OTUs) at a 97% similarity level for 16S rRNA and at a 95% similarity level for mcrA and pmoA. Singleton OTUs (OTUs with only 1 sequence) were removed, and the data were normalized by subsampling to the same size, which was 1129 for 16S rRNA (average length ~212 bp) for both lakes, 144 for pmoA (~243 bp) for Mekkojärvi, and 696 and 310 for mcrA (~243 bp) for Mekkojärvi and Alinen-Mustajärvi, respectively. Sequence variation was adequately covered in these libraries, as shown by Good’s coverage, an estimate of the proportion of amplified gene amplicons represented by sequence libraries for each sample that varied from 0.84 to 0.99 for 16S rRNA, 0.95 to 1 for mcrA, and 0.92 to 1 for pmoA. The size of 2 pmoA and 5 mcrA libraries fell below the above limits, and of these, only 3 mcrA libraries (with >75 sequences) were included for further calculations of relative abundances of OTUs, while the others were discarded.

Methanotrophic OTUs belonging to *Methylococcales* in 16S rRNA and pmoA libraries were classified to
genus level by searching their representative sequences against the NCBI nt/nr-database using standard nucleotide (blastn) and translated BLAST (blastx), respectively, as well as via phylogenetic tree analyses. Phylogenetic tree analyses, including representative sequences of OTUs, and database sequences of known Methylococcales were performed with Mothur-aligned nucleotide sequences for 16S rRNA and ClustalW-aligned deduced amino acid sequences for pmoA using the maximum likelihood algorithm (Jones–Taylor–Thornton [JTT] model for pmoA and the generalized time reversible [GTR] model for 16S rRNA) with 100 bootstraps in Mega 6.0 (Tamura et al. 2013). Besides analysing methanotrophs, bacterial 16S rRNA and 16S rRNA gene OTUs were classified into other functional groups based on previous literature. Cyanobacteria, as well as strictly anaerobic, anoxicogenic phototrophic H2S, and Fe2+-oxidizing Chlorobium (Van Gemerden & Mas 1995, Heising et al. 1999), were specifically analysed from both lakes. In addition, the higher depth resolution sampling in Alinen-Mustajärvi allowed the comparison of the depth distribution of methanotrophs with that of aerobic, i.e. nitrifying (Alawi et al. 2007) and Fe2+-oxidizing (Hedrich et al. 2011, Moya-Beltrán et al. 2014), and anaerobic, i.e. SO42−-reducing (Postgate & Campbell 1966, Finster 2008, Kuever 2014, Hausmann et al. 2016) and Fe3+-reducing (Lovley 2006), bacteria.

Shotgun metagenomic analyses

The samples for shotgun sequencing were taken from 0.2 µm polycarbonate filters, and the DNA was extracted using the PowerSoil DNA Isolation Kit (MO BIO). The preparation of the shotgun metagenomic libraries and sequencing (paired-end sequencing on the Illumina HiSeq2500 platform) are described in detail in Supplement 1. The sequencing produced a total of 120.5 Gb of sequence data. Reads were quality-filtered using Sickle (version 1.33; https://github.com/najoshi/sickle) and subsequently assembled with Ray (version 2.3.1) (Boisvert et al. 2010). Assembled contigs were cut into 1000 bp pieces and scaffolded with Newbler (454 Life Sciences, Roche Diagnostics). The mapping of the original reads to the Newbler assembly was done using Bowtie2 (version 2.15.0) (Langmead & Salzberg 2012), while duplicates were removed using Picard tools (version 1.101; https://github.com/broadinstitute/picard), and BEDTools (Quinlan & Hall 2010) was used for computing coverage. The raw reads from the shotgun sequencing were screened for methanotrophs using Kaiju (Menzel et al. 2016) with default settings against the complete NCBI RefSeq database. Furthermore, the functional potential of the metagenomes was assessed from the assembled data using the hidden Markov models (HMM) of the Pfam and TIGRFAM databases (Finn et al. 2007, Selengut et al. 2007) and HMMER3 software (version 3.1b2) (Durbin et al. 2002). The placement of the MAGs in the microbial tree of life was estimated using PhyloPhlAn (version 1.1.0) (Segata et al. 2013). All of the MAGs were also annotated using Prokka (version 1.11) (Seemann 2014). Furthermore, pmoA sequences of the methanotroph MAGs were analysed via phylogenetic tree analyses as explained above. In this study, the metagenomic analysis was focused solely on methanotrophs. A more general view on the metagenomic dataset will be given elsewhere (S. Peura et al. unpubl. data).

Sequence data accession numbers

Sequencing data were deposited to the NCBI Sequence Read Archive under study accession numbers SRP110764 for amplicon sequence data and SRP076290 for shotgun metagenomics data.

