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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 (^{13}C-CH_4 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 (SO_4^{2-}, Fe^{3+}, Mn^{4+}, and anthraquinone-2,6-disulfonate), except for NO_3^-, 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 NO_x^- 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 (CH_4), 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

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(Conrad 1999). Although lakes occupy only 3.7% of the global non-glaciated land area (Verpoorter et al. 2014), their CH_4 emissions are estimated to be as high as 6 to 24% of the total natural CH_4 release (Bastviken et al. 2004, 2011). The numerous lakes and ponds in the northern areas (north of 50° N) with annual CH_4 emissions of ~16.5 Tg (6 to 7% of natural release) are especially significant components of the global CH_4 budget (Wik et al. 2016). Thus, knowledge about CH_4 cycling in lakes, especially in northern areas, is essential to better constrain its global input and will ultimately aid in predicting climate change.

CH_4 emissions from natural ecosystems are largely regulated by aerobic oxidation by methane oxidizing bacteria (MOB), utilizing O_2 as an electron acceptor (EA) (Hanson & Hanson 1996), or through anaerobic oxidation of methane (AOM) by anaerobic methanotrophic archaea (ANME archaea), utilizing alternative inorganic (NO_3^-, SO_4^{2-}, Mn^{4+} or Fe^{3+}) 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 CH_4 in anaerobic conditions using the nitric oxide dismutase enzyme (Ettwig et al. 2010). Some methanogens also oxidize small amounts of CH_4 without external EAs during trace methane oxidation due to enzymatic backflux (Moran et al. 2005, Timmers et al. 2017). While AOM coupled with SO_4^{2-} reduction by ANME archaea is an efficient CH_4 sink in oceanic sediments and waters (Knittel & Boetius 2009), a variety of EAs, i.e. SO_4^{2-}, Fe^{3+}, and NO_3^-/NO_2^-, have been shown to be important drivers of the AOM process in freshwater sediments (Sivan et al. 2011, Deutzmann et al. 2014, á Norði & 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 CH_4 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 CH_4 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 O_2 to activate CH_4 and are suggested to further support their metabolism by fermentation (Kalyuzhnaya et al. 2013) or by anaerobic respiration using alternative EAs, i.e. NO_3^-, NO_2^- 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 O_2 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, CH_4 accumulates in the anoxic hypolimnion (Houser et al. 2003, Kankaala et al. 2007), and CH_4 oxidation taking place in the water column acts as an efficient CH_4 sink (Kankaala et al. 2006, Peura et al. 2012). In fact, isotopic profiling shows that a substantial part of CH_4 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 CH_4 oxidation and AOM in the water columns of boreal lakes remain unresolved.

We studied the contribution of aerobic CH_4 oxidation and AOM in water columns of 2 boreal oxygen-stratified lakes by geochemical profiling and by conducting water sample incubations amended with ^{13}C-labeled CH_4 and various EAs. CH_4-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 CH_4 oxidation below the oxycline (oxic–anoxic interface) of water column of these boreal, CH_4-rich lakes.

MATERIALS AND METHODS

Study lakes and sampling

The study lakes — Lake Mekkojärvi (61° 13′ N, 25° 8′ E) (area 0.004 km^2, 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 km^2, 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 O_2 and temperature profiles were measured using a YSI 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 CH_4, CO_2 and sulfide, and $^{13}C/^{12}C$ of dissolved inorganic carbon (DIC) for both lakes. In addition data was collected on total dissolved Fe and Mn for Mekkojärvi, and on $^{13}C/^{12}C$ of CH_4 and concentrations of inorganic nutrients (NO_3^-+NO_2^-, NH_4^+, PO_4^{2-}), SO_4^{2-}, total N, total P, DOC and particulate organic carbon (POC) for Alinen-Mustajärvi. For CH_4 oxidation experiments, water was collected from the epi- (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 CH_4 oxidation

To test the effects of EAs on the anaerobic CH_4 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 ^{13}C-labeled CH_4 and 1 replicate with ^{14}C-labeled CH_4. 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 ^{13}C-content of DIC, concentrations of CH_4 and CO_2, 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 ^{13}C-CH_4 (6 bottles), isotopically natural CH_4 (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 CH_4-concentration were taken 14 times, while those for ^{13}C-DIC and sulfide were taken 5 and 2 times, respectively, from the bottles amended with ^{13}C-CH_4 or normal CH_4, during the 6.5 mo pre-incubation. One further sampling of CH_4 and ^{13}C-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 CH_4 and 2 with ^{13}C-CH_4) 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

Table 1. Details of CH_4 oxidation experiments carried out in 2 boreal lakes in Finland. In Lake Mekkojärvi experiments, inorganic electron acceptors (EAs) consisted of a mixture of 5 mM NO_3^-, 1 mM SO_4^{2-}, 10 mM Mn^{4+}, and 0.5 mM Fe^{3+}; while 4 mM disodium anthraquinone-2,6-disulfonate was used as an organic EA. The final column shows the number of replicates amended with ^{13}C-labeled CH_4. In addition, there were control treatments without ^{13}C-labeled CH_4 (see 'Materials and methods')

