



## RESEARCH ARTICLE

10.1029/2020JG006190

### Special Section:

Winter limnology in a changing world

### Key Points:

- Positive relationship between methane oxidation and phosphorus observed among lakes was supported by a phosphorus amendment experiment
- The methanotrophic community composition was not affected by phosphorus amendments
- Enhanced methane oxidation in nutrient-rich lakes may neutralize more methane trapped under the ice and reduce ice-off emissions

### Supporting Information:

Supporting Information may be found in the online version of this article.

### Correspondence to:

H. O. Sawakuchi,  
[henrique.sawakuchi@liu.se](mailto:henrique.sawakuchi@liu.se)

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### Author Contributions:

**Conceptualization:** Stefan Bertilsson, David Bastviken

**Data curation:** Henrique O. Sawakuchi, Gaëtan Martin

**Formal analysis:** Henrique O. Sawakuchi, Gaëtan Martin

**Funding acquisition:** Sari Peura, Stefan Bertilsson, Jan Karlsson, David Bastviken

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# Phosphorus Regulation of Methane Oxidation in Water From Ice-Covered Lakes

Henrique O. Sawakuchi<sup>1,2</sup> , Gaëtan Martin<sup>3</sup> , Sari Peura<sup>3</sup> , Stefan Bertilsson<sup>4</sup>, Jan Karlsson<sup>2</sup> , and David Bastviken<sup>1</sup> 

<sup>1</sup>Department of Thematic Studies—Environmental Change, Linköping University, Linköping, Sweden, <sup>2</sup>Department of Ecology and Environmental Science, Climate Impacts Research Centre (CIRC), Umeå University, Umeå, Sweden, <sup>3</sup>Department of Forest Mycology and Plant Pathology, Science for Life Laboratory, Swedish University of Agricultural Sciences, Uppsala, Sweden, <sup>4</sup>Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences, Uppsala, Sweden

**Abstract** Winter methane (CH<sub>4</sub>) accumulation in seasonally ice-covered lakes can contribute to large episodic emissions to the atmosphere during spring ice melt. Biological methane oxidation can significantly mitigate such CH<sub>4</sub> emissions, but despite favorable CH<sub>4</sub> and O<sub>2</sub> concentrations, CH<sub>4</sub> oxidation appears constrained in some lakes for unknown reasons. Here we experimentally test the hypothesis that phosphorus (P) availability is limiting CH<sub>4</sub> oxidation, resulting in differences in ice-out emissions among lakes. We observed a positive relationship between potential CH<sub>4</sub> oxidation and P concentration across 12 studied lakes and found an increase in CH<sub>4</sub> oxidation in response to P amendment, without any parallel change in the methanotrophic community composition. Hence, while an increase in sedimentary CH<sub>4</sub> production and ebullitive emissions may happen with eutrophication, our study indicates that the increase in P associated with eutrophication may also enhance CH<sub>4</sub> oxidation. The increase in CH<sub>4</sub> oxidation may hence play an important role in nutrient-rich ice-covered lakes where bubbles trapped under the ice may to a greater extent be oxidized, reducing the ice-out emissions of CH<sub>4</sub>. This may be an important factor regulating CH<sub>4</sub> emissions from high latitude lakes.

**Plain Language Summary** Methane produced by microorganisms in lake sediments accumulates under the ice in seasonally ice-covered lakes and is released into the atmosphere when the ice melts. A specialized group of bacteria can consume methane in the water and significantly reduce methane emissions. In some lakes, however, this consumption is limited for unclear reasons. A recent study suggests that phosphorus, an essential nutrient for plants and algae in aquatic environments, could cause this limitation. Here, we tested the influence of phosphorus on the bacterial consumption of methane in 12 lakes with different phosphorus concentrations and made experiments with the addition of phosphorus. Our results indicate that methane consumption was higher in lakes with more phosphorus in the water and that phosphorus addition enhanced the consumption of methane. Despite higher production of methane expected from nutrient-rich lakes, our study shows that increased consumption of methane may reduce part of the enhanced production, limiting the emissions. This neutralization may be higher in seasonally frozen lakes where methane is trapped under the ice, allowing more time for methane consumption.

## 1. Introduction

Lakes are important global sources of CH<sub>4</sub> to the atmosphere. Climate change, especially in northern lakes, may increase CH<sub>4</sub> emissions by up to 54% (Wik et al., 2016). Furthermore, this increase may be exacerbated by eutrophication (Beaulieu et al., 2019; DelSontro et al., 2018). Nearly half of the global lake surface area is located in temperate and boreal zones where lakes are ice-covered for some period in the winter (Downing et al., 2006; Matthews et al., 2020). Under the ice, CH<sub>4</sub> bubbles released from the sediment can accumulate throughout the winter and result in high ice-out release in spring (Wik et al., 2016).

The observations of CH<sub>4</sub>-rich bubbles under lake ice at the end of the winter and associated emissions upon ice-out are mysterious for several reasons. (a) This CH<sub>4</sub> is exposed to oxic water for extended periods, which should create favorable conditions for CH<sub>4</sub> oxidation, known to have the capacity to deplete water column

**Investigation:** Henrique O. Sawakuchi, Gaëtan Martin

**Methodology:** Henrique O. Sawakuchi, Gaëtan Martin, Sari Peura, Stefan Bertilsson, Jan Karlsson, David Bastviken

**Project Administration:** Henrique O. Sawakuchi

**Visualization:** Henrique O. Sawakuchi, Gaëtan Martin

CH<sub>4</sub> to very low levels near the oxycline and found to be more sensitive to substrate availability than temperature (Bastviken et al., 2008; Duc et al., 2010; Kankaala et al., 2006). (b) Bubbles trapped under the ice spend a long time in the water, and over time a small fraction (8%) can be encapsulated and stored in growing ice or exchange gases with the water, dissolving towards equilibrium (80%), where it can be oxidized (Greene et al., 2014; Sepulveda-Jauregui et al., 2015). (c) Ebullition from sediments should decrease during the winter as shallow sediments cool down and if the availability of labile organic substrates from primary production fueling methanogenesis get progressively reduced (Wik et al., 2018). This production of CH<sub>4</sub> in sediment is the main CH<sub>4</sub> source in most lakes, except for the limited number of lakes experiencing continuous seepage of CH<sub>4</sub> of geogenic origin or from deep melting permafrost (Walter et al., 2008). Therefore, extensive renewal of under-ice CH<sub>4</sub> bubbles in the late phase of the ice-covered winter period is unlikely in most lakes. Despite this, CH<sub>4</sub>-rich water under the ice and high ice-out emissions have been observed in some lakes (Sepulveda-Jauregui et al., 2015).

