RESEARCH ARTICLE

10.1002/2016JG003382

Blaize A. Denfeld and Monica Ricão Canelhas shared first authorship.

Key Points:

- CH₄ oxidation in lake water from three out of seven ice-covered lakes occurred after one month of incubation at 2°C
- Factors other than CH₄ availability, temperature, and presence of methanotrophs, limited CH₄ oxidation
- CH₄ oxidation limitation was partially explained by phosphate availability and bacterial community interactions

Correspondence to:

B. A. Denfeld,
blaize.denfeld@umu.se

Citation:

Denfeld, B. A., M. Ricão Canelhas, G. A. Weyhenmeyer, S. Bertilsson, A. Eiler, and D. Bastviken (2016), Constraints on methane oxidation in ice-covered boreal lakes, *J. Geophys. Res. Biogeosci.*, 121, 1924–1933, doi:10.1002/2016JG003382.

Received 16 FEB 2016

Accepted 6 JUL 2016

Accepted article online 12 JUL 2016

Published online 22 JUL 2016

Constraints on methane oxidation in ice-covered boreal lakes

Blaize A. Denfeld^{1,2}, Monica Ricão Canelhas¹, Gesa A. Weyhenmeyer¹, Stefan Bertilsson^{1,3}, Alexander Eiler¹, and David Bastviken⁴
¹Department of Ecology and Genetics/Limnology, Uppsala University, Uppsala, Sweden, ²Now at Department of Ecology and Environmental Science, Umeå University, Umeå, Sweden, ³Science for Life Laboratory, Uppsala University, Uppsala, Sweden, ⁴Department of Thematic Studies-Environmental Change, Linköping University, Linköping, Sweden

Abstract Boreal lakes can be ice covered for a substantial portion of the year at which time methane (CH₄) can accumulate below ice. The amount of CH₄ emitted at ice melt is partially determined by the interplay between CH₄ production and CH₄ oxidation, performed by methane-oxidizing bacteria (MOB). Yet the balance between oxidation and emission and the potential for CH₄ oxidation in various lakes during winter is largely unknown. To address this, we performed incubations at 2°C to screen for wintertime CH₄ oxidation potential in seven lakes. Results showed that CH₄ oxidation was restricted to three lakes, where the phosphate concentrations were highest. Molecular analyses revealed that MOB were initially detected in all lakes, although an increase in type I MOB only occurred in the three lake water incubations where oxidation could be observed. Accordingly, the increase in CO₂ was on average 5 times higher in these three lake water incubations. For one lake where no oxidation was measured, we tested if temperature and CH₄ availability could trigger CH₄ oxidation. However, regardless of incubation temperatures and CH₄ concentrations, ranging from 2 to 20°C and 1–500 μM, respectively, no oxidation was observed. Our study indicates that some lakes with active wintertime CH₄ oxidation may have low emissions during ice melt, while other and particularly nutrient poor lakes may accumulate large amounts of CH₄ below ice that, in the absence of CH₄ oxidation, will be emitted following ice melt. This variability in CH₄ oxidation rates between lakes needs to be accounted for in large-scale CH₄ emission estimates.

1. Introduction

Lakes are significant contributors to the global methane (CH₄) budget, contributing up to 16% of the total CH₄ emissions into the atmosphere [Bastviken *et al.*, 2011]. High-latitude lakes represent a large fraction of all lakes on Earth [Verpoorter *et al.*, 2014] and can be ice covered for many months of the year [Prowse *et al.*, 2012]. During this time, CH₄ produced in anoxic sediments and exported to the water column by ebullition or diffusion accumulates in the water column below ice [Michmerhuizen *et al.*, 1996; Phelps *et al.*, 1998]. At the same time, in aerobic waters below ice, biological CH₄ oxidation has the potential to decrease concentrations of CH₄. Since CH₄ emissions from lakes can be substantial during the ice melt period, accounting for 3–84% of the annual CH₄ released from lakes [Karlsson *et al.*, 2013], understanding the constraints of CH₄ oxidation prior to ice melt is important. However, current knowledge on CH₄ oxidation during winter is sparse with no consensus on the rate of CH₄ oxidation [Michmerhuizen *et al.*, 1996; Kankaala *et al.*, 2006; Martinez-Cruz *et al.*, 2015], making it difficult to account for the amount of CH₄ that is oxidized below ice prior to ice melt.

CH₄ oxidation is carried out by methane-oxidizing bacteria (MOB), which use CH₄ as a carbon and energy source. Aerobic CH₄ oxidation typically takes place where both CH₄ and oxygen (O₂) are present in large enough quantities and has been suggested to be primarily limited by CH₄ substrate supply [cf. Duc *et al.*, 2010]. The highest rates of CH₄ oxidation in lakes are often found in transition zones between anoxic and oxic environments, where MOB profit from high CH₄ concentrations while also having access to O₂, the thermodynamically most favorable electron acceptor [Carini *et al.*, 2005; Bastviken, 2009]. Conditions at the water-ice interface can also be favorable for CH₄ oxidation when CH₄ ebullition from anoxic sediments elevates CH₄ concentrations in oxic surface waters [Ricão Canelhas *et al.*, 2016]. However, studies investigating CH₄ oxidation in cold surface waters below ice are limited, while the direct effect of temperature on CH₄ oxidation has been a matter of debate. Some studies suggest that CH₄ oxidation is indirectly temperature dependent, since CH₄ production is temperature related and CH₄ oxidation is driven by CH₄ availability [Segers, 1998; Kankaala

Figure 1. Locations and relative size of the seven ice-covered lakes in central Sweden from which water for CH_4 oxidation incubations were collected. Note distance between lakes is not to scale.

et al., 2006; Duc *et al.*, 2010; Borrel *et al.*, 2011]. Other studies conversely suggest that CH_4 oxidation is directly related to temperature since cold temperatures may restrain overall microbial activity [Sundh *et al.*, 2005; Martinez-Cruz *et al.*, 2015].

The aim of this study was to test the CH_4 oxidation potential in surface water collected during winter below lake ice. We performed a comparative lake screening for wintertime CH_4 oxidation potential in seven lakes characterized by varying water chemistry. In addition, for one of the seven lakes, a more detailed incubation at different temperatures and CH_4 concentrations was carried out to test if temperature and CH_4 availability could trigger CH_4 oxidation in a single system. In all incubations CH_4 oxidation was determined by tracking changes in CH_4 concentration over time. In addition, microbial community composition analysis was conducted to identify the presence of MOB at the start and the end of the incubation.