Lake	Depth zone	Pre-incubation	Treatments	Conditions (light, temperature, time)	No.
Mekkojärvi	Epilimnion	No	CH_4	Dark, +10°C, 21 d	2
	Metalimnion	No	CH_4		
			CH_4 + inorg. EAs		
			CH_4 + org. EAs		
	Hypolimnion	No	CH_4		
			CH_4 + inorg. EAs		
			CH_4 + org. EAs		
Alinen-Mustajärvi	Hypolimnion	Yes[a]	CH_4	Dark, +6.1°C, 134 d	5
			CH_4 + 1 mM NO_3^-		
			CH_4 + 1 mM SO_4^{2-}		
			CH_4 + 3 mM Fe^{3+}		
			CH_4 + 1 g l^{-1} humic acid		
			CH_4 + 1 g l^{-1} humic acid + 3mM Fe^{3+}		
			CH_4 + O_2	Dark, +6.1°C, 27 d	
			CH_4	Light, +6.1°C, 134 d	
			CH_4	Red light, +6.5°C, 134 d	

[a]A 6.5 mo pre-incubation of concentrated water samples was done before the EA and light experiments for stable isotope probing (SIP) of DNA and RNA. However, SIP failed due to an insufficient amount of extracted DNA and RNA

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

$^{13}C/^{12}C$ 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 $\delta^{13}C$ values for water column data and as excess concentration of ^{13}C-CO_2 or ^{13}C-DIC for incubations (i.e. the concentration of ^{13}C produced solely from the added ^{13}C-CH_4) according to Supplement 1.

The accumulation of excess ^{13}C-CO_2 or ^{13}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}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}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}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, anoxygenic phototrophic H_2S, and Fe^{2+}-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 Fe^{2+}-oxidizing (Hedrich et al. 2011, Moya-Beltrán et al. 2014), and anaerobic, i.e. SO_4^{2-}-reducing (Postgate & Campbell 1966, Finster 2008, Kuever 2014, Hausmann et al. 2016) and Fe^{3+}-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 data were then normalized using the counts of 139 single copy genes as described previously (Rinke et al. 2013). The assembled contigs were binned with MetaBAT (version 0.26.3) (Kang et al. 2015) to reconstruct the genomes of the most abundant lake microbes, i.e. metagenome assembled genomes (MAGs). The quality of the MAGs was evaluated using CheckM (version 1.0.6) (Parks et al. 2015). The cut-offs for high-quality MAGs were set to ≥40% for completeness and ≤5% for contamination.

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 ^{13}C-CO_2 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).