Recently, a study found an unexpected absence of CH<sub>4</sub> oxidation in water from ice-covered lakes, while CH<sub>4</sub> oxidation was extensive in other nearby lakes, highlighting that information regarding the factors controlling winter CH<sub>4</sub> oxidation in lakes is still scarce and contradictory (Denfeld et al., 2018). Martinez-Cruz et al. (2015) observed that the winter CH<sub>4</sub> oxidation variability was mainly related to O<sub>2</sub> availability in Alaskan lakes, which is in agreement with previous observations (Bastviken, 2009; Segers, 1998), but cannot explain the range of potential CH<sub>4</sub> oxidation found in oxic surface waters under the ice (Denfeld et al., 2016). It has been suggested that high O<sub>2</sub> concentration may inhibit CH<sub>4</sub> oxidation (Reis et al., 2020), and light is another factor suggested to inhibit CH<sub>4</sub> oxidation (Dumestre et al., 1999; Murase & Sugimoto, 2005). However, a persistent ice cover may both reduce the O<sub>2</sub> and attenuate light below inhibitory levels in the water column. Under such conditions, Denfeld et al. (2016) found that the lakes with extensive CH<sub>4</sub> oxidation in under-ice lake water had higher phosphate (PO<sub>4</sub><sup>3-</sup>) concentrations than lakes where CH<sub>4</sub> oxidation was absent. Phosphorus (P) is a major nutrient required for all life forms (Westheimer, 1987) and could potentially limit methanotrophic activity in lakes (Denfeld et al., 2016). Further, correlation between CH<sub>4</sub> oxidation and P concentration has been previously observed in soils (Chauhan et al., 2012; Gray et al., 2014).

Clearly, under-ice CH<sub>4</sub> oxidation can be an important constraint for ice-off CH<sub>4</sub> emissions, but for unknown reasons, CH<sub>4</sub> oxidation activity seems to vary widely among lakes. The present study aimed to estimate CH<sub>4</sub> oxidation in lakes covering a range of total P availability and experimentally assess cause-effect relationships between P concentration and CH<sub>4</sub> oxidation to test the hypothesis that P availability favors CH<sub>4</sub> oxidation at low-temperature conditions representative of under-ice conditions in boreal lakes.

## 2. Materials and Methods

### 2.1. Lakes and Sampling

We visited 12 lakes in southeast Sweden in March 2018 and collected 4.5 liters of water 10 cm below the ice after carefully drilling the ice with a manual ice auger. Water was sampled at the deepest point for lakes with bathymetric information and near the center when depth information was not available. Ice thickness ranged from 24 to 41 cm. Lakes size ranged from 0.02 to 23.7 km<sup>2</sup> and have different trophic states (oligotrophic, mesotrophic, and eutrophic). The ice-cover period for these lakes is between December and April (SMHI, 2006). Seven of the lakes had been included in a previous study addressing the impact of winter conditions on CH<sub>4</sub> oxidation (Denfeld et al., 2016).

Samples (5 ml) for the in-situ concentration of CH<sub>4</sub> were collected with a syringe connected to a tube placed under the ice and immediately transferred to 20 ml glass vials filled with high purity nitrogen gas (N<sub>2</sub>) and with 150 μL H<sub>3</sub>PO<sub>4</sub> (85%) to preserve the sample. Water samples were also collected for total P (TP) and dissolved organic carbon (DOC). DOC samples were filtered through combusted GFF filters into glass vials. Non-filtered water samples were collected in acid-washed (HCl 10%) HDEP bottles and preserved with H<sub>2</sub>SO<sub>4</sub> for TP analysis. Water for the incubation experiments was pumped directly from the lake into acid-washed cubitainers (4.5 L) using a battery-operated Masterflex peristaltic pump (Cole-Parmer) and kept at 4°C. Water temperature, pH, dissolved oxygen, and electric conductivity were measured using multiparameter probes (YSI EXO2 and Aquaread AP-5000). Lake water was analyzed for DOC using a

TOC analyzer (Shimadzu TOC-V<sub>CSH</sub>). TP and PO<sub>4</sub><sup>3-</sup> were analyzed colorimetrically using an AutoAnalyzer (BRAN + LUBBE AutoAnalyzer3).

## 2.2. Methane Oxidation Assessment and P Amendment

Acid washed (10% HCl) and combusted (550°C for 4h) 120 ml glass vials were rinsed and filled with 80 ml of lake water. For each lake, 10 vials were prepared using water from the same cubitainer, and 250 μL of an aqueous PO<sub>4</sub><sup>3-</sup> solution (NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O) was added in five of them to increase the PO<sub>4</sub><sup>3-</sup> concentration by 500 μg/L. In total, 120 vials were incubated for the experiment. Vials were capped with acid-washed and autoclaved 10 mm butyl rubber stoppers and sealed with aluminum crimps. Before incubation, vials' headspaces were flushed for 5 min with high purity carbon-free synthetic air to remove CH<sub>4</sub> and CO<sub>2</sub> and standardize the concentration of O<sub>2</sub>. After that, 1.8 ml of 99% CH<sub>4</sub> was added to the headspace to reach a CH<sub>4</sub> concentration of 100 μM CH<sub>4</sub> in the water at 4°C, according to Henry's law. Incubations were carried out in the dark at 4°C on a shaking table to gently mix the water 15 min every two hours. Previous similar experiments to assess the potential CH<sub>4</sub> oxidation in water from ice-covered lakes incubated at 2–4°C have reported a delayed start (lag-phase) varying from 7 to 12 days (Canelhas et al., 2016; Martinez-Cruz et al., 2015). Thottathil et al. (2019) observed that lake water incubations to measure CH<sub>4</sub> oxidation at low temperatures (5°C) require incubations over several weeks before a significant change in CH<sub>4</sub> concentration can be detected. Here, CH<sub>4</sub> headspace concentration in the vials were monitored during 83–88 days and samples taken approximately every two weeks. Long-term incubations (up to 90 days) were previously done to evaluate the influence of light and inorganic nitrogen on CH<sub>4</sub> oxidation (Murase & Sugimoto, 2005).

Gas samples from the headspace in the vials were taken by adding, mixing, and withdrawing 3 ml of synthetic air, using a 5 ml syringe, for analysis by gas chromatography (GC). This procedure was done to avoid pressure changes during the experiment and allow monitoring the CH<sub>4</sub> concentration over time in the same vial. Dilution effects by sampling were corrected based on blank control vials prepared with Milli-Q water and run along with the lake water vials throughout the whole experiment. In total, 12 blank vials were prepared with the same amount of CH<sub>4</sub>, and with or without the addition of PO<sub>4</sub><sup>3-</sup>, and monitored together. Thus, the decrease in CH<sub>4</sub> in the blank vials was due to dilution caused by the addition of synthetic air when sampling. Analysis of CH<sub>4</sub> for all samples was made using a GC with a flame ionization detector and a methanizer (Agilent 7890; 60/100 poropakQ, 6 ft × 1/8 in column, manual injection via a sample loop), with a detection limit of 4.4 × 10<sup>-7</sup> mmol of CH<sub>4</sub>.