Statistical analyses

The differences in 13C-CO2 production rates between treatments in Alinen-Mustajärvi were examined separately for each of the 3 time periods during the incubation (Periods 1 to 3, see above), using a 1-way analysis of variance (p < 0.05) followed by pairwise post-hoc tests, using the least significant difference (LSD) technique with Hochberg-Bonferroni-corrected α-values. The analyses were performed using IBM SPSS Statistics version 23. The results of Lake Mekkojärvi experiments were only interpreted visually, due to low sample size (n = 2).
RESULTS

Physicochemical conditions in the water column of the study lakes

The study lakes were acidic (pH ≤ 6). The temperature stratification was stronger in Alinen-Mustajärvi than in Mekkojärvi (Figs. S1 & S2A; all supplementary figures are available in Supplement 2 at www.int-res.com/articles/suppl/a081p257_supp.pdf). Both lakes were steeply oxygen-stratified. The oxycline, which divided the water column into oxic epilimnion and anoxic meta- and hypolimnion, was at 1.3 m from the surface in Mekkojärvi and at 2.3 m in Alinen-Mustajärvi (Fig. 1A,C). ORP decreased only very slightly in the metalimnion before reaching the redoxcline in the hypolimnion, where a drastic decrease in ORP took place (Fig. 1A,C). In Alinen-Mustajärvi, the change in ORP was accompanied by a decrease in SO$_4^{2-}$ and an increase in dissolved sulfide (Fig. S2A). In Mekkojärvi, sulfide was also much higher in the meta- and hypolimnion than in epilimnion, and both Fe and Mn increased towards the bottom (Fig. S1). Furthermore, there was vertical variation in NO$_3^-+NO_2^-$, NH$_4^+$, total-N, PO$_4^{3-}$, total-P, DOC, and POC in Alinen-Mustajärvi (Fig. S2B,C).

In Mekkojärvi, the concentrations of CH$_4$ and CO$_2$, and $\delta^{13}$C of DIC were higher in the hypolimnion than in other layers (Fig. 1B). In Alinen-Mustajärvi, the concentration and $\delta^{13}$C of CH$_4$ were stable in the epilimnion and in the upper parts of the metalimnion (Fig. 1D,E). However, CH$_4$ concentration started to increase towards the bottom in the lower part of metalimnion. At the same time, $\delta^{13}$C of CH$_4$ peaked in the lower part of metalimnion, then decreased considerably towards the upper part of the hypolimnion, and was at stable low levels below 5 m depth (Fig. 1D,E). CO$_2$ concentration was quite stable in the upper part of the epilimnion, then increased gradually towards the middle part of the metalimnion, and was quite stable until 4.5 m depth in the hypolimnion. Below 4.5 m depth, a substantial increase in CO$_2$ took place towards the bottom (Fig. 1D). In contrast, $\delta^{13}$C of DIC fluctuated in the water column, with lower values in the lower part of epilimnion and at the interface between meta- and hypolimnion, and higher values in the upper part of the epilimnion, in the middle of the metalimnion and at the bottom (Fig. 1D).
Fig. 2. Relative abundances of components of the microbial community in Lake Meikkojärvi, Finland: (A) *Methylococcales* and anoxygenic phototrophic H₂S and Fe²⁺-oxidizing (*Chlorobium*) bacteria; (B) dominant OTUs of *Methylococcales* (and their affiliation) based on the 16S rRNA gene and 16S rRNA; (C) dominant OTUs of *Methylococcales* based on the *pmoA* gene and mRNA transcripts. Values are shown for samples collected *in situ* and after experimental incubation (21 d) of water samples collected from the epilimnion, metalimnion, and hypolimnion and amended with ¹³C-CH₄, ¹³C-CH₄ plus a mixture of inorganic electron acceptors (IEA: NO₃⁻, SO₄²⁻, Fe³⁺ and Mn⁴⁺), and ¹³C-CH₄ plus an organic EA (OEA: di-sodium anthraquinone-2,6-disulfonate). Data are presented as average ± SD when n = 2, otherwise n = 1.
Microbes in the study lakes analysed by DNA- and RNA-based amplicon sequencing

