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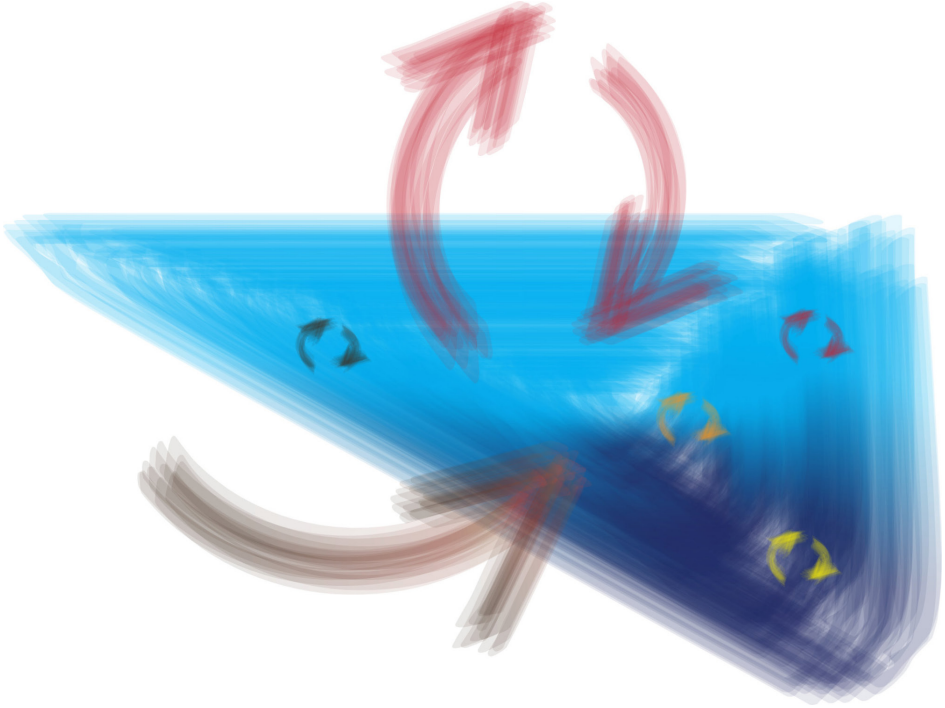
Gaëtan Martin



Microbial mitigation of greenhouse gas emissions from boreal lakes

Gaëtan Martin
Faculty of Forest Sciences
Department of Forest Mycology and Plant Pathology
Uppsala

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Cover: Carbon cycling in lakes encompasses a multitude of processes
(Image: Gaëtan Martin)

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Abstract

The climate change crisis has drawn the attention of both the public and scientific community to the carbon cycle and particularly to the importance of greenhouse gases (GHG) carbon dioxide (CO_2) and methane (CH_4). CO_2 has been a key component of Earth´s climate regulation throughout its geological history and is now the main driver of the current change in climate. CH_4 has been responsible for a quarter of the cumulative radiative forcing observed so far. Recent studies suggest that lakes could be a major source of both CO_2 and CH_4. Boreal lakes are of special interest as they represent 27% of the global lake area, and their production of CO_2 and CH_4 are expected to increase in the future.

This project aimed to investigate microbial processes with the potential to limit the emissions of GHGs from boreal lakes. For that purpose, the impact of an increase in phosphorus (P) concentration in the water on CH_4 oxidation under the ice was investigated as well as the community composition of the methanotrophic guild. We also looked at the potential importance of chemolithoautotrophic microorganisms in fixing CO_2 in the water column. Using a combination of geochemical analysis, genomic studies, and in vivo assays, we showed that P amendment has the potential to increase methane oxidation, possibly limiting the expected increase in CH_4 emissions due to anthropogenic fertilization of boreal lakes. We also showed that methanotrophic community structure in boreal lakes is driven by CH_4 concentration and that alphaproteobacterial methanotrophs might play an important role in removing CH_4 from surface waters. Finally, we showed that dark carbon fixation is a common trait in boreal lakes and that it seems related to the iron cycle.