To calculate CH<sub>4</sub> oxidation based on the decline in headspace CH<sub>4</sub> over time, we used the decay constant ( $\lambda$ ) as the specific rate of methane oxidation (SRMOx) according to the following equation:

$$C_t = C_0 e^{-\lambda t} \quad (1)$$

$\lambda$  is the decay constant representing the fraction of the CH<sub>4</sub> being oxidized per time unit (day<sup>-1</sup>; dimensionless),  $C_t$  is the concentration at time  $t$  (day), and  $C_0$  is the starting concentration after the lag-phase.  $\lambda$  was determined from the best fit of Equation 1 relative to the time series of measurements in each incubation bottle. The fit error was estimated using the R<sup>2</sup> of the linear fit after log transformation.

For each vial, we considered that a lag-phase occurred during the analysis period when the concentration in the vial remained inside the confidence interval (95%) of the starting CH<sub>4</sub> concentration. A lag-phase of 12–23 days was seen in most vials. However, cases without lag-phase or where no CH<sub>4</sub> oxidation was recorded were also observed. We focused on the period of decline even if occurring at different times in different bottles. Hence, the CH<sub>4</sub> oxidation capacity of each vial, was calculated using the time after the lag-phase period.

Our incubations had a large reservoir of O<sub>2</sub> in the air headspace volume (40 ml headspace and 80 ml water). The aeration of the vials by air purging at the start of the experiment equilibrated the water with O<sub>2</sub> in the air, leading to an expected O<sub>2</sub> concentration of approximately 13 mg/L in all vials. Assuming that the decrease in O<sub>2</sub> is mainly attributed to DOC degradation we estimated the maximum expected O<sub>2</sub> decrease during incubation as follows: A previous long-term incubation (426 days) of lake water representing a similar range of DOC and TP levels as in this study (including one of the lakes in this study, Lillsjön) at 15°C, found a total DOC decrease of 30% (Bastviken et al., 2004). If assuming a 1:1 molar O<sub>2</sub> consumption per DOC-C mineralized, a 30% DOC mineralization with our highest starting DOC concentration (33.1 mg/l)

would have depleted a maximum of 10% of the O<sub>2</sub> reservoir in our vials. Our incubation over much shorter time (88 days) and at 4°C makes such high mineralization and O<sub>2</sub> consumption unrealistic and a more likely maximum O<sub>2</sub> consumption is in the order of 2.5% during the incubation (assuming a factor of 2 reduction from the time difference and another factor of 2 reduction from the temperature difference). Hence, the O<sub>2</sub> reservoir in the vial headspace kept the O<sub>2</sub> levels high and similar across all vials.

### 2.3. Microbial Community Characterization

To assess the impact of the P amendment on the microbial communities and test whether differences in SRMOx may be explained by change in the methanotroph communities we sampled and analyzed independently each replicate at the end of the incubation. For that purpose, 40 ml of water were sampled from each vial and stored at –20°C for further DNA analysis. Once thawed, each sample was filtered through separate 0.22 µm Durapore® Membrane Filters (Merck, Darmstadt, Germany). Filters were stored overnight at –20°C and DNA extracted using the DNeasy PowerSoil Kit (Quiagen, Hilden, Germany). Library preparation for 16S rRNA gene analysis was done following a two-steps Polymerase Chain Reaction (PCR) protocol, as described in Sinclair et al., 2015. All PCRs were conducted in 20 µl of volume using 0.02 U/µl Phusion high fidelity DNA polymerase, 1X Q5 reaction buffer (NEB, UK), 0.25 µM primers and 200 µM dNTP mix and 1 µl DNA template.

The first step was performed in triplicate with primers 341F (3'-CACTCTTCCCTACACGACGCTCTTC-CGATCTNNNNCTACGGGNGGCWGCAG-5') and 805NR (3'-AGACGTGTGCTCTTCCGATCTGACTAC-NVGGGTATCTAATCC-5') (Herlemann et al., 2011). The thermal program consisted of 20 cycles with an initial 98°C denaturation step for 10 min, a cycling program of 98°C for 10 s, 48°C for 30 s, and 72°C for 30 s and a final elongation step at 72°C for 2 min. Triplicate PCR reactions were then pooled and purified with magnetic beads (Sera-Mag™ Select, GE Healthcare, Chicago, United States of America), and 2 µl of the purified products were used as a template for a second stage PCR, where indexed primers were added. The second thermal program consisted of 15 cycles with an initial 98°C denaturation step for 30 s, a cycling program of 98°C for 10 s, 66°C for 30 s, and 72°C for 30 s and a final elongation step at 72°C for 2 min. Following amplification, PCR products were again purified with magnetic beads and quantified with Qubit™ using the Qubit™ dsDNA HS Assay Kit (Invitrogen™). Finally, 15.6 µg of each indexed and purified PCR product were pooled before submission of the sample to the Science for Life Laboratory SNP/SEQ sequencing facility hosted by Uppsala University (Uppsala, Sweden). Sequencing was done using Illumina Miseq in paired-end mode with 300bp and v3 chemistry.

Sequence processing was performed with Mothur 1.41.0 following the MiSeq SOP (Kozich et al., 2013), with the exception that clustering to operational taxonomic units (OTUs) was done using VSEARCH (Rognes et al., 2016) as implemented in Mothur. The OTU table, consensus taxonomy table, and a phylogenetic tree created using Mothur were analyzed in R using the phyloseq package (McMurdie & Holmes, 2013). Rarefaction was applied to the OTU table before further analysis. Sampling size for the rarefaction was set at 90% of the number of sequences present in the sample with the lowest amount of sequences, and set seed (1) was used to initialize repeatable random subsampling, retained for further analysis. Following rarefaction, all OTUs with less than 10 reads in the subsampled data were removed from the OTU table. OTUs were considered methanotrophic if they were assigned to one of the known aerobic methanotrophic taxa. Those taxa are the orders Methylococcales and Methyloacidiphilales, as well as the genera *Methylocystis*, *Methylosinus*, *Methylocapsa*, *Methylocella*, *Methyloferula*, *Candidatus Methyloimabilis*, and *Candidatus Methanoperedenaceae*.