2. Materials and Methods

2.1. Lake Survey Sampling

For the CH_4 oxidation incubation experiment, water was collected from seven ice-covered lakes in early 2015: Björklinge-Långsjön, Erken, Fyrsjön, Lumpen, Lötsjön, Malstasjön, and Plåten (Figure 1). The lakes were chosen based on differences in morphometry and water chemistry.

Water for the incubations was collected in polypropylene bottles from a drilled hole in the ice at a depth of 0.5 m below the bottom of the ice. The depth of 0.5 m was chosen to avoid sampling water that had been disturbed by the drilling of the ice. In situ water temperature, O_2 , and conductivity were measured using a HQ40d Portable Multiparameter sonde (HACH). pH, CH_4 concentration and dissolved organic carbon (DOC) of the lake water were measured upon returning from the field. CH_4 measurements were made using the headspace technique, where a 60 mL polypropylene syringe (Plastipak- Becton-Dickson) was first filled with bubble free lake water. A headspace of ambient air was immediately introduced and the syringe was shaken for at least 1 min to equilibrate dissolved CH_4 from the water with the headspace. The resulting headspace was transferred to a closed serum vial filled with a saturated NaCl solution (sample injection replaced NaCl solution in the vial as described in detail in Bastviken *et al.* [2010]) to preserve the sample until it was analyzed. Analyses were performed on a gas chromatograph (GC) (Agilent Technologies 7890A GC Systems) equipped with a flame ionization detector. CH_4 concentrations were calculated according to Henry's law, correcting for temperature according to Lide and Frederikse [1995] and corrected for CH_4 in ambient air (also measured on the GC). For DOC analyses water was filtered through a precombusted 0.7 μm glass fiber filter (Whatman GF/F) and then measured on a total organic carbon (TOC-V) analyzer (Shimadzu scientific Instruments).

2.2. CH₄ Oxidation Potential in Multiple Lakes

Incubations were performed in 120 mL glass bottles that had been acid washed overnight in 10% HCl, subsequently rinsed repeatedly with Q-grade water (Milli-Q Advantage A 10 ultrapure water purification system) and finally muffled for 4 h at 450°C. The clean bottles were filled with 60 mL of lake water and capped with 10 mm thick butyl rubber stoppers and aluminum crimp seals. The headspace in the vials was then evacuated and pressure equilibrated with outside air by inserting a needle through the stopper for 2 min. CH₄ concentration in the ambient air was measured to determine the exact amount of CH₄ in the headspace at the start of the experiment. To obtain the desired CH₄ concentration of 100 μM CH₄ in the water of each incubation bottle, 16 mL of a predetermined CH₄ gas stock was first injected, using a syringe connected to a needle, and mixed with the headspace. Subsequently, 16 mL of headspace gas was taken out with the same syringe to maintain the atmospheric pressure in the bottles and used as sample for initial stable isotope analysis (detailed below). The CH₄ concentration of the gas stock was determined by the partitioning of CH₄ between the headspace and the water according to Henry's Law.

For the lake survey water samples, three replicate bottles and one autoclaved lake water control bottle were set up for each lake. Bottles were incubated at 2°C and placed in a water bath equipped with water pumps that created constant whirl movement and mixing of the vials for 15 min every second hour. Initial CH₄ and carbon dioxide (CO₂) were measured in the headspace after 1 day (start) and after 26 days (end) for most lakes (Björklings-Långsjön after 28 days). In addition, gas for stable isotope analysis and water for the analysis of nitrate (NO₃⁻), phosphate (PO₄³⁻), and sulfate (SO₄²⁻), bacterial abundance, and bacterial community composition were sampled at the start and end of the incubation (described below).

2.3. Temperature and CH₄ Concentration Effects in Björklings-Långsjön

To test if CH₄ concentration and temperature trigger the oxidation of CH₄, incubations were set up with water collected below ice from Björklings-Långsjön. Since Björklings-Långsjön is a mesotrophic lake that had intermediate levels of NO₃⁻, PO₄³⁻, and CH₄ but did not show any sign of CH₄ oxidation in the survey (see section 3), it was a suitable candidate for the detailed incubation. Björklings-Långsjön is situated in central Sweden (Figure 1), has clear water, and is surrounded mostly by forest and agriculture (~40% each). The lake is groundwater fed, draining catchment soils with a marine origin, which causes it to have higher conductivity and a greater buffering capacity. Incubations were set up in the same manner as detailed above. However, in this case, bottles were amended with four different CH₄ concentrations: 1, 10, 100, and 500 μM, with five replicate bottles and one control bottle with autoclaved lake water for each concentration. Bottles were placed in four temperature control chambers, set to 2, 4, 10, and 20°C, equipped with water baths (i.e. 4 × 4 treatments and in total 96 bottles including controls).

Over the first 2 weeks, headspace CH₄ and CO₂ were regularly monitored every second to third day. Subsequently, the interval between sampling events was increased to approximately 1 week, with a total length of the incubation time of 53, 52, 44, and 43 days for 2, 4, 10, and 20°C, respectively. In order to avoid underpressure in the vials when withdrawing samples repeatedly, 1 mL of atmospheric air stock was injected into the vial and mixed with the headspace before collecting 1 mL of headspace gas to be analyzed for CH₄ on the GC. Prior to injection into the GC, all samples were manually diluted with N₂ (Air Liquide). The ambient atmospheric pressure and the dilutions from air stock additions, as well as dilution with N₂, were accounted for when calculating CH₄ and CO₂ concentration in the headspace.

2.4. Chemical and Biological Analyses of the Incubations

As an additional measure of CH₄ oxidation, gas samples for isotope analysis of δ¹³C-CH₄ (i.e., ¹³C/¹²C) were taken at the start and end of the incubation experiment. A change in the isotope composition with an enrichment of the heavier ¹³C isotope indicates methanotrophic bacterial activity. Briefly, gas samples of 16 mL were injected into 12 mL Exetainer® vials (Labco Ltd, High Wycombe, UK). Stable isotope analysis was carried out at the Stable Isotope Facility at UC Davis following standard procedures (<http://stableisotopefacility.ucdavis.edu>) using an isotope ratio mass spectrometer (ThermoScientific DELTA V Plus, Bremen, Germany).