The sample storage and nucleic acid extraction methods differed between lakes (see ‘Materials and methods’). Therefore, detailed comparisons of relative abundances of microbial groups between the study lakes were not made. The methanotrophic bacterial community was dominated by gamma proteobacterial MOB of the order Methylococcales (i.e. MOB Type I) (Figs. 2 & 3A). Alphaproteobacterial MOBs (i.e. MOB Type II) were very rare in Mekkojärvi (<0.3% of bacteria in situ and <1.7% of bacteria in incubated samples) and absent in Alinen-Mustajärvi amplicon libraries. Verrucomicrobial MOBs or putative aerobic CH₄-oxidizing bacteria belonging to phylum NC10 were not detected. Detailed phylogenetic analyses showed that the in situ Methylococcales community in Mekkojärvi was dominated by Methylobacter, i.e. 16S rRNA gene OTU 3 and pmoA OTU 1 (Figs. 2B,C, 4 & 5). In contrast, in Alinen-Mustajärvi, a putative novel Methylococcales group, represented by 16S rRNA gene OTU 9, substantially outnumbered the 2 other most abundant Methylobacter OTUs, OTUs 3 and 27 (Figs. 3A & 4). OTU 9 was very rare in Mekkojärvi (<0.1% in hypolimnion). To increase the confidence in the phylogenetic assignment of the dominant OTUs, the phylogenetic analyses of 16S rRNA genes were also performed using longer clone library sequences, which were previously collected from the study lakes, and contained more information than the shorter amplicon sequences (Figs. S3 & S4). The analysis of AM949373 (469 bp) and HE616477 (828 bp) that shared 99% and 100% similarity with representative sequences of OTUs 3 and 27, respectively, gave further confirmation that these OTUs represented Methylobacter, as they had 98% similarity with their closest database representative, which was Methylobacter psychrophilus (Figs. 4, S3 & S4). In addition, a representative sequence of OTU 9 and a highly similar (99.7% similarity) clone library sequence, HE616416 (830 bp), previously collected from the water column of Alinen-Mustajärvi, were identically positioned in the phylogenetic tree, being 93.1% and
89.9% similar, respectively, to the closest known Methylococcales genus, *Methylothericola* (Figs. 4 & S4). Since these similarities were less than the widely used 95% similarity threshold for classification of sequences into different genera, this group very likely belonged to a novel genus. Since OTU 9 representative sequence and the clone library sequence HE616416 shared 93% and 90% similarity, respectively, with the closest environmental database sequences from wet environments, i.e. wetland, lake sediment, rice rhizosphere, and subsurface geothermal water (data not shown), OTU 9 was given the following candidate names for genus and species: *Candidatus Methylo moundiphilus alinensis*. *Methylo* denotes potential consumption of methyl-compounds, *umidi* (from Latin *umida*, which means ‘wet’), and *philus* (from Greek *philos*, which means ‘friend, loving’) denotes the preference for wet environments. Thus, *Methylo moundiphilus* is a methyl-using bacterium that prefers wet environments, and the species
Fig. 5. Phylogenetic tree of deduced amino acid sequences of the \textit{pmoA} gene of \textit{Methylococcales} (i.e. Type I MOB divided into clusters Ia and Ib), showing the phylogenetic positions of representative sequences of most abundant OTUs from Lake Mekkojärvi as well as sequences from metagenomic bins from Lake Alinen-Mustajärvi. The tree was constructed using the maximum-likelihood algorithm with the JTT substitution model. The length of amino acid sequences is 75. A tree with longer sequences validating the phylogenetic position of metagenomic bins 10 and 140 is presented in Fig. S9 in Supplement 2. The scale bar indicates the number of substitutions per site. The numbers at the nodes indicate the percentage of occurrence in 100 bootstrapped trees (bootstrap values >50% are shown).
name *alinensis* denotes the lake in which it was first detected, Lake Alinen-Mustajärvi.

MOBs were present both above and below the oxycline, down to the deepest sampling depths in both lakes. Based on the results from Mekkojärvi, they were also actively transcribing *pmoA* (Fig. 2C). In Mekkojärvi, the *in situ* relative abundance of MOBs was highest in the metalimnion and lowest in the hypolimnion, based on both the 16S rRNA and 16S rRNA gene sequences. The relative abundance of putative anoxygenic phototrophic H$_2$S and Fe$^{2+}$-oxidizing *Chlorobium* increased from the epilimnion to the hypolimnion (Fig. 2A). *Cyanobacteria* were present below the oxycline in the meta- and hypolimnion but with low relative abundance (<0.8% of 16S rRNA gene sequences) (data not shown).

The higher depth resolution sampling in Alinen-Mustajärvi revealed the total *Methylococcales* and *Ca* M. alinensis maximum to be below the oxycline, at 3.5 m in the metalimnion, which corresponded to depths where CH$_4$ concentration increased towards the bottom, CO$_2$ concentration was stable, and $\delta^{13}$C of CH$_4$ and DIC reached their maximum and minimum, respectively (Figs. 1D,E & 3A). The putative anaerobic Fe$^{3+}$-reducing bacteria (mainly *Geothrix*) and aerobic NO$_2^-$-oxidizing bacteria (mostly *Candidatus Nitrotoga*) peaked at the same depth (Fig. 3B). The 2 most abundant *Methylobacter*-OTUs peaked lower in the water column than *Ca* M. alinensis, at the same depth (4.5 m) as the putative SO$_4^{2-}$-reducing (mostly *Desulfovibrio* and *Desulfobulbaceae*) and anoxygenic phototrophic H$_2$S, and Fe$^{3+}$-oxidizing bacteria (*Chlorobium*) (Fig. 3). Putative aerobic Fe$^{2+}$-oxidizing bacteria (mainly *Ferrovum*) were generally more numerous higher in the water column than any other studied group (Fig. 3). *Cyanobacteria* were present in the meta- and hypolimnion but with low relative abundance (<0.8% of 16S rRNA gene sequences).

The final *mcrA* dataset consisted only of methanogenic archaea, which were present both above and below the oxycline in both lakes, and were actively transcribing *mcrA* in each study layer of Mekkojärvi (Figs. S5 & S6). However, in the raw data preceding singleton-removal and subsampling, ANME archaea belonging to ANME 2D had a marginal abundance (maximum 0.3% of *mcrA* sequences) in some incubated metalimnion and hypolimnion samples of Mekkojärvi. Yet, they neither transcribed *mcrA* in any of the samples nor were present *in situ* in the study lakes.
Methanotrophs in Lake Alinen-Mustajärvi studied by shotgun metagenomic analysis

The oxycline was located slightly deeper (2.9 m), when the sampling for the metagenomic analyses were conducted (i.e. 2 wk after sampling for other analyses) (Fig. S7A). The size of the metagenomic libraries varied from ~5 to ~11 Gb, and their coverage from ~40 to ~75% (Fig. S7B). In accordance with 16S rRNA gene amplicon results, anaerobic methanotrophs were not detected, and Methylcoccales were the dominant MOB group, having the highest relative abundance below the oxycline in the metalimnion (Fig. 6A). Alphaproteobacterial and verrucomicrobial MOBs were also detected in Alinen-Mustajärvi, but they were rare (Fig. 6A). Vertical variation in the abundances of pmoA, as well as genes coding for particulate methane monoxygenase Subunits b (pmoB) and c (pmoC), followed that of Methylcoccales (Fig. 6B).