### 2.4. Data Analysis

The overall effect of lake TP in SRMOx was tested by linear regression analysis using log-transformed SRMOx to achieve normality and homogeneity of variance of residuals. The difference between non-amended and P-amended incubations in each lake was assessed by a two-sided Wilcoxon rank-sum test. The potential influence of in situ water chemistry parameters on the SRMOx was assessed by the two-sided Spearman correlation test using SRMOx observed in non-amended incubations. Linear regression, Wilcoxon rank-sum test, and Spearman correlation test were performed in R using the built-in Stats Package (R Core

**Table 1**  
*In-Situ Concentrations of Dissolved Molecular Oxygen (DO), Dissolved Organic Carbon (DOC), Methane (CH<sub>4</sub>), Total Phosphorus (TP), and in the Water 10 cm Below the Ice*

Lake ID	Lakes	DO (mg/L)	DOC (mg/L)	CH <sub>4</sub> (μM)	TP (μg/L)
MOX01 <sup>a</sup>	Lumpen	6.7	25.2	0.08	14.9
MOX02 <sup>a</sup>	Björklinge-Långsjön	11.8	2.6	1.14	9.9
MOX03 <sup>a</sup>	Erken	13.4	4.9	0.12	24.6
MOX04 <sup>a</sup>	Malstasjön	3.8	9.9	0.07	74.3
MOX05 <sup>a</sup>	Fyrsjön	8.6	6.3	0.07	10.5
MOX06 <sup>a</sup>	Plåten	3.0	23.6	0.04	14.7
MOX07 <sup>a</sup>	Lötsjön	8.0	5.2	0.06	24.9
MOX08	Glimmingen	11.3	8.3	0.11	5.3
MOX09	Stortoveln	10.6	7.1	0.24	5.3
MOX10	Bengtsgolen	0.0	33.1	0.14	34.7
MOX11	Lillsjön	5.9	25.5	0.16	15.9
MOX12	Parsen	4.2	16.7	0.1	15.8

<sup>a</sup>Lakes where under-ice CH<sub>4</sub> oxidation was evaluated in Denfeld et al., 2016.

Team, 2019). To test the effect of the lake of origin and treatment on total and methanotrophic communities, we used PERMANOVA (Adonis, vegan package, R) based on Bray distance matrices, with 10,000 free permutations and number of groups minus one degree of freedom. When the p-value was between 0.1 and 0.001, we ran 10<sup>6</sup> extra permutations.

### 3. Results

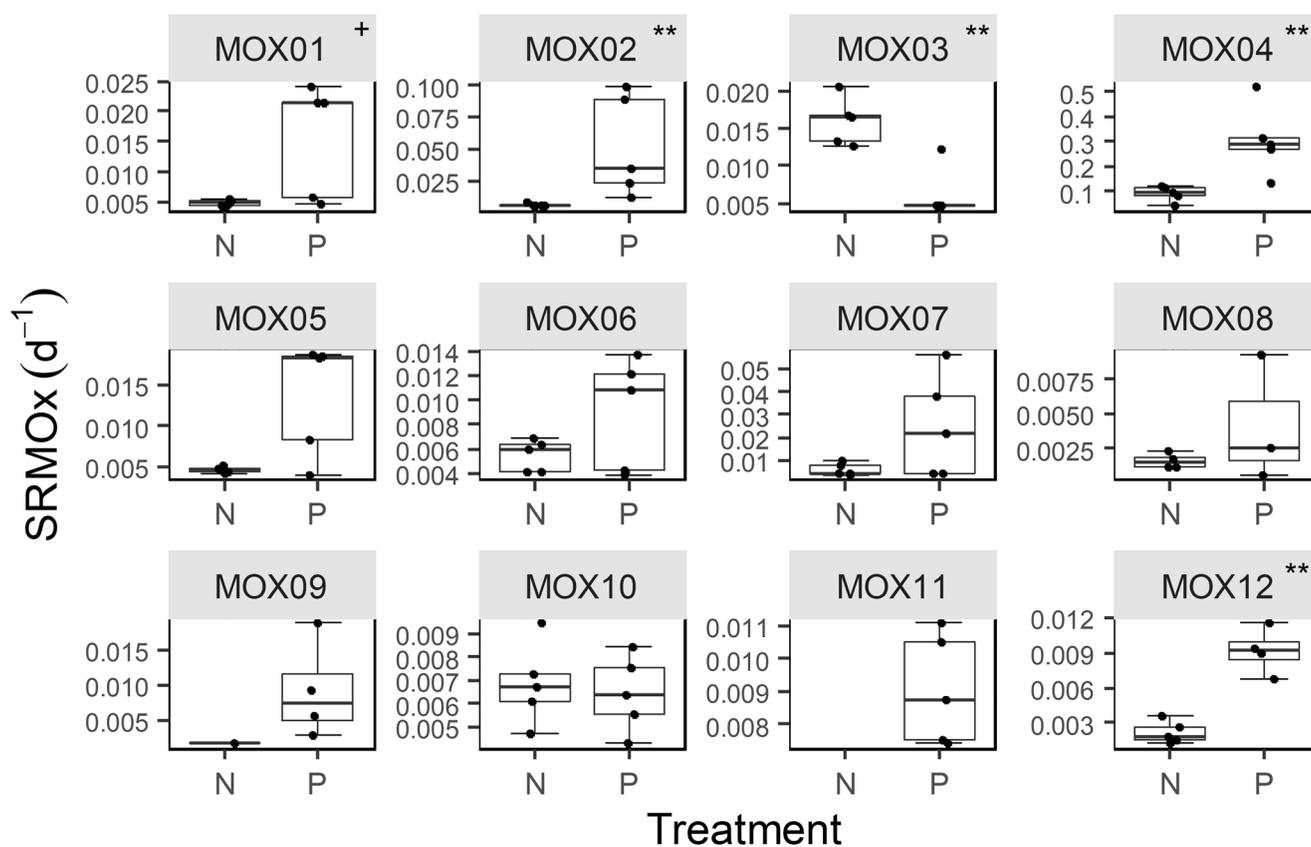
In-situ CH<sub>4</sub> concentrations were higher than atmospheric saturation (~5 nM at 0°C) for all lakes, with concentrations ranging from 0.04 to 1.1 μM (Table 1). Total P (TP) concentrations in the studied lakes ranged from 5.3 to 74.3 μg/L, classifying the lakes as oligotrophic, mesotrophic, and eutrophic (Tables 1 and S1). Dissolved O<sub>2</sub> concentration also varied between lakes, but only one lake (MOX10, Bengtsgolen) was anoxic. There were large variability in DOC (2.6–33.1 mg/L, Table 1) and other in-situ physicochemical parameters (Table S1) among the lakes. During the incubation, with exception of Lake MOX03, the change in CH<sub>4</sub> concentrations in the P-amended vials was larger than in the non-amended. The largest change in CH<sub>4</sub> concentration was observed in MOX04 vials where 98.7% and 99.9% of the initial amount of CH<sub>4</sub> were consumed in the non-amended and P-amended vials, respectively, with the lowest concentration reaching 2 × 10<sup>-5</sup> mmol of CH<sub>4</sub> (Table S2). For individual lakes, we observed a large variability of SRMOx ranging from 0 (no CH<sub>4</sub> oxidation registered for Lake MOX11) to 0.09 days<sup>-1</sup> for non-amended