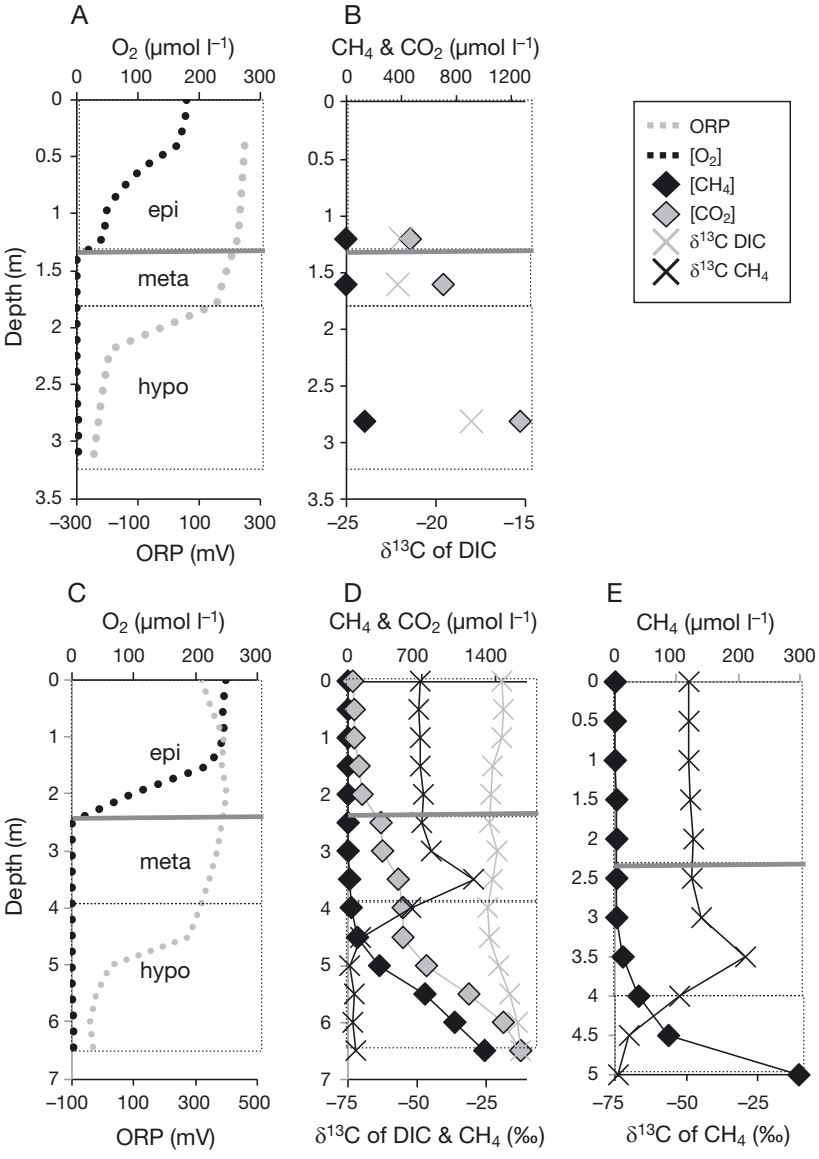

Fig. 1. Vertical depth profiles measured in 2 boreal lakes in Finland: (A) oxidation-reduction potential (ORP) and O_2 concentration in Lake Mekkojärvi; (B) $\delta^{13}C$ of dissolved inorganic carbon (DIC), and concentrations of CH_4 and CO_2 in Lake Mekkojärvi; (C) ORP and O_2 concentration in Lake Alinen-Mustajärvi; (D) $\delta^{13}C$ of DIC and CH_4, and CH_4 and CO_2 concentrations in Lake Alinen-Mustajärvi; (E) $\delta^{13}C$ and concentration of CH_4 at a higher resolution for the 0–5 m layer in Lake Alinen-Mustajärvi. Oxycline depth is denoted with a grey line. The epi- (above the oxycline) as well as meta- and hypolimnion (below the oxycline) zones are indicated with dashed line boxes