Keywords: Methane, chemolithoautotrophy, phosphorus, boreal lakes, iron

Author’s address: Gaëtan Martin, Swedish University of Agricultural Sciences, Department of Forest Mycology and Plant Pathology, Uppsala, Sweden

Microbial mitigation of greenhouse gas emissions from boreal lakes

Abstract

Klimatkrisen har uppmärksammat betydelsen av kolets kretslopp och växthusgaser och då främst koldioxid (CO_2) och metan (CH_4). Koncentrationen av koldioxid i atmosfären har varit en avgörande faktor för hur klimatet utvecklats under jordens historia och dess ökning är den främsta anledningen till den accelererande klimatförändringen. Metan beräknas ligga bakom en fjärdedel av den sammanlagda hittillsvarande växthuseffekten. Senare tids studier visar att sjöar kan utgöra en stor källa för det samlade utsläppet av både koldioxid och metan. Boreala sjöar är av särskilt intresse eftersom de motsvarar 27% av jordens sjöars yta, och för att deras utsläpp av koldioxid och metan förväntas öka i med varmare klimat.

Syftet med min avhandling var att undersöka mikrobiella processer som potentiellt skulle kunna minska utsläppen av växthusgaser från nordliga sjöar på norra halvklotet, så kallade boreala sjöar. För att göra detta undersökte jag hur en ökad fosforkoncentration i sjövatten påverkar oxidering av metan under is och sammansättningen av mikroorganismer som livnär sig av metan, så kallade metanotrofiska mikroorganismer. Jag studerade också betydelsen av kemolitoautotrofa mikroorganismer som kan binda koldioxid i vatten utan solljus. Med hjälp av geokemiska analyser, genetiska studier och experiment i fält fann jag att högre halter av fosfor kan öka oxideringen av metan, vilket skulle kunna minska den förväntade ökningen av metanutsläpp som orsakas av övergödning. Jag visade också att artsammansättningen av metanotrofiska mikroorganismer drivs av metankoncentration och att metanotrofa alphaproteobakterier kan spela en viktig roll för att bryta ner metan i ytvatten. Slutligen kunde jag konstatera att det är vanligt att koldioxid binds och fixeras i vatten dit solljuset inte når och att processen verkar vara kopplad till järnets omsättning.

Author’s address: Gaëtan Martin, Swedish University of Agricultural Sciences, Department of Forest Mycology and Plant Pathology, Uppsala , Sweden

Dedication

A mes parents qui m’ont soutenu dans tous mes projets, même les plus saugrenus (à part le basket, mais bon, ils avaient raison)

Contents

List of publications

This thesis is based on the work contained in the following scientific articles, referred to by Roman numerals in the text:

I. Buck Moritz, Sarahi L. Garcia, Leyden Fernandez, **Gaëtan Martin**, Gustavo A. Martinez-Rodriguez, Jatta Saarenheimo, Jakob Zopfi, Stefan Bertilsson, and Sari Peura. (2021). Comprehensive Dataset of Shotgun Metagenomes from Oxygen Stratified Freshwater Lakes and Ponds. Scientific Data 8 (1): 131

II. Henrique O. Sawakuchi, **Gäetan Martin**, Sari Peura, Stefan Bertilsson, Jan Karlsson, David Bastviken (2021). Phosphorus regulation of methane oxidation in water from ice-covered lakes. Journal of Geophysical Research: Biogeosciences 126

III. **Gaëtan Martin**, Antti Juhani Rissanen, Sarahi L. Garcia, Maliheh Mershad, Moritz Buck, Sari Peura (2021). *Candidatus* Methylumidiphilus drives peaks in methanotrophic relative abundance in stratified lakes and ponds across Northern landscapes. Frontiers in Microbiology 12: 2077

IV. **Gaëtan Martin**, Antti Juhani Rissanen, Sarahi L. Garcia, Sari Peura Dark carbon fixation is a common process in stratified boreal lakes. (manuscript)

The contribution of Gaëtan Martin to the articles included in this thesis was as follows:

I. Selected some of the sampling sites and collected a significant part of the samples. Participated in the manuscript writing.