From a total of 8 MAGs affiliated to MOBs, 4 were of high quality, i.e. Bins 10 (95.3% complete, 4.8% contaminated), 126 (95.8%, 0.7%), 140 (66.7%, 0%), and 149 (94.1%, 1.4%) and will be considered further (Fig. S8); they all belonged to Methylcoccales. Three of them had their highest relative abundance below the oxycline, Bins 10 and 140 in the metalimnion and Bin 149 in the hypolimnion, while Bin 126 had its highest abundance in the epilimnion (Fig. 6C). The 16S rRNA gene sequences of the bins were not obtained. However, PhyloPhlAn, which uses whole-genome sequence data, placed the most dominant bin, Bin 10, closest to Methylococcales (Figs. 4, S3, & S4). Hence, it is likely that Bins 126, 140, and 149 represented species that have no genomes from isolated organisms (Fig. S8). However, although we could not recover a pmoA gene for Bin 149, the analysis of pmoA genes of Bins 126 and 140 suggested them to be most closely related to Methylobacter (Figs. 5 & S9). Although it is possible that Methyloccoxis can obtain their pmoA gene via lateral gene transfer from other Methylcoccales (Oswald et al. 2017), neither of the 16S rRNA gene OTUs in Alinen-Mustajärvi were affiliated with Methyloccoxis (Figs. 4, S3, & S4). Hence, it is likely that Bins 126, 140, and 149 represented species that have no genomes from isolated organisms available (e.g. Methyloccoxis psychrophilus). Due to this uncertainty, these bins were not assigned to genera. Interestingly, the bins that thrived below the oxycline (i.e. Bins 10, 140, and 149) contained genes coding for denitrification enzymes, i.e. narG (nitrate reductase) in Bins 10 and 149, nirS (nitrite reductase) in Bin 140, and norB (nitric oxide reductase) in Bin 10, while the genetic denitrification potential was not detected in Bin 126 that was most abundant in the epilimnion (Fig. 6C).

### Table 2. Potential CH₄ oxidation rates, measured as excess ¹³C-DIC production during incubation (21 d) of water samples collected from the epi-, meta-, and hypolimnion of Lake Mekkojärvi, Finland, and subjected to different treatments. Minimum and maximum values of excess ¹³C-DIC production are shown (n = 2 replicates per treatment). Inorganic electron acceptors (EA) included a mixture of NO₃⁻, SO₄²⁻, Fe³⁺, and Mn⁴⁺; while disodium anthraquinone-2,6-disulfonate was used as an organic EA

<table>
<thead>
<tr>
<th>Depth zone</th>
<th>Treatment</th>
<th>Excess ¹³C-DIC production (min; max) (nmol l⁻¹ d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epilimnion</td>
<td>CH₄</td>
<td>479.5; 916.5</td>
</tr>
<tr>
<td>Metalimnion</td>
<td>CH₄</td>
<td>977.4; 1140.7</td>
</tr>
<tr>
<td></td>
<td>CH₄ + inorg. EAs</td>
<td>132.0; 156.6</td>
</tr>
<tr>
<td></td>
<td>CH₄ + org. EAs</td>
<td>59.0; 134.6</td>
</tr>
<tr>
<td>Hypolimnion</td>
<td>CH₄</td>
<td>1093.4; 1147.5</td>
</tr>
<tr>
<td></td>
<td>CH₄ + inorg. EAs</td>
<td>691.9; 839.5</td>
</tr>
<tr>
<td></td>
<td>CH₄ + org. EAs</td>
<td>33.2; 34.2</td>
</tr>
</tbody>
</table>

Variation in potential CH₄ oxidation and in microbial community structure in the incubation experiments

In Mekkojärvi, potential CH₄ oxidation based on the accumulation of excess ¹³C-DIC in incubations
was generally higher in the meta- and hypolimnion than in the epilimnion (Table 2). The addition of inorganic and organic EAs decreased the potential CH₄ oxidation (Table 2). However, the decrease was considerably lower (32%) in the treatment with inorganic EAs in the hypolimnion than in the other treatments (86 to 97%) (Table 2).