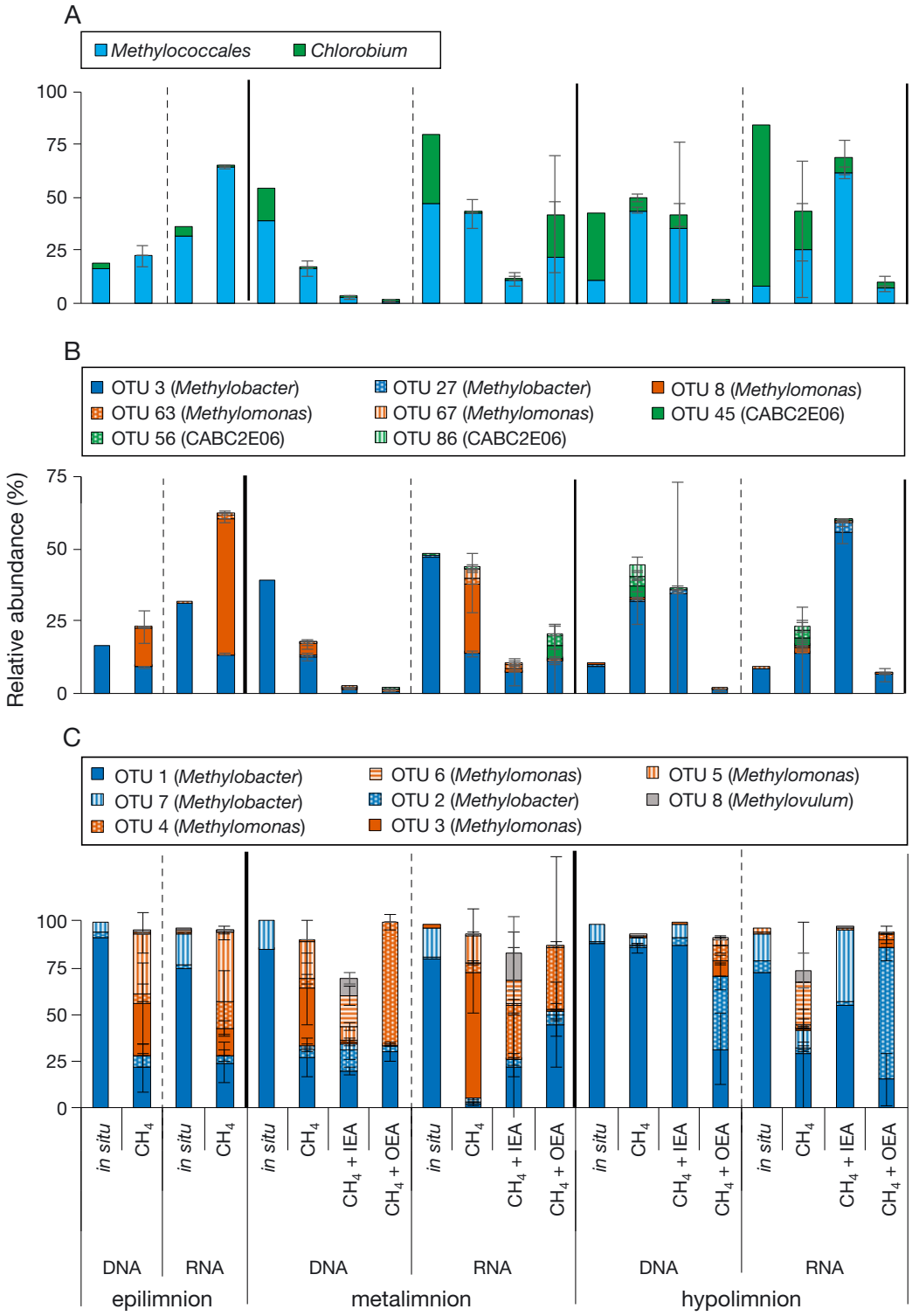

Fig. 2. Relative abundances of components of the microbial community in Lake Mekkojärvi, Finland: (A) *Methylococcales* and anoxygenic phototrophic H_2S and Fe^{2+}-oxidizing (*Chlorobium*) bacteria; (B) dominant OTUs of *Methylococcales* (and their affiliation) based on the 16S rRNA gene and 16S rRNA; (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 epi-, meta-, and hypolimnion and amended with ^{13}C-CH_4, ^{13}C-CH_4 plus a mixture of inorganic electron acceptors (IEA: NO_3^-, SO_4^{2-}, Fe^{3+} and Mn^{4+}), and ^{13}C-CH_4 plus an organic EA (OEA: di-sodium anthraquinone-2,6-disulfonate). Data are presented as average ± SD when n = 2, otherwise n = 1

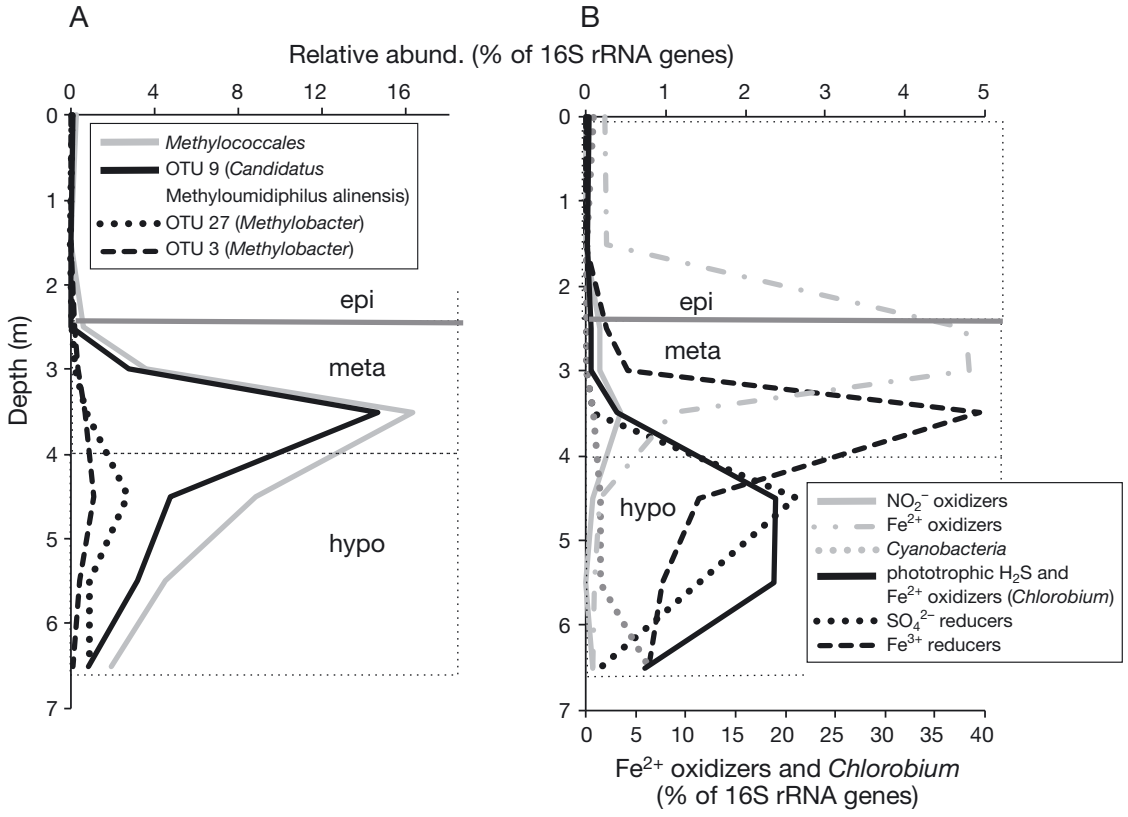

Fig. 3. Vertical depth profiles of relative abundances, measured as % of 16S rRNA gene amplicons, of components of the microbial community in Lake Alinen-Mustajärvi, Finland: (A) total *Methylococcales*, and 3 dominant *Methylococcales* OTUs (and their taxonomic affiliation); (B) *Cyanobacteria*, aerobic NO_2^- and Fe^{2+}-oxidizing bacteria, anaerobic Fe^{3+} and SO_4^{2-}-reducing bacteria as well as anoxygenic phototrophic H_2S and Fe^{2+}-oxidizing bacteria. Oxycline depth is denoted with a grey line. Epi- (above the oxycline) as well as the meta- and hypolimnion (below the oxycline) zones are indicated with dashed line boxes. Note the different *x*-axes for Fe^{2+} oxidizers and *Chlorobium* in (B)

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 gammaproteobacterial 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 anaerobic CH_4-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