II. Participated in the development of the project, helped with the sampling and preparation of the incubation, and performed all the analyses of the microbial community. Collaborated in writing the first version of the manuscript as well as in answering the reviewers' requests.

III. Took part in designing the study, run all the analyses and wrote the first version of the manuscript.

IV. Designed the project and led the field campaigns and samples analyses. Analyzed the environmental and genomic data and wrote the first draft of the manuscript.

Abbreviations

Greenhouse gases	GHG
Chemolithoautotrophs organisms	ChLithO
Methane oxidising organisms	MO
Methane oxidation	MOx
Dissolved organic carbon	DOC
Dark carbon fixation	DF
Dissolved Nitrogen	DN
Exopolysaccharides	EPS

1. Background and context of the present work

1.1 Two ends for a cycle

At the core of this work are two carbon compounds that are simultaneously very similar and radically different. Carbon dioxide (CO_2) and methane (CH_4) are two one-carbon molecules standing at each extremity of the redox ladder of carbon-based molecules. The first is the most oxidised form of carbon while the second is the most reduced. Despite that radical difference, they both are final product of the food web, one in oxic condition (CO_2) and the other in highly reduced, anoxic conditions (CH_4). The idea that two different molecules can be at the end of the same cycle is a nice introduction to the complexity of the relations between microbial ecology, physiology, and biogeochemistry at the centre of my thesis. A big part of the intersection between ecology and biogeochemistry is understanding how energy, nutrients and biomass are moved from one compartment of an ecosystem to another. These transfers of energy from one compartment to the next are strongly linked with the carbon cycle. In short, primary producers use light or redox reactions as a source of energy to transform energy-poor inorganic carbon into energy-rich biomass. This biomass will provide energy and carbon into the food web (e.g., grazer, predator, decomposer). To obtain energy, these organisms will oxidise the biomass through respiration or fermentation. In oxic condition, respiration dominates and the final product of it is CO_2. But in the absence of oxygen, fermentation is used to produce energy, releasing hydrogen (H_2). In those anoxic conditions, some microbes

can combine oxidised C (i.e., CO_2) with H_2 to produce extra energy and CH_4. Both CH_4 and CO_2 can then be turned back into biomass.

In both cases, we are now back at the beginning of the cycle. In both cases, the balance between the work of the primary producers and the consumers is critical for our future. Indeed, both CO_2 and CH_4 are potent greenhouse gases (GHG). If the primary production rate of a system is higher than the degradation of biomass into CO_2 and CH_4, the system is a GHG sink. If the production of CO_2 or CH_4 is faster than their transformation into biomass, the system is a GHG source. So, as they are chemically and energetically opposed and produced in very different environments, their ecological relevance is similar both in their role as C source and their potential impact on the climate. Their differences are nevertheless critical. CH_4 has a higher warming potential but a shorter lifespan (Etminan et al. 2016), and, even if some methanotrophs are active in fully oxic environment, its biological relevance is mostly associated to prokaryotes living in anoxic or microoxic environment.

On the other hand CO_2 is not as efficient in keeping heat in the atmosphere but its abundance and stability make it the most important GHG on our planet (Berner et al. 1983). Its prevalence is also more widely spread as the transformation of biomass to CO_2 can be performed by a wide range of organisms belonging to all kingdoms. Furthermore, it is not only the end product of aerobic and anaerobic respiration, but also a potential end product of fermentation and a C source for CH_4 production.

1.2 Where two worlds meet

Both the compounds and the organisms that rely on CO_2 and CH_4 for their survival seem to belong to different worlds. CO_2 production is mostly associated with oxidizing conditions (which can also be found in anoxic systems) whereas biologically produced CH_4 is widely associated with reduced environments. But when we look at their transformation into biomass things are different. The most common and best-known process for CO_2 assimilation to biomass is oxygenic photosynthesis, where solar energy is used to combine CO_2 with water, producing reduced organic matter and O_2, perpetuating the oxic conditions that most complex life rely on. Even if photosynthesis is the best-known carbon fixation process, it is known since

the times of Winogradsky that some organisms, called chemolithoautotrophs, can turn mineral carbon into biomolecules in the absence of light (Ackert 2006). Chemolithoautotrophic organisms (ChLithO) are prokaryotes (i.e., bacteria and archaea) that use energy from the oxidation of reduced compounds to get the energy and reducing power necessary to convert inorganic C into biomass. ChLithO is a diverse group of microorganisms regarding phylogeny, physiology and ecology (Enrich-Prast et al. 2009). To strive, they need the simultaneous presence of oxidized and reduced compounds.