The water chemistry of incubation water was characterized at the start and end of the experiment. Water for analyses of NO₃⁻, PO₄³⁻, and SO₄²⁻ was first prefiltered through rinsed 0.2 μm membrane filters (Pall Corporation) and then analyzed by ion chromatography (IC) on a Metrohm IC system (883 Basic IC plus

and 919 Autosampler Plus) fitted with a Metrosep A Supp 4/5 guard column and a Metrosep A Supp 5 analytical column (150 × 4.0 mm).

Further, water for analysis of bacterial abundance was collected at the start and end of all incubations. Briefly, cells were fixed with 0.2 μm filtered formaldehyde (2% final concentration) and stored at 4°C prior to analyses. Cells were stained with the fluorescent nucleic acid stain Syto13® (Molecular probes, Invitrogen, Carlsbad, CA, USA) according to the protocol of *del Giorgio et al.* [1996] and were then counted with a flow cytometer equipped with a 488 nm blue solid state laser (Cyflow Space, Partec, Görlitz, Germany) using green fluorescence for triggered particle scoring. Cell counts were analyzed using Flowing Software version 2.5 (Perttu Terho, Centre for Biotechnology, Turku Finland).

Cells for DNA extractions and analyses of bacterial community composition were captured by filtering 100 mL of water at the start of the incubation and 40 mL at the end through 0.2 μm Supor 200 filters (Pall Corporation, Port Washington, NY, USA). The filters were stored at −80°C. The Power Soil DNA isolation kit was used to extract DNA as recommended by the manufacturer (MoBio Laboratories, Carlsbad, CA, USA). Bacterial 16S rRNA genes were amplified by PCR and analyzed as described in detail in *Sinclair et al.* [2015]. Barcoded amplicons from individual samples were pooled and sequenced with 2 × 300 cycles using the Illumina MiSeq platform at the SciLifeLab SNP/SEQ sequencing facility hosted by Uppsala University. Raw sequence data were quality filtered using an in-house pipeline [*Sinclair et al.*, 2015], and sequences were grouped in operational taxonomic units (OTUs) using UPARSE with a 3% dissimilarity threshold [*Edgar*, 2013] and taxonomically annotated using CREST with SilvaMod [*Lanzén et al.*, 2012]. Estimates of relative abundances (%) of methanotrophs in the bacterial community were performed after random resampling of the OTU table to the smallest sample size using R (version 3.1.1) and the Vegan package [*Oksanen et al.*, 2013]. Raw sequence data have been deposited at the Sequence Read Archive (SRA) in GenBank® under accession number SRP063993.

2.5. CH₄ Oxidation

To quantify the proportion of change in CH₄ concentration from the start to the end of incubation experiments, we calculated the relative change (ΔCH₄), expressed as the fraction of the initial CH₄ being oxidized, according to the equation:

$$\Delta\text{CH}_4 = \frac{\text{CH}_4(\text{END}) - \text{CH}_4(\text{START})}{\text{CH}_4(\text{START})}, \quad (1)$$

where CH₄ (START) is the concentration on day 1 and CH₄ (END) is the concentration at the end of the incubation after 26 days for all lakes, except Björklings-Långsjön (after 28 days). We also estimated the relative change in CO₂ concentrations, using equation (1), where we replaced CH₄ by CO₂.

For the lake survey (seven lakes incubated with 100 μM at 2°C, three bottles, and one control per lake) an analysis of variance (ANOVA) was run to test for a significant difference between ΔCH₄ (dependent variable) of the lake to the grouped controls, indicating CH₄ oxidation. Lake was set as the independent variable (eight groups: Björklings-Långsjön (*n* = 5), Erken (*n* = 3), Fyrsjön (*n* = 3), Lumpen (*n* = 3), Lötsjön (*n* = 3), Malstasjön (*n* = 3), Plåten (*n* = 3), and control (*n* = 7)). A post hoc Tukey-Kramer test was used to determine which lakes differed from the control. We assumed that significant CH₄ oxidation occurred with *p* value < 0.05. The residuals of the ANOVA analysis were tested for normal distribution (Shapiro-Wilk, *p* value > 0.05) and homogeneity of variance (Levene's test, *p* value > 0.05).

In the extended incubation with Björklings-Långsjön water, it was assessed whether CH₄ oxidation had occurred in any of the different treatments, using separate ANOVA tests for each temperature room (2, 4, 10, and 20°C). For all four ANOVA tests, ΔCH₄ was set as the dependent variable and treatment (five groups: 1 μM (*n* = 5), 10 μM (*n* = 5), 100 μM (*n* = 5), and 500 μM (*n* = 5) and control (*n* = 4)) was set as the independent variable. Again, post hoc Tukey-Kramer tests were used to determine which treatment had significant CH₄ oxidation.

Since CH₄ oxidation causes isotopic fractionation, δ¹³C-CH₄ was used as an independent confirmation of CH₄ oxidation in lakes Malstasjön, Erken and Lötsjön. Based on the incubations, we could also empirically determine the isotope fractionation factor (α value) at 2°C according to *Coleman et al.* [1981]. For each bottle the fraction (%) of the CH₄ oxidized under closed system conditions (*f*_{closed}) was calculated from the isotope fractionation factor (α) using the Rayleigh model for a closed system [*Liptay et al.*, 1998].