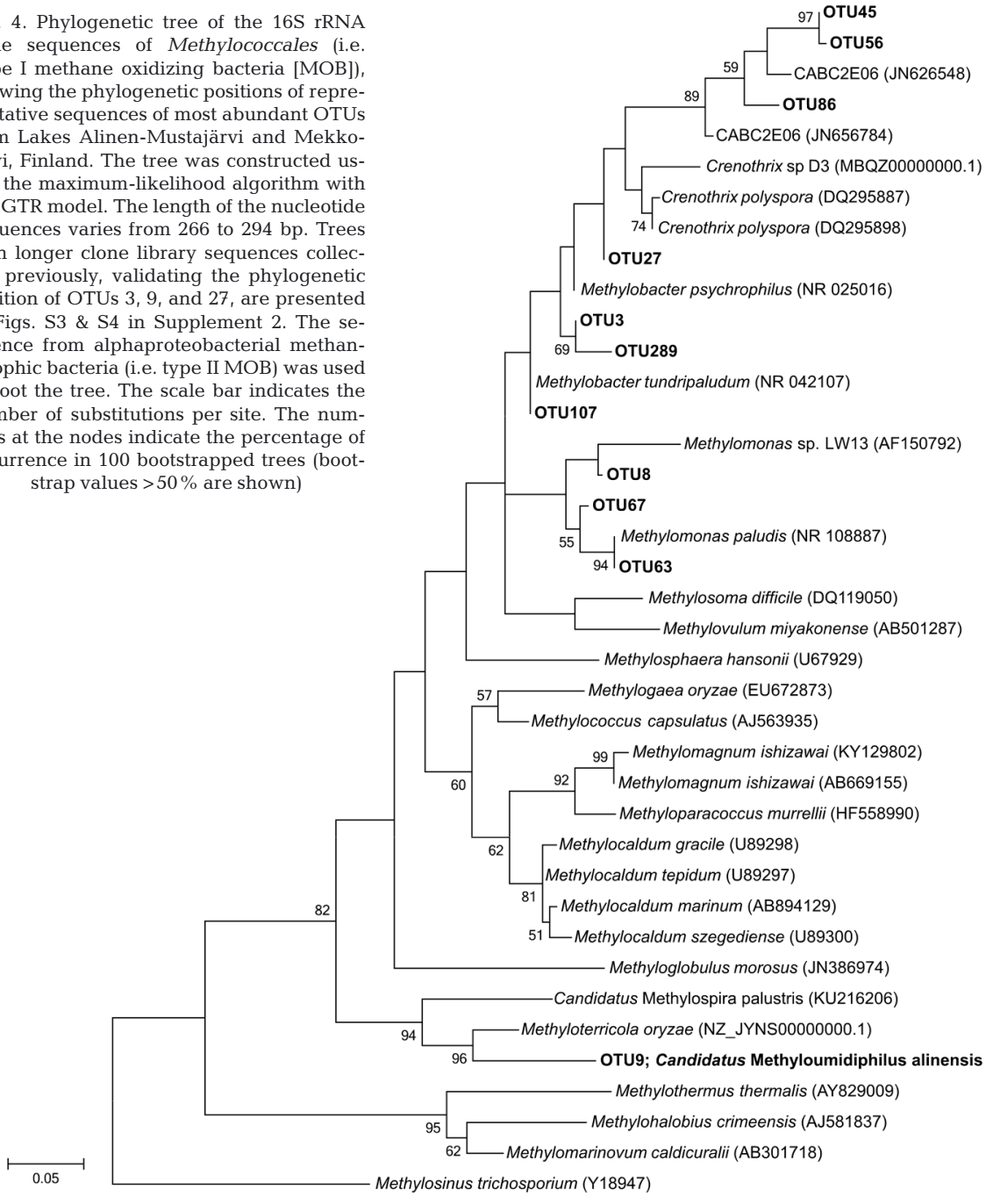

Fig. 4. Phylogenetic tree of the 16S rRNA gene sequences of *Methylococcales* (i.e. Type I methane oxidizing bacteria [MOB]), showing the phylogenetic positions of representative sequences of most abundant OTUs from Lakes Alinen-Mustajärvi and Mekkojärvi, Finland. The tree was constructed using the maximum-likelihood algorithm with the GTR model. The length of the nucleotide sequences varies from 266 to 294 bp. Trees with longer clone library sequences collected previously, validating the phylogenetic position of OTUs 3, 9, and 27, are presented in Figs. S3 & S4 in Supplement 2. The sequence from alphaproteobacterial methanotrophic bacteria (i.e. type II MOB) was used to root the tree. 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)

89.9% similar, respectively, to the closest known *Methylococcales* genus, *Methyloterricola* (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* Methyloumidiphilus 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, *Methyloumidiphilus* is a methyl-using bacterium that prefers wet environments, and the species