vials and from 0.006 to 0.306 days<sup>-1</sup> for P-amended vials. The overall average fit error based on the R<sup>2</sup> of the linear fit of log transformed data was 0.93 ± 0.09, ranging from 0.50–1.00 for individual vials. Despite the consistently higher mean SRMOx in P-amended vials for most of the lakes, the difference was significant for only four lakes (Wilcoxon, *p* < 0.05) (Table S3). Methane oxidation rate ranged from 2.5–13.5 mmol m<sup>-3</sup> d<sup>-1</sup> and 3.9–29.4 mmol m<sup>-3</sup> d<sup>-1</sup> for non-amended and P-amended, respectively (Table S4).

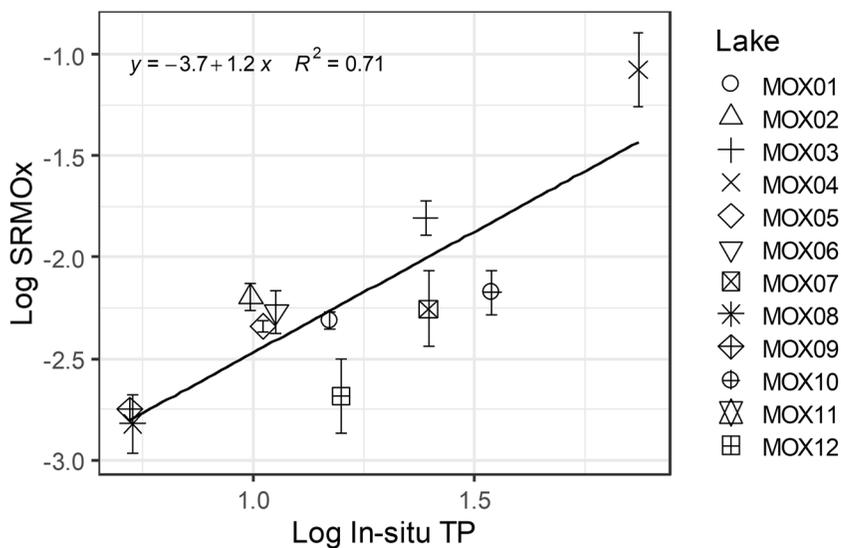
Overall, higher SRMOx was observed in P-amended incubations compared to the non-amended ones (0.041 ± 0.094 days<sup>-1</sup> and 0.014 ± 0.027 days<sup>-1</sup>, respectively; Wilcoxon, *p* < 0.001). Single lake evaluation shows an enhancement in P-amended vials in 11 out of the 12 studied lakes, the only exception was for MOX03, where higher SRMOx was observed in the non-amended vials (Figure 1, Table S3). However, despite the overall enhancement in CH<sub>4</sub> oxidation attributed to P amendment in the single lakes, in approximately half of the lakes, not all P-amended replicates showed an increase in SRMOx, resulting in five lakes where the difference was statistically significant.

In addition to the P-amendment experiment we observed a positive relationship between the natural (i.e., non-amended) SRMOx and in-situ TP concentration (*R*<sup>2</sup> = 0.71, *p* = 0.001, Figure 2). Despite that the highest TP and SRMOx observed in lake MOX04 seems to be driving the observed trend, a significant positive effect remains if this lake is removed from the evaluation (*R*<sup>2</sup> = 0.50, *p* = 0.022). However, we acknowledge that more data from eutrophic and hypereutrophic lakes would have improved our evaluation. We did not find any significant effect between DOC and SRMOx (*R*<sup>2</sup> = 0.006, *p* = 0.82).

After trimming and clustering the number of reads in each sample ranged from 43,452 to 100,884 (Table S5). After rarefaction and removal of rare OTUs (represented by less than 10 reads across all samples), the combined data set consisted of 4,692,720 reads grouping into 2,663 OTUs, used for further analyses. Methanotrophs represented 0.02%–69.4% of all reads in individual samples (Figure S2). Methanotroph reads were assigned to 228 OTUs almost exclusively affiliated with Gammaproteobacteria (99.3% of the analyzed methanotroph reads), where 94.5% were accounted by the five most abundant OTUs. The five most abundant OTUs were closely related to the two cultivated psychrophilic methanotrophs belonging to the Methylococcaceae family, *Methylobacter tundripaludum* and *Methylovulum psychrotolerans*, with 16S rRNA sequence identities ranging from 95.5% to 99.6% (Table 2). The proportion of reads assigned to methanotrophs in relation to the total microbial community reads is what we call proportion of methanotrophs.



**Figure 1.** Comparison of the specific methane oxidation rate (SRMOx) observed among non-amended (N) and P-amended (P) vials. CH<sub>4</sub> oxidation was not observed in non-amended vials of MOX11. Note that the y-axis scale is adjusted for the values observed for each lake. Dots show the SRMOx values of replicates where CH<sub>4</sub> oxidation was observed. The thick line in the middle of the box is the median, and the lower and upper boundaries represent 25th and 75th percentiles, points outside the box, and the error bar correspond to 1.5\* inter-quartile range from the hinge. Symbols on the right upper corner of the panels represent the p-value ranges (0–0.001 = '\*\*\*', 0.001–0.01 = '\*\*', 0.01–0.05 = '\*', 0.05–0.1 = '+', 0.1–1.0 = "No symbol"), based on Two-sided Wilcoxon rank-sum test presented on the Table S3.



**Figure 2.** Linear relationship between the log-transformed mean specific rates of methane oxidation (SRMOx) for non-amended vials and in-situ total phosphorus (TP) concentrations. The error bar shows the standard deviation of the mean for the replicates.