Table 1. Geographical Coordinates (North—N and East—E), Lake Area (LA), Average Depth (Zavg), Water Temperature (Temp), Oxygen (O₂), Conductivity (Cond), pH, CH₄ Concentration (CH₄), and Dissolved Organic Carbon (DOC) Reported for the Seven Sampled Lakes (ID) (Figure 1)^a

Lake	ID	Coordinates (N, E)	LA (km ²)	Zavg (m)	Temp (°C)	O ₂ (mg L ⁻¹)	Cond (μS cm ⁻¹)	pH	CH ₄ (μM)	DOC (mg L ⁻¹)
Malstasjön	M	59.77, 18.64	0.24	3.4	0.2	9.6	307	7.9	0.97	15.4
Erken	E	59.84, 18.63	23.7	9.0	0.3	13.4	239	8.1	1.11	10.7
Lötsjön	Ls	59.87, 17.95	0.58	6.0	0.4	12.7	201	7.9	0.04	9.7
Björklinge	BL	60.03, 17.57	2.46	6.1	nd	nd	nd	8.0 ^b	0.43	6.8
Plåten	P	59.86, 18.54	0.02	2.9	0.7	5.7	93	6.7	0.04	28.0
Fyrsjön	F	59.79, 18.51	0.16	2.6	0.7	9.9	203	7.8	0.61	14.3
Lumpen	L	59.96, 17.28	0.24	1.3	1.1	7.2	62	6.5	0.44	27.1

^aLake water chemistry was measured from water collected at 0.5 m below the bottom of the ice. Lakes with detectable CH₄ oxidation are in bold.^bMeasured below ice in winter of 2012; nd = not determined.

2.6. Bacterial Community Composition

Differences in bacterial community composition at the start and the end of the incubations were assessed using Bray-Curtis dissimilarity measures on rarefied OTU tables and were visualized in a nonmetric multidimensional scaled plot. Further, differences in start and end bacterial community composition were compared between lakes where CH₄ oxidation was observed with lakes where CH₄ oxidation was not observed. The difference in end bacterial community composition comparison between lakes was run an additional time with MOB excluded from the test. The compositional difference, from the Bray-Curtis dissimilarity, between lakes was tested with permutational multivariate analysis of variance (PERMANOVA) analysis (R software), in order to reveal significant differences in community composition. To determine if the relative abundance in MOB at the end of the incubation was significantly higher in lakes with observed oxidation compared to lakes where oxidation was not observed, we used a nonparametric Wilcoxon test (nonnormally distributed data, Shapiro-Wilk's test result: $p < 0.01$). An indicator species test was also performed, using the indicpecies package with the R software (version 3.1.1), to compare the co-occurring communities of the lakes that showed oxidation with the emerging communities in lakes where no CH₄ oxidation was observed. The indicator species test classifies an OTU as exclusive when it is consistently found in either group of lakes. The identified OTUs indicate high association with the sample group and can be used to characterize the sample [Dufrêne and Legendre, 1997]. Prior to analysis, OTUs classified as known methanotrophs were removed, in order not to bias the lakes that had clearly shown oxidation from the ones that had not, and the OTU table was subsampled in order to perform the indicator species test. The test results are given as indicator value (indval) ranging from 0 to 1, with higher values representing a better prediction indication. The significance of the indicator value was then tested using a permutation test (permutations = 999) and the p value was adjusted for repeated measurements.

3. Results

For all seven lakes, the surface waters sampled below the ice were supersaturated in CH₄ (relative to atmospheric levels) and contained MOB (Tables 1 and 2). The water was also oxic in the six lakes where O₂ was measured (Table 1). Despite these properties, relative change in CH₄ concentration at the end of the

Table 2. Relative Change in CH₄ and CO₂ (Calculated According to Equation (1)) and Start and End Relative Abundance of MOB (Reported as the Percent of the Total Community) in the Lake Survey Incubation (at 2°C, 100 μM)^a

ID	ΔCH ₄	ΔCO ₂	Start MOB (%)	End MOB (%)	CH ₄ oxidation (%)
M	-0.37	1.70	2.1	60.6	41
E	-0.17	2.75	11.5	51.2	21
Ls	-0.08	0.90	3.5	36.8	8
BL	0.01	0.52	1.6	0.1	nd
P	-0.01	0.30	1.1	0.4	nd
F	-0.02	0.29	1.2	0.7	nd
L	0.02	0.34	4.1	1.2	nd

^aLakes with detectable CH₄ oxidation are in bold and the corresponding average fractions of CH₄ oxidation is reported; nd = not determined. For lake abbreviations refer to Table 1.

Figure 2. Nonmetric multidimensional scaling showing the difference between the start and end bacterial community composition in the experiment. Solid symbols represent lake water at the start (day 1) and open symbols represent replicates of lake water incubations at the end (day 26) of the incubation. For full lake names see Table 1.

incubations was only significantly different from the control in the lakes Malstasjön, Erken, and Lötsjön (ANOVA, Tukey-Kramer adjusted p value < 0.01), indicating CH_4 oxidation occurred in three of the seven lakes (Table 2). In lake Björklinge-Långsjön, where no oxidation was observed at 2°C , $100\ \mu\text{M}$, the restriction of CH_4 oxidation was confirmed at different temperatures (2, 4, 10, and 20°C) and CH_4 concentrations (1, 10, 100, and $500\ \mu\text{M}$). None of these treatments were statistically different from the control (ANOVA, Tukey-Kramer adjusted p value > 0.05).

The enrichment in ^{13}C , i.e., increasing $\delta^{13}\text{C}\text{-CH}_4$ values, further confirmed methanotrophic activity in lakes Malstasjön, Erken, and Lötsjön. In these three lakes the fraction of CH_4 oxidized during the 26 day incubation, determined by ^{13}C fractionation for a closed system, ranged from 7 to 42%, with an average of 41, 21, and 8%, respectively, for lakes Malstasjön, Erken, and Lötsjön (Table 2). The isotope fractionation factor (α) at 2°C , used to determine the fraction of CH_4 oxidized, ranged between 1.017 and 1.038.

Figure 3. Relative abundance (%) of the dominant MOB (*Methylococcaceae*) increase in the three lakes with significant CH_4 oxidation (M, E, and Ls) and methylophilic (*Methylophilaceae*) in all seven lakes surveyed from the start (S) to the end (E) of the incubation in CH_4 -amended lake water incubated at 2°C . End values are reported as mean of three vials. For full lake names see Table 1.

The CO_2 concentration in the headspace of the bottles in all incubations increased from the start to the end. However, the ΔCO_2 was on average 5 times higher in lakes where CH_4 was oxidized (mean $\pm$ SD, ΔCO_2 : 1.78 ± 0.93) compared to lakes where no oxidation was observed (mean $\pm$ SD, ΔCO_2 : 0.36 ± 0.11) (Table 2).