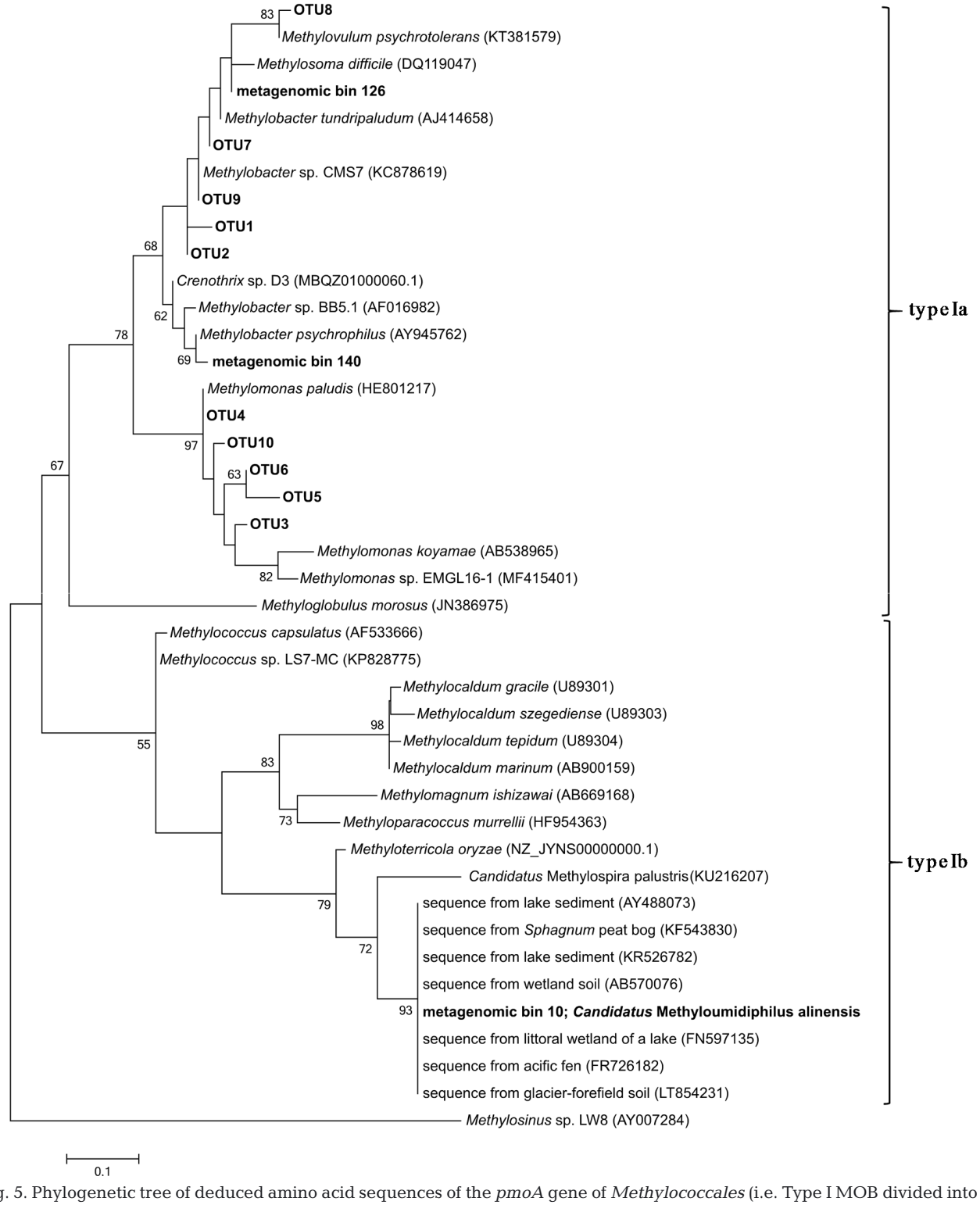

Fig. 5. Phylogenetic tree of deduced amino acid sequences of the *pmoA* gene of *Methylococcales* (i.e. Type I MOB divided into clusters Ia and Ib), showing the phylogenetic positions of representative sequences of most abundant OTUs from Lake Mekkojärvi as well as sequences from metagenomic bins from 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 sequence from alphaproteobacterial methanotrophic bacteria (i.e. Type II MOB) was used to root the tree. 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)