**Table 2**

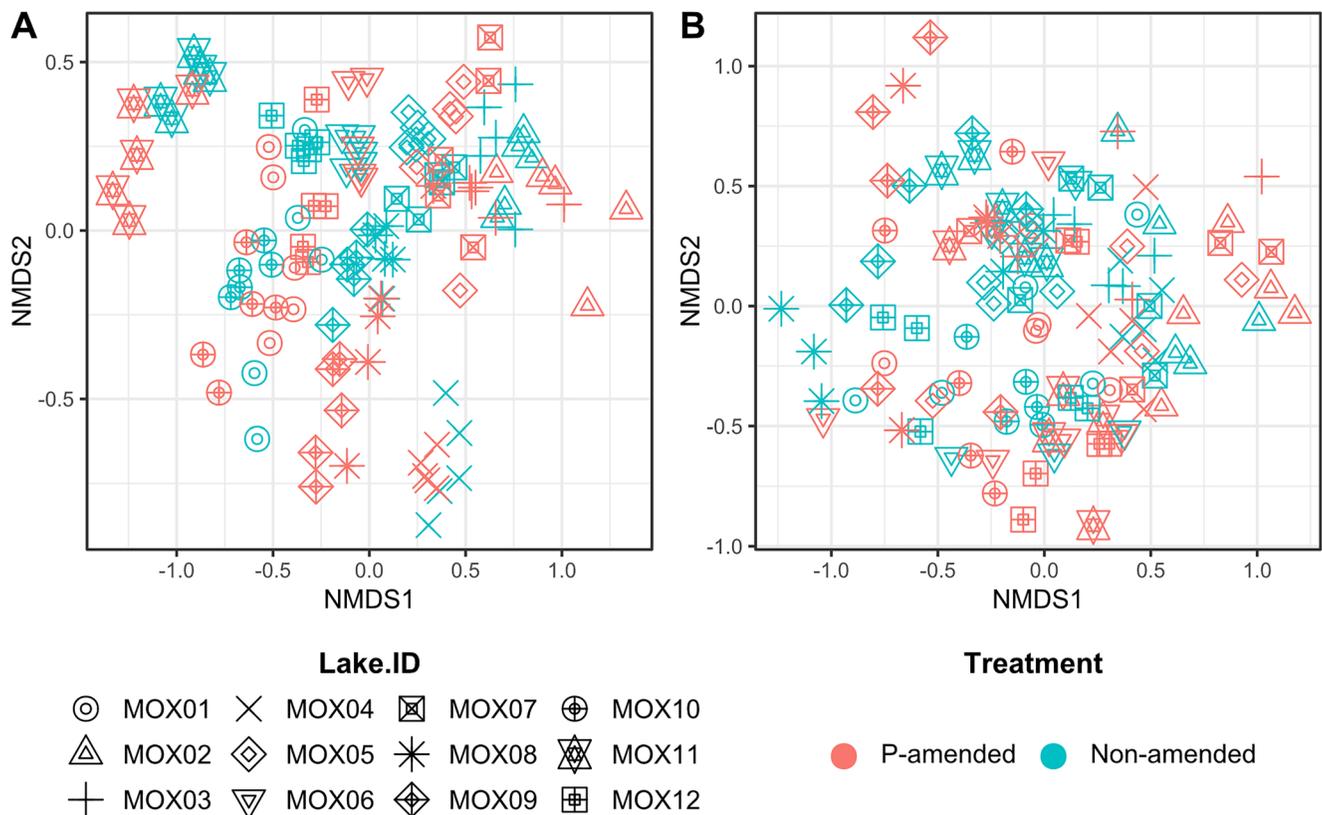
Similarity of the Most Common Operational Taxonomic Units With Their Closest Isolated Organism and Their Occurrence Across Vials, Treatments, and Lakes

OTU	Closest cultivated relative	Identity (%)	Proportion of reads (%)	Occurrence		
				All vials	Treatment	Lake
otu_0003	<i>Methylobacter tundripaludum</i>	98.7	36.0	118/120	24/24	12/12
otu_0004	<i>Methylovulum psychrotolerans</i>	96.8	31.3	99/120	24/24	12/12
otu_0010	<i>Methylovulum psychrotolerans</i>	99.4	13.3	96/120	23/24	10/12
otu_0014	<i>Methylobacter tundripaludum</i>	95.5	7.7	86/120	24/24	12/12
otu_0018	<i>Methylobacter tundripaludum</i>	95.5	6.6	71/120	21/24	10/12

Note. Identity indicates the similarity of the OTU with a cultivated organism based on 16S rRNA gene amplicons. The proportion of reads is relative to the total number of reads (789,759) identified as methanotrophs.

The overall relative proportion of methanotrophs in the non-amended incubations was not correlated to the SRMOx or the lake TP concentration. We also did not find any difference in the average proportion of methanotrophs between P-amended and non-amended vials ( $0.20 \pm 0.18$  and  $0.14 \pm 0.16$ , respectively; Wilcoxon,  $p = 0.087$ ).

Analysis of the integrated influence of lake and treatment illustrated by non-metric multidimensional scaling (NMDS) indicate an influence on the total bacterial community composition (PERMANOVA  $p < 0.001$ ) with a stronger influence of the lake of origin on the bacterial community composition than for the P-amendment, as reflected by their respective  $R^2$  values of 0.42 and 0.03 (Figure 3a). When only the



**Figure 3.** Overall beta-diversity for total bacterial and methanotrophic community. Beta-diversity analysis using non-metric multidimensional scaling (NMDS) for total bacterial (a) and methanotrophic (b) communities.

methanotrophs are considered, only the lake ID has an effect on the community composition ( $p < 0.001$ ,  $R^2 = 0.34$ ,  $F = 5.34$ ), whereas treatment has no effect ( $p = 0.37$ ,  $R^2 = 0.01$ ,  $F = 1.07$ ) (Figure 3b).

Single lake evaluation of the total bacterial beta-diversity suggested that for seven lakes, the addition of P affected the community composition as the samples tended to group according to treatment with ADONIS  $p$ -value below 0.05 (Figure S1a).

Even if NMDS plots suggested an effect of P-amendment on the methanotrophic communities in lakes MOX10 and MOX11, all ADONIS  $p$ -values were above 0.05 (Figure S1b).

#### 4. Discussion

The observed  $\text{CH}_4$  and  $\text{O}_2$  levels correspond to previously reported values from ice-covered lakes (Denfeld et al., 2016; Samad & Bertilsson, 2017; Sepulveda-Jauregui et al., 2015). SRMOx of  $0.074 \text{ days}^{-1}$  reported for under-ice water samples from Lake Erken (Canelhas et al., 2016) was within the overall range of SRMOx observed in our study, but this value is higher than our SRMOx estimate for the same lake (MOX03). Previously reported summertime surface water SRMOx for temperate and boreal lakes varying from  $0.04$  to  $0.94 \text{ days}^{-1}$  was, in general, higher than what we observed in winter (Bastviken et al., 2008; Thottathil et al., 2019). However, seasonal variation indicating higher  $\text{CH}_4$  oxidation in summer compared with winter has already been reported for arctic lakes (Martinez-Cruz et al., 2015), yet our potential methane oxidation rates were within the range ( $0.01$ – $36.1 \text{ mmol m}^{-3} \text{ d}^{-1}$ ) previously observed in incubations at low temperature (i.e.,  $2^\circ\text{C}$ – $5^\circ\text{C}$ ) for northern lakes (Martinez-Cruz et al., 2015; Sepulveda-Jauregui et al., 2018; Thottathil et al., 2018).