The composition of bacterial communities differed significantly between the start and end of the incubation for all seven lakes (PERMANOVA, $F=3.85$, $R^2=0.12$, $p=0.003$; Figure 2). Comparing bacterial communities in lakes where CH_4 oxidation was observed with bacterial communities in lakes where CH_4 oxidation was not observed, we found that start communities were not significantly different (PERMANOVA, $F=0.71$, $R^2=0.12$, $p=0.75$), whereas end communities were significantly different (PERMANOVA, $F=15.64$, $R^2=0.49$, $p=0.002$). In order to investigate if the difference in bacterial community at the end of the incubation could be related to taxa other than MOB, we also investigated differences in community composition with MOB excluded from the test. Also here, bacterial communities at the end of the experimental incubation

Table 3. Indicator Species of key Non-MOB Bacterial Groups (Taxa) for Lakes Where CH₄ Oxidation Was Observed (Bold) and for Lakes Where CH₄ Oxidation Was Not Observed^a

Taxonomical Assignment	Indval	<i>p</i> Value	Freq
<i>β-proteobacteria-Methylophilaceae</i>	0.99	0.03	14
<i>β-proteobacteria-Methylophilaceae</i>	0.99	0.03	10
<i>Flavobacteria-Cryomorphaceae</i>	0.87	0.03	11
<i>β-proteobacteria-Methylophilaceae</i>	0.84	0.03	19
<i>Sphingobacteria-Sphingobacteriaceae</i>	0.83	0.03	15
<i>β-proteobacteria-Oxalobacteraceae</i>	0.79	0.03	16
<i>Actinobacteria-Sporichthyaceae</i>	0.78	0.04	20
<i>β-proteobacteria-Methylophilaceae</i>	0.77	0.03	10
<i>γ-proteobacteria-Moraxellaceae</i>	0.77	0.03	9
<i>Actinobacteria-Sporichthyaceae</i>	0.68	0.03	23
<i>Actinobacteria-Sporichthyaceae</i>	0.67	0.03	6
<i>β-proteobacteria-Comamonadaceae</i>	0.78	0.03	23
<i>Phycisphaerae-Phycisphaeraceae</i>	0.74	0.03	20
<i>β-proteobacteria-Comamonadaceae</i>	0.66	0.03	23

^aThe test results are given as an indicator value for each taxa (indval), the adjusted *p* value depicts the significance level of the permutational test (significance level < 0.05), and the relative frequency (freq) is reported as the number of times the taxa appeared in the sample.

remained significantly different when comparing lakes with and without observed CH₄ oxidation (PERMANOVA, $F = 5.7$, $R^2 = 0.26$, $p = 0.001$).

MOB were represented by the families *Methylocystaceae*, *Methylococcaceae*, and *Methylacidiphilaceae* in all lakes at the start of the incubation. The relative abundance of MOB only increased in the three lakes, where significant CH₄ oxidation was observed (Figure 3), which was explained by a significant increase in *Methylococcaceae* (nonparametric Wilcoxon test: $p < 0.0001$).

An indicator species test revealed 11 indicator OTUs that were typical for the lakes featuring CH₄ oxidation, whereas three OTUs were indicative for lakes where CH₄ oxidation was not observed (Table 3). In the group of lakes with CH₄ oxidation, 4 out of the 11 indicator OTUs were methylotrophs from the facultative methylotroph group *Methylophilaceae*. Accordingly, an increase in relative abundance of both *Methylococcaceae* and *Methylophilaceae* was observed in lakes where CH₄ oxidation occurred (Figure 3).

Among the lake water characteristics measured, initial PO₄^{3−} concentrations stood out as being higher in the three lakes with significant CH₄ oxidation compared to the lakes where CH₄ oxidation was not observed (Table 4). In the mentioned three lakes, most of the measured inorganic nutrients were depleted during the month-long incubation except for SO₄^{2−} which remained at a stable level. In all three lakes PO₄^{3−} decreased to below detection limit (<10 μg L^{−1}), while NO₃[−] decreased by 23, 99, and 16%, for Malstasjön, Erken, and Lötsjön, respectively (Table 4).

4. Discussion

The lake screening incubations revealed that wintertime CH₄ oxidation potential among lakes varied greatly; some lakes had a high potential for CH₄ oxidation, while in other lakes CH₄ oxidation was not observed even

Table 4. Start and End Nitrate (NO₃[−]), Phosphate (PO₄^{3−}), Sulfate (SO₄^{2−}), and Total Bacterial Abundance (Abund) in the Different Lake Water Incubations^a

ID	Start NO ₃ [−] (mg L ^{−1})	End NO ₃ [−] (mg L ^{−1})	Start PO ₄ ^{3−} (μg L ^{−1})	End PO ₄ ^{3−} (μg L ^{−1})	Start SO ₄ ^{2−} (mg L ^{−1})	End SO ₄ ^{2−} (mg L ^{−1})	Start Abund (10 ⁵ cells mL ^{−1})	End Abund (10 ⁵ cells mL ^{−1})
M	10.40	8.03	74.1	<10	42.7	42.0	2.78	1.20
E	0.83	0.01	96.1	<10	29.3	29.3	1.63	7.80
Ls	1.39	1.17	25	<10	5.7	5.6	4.77	5.85
BL	1.13	0.89	16	<10	69.4	70.5	4.43	8.95
P	0.90	0.88	<10	<10	11.9	11.8	8.59	7.77
F	1.00	1.04	<10	<10	13.2	13.5	2.83	4.28
L	1.27	1.32	<10	<10	6.1	6.0	4.66	2.60

^aLakes (ID) with detectable CH₄ oxidation are in bold. For lake abbreviations refer to Table 1.