The microbial community variations between the treatments were studied by amplicon sequencing in Mekkojärvi experiments. Besides the Methylococcales OTUs that dominated in situ (i.e. 16S rRNA OTU 3 and pmoA OTU 1), there were also other OTUs that were increasingly present after the experimental incubations in Mekkojärvi (Fig. 2B,C). However, in this study, we focused specifically on the effects of EAs on the MOB OTUs that dominated in situ as well as on total Methylococcales. EA-amended samples generally had fewer Methylococcales than the CH₄ treatment, except for the inorganic EA-induced increase at the level of 16S rRNA in the hypolimnion (Fig. 2A). Organic EAs in general decreased the relative abundance of Methylococcales OTU 3 when compared to the CH₄ treatment (Fig. 2B). In contrast, inorganic EAs increased the relative abundance of OTU 3 in the hypolimnion but slightly decreased it in the metalimnion at the level of 16S rRNA (Fig. 2B). In addition, there was no inorganic EA-driven change in OTU 3 abundance in the hypolimnion but a decrease in metalimnion at the 16S rRNA gene level (Fig. 2B). Compared to the CH₄ treatment, organic EAs decreased the relative abundance of Methylobacter OTU 1 at the level of both the pmoA gene and its mRNA transcripts in the hypolimnion, whereas they did not affect it at the level of pmoA gene, but even increased it at the level of mRNA transcripts in the metalimnion (Fig. 2C). In contrast, inorganic EAs increased the relative abundance of both the relative abundance of pmoA gene and its mRNA transcripts in the dark, and CH₄ under red and normal light; (B) CH₄ + O₂ in the dark, and CH₄ under red light. Note the significant accumulation of CH₄ in the hypolimnion (5.5 m) of Lake Alinen-Mustajärvi and subjected to different treatments: (A) CH₄ and CH₄+NO₃ − in the dark, and CH₄ under red and normal light; (B) CH₄ + O₂ in the dark, and CH₄ under red light. Note the significant accumulation of CH₄ in the hypolimnion (5.5 m) of Lake Alinen-Mustajärvi, Finland, and subjected to different treatments (n = 5 replicates per treatment). Potential CH₄ oxidation could not be assessed for samples amended with humic acids (CH₄ + humic acids and CH₄ + humic acids + Fe³⁺) due to CO₂ concentrations being below the detection limit of the IRMS. Different letters in the final column indicate significant differences in CH₄ oxidation between treatments (1-way ANOVA, p < 0.05). CH₄ oxidation rates in treatments with added O₂ were many-fold higher than in other treatments and were therefore excluded from the statistical test.

Table 3. Potential CH₄ oxidation rates, measured using an isotope ratio mass spectrometer (IRMS) as average (±SD) excess ¹³C-CO₂ production at different time periods during incubations of concentrated water samples collected from the hypolimnion (depth 5.5 m) of Lake Alinen-Mustajärvi, Finland, and subjected to different treatments (n = 5 replicates per treatment). Potential CH₄ oxidation could not be assessed for samples amended with humic acids (CH₄ + humic acids and CH₄ + humic acids + Fe³⁺) due to CO₂ concentrations being below the detection limit of the IRMS. Different letters in the final column indicate significant differences in CH₄ oxidation between treatments (1-way ANOVA, p < 0.05). CH₄ oxidation rates in treatments with added O₂ were many-fold higher than in other treatments and were therefore excluded from the statistical test.

<table>
<thead>
<tr>
<th>Time period (d)</th>
<th>Treatment</th>
<th>Excess ¹³C-CO₂ production (nmol l⁻¹ d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–21</td>
<td>CH₄</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>CH₄ + NO₃⁻</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>CH₄ in red light</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>CH₄ in light</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>CH₄ + SO₄²⁻</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>CH₄ + Fe³⁺</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>21–71</td>
<td>CH₄</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>CH₄ + NO₃⁻</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>CH₄ in red light</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>CH₄ in light</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>CH₄ + SO₄²⁻</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>CH₄ + Fe³⁺</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>71–134</td>
<td>CH₄</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>CH₄ + NO₃⁻</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>CH₄ in red light</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>CH₄ in light</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>CH₄ + SO₄²⁻</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>CH₄ + Fe³⁺</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>0–6</td>
<td>CH₄ + O₂</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>6–9</td>
<td>CH₄ + O₂</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>9–27</td>
<td>CH₄ + O₂</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

Fig. 7. Accumulation of excess ¹³C-CO₂ during incubation of concentrated water samples (average ± SD, n = 5) collected from the hypolimnion (5.5 m) of Lake Alinen-Mustajärvi and subjected to different treatments: (A) CH₄ and CH₄+NO₃⁻ in the dark, and CH₄ under red and normal light; (B) CH₄ + O₂ in the dark, and CH₄ under red light.
active abundance of OTU 1 at the level of pmoA transcripts, whereas they did not generally affect it at the level of pmoA genes (Fig. 2C).