We found a similar variability in  $\text{CH}_4$  oxidation among the lakes previously studied by Denfeld et al. (2016), with increasing  $\text{CH}_4$  oxidation from MOX07 (Lötsjön), MOX03 (Erken), and MOX04 (Malstasjön). Variability in  $\text{CH}_4$  oxidation among 30 ice-covered lakes was also observed in Alaska, where the causes of such variability were attributed to  $\text{CH}_4$  or  $\text{O}_2$  limitations (Martinez-Cruz et al., 2015). Thottathil et al. (2018) found a positive correlation between  $\text{CH}_4$  oxidation and DOC for 14 boreal lakes during the summer, however, this correlation was argued to be attributed to indirect factors such as light inhibition and  $\text{O}_2$  availability, which agrees with the lack of correlation between DOC and SRMOx in this study. In our experiment, both  $\text{CH}_4$  and  $\text{O}_2$  were amended to the vials at the start of the incubation that was carried out in the dark. Hence, the variability observed among our lakes was not expected to be related to lack of substrate or light inhibition.

The positive relationship we observed between SRMOx and in-situ TP concentrations in the lakes supports the potential influence of P on  $\text{CH}_4$  oxidation pointed out by Denfeld et al. (2016). Previously, correlation between  $\text{CH}_4$  oxidation and P has also been reported in Everglade dry wetlands soils (Chauhan et al., 2012), arctic permafrost soils (Gray et al., 2014), tropical forest soils (Zhang et al., 2011), and in temperate drainage ditches (Veraart et al., 2015). In addition to the positive relationship between SRMOx and P in natural conditions, our experimental results suggest an enhancement of SRMOx attributed to P amendment, providing mechanistic support to the positive relationship between SRMOx and lake TP concentration below the ice (Figures 1 and 2).

Despite a change in the whole microbial community composition observed between treatments, the methanotrophic community composition seemed unaffected by P, regardless of lake, and was systematically dominated by only a few OTUs closely related to psychrophilic methanotrophs (Table 2). The dominance of Methylococcaceae in the methanotrophic community at the end of our incubation experiment is in line with previous assessments of winter  $\text{CH}_4$  oxidation in some of our studied lakes, where a significant increase in relative abundance of Methylococcaceae was observed during incubations (Canelhas et al., 2016; Denfeld et al., 2016). The dominance of Type I methanotrophs in previous studies where a phosphorus- $\text{CH}_4$  oxidation relationship was observed (Gray et al., 2014; Veraart et al., 2015) is also in line with our findings. The dominance of these type I psychrophilic methanotrophs has also been observed in other boreal and arctic freshwater bodies (Crevecoeur et al., 2019; Rissanen et al., 2018) and from other different cold environments like groundwater, northern taiga, tundra soils, polar lakes and permafrost sediments (Kalyuzhnaya et al., 1999; Khmelenina et al., 2002; Trotsenko & Khmelenina, 2005; Vecherskaya et al., 1993).

Regardless the positive correlation observed between  $\text{CH}_4$  oxidation and TP concentration we did not find a significant correlation between the relative abundance of methanotrophs and SRMOx or TP concentration. This, combined with the dominance of an OTU associated with psychrophilic methanotrophs, suggests that low temperature and possibly high  $\text{CH}_4$  and  $\text{O}_2$  concentrations might have a stronger effect than P on the methanotrophic community selection. The observed lack of correlation between the relative abundance of methanotrophs and SRMOx could be attributed to a similar increase in the abundance of all bacteria, resulting in unchanged relative abundance. The observed increase in  $\text{CH}_4$  oxidation attributed to P availability could be related to an increase in the absolute abundance, despite the absence of relative increase of methanotrophs in P amended samples, yet we do not have the data to verify this. It is also possible that only the activity of methanotrophs was increased. Several studies where nutrient concentrations have been manipulated report a decoupling of bacterial production and abundance (DeBruyn et al., 2004; Vrede et al., 1999), suggesting that a system could respond with an increase in production, or other processes like  $\text{CH}_4$  oxidation, without any parallel increase in their abundances. This decoupling was observed for MOB in a boreal lake and in a rice paddy where  $\text{CH}_4$  oxidation did not seem to be attributed to MOB abundance (van Grinsven et al., 2021; Zheng et al., 2013), suggesting that the simple change in activity could also explain the increased  $\text{CH}_4$  oxidation observed in our experiment.

The effect of P on the SRMOx could be indirect via stimulation of the combined microbial community, rather than a direct and specific P limitation experienced by MOB. It was indeed pointed out that methanotrophs may benefit from the release of compounds, such as vitamins, amino acids, and organic acids by other bacteria (Iguchi et al., 2011; Xing et al., 2006), while non-methanotrophic bacteria could benefit from enhanced exopolysaccharide production by type I methanotrophs under high nutrient (e.g., P) and substrate availability (Malashenko et al., 2001). This potential systemic effect of the combined microbial community and mutualistic interactions was also observed in experiments showing that MOB (*Methylomonas spp.*) increased their activity (consuming more  $\text{CH}_4$ ) when the richness of heterotrophic bacteria increased (Ho et al., 2014). The increase in activity observed in our experiment could be then attributed to a systemic effect of P on the microbial community methanotroph activity rather than a direct use of P by methanotrophs. However, if neither indirect or direct P effects can be excluded, our results suggest that the influence of such interactions and secondary metabolites on  $\text{CH}_4$  oxidation could be important in nutrient-rich lakes where a more diverse microbial community could be sustained, in relation to lakes with lower trophic state (Kiersztyn et al., 2019).