after 1 month of incubation. These patterns were confirmed by multiple independent measurements; CH₄ concentration changes, $\delta^{13}\text{C}\text{-CH}_4$ values, and increase in abundance of MOB. Previous studies have found CH₄ oxidation in lakes to be limited by CH₄ availability [e.g., Kankaala *et al.*, 2006; Duc *et al.*, 2010] with greatest wintertime CH₄ oxidation found in relatively deep waters where CH₄ concentrations are highest [e.g., Bastviken *et al.*, 2002]. Since the CH₄ concentration chosen for the lake survey incubation (100 μM) was higher than CH₄ concentrations in most ice-covered boreal lakes (mean CH₄ concentration of 3.39 and 53.62 μM for surface and bottom waters, respectively [Juutinen *et al.*, 2009]), the potential CH₄ concentration limitation threshold should have been exceeded, and therefore, CH₄ oxidation was expected. However, our study revealed that elevated CH₄ concentrations were not sufficient to activate CH₄ oxidation in more than half of the studied lakes, in spite of ubiquitous presence of MOB (Figure 3). Similarly, lake water from Björklinge-Långsjön incubated at varying CH₄ concentrations, spanning from concentrations (1 μM) similar to the in situ conditions (Table 1) to well above a potential CH₄ limitation threshold (500 μM), did not show CH₄ oxidation, further confirming that CH₄ oxidation was not dependent on CH₄ availability. Additionally, it has been argued that CH₄ oxidation potential can vary with temperature [e.g., Martinez-Cruz *et al.*, 2015]. For example, He *et al.* [2012] found that CH₄ oxidation was highest at incubation temperatures most similar to in situ conditions for that study (21°C), while CH₄ oxidation at temperatures of 4 and 10°C was very low. Despite incubating lake water at near in situ temperature in the present study (~2°C) and also at 4, 10, and 20°C, in the case of Björklinge-Långsjön, CH₄ oxidation could still not be observed for any of the tested temperatures or CH₄ substrate levels. Hence, our results indicate that factors other than temperature, CH₄ availability, and MOB presence may limit CH₄ oxidation during winter below ice.

Other variables shown to limit CH₄ oxidation have been previously reviewed [Hanson and Hanson, 1996; Bastviken, 2009] and include O₂ availability, pH, salinity, light intensity, inorganic nitrogen concentrations (NH₄⁺ or NO₃⁻), and zooplankton predation on bacteria, including the rather large methanotrophs. Since our study focused on CH₄ oxidation during winter, when the lakes were covered by ice and snow, the effects of light and zooplankton predation on CH₄ oxidation may have been reduced. The pH and salinity (conductivity used as a proxy) varied between lakes, but no clear pattern was observed between the lakes with CH₄ oxidation and the lakes where this process was not detected. The effects of NH₄⁺ and NO₃⁻ on CH₄ oxidation have been contradictory since both inhibition and stimulation of oxidation has been reported previously (reviewed in Bodelier and Laanbroek [2004]). Furthermore, a clear relationship between NO₃⁻ and CH₄ oxidation was not found in this study, and it therefore appears that CH₄ oxidation was limited by some other factor than previously proposed.

Phosphorus availability played an important role in CH₄ oxidation and heterotrophic respiration since the three lakes with the highest PO₄³⁻ concentrations were the only lakes where CH₄ oxidation was observed and had highest ΔCO_2 (Table 2). Furthermore, even though MOB were present in all lakes at the start of the incubation, MOB abundance only increased in the three lakes with highest PO₄³⁻ concentrations (Figure 3). MOB are considered slow growing bacteria with low growth rates [van Bodegom *et al.*, 2001] and often need several weeks to replicate [Horz *et al.*, 2002]. Since all organisms require phosphorus for cell division, energy transformations, and cell maintenance, MOB may be out competed for nutrients by faster growing heterotrophic bacteria. Accordingly, the PO₄³⁻ available in the three lakes (Table 4) where CH₄ oxidation was detected could potentially have provided sufficient phosphorus to sustain both fast growing heterotrophs and slow growing MOB. Higher productivity and organic carbon turnover rates, as shown by higher CO₂ production in lakes with observable CH₄ oxidation (Table 2), supports this view. In addition, CH₄ oxidation likely contributed substantially to the increase in total CO₂ production over the incubation time in CH₄ oxidizing incubations.

A methanotroph-methylotroph relationship was observed in our incubations, as methylotrophs from the *Methylophilaceae* family were identified as an indicator in lakes with CH₄ oxidation (Table 3) and since their relative abundance increased toward the end of the incubation in these lakes (Figure 3). The relationship between methanotrophs and methylotrophs may be beneficial, since it has been suggested that high methanol concentrations may inhibit methanotrophs and co-occurrence with methylotrophs able to assimilate methanol would thus reduce this compound to levels that would enable the methanotrophs to thrive [Anthony, 1982]. To what extent this happens in the natural environment, with nutrient patches and micro niches, is difficult to predict and requires further investigation.

In addition to phosphorus availability and microbial community interactions, other factors may have further enhanced MOB growth and subsequent CH_4 oxidation, as e.g., Lake Erken had the highest PO_4^{3-} at the start of the incubation but did not have the highest rate of CH_4 oxidation. Rather, Lake Malstasjön had the highest rate of CH_4 oxidation and this was also manifested in higher relative MOB abundance (Figure 3). Among the lakes studied by Bastviken *et al.* [2002] biomass of MOB has previously been shown to correlate positively with CH_4 oxidation [Sundh *et al.*, 2005], a result that is similar to the observations in the present study. Besides the nutrients measured in this study (PO_4^{3-} , NO_3^- , SO_4^{2-}), there are several other elements that may play a role in the enzyme activity of MOB. For example, the methane monooxygenase enzyme is limited by copper availability [Semrau *et al.*, 1995] and in the carbon assimilation pathway the hexulose phosphate synthase enzyme is enhanced by magnesium and manganese [Ferenci *et al.*, 1974].

As well as nutrient effects, different groups of MOB are shown to be active in different environments and such biogeographical patterns may also affect CH_4 oxidation. MOB can be divided into three functional groups depending on the formaldehyde fixation pathway (type I, type II, and type X—a subgroup of type I [Bowman, 2006]). Metabolic specialization in MOB to different temperature optima between population subgroups, e.g., type I and type II [Hanson and Hanson, 1996] has been proposed, which could make them particularly sensitive to sudden temperature and environmental changes. In the three CH_4 oxidizing lakes we found that the same type I MOB, *Methylococcaceae*, dominated at the end of the incubation. This agrees with previous studies that have detected type I MOB more often in lakes with low temperature [Sundh *et al.*, 2005], while type II MOB have been reported in tropical lakes [Dumestre *et al.*, 2001].