Based on the accumulation of excess $^{13}$C-DIC, potential CH$_4$ oxidation took place during the 6.5 mo pre-incubation of the hypolimnion samples (5.5 m depth) of Alinen-Mustajärvi (Fig. S10). Concurrent accumulation of sulfide and CH$_4$ indicated that anaerobic conditions prevailed during this period (Fig. S10). Despite the long pre-incubation, it was confirmed by 16S rRNA gene amplicon sequencing that the same Methylococcales OTUs that dominated in situ (i.e. OTUs 3, 9 and 27) dominated the pre-incubation bottle 1 mo before the onset of the EA and light experiments (thus, after 5.5 mo pre-incubation). The relative abundance of Methylococcales was slightly lower in the pre-incubation bottle (3.7%) than in situ (4.5%), but these numbers are not directly comparable due to the different sample storage and DNA extraction methods. Compared to the treatment with only CH$_4$, the amendment of O$_2$ substantially increased the potential CH$_4$ oxidation (based on the accumulation of excess $^{13}$C-CO$_2$) (Fig. 7, Table 3). In addition, normal light enhanced potential CH$_4$ oxidation during the first 21 d, while red light increased it during the later stages of incubation (Fig. 7, Table 3). In contrast, the potential CH$_4$ oxidation rate was not affected by NO$_3^−$ and was significantly decreased (i.e. not observed to take place at all) in samples amended with Fe$^{3+}$ or SO$_4^{2−}$ (Table 3). However, despite similar CH$_4$ oxidation rates, the addition of NO$_3^−$ generally led to a higher concentration of excess $^{13}$C-CO$_2$ than the addition of CH$_4$ alone (Fig. 7). CH$_4$ oxidation could not be assessed for samples amended with humic acids due to the CO$_2$ concentration being below the detection limit of IRMS.

**DISCUSSION**

In this study, we demonstrated active CH$_4$ oxidation below the oxic–anoxic interface in 2 boreal humic oxygen-stratified lakes, supporting previous findings about these environments (Kankaala et al. 2006; Peura et al. 2012, Nykänen et al. 2014). Incubations without EA amendments led to slightly higher CH$_4$ oxidation potential in water samples collected from below rather than above the oxycline in Mekkojärv (Table 2). MOBs also actively transcribed pmoA at all depth layers in Mekkojärv (Fig. 2C). In addition, as microbial CH$_4$ oxidation fractionates against the heavier isotope, enriching the residual CH$_4$ in $^{13}$C (Whiticar 1999), the concurrent upward decrease in CH$_4$ concentration and increase in its $\delta^{13}$C in the 5 to 3.5 m layer confirms previous findings that in situ CH$_4$ oxidation was most active below the oxycline in Alinen-Mustajärvi (Fig. 1E) (Peura et al. 2012). As oxidation of CH$_4$ produces CO$_2$ with a lower $\delta^{13}$C value than oxidation of organic matter, the lowest $\delta^{13}$C of DIC observed at the same depth layers further support active CH$_4$ oxidation (Fig. 1D).

As hypothesized, the presence of Methylococcales and the lack of NC10 bacteria in the bacterial 16S rRNA data, as well as the lack of ANME archaea in the mcrA data, indicate that aerobic MOBs were the dominant methanotrophs below the oxycline in these boreal lakes, in accordance with evidence from temperate lakes (Blees et al. 2014, Milucka et al. 2015, Oswald et al. 2015, 2016a,b). However, it has to be acknowledged that the PCR amplicon sequencing approach, despite adequately resolving the sequence diversity in the amplicon pool, suffers from PCR-associated problems (e.g. primer bias and amplicon length), which can affect the view on microbial diversity (Hong et al. 2009, Engelbrektson et al. 2010). Therefore, we used PCR-free shotgun metagenomic analysis to confirm our findings by showing exclusive dominance of MOBs, mainly Methylococcales, in the methanotrophic community in Alinen-Mustajärvi. The general lack of EA-induced CH$_4$ oxidation in anaerobic incubations gave further support for the lack of activity of the typical AOM organisms (i.e. ANME archaea and NC10 bacteria). In general, their activity seems to be limited to sediments in lakes (Deutzmann et al. 2014, á Norði & Thamdrup 2014), which is probably due to lower environmental stability in water columns, which is less suitable for these slow-growing organisms.

PAR is known to be above the lowest threshold for oxygenic photosynthesis and chlorophyll a to be present below the oxycline in both study lakes during summer days (see ‘Materials and methods’). Accordingly, this study found potentially photosynthetic Cyanobacteria below the oxycline in both study lakes. In addition, isotopic data indicated active CH$_4$ oxidation in Alinen-Mustajärvi, and the relative abundance of MOBs was highest below the oxycline in both lakes (Figs. 1–3 & 6). Together with the results on light-enhanced potential CH$_4$ oxidation in the hypolimnion of Alinen-Mustajärvi (Table 3), this suggests that oxygenic photosynthesis-driven CH$_4$ oxidation by MOBs is potentially responsible for a major part of CH$_4$ consumption below the oxycline in shallow humic lakes of the boreal zone during summer days. This finding is in agreement with the previous results from temperate lakes and
further confirms our hypothesis on MOBs dominating water column methanotrophic activity below the oxycline in oxygen-stratified lakes (Milucka et al. 2015, Oswald et al. 2015).

Interestingly, the relative abundance of MOBs in Alinen-Mustajärvi peaked below the other major aerobic bacterial group, Fe^{2+}-oxidizing bacteria, at the same depth layers as anaerobic bacteria (Fig. 3), in accordance with results from temperate Lake Rotsee, Switzerland (Oswald et al. 2015, Brand et al. 2016). This depth distribution pattern was very likely due to the inhibitory effect of light on MOB activity, which can take place in PAR as low as 4 µmol photons m^{-2} s^{-1} (Dumestre et al. 1999, Murase & Sugimoto 2005). Indeed, the estimated PAR was below this limit (maximum ~3.5 µmol photons m^{-2} s^{-1} at 3.5 m depth) at the 5 to 3.5 m depth layer in Alinen-Mustajärvi, where CH_{4} oxidation and the relative abundance of MOBs was highest (Figs. 1D,E & 3A).