Similarly, most methanotrophs can use the energy yield of the CH_4/O_2 redox pair to fix carbon. The simultaneous presence of compounds normally present in reducing or oxidizing conditions is commonly found at the interface between oxic and anoxic environments. Such environment can be found in many systems, including soils, sediments, water columns, or intestinal tract of animals, including humans (Brune et al. 2000; Kirf et al. 2015; Uteau et al. 2015; Shin et al. 2019). In water, such interfaces are found at the top layer of sediment or the chemocline in the water column of oxygen stratified lakes (Camacho et al. 2001; Santoro et al. 2013). Whereas the stability and depth of stratification depend on several parameters, such as temperature, salinity, watercolor, lake morphology, climate, etc., the different layers of stratified lakes tend to share a few common features (Figure 1). The surface layer, epilimnion, is rich in oxygen while at the lake bottom, in the hypolimnion, anoxic conditions often prevail. Between these two layers, a thin but distinct layer develops: this is the metalimnion. At this interface between the oxic and anoxic waters, a steep and stable gradient of oxygen, salinity and redox couples is generally observed: this is the chemocline (Børsheim et al. 1985; Camacho et al. 2001). Chemoclines are hotspots for both ChLithO and methanotroph activity (Camacho et al. 2001; Sundh et al. 2005; Santoro et al. 2013; Rissanen et al. 2018). But despite being a hot spot, chemocline is often neglected as many works focus on surface water and phototrophic primary production.

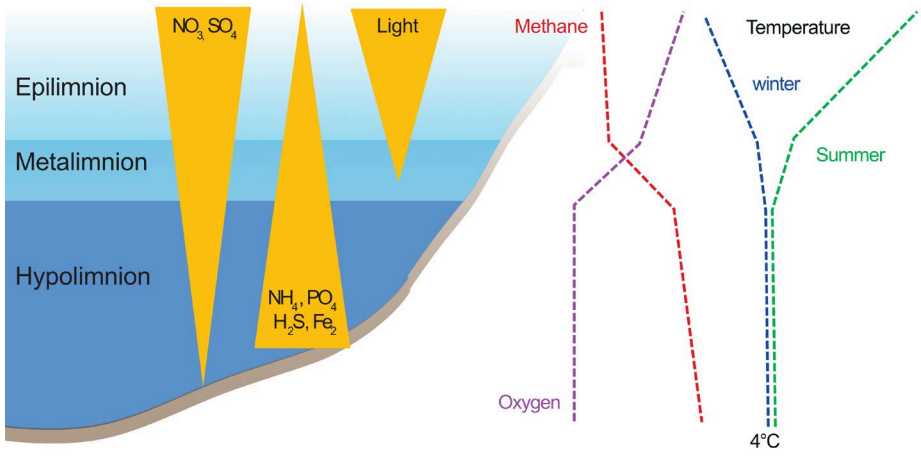

Figure 1: Schematic representation of a stratified lake. During stratification, the lake water column is divided into three distinct layers. The surface layer, epilimnion, is rich in oxygen, while at the lake bottom, in the hypolimnion, anoxic conditions prevail. Between these two layers, a thin but distinct layer develops: the metalimnion. During winter, when the lake freezes, the temperature of the layers is inverted, with the warmer layer being the hypolimnion (Martin et al. 2021).