A multitude of factors can influence wintertime CH_4 oxidation, and since it is so far not known how these factors interact, further studies are needed. Specifically, multifactorial experimental studies would be beneficial to resolve the constraints of nutrient limitation on wintertime CH_4 oxidation across different lakes by enriching lake water incubations with different amounts of nutrients to determine if there is a nutrient limitation threshold. In vitro studies have shown that cultures of methanotrophs can either be enhanced or inhibited by the addition of micronutrients [Boiesen *et al.*, 1993]; however, the extent to which these dependencies translates to environments where complex bacterial communities also will react to changes in nutrient additions needs further investigation. Complex interactions between different factors such as CH_4 and nutrient availability as well as temperature should then also be considered.

To conclude, factors other than CH_4 availability and temperature limited wintertime CH_4 oxidation in our lake water incubations. Based on our results, we propose that phosphorus availability, at least partially, limits MOB growth and thus also CH_4 oxidation. We also suggest that interactions between different bacterial groups may play a role as indicated by observed community changes. Overall, this suggests that the potential for CH_4 oxidation below ice varies between different lakes and likely depends on nutrient availability and bacterial community interactions. In a broader context, the presence or absence of wintertime CH_4 oxidation is a critical factor for accurately estimating CH_4 emissions during ice melt, which have been suggested to be important. Our study, therefore, indicates that CH_4 emissions could vary between lakes, as nutrient poor lakes may have lower wintertime CH_4 oxidation compared to nutrient rich lakes. Future changes in climate and land use may alter the availability of both nutrients and other microbial substrates in lakes. For this reason, it is important to have a solid understanding of linkages between abiotic drivers, the microbial systems, and CH_4 oxidation, not only for understanding present day CH_4 dynamics below ice but also for predicting future CH_4 emissions from seasonally ice-covered boreal lakes.

References

- Anthony, C. (1982), *The Biochemistry of Methyloprophs*, Academic Press, London.
- Bastviken, D. (2009), Methane, in *Encyclopedia of Inland Waters*, edited by G. Likens, pp. 783–805, Elsevier, Oxford, New York.
- Bastviken, D., J. Ejlertsson, and L. Tranvik (2002), Measurement of methane oxidation in lakes: A comparison of methods, *Environ. Sci. Technol.*, 36(15), 3354–3361.
- Bastviken, D., A. L. Santoro, H. Marotta, L. Q. Pinho, D. F. Calheiros, P. Crill, and A. Enrich-Prast (2010), Methane emissions from pantanal, South America, during the low water season: Toward more comprehensive sampling, *Environ. Sci. Technol.*, 44(14), 5450–5455, doi:10.1021/es1005048.
- Bastviken, D., L. J. Tranvik, J. A. Downing, P. M. Crill, and A. Enrich-Prast (2011), Freshwater methane emissions offset the continental carbon sink, *Science*, 331, 50.
- Bodilier, P. L. E., and H. J. Laanbroek (2004), Nitrogen as a regulatory factor of methane oxidation in soils and sediments, *FEMS Microbiol. Ecol.*, 47(3), 265–277, doi:10.1016/S0168-6496(03)00304-0.

Acknowledgments

We thank Jan Johansson for the help with field sampling. We thank Henrik Reyier and Lena Lundman for laboratory support at Linköping University, Sivakiruthika Natchimuthu for GC support, Omneya Ahmed for amplification and barcoding work, and Christoffer Bergvall for Ion Chromatography expertise. This work was funded by the Malméns foundation (grants to M.R.C. and B.D.), the Swedish Research Council, and the Swedish Research Council FORMAS (grants to S.B. and G.W. for the overall project and to D.B. regarding analyses and laboratory support at Linköping University), the NordForsk approved Nordic Centre of Excellence “CRAICC” (grant to G.W.), and Carl Tryggers stiftelse (grant to A.E.). DNA sequencing was performed at the SciLifeLab SNP/SEQ facility hosted by Uppsala University, and bioinformatic analyses were carried out using high-performance computing resources made available via Uppsala Multidisciplinary Center for Advanced Computational Science (UPPMAX). Raw sequence data have been deposited at the Sequence Read Archive (SRA) in GenBank® under accession number SRP063993. All other data referred to in this article can be obtained by contacting the corresponding author at blaise.denfeld@umu.se.