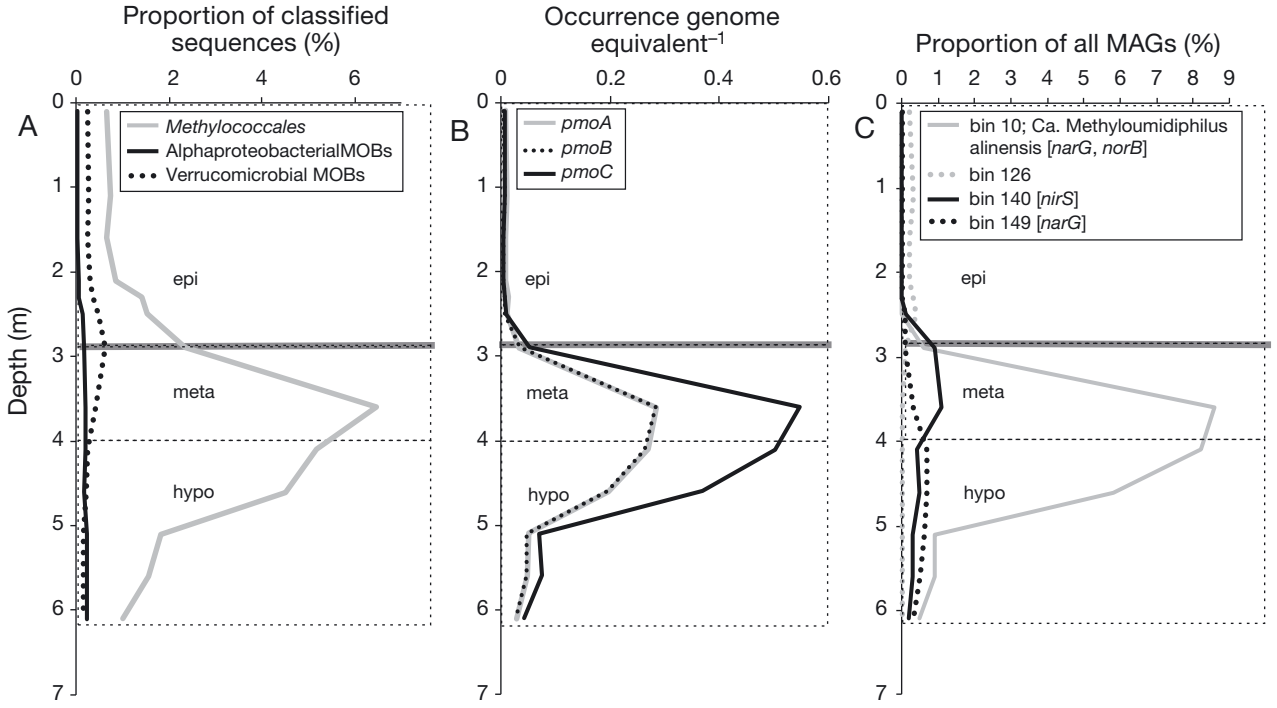

Fig. 6. Vertical depth profiles from Lake Alinen-Mustajärvi, Finland based on shotgun metagenomic analysis for (A) different groups of aerobic methanotrophic bacteria (MOB); (B) genes coding for particulate methane monooxygenase Subunits a (*pmoA*), b (*pmoB*), and c (*pmoC*); (C) metagenome assembled genomes (MAGs; i.e. metagenomic bins) of *Methylococcales*. The denitrification genes found within the MAGs are denoted in brackets after the name of each bin in (C). Oxycline depth is denoted with a grey line. The epi- (above the oxycline) as well as meta- and hypolimnion (below the oxycline) zones are indicated with dashed line boxes

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_2S 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 (<0.3% of 16S rRNA 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_2S, 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 and *mcrA* gene amplicon results, anaerobic methanotrophs were not detected, and *Methylococcales* 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 monooxygenase Subunits b (*pmoB*) and c (*pmoC*), followed that of *Methylococcales* (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 *Methylococcales*. 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 *Methyloterricola oryzae* (Fig. S8), which is in accordance with the phylogenetic position of the most dominant *Methylococcales*-OTU, OTU 9 (Figs. 4 & S4). Furthermore, the deduced amino acid sequence of *pmoA* of Bin 10 was most similar to *Methyloterricola* (Figs. 5 & S9). Altogether, this suggests that Bin 10 and the 16S rRNA gene OTU 9 represent the same species. However, in accordance with the 16S rRNA gene results, the deduced amino acid sequence of *pmoA* of Bin 10 was still quite distantly related to *M. oryzae*, sharing only 90% similarity (Figs. 5 & S9). This confirms that OTU 9 and metagenomic Bin 10 represent a novel genus and species of *Methylococcales*. The deduced amino acid *pmoA* sequence of Bin 10 shared 97 to 100% similarity with the closest environmental database sequences, which were dominantly from wet environments (peatlands, wetlands, lake and river sediments) (Figs. 5 & S9), which further supports our choice of name for this novel genus (see 'Microbes in the study lakes analysed by DNA- and RNA-based amplicon sequencing' above). In contrast to Bin 10, PhyloPhlAn placed the other *Methylococcales* bins closest to *Crenothrix* but to a branch without any 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 *Crenothrix* can obtain their *pmoA* gene via lateral gene transfer from other *Methylococcales* (Oswald et al. 2017), neither of the 16S rRNA gene OTUs in Alinen-Mustajärvi were affiliated with *Crenothrix* (Figs. 4, S3, & S4). Hence, it is likely that Bins 126, 140, and 149 represented species that have no genomes or isolated members available (e.g. *Methylobacter 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 CH_4 oxidation rates, measured as excess ^{13}C-dissolved inorganic carbon (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 exceess ^{13}C-DIC production are shown (n = 2 replicates per treatment). Inorganic electron acceptors (EA) included a mixture of NO_3^-, SO_4^{2-}, Fe^{3+}, and Mn^{4+}; while disodium anthraquinone-2,6-disulfonate was used as an organic EA

Depth zone	Treatment	Excess ^{13}C-DIC production (min; max) (nmol l^{-1} d^{-1})
Epilimnion	CH_4	479.5; 916.5
Metalimnion	CH_4	977.4; 1140.7
	CH_4 + inorg EAs	132.0; 156.6
	CH_4 + org. EAs	59.0; 134.6
Hypolimnion	CH_4	1093.4; 1147.5
	CH_4 + inorg. EAs	691.9; 839.5
	CH_4 + org. EAs	33.2; 34.2

Variation in potential CH_4 oxidation and in microbial community structure in the incubation experiments

In Mekkojärvi, potential CH_4 oxidation based on the accumulation of excess ^{13}C-DIC in incubations