We acknowledge that the lack of absolute abundance of methanotrophs limited a clear understanding about to what extent the increase in  $\text{CH}_4$  oxidation due to P was attributed to an increase in the methanotrophic density or due to an increase in activity. Similarly, a time series would have been beneficial, but the incubation volume and microbial biomass concentration did not allow for multiple DNA sampling timepoint. Additionally, our incubations were set to be similar to the condition observed in the surface water below the ice where  $\text{O}_2$  was present in most lakes. Inhibition of  $\text{CH}_4$  oxidation by high  $\text{O}_2$  concentration have been recently suggested as a factor influencing  $\text{CH}_4$  oxidation in lakes (Reis et al., 2020; Thottathil et al., 2019). The  $\text{O}_2$  reservoir in the vial headspace kept the  $\text{O}_2$  levels high (i.e., most likely never below 97.5% of the starting levels) and similar across all vials. Thereby possible differences in  $\text{O}_2$  inhibition of  $\text{CH}_4$  oxidation among vials could not have been large enough to explain the results. As  $\text{O}_2$  levels were high during incubation we cannot exclude  $\text{O}_2$  inhibition of  $\text{CH}_4$  oxidation. However, maximum  $\text{CH}_4$  oxidation observed at high  $\text{O}_2$  saturation suggest that  $\text{O}_2$  inhibition is not always happening, and that this potential inhibition remain unclear (van Grinsven et al., 2020). Nevertheless, any potential  $\text{O}_2$  inhibition did not prevent the P-induced stimulation of the  $\text{CH}_4$  oxidation. We also do not discard the possibility that the higher  $\text{O}_2$  concentration in our experiment in comparison with in-situ conditions could have stressed the methanotrophic community adapted to low  $\text{O}_2$  concentration, limiting their capacity to oxidize  $\text{CH}_4$  in the experiment. Further work on how the magnitude of the P-stimulation of  $\text{CH}_4$  oxidation is interacting with levels of  $\text{O}_2$  or other factors influencing  $\text{CH}_4$  oxidation, would be interesting.

Our P amendment experiment indicate that the correlation between SRMOx and TP observed in field data may be causal. Our study further suggests that the increase in potential  $\text{CH}_4$  oxidation along the trophic gradient of lakes observed by Sepulveda-Jauregui et al. (2018) could be attributed to the P availability in the water. However, the synergistic effect of increased nutrient concentration and water temperature were found

to intensify the potential CH<sub>4</sub> production in microcosm lake sediment experiments (Sepulveda-Jauregui et al., 2018), and ebullitive CH<sub>4</sub> emissions from mesocosm experiments (Davidson et al., 2018) suggest that despite the higher offset by oxidation, CH<sub>4</sub> emissions could still increase with warming and eutrophication. It is worth highlighting that enhanced CH<sub>4</sub> oxidation can significantly reduce the amount of CH<sub>4</sub> in the water column, reducing the emissions through diffusion. Hence, it is important to note that assessments of CH<sub>4</sub> fluxes from eutrophic systems should be designed to appropriately cover ebullitive emissions of CH<sub>4</sub> to prevent underestimation of the total CH<sub>4</sub> emissions. The P-CH<sub>4</sub> oxidation link could be an important factor controlling diffusive fluxes during the open water season. However, we argue that this importance could be more significant during winter when CH<sub>4</sub> oxidation also mitigates ebullition by oxidation of CH<sub>4</sub> in bubbles trapped under the ice and diffusing back into the water.

Similarly, photosynthetic consumption of P could cause P deficiencies in the surface lake water column, leading to decreasing methane oxidation rates. Such a P deficiency, rather than direct light inhibition, could be a complementary or alternative explanation to the proposed enzymatic inhibition of CH<sub>4</sub> oxidation by light (Dumestre et al., 1999; Murase & Sugimoto, 2005; Shelley et al., 2017). An increase in phytoplankton associated with a decrease in the relative proportion of MOB and a consequent increase of in situ CH<sub>4</sub> concentration was observed after the experimental removal of the snow cover on a seasonally ice-covered lake (Garcia et al., 2019). Similarly, MOB expansion in the water column has also been observed during the ice season as compared to summer when MOB were largely restricted to the deeper zones of the lakes (Samad & Bertilsson, 2017). During summer, competition for P would be higher near the surface while P concentrations usually increase in the anoxic zone of the water column.

Wintertime has been highlighted as more important for the carbon cycle than previously assumed (Denfeld et al., 2018; Sharma et al., 2020), and our results bring evidence that P availability could increase winter CH<sub>4</sub> oxidation with high potential for mitigating CH<sub>4</sub> emissions to the atmosphere. The identification of P as a controlling factor of CH<sub>4</sub> oxidation can improve predictions of ice-off and open water emissions and, thus, annual CH<sub>4</sub> emissions from lakes. This study also provides perspectives for the future. The predicted reduction in the length of the ice-covered season and snow coverage (Sharma et al., 2019) could reduce bubble trapping by ice and thereby reduce the mitigative capacity of methane emissions in winter, especially in nutrient-rich (i.e., eutrophic) lakes where ebullition is suggested to be highest (Beaulieu et al., 2019) and P would not limit CH<sub>4</sub> oxidation. This can result in overall higher annual emissions from lakes, and contribute as a additional CH<sub>4</sub> feedback to global warming (Dean et al., 2018).

## 5. Conclusion

Based on the overall positive response of CH<sub>4</sub> oxidation to phosphate addition observed in the experiment, we conclude that when CH<sub>4</sub> and O<sub>2</sub> are not limiting factors, CH<sub>4</sub> oxidation in lakes could be constrained by P availability. We found that limited CH<sub>4</sub> oxidation could be expected during winter in nutrient-poor lakes, potentially explaining why large emissions during ice-off may happen in some lakes but not in others. On the other hand, high P availability could increase CH<sub>4</sub> oxidation capacity, especially in winter, when ebullitive emissions are trapped under the ice and subjected to CH<sub>4</sub> oxidation, with the potential to reduce the ice-out share of the annual emissions. We do not exclude the possibility that other variables alone or in combination with P may also play a role in controlling CH<sub>4</sub> oxidation. We also acknowledge that P may favor CH<sub>4</sub> emissions by increasing eutrophication, which negatively influences O<sub>2</sub> availability due to increased heterotrophic respiration, creating conditions that could limit under ice CH<sub>4</sub> oxidation resulting in higher ice-off emissions. However, despite the seemingly important role P has on winter CH<sub>4</sub> oxidation, its influences on the balance between CH<sub>4</sub> production and CH<sub>4</sub> oxidation needs further investigation across different seasons and lake types, helping understand the regulations of the net CH<sub>4</sub> balance and improve estimates of annual CH<sub>4</sub> emissions from lakes.

## Conflict of Interest

The authors declare no conflicts of interest relevant to this study.

## Data Availability Statement

Sequences and associated metadata are deposited in NCBI's Sequence Read Archive and BioSample under accession number PRJNA638356 and can be accessed through the link: <https://www.ncbi.nlm.nih.gov/bio-project/PRJNA638356>. All data used in the paper appear as metadata of each sample.

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