- Boiesen, A., E. Arvin, and K. Broholm (1993), Effect of mineral nutrients on the kinetics of methane utilization by methanotrophs, *Biodegradation*, 4(3), 163–170, doi:10.1007/BF00695118.
- Borrel, G., D. Jézéquel, C. Biderre-Petit, N. Morel-Desrosiers, J. P. Morel, P. Peyret, G. Fonty, and A. C. Lehours (2011), Production and consumption of methane in freshwater lake ecosystems, *Res. Microbiol.*, 162(9), 833–847, doi:10.1016/j.resmic.2011.06.004.
- Bowman, J. (2006), The methanotrophs—The families methylococcaceae and methylocystaceae, in *Prokaryotes*, pp. 266–289, Springer, New York.
- Carini, S., N. Bano, G. LeCleir, and S. B. Joye (2005), Aerobic methane oxidation and methanotroph community composition during seasonal stratification in Mono Lake, California (USA), *Environ. Microbiol.*, 7(8), 1127–1138, doi:10.1111/j.1462-2920.2005.00786.x.
- Coleman, D. D., J. B. Risatti, and M. Schoell (1981), Fractionation of carbon and hydrogen isotopes by methane-oxidizing bacteria, *Geochim. Cosmochim. Acta*, 45(7), 1033–1037, doi:10.1016/0016-7037(81)90129-0.
- del Giorgio, P. A., D. F. Bird, Y. T. Prairie, and D. Planas (1996), Flow cytometric determination of bacterial abundance in lake plankton with the green nucleic acid stain SYTO 13, *Limnol. Oceanogr.*, 41(4), 783–789.
- Duc, N. T., P. Crill, and D. Bastviken (2010), Implications of temperature and sediment characteristics on methane formation and oxidation in lake sediments, *Biogeochemistry*, 100(1), 185–196, doi:10.1007/s10533-010-9415-8.
- Dufréne, M., and P. Legendre (1997), Species assemblages and indicator species: The need for a flexible asymmetrical approach, *Ecol. Monogr.*, 67, 345–366.
- Dumestre, J. F., E. O. Casamayor, R. Massana, and C. Pedrós-Alió (2001), Changes in bacterial and archaeal assemblages in an equatorial river induced by the water eutrophication of Petit Saut dam reservoir (French Guiana), *Aquat. Microb. Ecol.*, 26(3), 209–221, doi:10.3354/ame026209.
- Edgar, R. C. (2013), UPARSE: Highly accurate OTU sequences from microbial amplicon reads, *Nat. Methods*, 10(10), 996–998, doi:10.1038/nmeth.2604.
- Ferenci, T., T. Strom, and J. R. Quayle (1974), Purification and properties of 3-hexulose phosphate synthase and phospho-3-hexuloisomerase from *Methylococcus capsulatus*, *Biochem. J.*, 144(3), 477–486.
- Hanson, R. S., and T. E. Hanson (1996), Methanotrophic bacteria, *Microbiol. Rev.*, 60(2), 439–471.
- He, R., M. J. Wooller, J. W. Pohlman, J. Quensen, J. M. Tiedje, and M. B. Leigh (2012), Diversity of active aerobic methanotrophs along depth profiles of arctic and subarctic lake water column and sediments, *ISME J.*, 6(10), 1937–1948, doi:10.1038/ismej.2012.34.
- Horz, H. P., A. S. Raghubanshi, J. Heyer, C. Kammann, R. Conrad, and P. F. Dunfield (2002), Activity and community structure of methane-oxidising bacteria in a wet meadow soil, *FEMS Microbiol. Ecol.*, 41(3), 247–257, doi:10.1016/S0168-6496(02)00300-8.
- Juutinen, S., M. Rantakari, P. Kortelainen, J. T. Huttunen, T. Larmola, J. Alm, J. Silvola, and P. J. Martikainen (2009), Methane dynamics in different boreal lake types, *Biogeosciences*, 6(2), 209–223, doi:10.5194/bg-6-209-2009.
- Kankaala, P., J. Huotari, E. Peltomaa, T. Saloranta, and A. Ojala (2006), Methanotrophic activity in relation to methane efflux and total heterotrophic bacterial production in a stratified, humic, boreal lake, *Limnol. Oceanogr.*, 51(2), 1195–1204.
- Karlsson, J., R. Giesler, J. Persson, and E. Lundin (2013), High emission of carbon dioxide and methane during ice thaw in high latitude lakes, *Geophys. Res. Lett.*, 40, 1123–1127, doi:10.1002/grl.50152.
- Lanzén, A., S. L. Jørgensen, D. H. Huson, M. Gorfer, S. H. Grindhaug, I. Jonassen, L. Øvreås, and T. Urich (2012), CREST—Classification resources for environmental sequence tags, *PLoS One*, 7, e49334, doi:10.1371/journal.pone.0049334.
- Lide, D. R., and H. Frederikse (1995), *Handbook of Chemistry and Physics*, 76th ed., CRC Press, Boca Raton, Fla.
- Liptay, K., J. Chanton, P. Czepliel, and B. Mosher (1998), Use of stable isotopes to determine methane oxidation in landfill cover soils, *J. Geophys. Res.*, 103(D7), 8243–8250, doi:10.1029/97JD02630.
- Martinez-Cruz, K., A. Sepulveda-Jauregui, K. Walter Anthony, and F. Thalasso (2015), Geographic and seasonal variation of dissolved methane and aerobic methane oxidation in Alaskan lakes, *Biogeosciences*, 12, 4595–4606, doi:10.5194/bg-12-4213-2015.
- Michmerhuizen, C. M., R. G. Striegl, and M. E. McDonald (1996), Potential methane emission from northern-temperate lakes following ice melt, *Limnol. Oceanogr.*, 41(5), 985–991.
- Oksanen, J., F. Guillaume Blanchet, R. Kindt, P. Legendre, P. R. Minchin, R. B. O'Hara, G. L. Simpson, P. Solymos, M. H. H. Stevens, and H. Wagner (2013), *Vegan: Community Ecology Package*, R package version 2.0-10.
- Phelps, A. R., K. M. Peterson, and M. O. Jeffries (1998), Methane efflux from high-latitude lakes during spring ice melt, *J. Geophys. Res.*, 103(D22), 29,029–29,036, doi:10.1029/98JD00044.
- Prowse, T., K. Alfredsen, S. Beltaos, B. Bonsal, C. Duguay, A. Korhola, J. McNamara, W. F. Vincent, V. Vuglinsky, and G. A. Weyhenmeyer (2012), Arctic freshwater ice and its climatic role, *Ambio*, 40(S1), 46–52, doi:10.1007/s13280-011-0214-9.
- Ricão Canelhas, M., B. A. Denfeld, G. A. Weyhenmeyer, D. Bastviken, and S. Bertilsson (2016), Methane oxidation at the water-ice interface of an ice-covered lake, *Limnol. Oceanogr.*, doi:10.1002/lno.10288.
- Segers, R. (1998), Methane production and methane consumption: A review of processes underlying wetland methane fluxes, *Biogeochemistry*, 41(1), 23–51.
- Semrau, J. D., D. Zolanz, M. E. Lindstrom, and S. I. Chan (1995), The role of copper in the pMMO of *Methylococcus capsulatus* Bath: A structural vs. catalytic function, *J. Inorg. Biochem.*, 58(4), 235–244, doi:10.1016/0162-0134(94)00056-G.
- Sinclair, L., O. A. Osman, S. Bertilsson, and A. Eiler (2015), Microbial community composition and diversity via 16S rRNA gene amplicons: Evaluating the illumina platform, *PLoS One*, 10(2), doi:10.1371/journal.pone.0116955.
- Sundh, I., D. Bastviken, and L. J. Tranvik (2005), Abundance, activity, and community structure of pelagic methane-oxidizing bacteria in temperate lakes, *Appl. Environ. Microbiol.*, 71(11), 6746–6752, doi:10.1128/AEM.71.11.6746-6752.2005.
- van Bodegom, P., F. Stams, L. Mollema, S. Boeke, and P. Leffelaar (2001), Methane oxidation and the competition for oxygen in the rice rhizosphere, *Appl. Environ. Microbiol.*, 67(8), 3586–3597, doi:10.1128/AEM.67.8.3586-3597.2001.
- Verpoorter, C., T. Kutser, D. A. Seekell, and L. J. Tranvik (2014), A global inventory of lakes based on high-resolution satellite imagery, *Geophys. Res. Lett.*, 41, 6396–6402, doi:10.1002/2014GL060641.