Furthermore, in Mekkojärvi, MOB relative abundance was lower in the epilimnion with higher PAR (~4.2 µmol photons m^{-2} s^{-1}) than in the metalimnion (~0.4 µmol photons m^{-2} s^{-1}) (Fig. 2A). By oxidizing CH_{4} below the oxycline, MOBs also potentially supported the daytime metabolism of anaerobes by sustaining anoxic conditions via immediately consuming the O_{2} generated by the oxygenic photosynthesis.

Since AOM organisms were not present, the measured CH_{4} oxidation in the dark anaerobic treatments could be due to both MOB activity as well as trace methane oxidation by methanogens (Moran et al. 2005). The general EA-induced decreases in CH_{4} oxidation actually indicate a contribution of trace methane oxidation, since it is known to decrease concurrently with methanogenesis, when methanogenic samples are exposed to increased availability of EAs other than CO_{2} (Tables 2 & 3) (Moran et al. 2005, Meulepas et al. 2010, Timmers et al. 2016). However, the concurrent transcription of pmoA of MOBs and mcrA of methanogens in Mekkojärvi experiments show that MOBs were also active in the dark and anaerobic incubations (Figs. 2C & S5). Nonetheless, except for the NO_{3}^{-}-treatment in Alinen-Mustajärvi experiments, EAs seemed to decrease the CH_{4} oxidation activity of MOBs, as suggested by the general EA-induced decreases in the relative abundance of Methylococcales (Fig. 2A). As NO_{3}^{-} addition would also be expected to decrease trace methane oxidation, the insignificant effect of NO_{3}^{-} on CH_{4} oxidation in Alinen-Mustajärvi experiments could actually stem from a simultaneous decrease in trace methane oxidation and an increase in MOB-driven methane oxidation (Table 3). The generally higher levels of excess ~^{15}C-CO_{2} in the CH_{4}+NO_{3}^{-} treatment compared with the treatment with only CH_{4} also imply that NO_{3}^{-} had an enhancing effect on CH_{4} oxidation (Fig. 7). Supporting this, NO_{3}^{-} was present (Fig. S2B), and our previous stable isotope study also indicated active denitrification in the water column of Alinen-Mustajärvi (Tirola et al. 2011). Furthermore, the Methylococcales MAGs, which were most abundant below the oxycline in Alinen-Mustajärvi, had genetic potential for partial denitrification (Fig. 6C). This suggests that boreal lake MOBs can potentially couple NO_{3}^{-} reduction with CH_{4} oxidation. Despite our efforts to prevent O_{2} contamination, we cannot completely rule out the possibility that trace amounts of O_{2} diffused to incubation bottles through or out of the septa (since O_{2} can be trapped within septa during sample preparations) during the incubations (De Brabandere et al. 2012). Therefore, more rigorous measures to prevent O_{2} contamination are needed in future studies to resolve whether NO_{3}^{-} reduction of MOBs was coupled with micro-aerobic CH_{4} activation (Kits et al. 2015b, Padilla et al. 2017) or with AOM that is independent of external O_{2}, e.g. via a similar mechanism to that carried out by bacteria in NC10 phyla (Ettwig et al. 2010).

The lack of SO_{4}^{2-}-induced CH_{4} oxidation by MOBs was expected based on previous studies (Oswald et al. 2015, 2016b). In contrast, results on Fe^{3+} and Mn^{4+} were not expected, since both of them enhanced the activity of Methylococcales in the temperate Lake Zug, Switzerland (Oswald et al. 2016b), while Fe^{3+} was also suggested to enhance CH_{4} oxidation by a mixed MOB-methanogen community in deep sediments of Lake Kinneret (Bar-Or et al. 2017). Accordingly, the decrease in CH_{4} oxidation by organic EAs was unexpected, as humic substances act as electron shuttles between Fe^{3+} (Mn^{4+}) and bacteria (Lovley et al. 1996), and the capability for Fe^{3+}/Mn^{4+} and organic EA respiration usually occur in the same species (Lovley 2006). However, despite decreasing the potential CH_{4} oxidation, the addition of inorganic EAs actually increased the relative pmoA expression of the Methylobacter MOBs that dominated in situ (Fig. 2C). Furthermore, the inorganic EA-induced decrease in the CH_{4} oxidation in the hypolimnion was actually very modest compared to other treatments, also coinciding with an increase in the relative abundance of in situ dominant MOB 16S rRNA (Fig. 2, Table 2). This implies that the metabolism of the in situ dominant MOBs in Mekkojärvi was enhanced by inorganic EAs; however, their effect on total potential CH_{4} oxidation was probably masked by the decrease in methanogen trace methane oxidation as explained...
above, as well as by the effect of other, naturally rare MOBs, which became prevalent and active during the incubation (Fig. 2B,C). Thus, further studies are still needed to assess the effect of EAs on CH₄ oxidation of in situ dominant MOBs in boreal lakes. These studies could utilize specific inhibitors for the activity of methanogenic archaea and MOBs to distinguish between different CH₄-oxidizing processes (Miller et al. 1998, Liu et al. 2011) as well as have a shorter incubation time than in this study to prevent the increase and activity of the undesired, naturally rare MOBs. A culture-dependent study approach (i.e. experiments with isolated lake MOBs) could be also adopted. It is also possible that the quality (e.g. oxidation state) of the utilized organic EAs differed from that of the organic EAs present in boreal lakes to an extent that would make them less usable for the lake microbes. Indeed, native oxidized organic matter was recently shown to increase bacterial activity in lake water columns (Lau et al. 2017). Therefore, further studies should also assess the effects of in situ organic EAs on MOB metabolism.