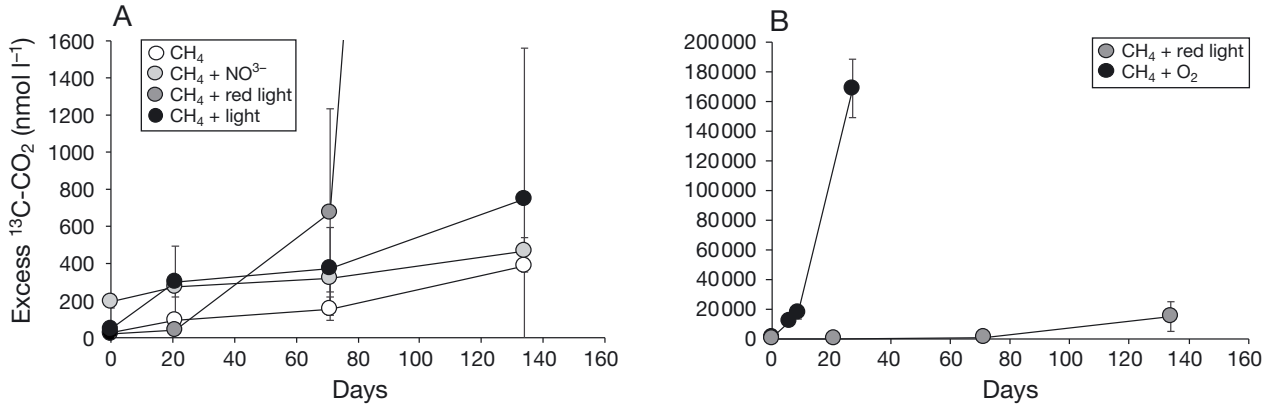

Fig. 7. Accumulation of excess ^{13}C-CO_2 during incubation of concentrated water samples (average ± SD, n = 5) collected from the hypolimnion (5.5 m) of Lake Alinen-Mustajärvi and subjected to different treatments: (A) CH_4 and CH_4+NO_3^- in the dark, and CH_4 under red and normal light; (B) CH_4 + O_2 in the dark, and CH_4 under red light. Note the significant accumulation of ^{13}C-CO_2 during the period 71–134 d in the red light treatment

was generally higher in the meta- and hypolimnion than in the epilimnion (Table 2). The addition of inorganic and organic EAs decreased the potential CH_4 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 CH_4 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 CH_4 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 CH_4 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 rel-

Table 3. Potential CH_4 oxidation rates, measured using an isotope ratio mass spectrometer (IRMS) as average (±SD) excess ^{13}C-CO_2 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 CH_4 oxidation could not be assessed for samples amended with humic acids (CH_4 + humic acids and CH_4 + humic acids + Fe^{3+}) due to CO_2 concentrations being below the detection limit of the IRMS. Different letters in the final column indicate significant differences in CH_4 oxidation between treatments (1-way ANOVA, $p < 0.05$). CH_4 oxidation rates in treatments with added O_2 were many-fold higher than in other treatments and were therefore excluded from the statistical test

Time period (d)	Treatment	Excess ^{13}C-CO_2 production (nmol l^{-1} d^{-1})	
0–21	CH_4	3.2 ± 0.3	a
	CH_4 + NO_3^-	3.6 ± 2.5	a
	CH_4 in red light	1.1 ± 0.3	a
	CH_4 in light	12.1 ± 9.2	b
	CH_4 + SO_4^{2-}	0	
	CH_4 + Fe^{3+}	0	
21–71	CH_4	1.3 ± 1.3	a
	CH_4 + NO_3^-	0.9 ± 1.5	a
	CH_4 in red light	12.7 ± 11.1	b
	CH_4 in light	2.2 ± 3.8	a
	CH_4 + SO_4^{2-}	0	
	CH_4 + Fe^{3+}	0	
71–134	CH_4	3.7 ± 0.6	a
	CH_4 + NO_3^-	2.3 ± 1.2	a
	CH_4 in red light	217.3 ± 160.0	b
	CH_4 in light	6.6 ± 12.5	a
	CH_4 + SO_4^{2-}	0	
	CH_4 + Fe^{3+}	0	
0–6	CH_4 + O_2	1714.6 ± 354.0	
6–9	CH_4 + O_2	2145.1 ± 1446.3	
9–27	CH_4 + O_2	8391.7 ± 1092.8	

ative 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 Mekkojärvi (Table 2). MOBs also actively transcribed *pmoA* at all depth layers in Mekkojärvi (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 δ^{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 δ^{13}C value than oxidation of organic matter, the lowest δ^{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 ^{13}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_x^- was present (Fig. S2B), and our previous stable isotope study also indicated active denitrification in the water column of Alinen-Mustajärvi (Tiirola 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_x^- 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_x^- 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 CH_4 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 CH_4-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*. Methyloumidiphilus alinensis suggests that it played a very important role in water column CH_4 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, Frindte 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 CH_4 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*, *Methylomonas*, and *Crenothrix*), the putative novel MOB lineage *Ca*. Methyloumidiphilus 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 NO_x^- reduction may support micro-aerobic or even anaerobic CH_4 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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