The dominance and vertical distribution patterns of the putative novel MOB lineage Ca. Methyloframidiphilus alinensis suggests that it played a very important role in water column CH₄ oxidation in Alinen-Mustajärvi (Figs. 1D,E & 3A). It belongs to MOB Type Ib (Figs. 5 & S9), a group that was previously considered to consist only of species that are adapted to thermal habitats (Danilova et al. 2016). However, our findings and recent isolation of Methyloterricola oryzae from stems of rice plants and enrichment of Ca. Methylospira palustris from peat bog together with the discovery of pmoA sequences that are closely related to these lineages from various non-thermal habitats have now changed this view (Danilova et al. 2016, Frindt et al. 2017). These results also suggest that there are even more undiscovered MOB Type Ib genera inhabiting non-thermal habitats. We actually noticed that 16S rRNA gene sequences from Ca. M. alinensis were assigned as ‘unclassified Gammaproteobacteria’ when using older Silva 119 (released 24 July 2014) and 123 (23 July 2015) databases, which suggests that previous 16S rRNA amplicon-based studies might have failed to assign members of this lineage to MOBs. Therefore, we supplemented the utilized Silva 128 database with the clone library sequence representing Ca. M. alinensis (HE616416) and made a preliminary screening for this lineage from a set of 16S rRNA gene amplicon libraries from water column samples of oxygen-stratified lakes. Besides the study lakes, Ca. M. alinensis was detected in Lakes Valkea-Kotinen and Valkea-Mustajärvi in Finland (Peura et al. 2012), Lakes 227 and 442 in Canada (Schiff et al. 2017), Lake Grosse Fuchskuhle in Germany (Garcia et al. 2013), and Lake Rotsee in Switzerland (Oswald et al. 2017). However, it had much lower relative abundance (0.0004 to 0.7% of bacterial 16S rRNA genes) in the other lakes compared with Alinen-Mustajärvi (up to 14.6%), which could be caused by database biases (i.e. insufficient representation of the sequence diversity of this lineage in the database).

However, these results suggest that Ca. M. alinensis is a common member of MOB communities in water columns of oxygen-stratified lakes. Further studies are needed to assess its importance in CH₄ oxidation in different ecosystems as well as the factors affecting its activity and distribution. Our results from the 2 study lakes suggest that Ca. M. alinensis prefers microaerobic/anaerobic conditions. However, its distribution pattern is different from that of the other ubiquitous water-column MOB genus, Methylobacter, suggesting niche differentiation between these bacterial genera. The preference of Ca. M. alinensis for higher water column layers and its rarity in Mekkojärvi, as compared to Methylobacter, can be due to it being more competitive in higher redox conditions or higher light radiation than Methylobacter, since both ORP and PAR decreased with depth and were higher in microaerobic/anaerobic layers of Alinen-Mustajärvi compared with Mekkojärvi.

CONCLUSION

Accumulating evidence from this and previous studies now suggests almost exclusive dominance of aerobic MOBs in the methanotrophic community and activity both above and below the oxycline in the water column of oxygen-stratified methane-rich lakes in the boreal and temperate zones. Besides the typical MOB-genera (e.g. Methylobacter, Methylo monas, and Crenothrix), the putative novel MOB lineage Ca. Methyloframidiphilus alinensis, found in this study, may be an important member of the MOB community in the water columns of oxygen-stratified lakes and has probably been undetected as a MOB in many previous 16S rRNA amplicon studies due to database biases. In contrast to MOBs, the activity of typical AOM bacteria (NC10 phyla) and archaea (ANME archaea) in lakes seems to be limited to sediments. The incubation results together with the detection of genetic denitrification potential in MAGs of MOBs also imply that NO₃⁻ reduction may support micro-aerobic or even anaerobic CH₄ oxidation activity of boreal lake MOBs. Furthermore, this study suggests that light-
driven oxygenic photosynthesis potentially supports aerobic CH$_4$ oxidation below the oxycline in boreal lakes, in accordance with results from temperate lakes. However, light radiation above a certain PAR limit may also inhibit MOB activity, as was also suggested by the vertical distribution of MOBs in the study lakes. Consequently, the projected water brownification that decreases light penetration, and therefore, oxygenic photosynthesis in lake water columns can either (1) decrease CH$_4$ oxidation, (2) not affect it due to ascent of the oxycline and the CH$_4$ oxidation layer, or (3) even increase it, due to the cessation of light inhibition of MOBs.

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