1.3 Small lakes, big effect

GHG emissions from freshwater systems have often been dismissed (Falkowski et al. 2000), until the fifth assessment report of the IPCC acknowledged the importance of lakes in the global carbon cycle (Ciais et al. 2013). Inland waters are generally considered net emitters of CO_2 as their surface water is commonly supersaturated in CO_2 (Sobek et al. 2005; Tranvik et al. 2009a; Raymond et al. 2013). There are also reports of supersaturation of CH_4 in surface water of lakes, including lakes in boreal landscapes (Kortelainen et al. 2001; Billett & Moore 2008; Blees et al. 2014). Several studies suggest that lakes and ponds are the dominant and underestimated sources of natural CH_4 at high northern latitudes (i.e., in boreal and arctic areas) (Bastviken et al. 2011; Wik et al. 2016b; a). Boreal lakes are of special interest as they represent 27% of the global lake area whereas boreal biome covers only 14% of the global land surface (Verpoorter et al. 2014). More critically, projections suggest that both their

CO_2 and CH_4 emissions will increase due to climate change and increase in human activities (Tranvik et al. 2009b; Bogard & del Giorgio 2016; Wik et al. 2016a; Hastie et al. 2018).

Boreal lakes are also peculiar in their chemistry and physics. Most lakes develop thermal stratification during the warm season, but their hypolimnion does not necessarily become anoxic. Indeed, light penetration allows oxygenic phototrophs to maintain oxic conditions below the thermocline (Fee 1976; Nürnberg & Shaw 1998). Furthermore, in clear oligotrophic lakes oxygen consumption is low. On the other hand, small colored lakes are more likely to possess an anoxic hypolimnion (Nürnberg & Shaw 1998) even when very shallow. In these lakes, chemocline can even be at a lower depth than the thermocline (Deshpande et al. 2015). This is the result of the combination of limited oxygenic photosynthesis due to low light penetration and higher heterotrophic respiration due to high dissolved organic carbon (DOC) content. In these small lakes, the area and color also strongly impact the depth and stability of the epilimnion (Fee et al. 1996). This, in turn has a strong impact on the photosynthetic primary production. Indeed, the limited depth of euphotic layer means that despite higher C fixation per unit of volume, the fixation rate per unit of lake area is lower in colored lakes (Nürnberg & Shaw 1998). A combination of browning, higher temperature, longer summer, increased human pressure and advance of the tree line is expected to increase the duration of both thermal and chemical stratification in boreal lakes. (Fee et al. 1996; Brothers et al. 2014; de Wit et al. 2016; Jenny et al. 2016; Woolway et al. 2021; Klaus et al. n.d.).

All these changes are expected to increase CO_2 emissions from boreal lakes as these features favor heterotrophic respiration over photosynthesis (Bogard & del Giorgio 2016; Hastie et al. 2018; Kuhn & Butman 2021). There is also a strong risk that CH_4 production in lakes will increase with climate change and human activities (Tranvik et al. 2009b; Wik et al. 2016b). This increase in emission is expected to be mostly driven by higher DOC and eutrophication supplying both organic matter and nutrients to methanogens and favorizing the formation of anoxic hypolimnion (Sepulveda-Jauregui et al. 2015, 2018).

1.4 Mind the gap

While it is generally accepted that small boreal lakes are and will be playing an important role in the natural cycle of GHGs, some of its main features are often dismissed. Indeed, as many studies focus on the net emissions of GHGs (Wik et al. 2016b; Nydahl et al. 2020) and develop models to predict future emissions and conditions (de Wit et al. 2016; Kiuru et al. 2018), little attention is paid to the hotspot in the metalimnion. This is particularly true regarding the potential mitigation of CO_2. Several works focusing on energy mobilization in boreal lakes do not consider the potential importance of dark carbon fixation (DF) (Andersson 1983; Salonen et al. 1992; Carpenter et al. 1998; Nürnberg & Shaw 1998; Ask et al. 2009; Kodama et al. 2012). However, the measured primary production seems unable to support the total carbon demand of bacteria (Kankaala et al. 2013). This is particularly striking as not only do boreal lakes offer ideal condition for ChLithO, but previous studies have also shown both a genetic potential for ChLithO (Taipale et al. 2009; Peura et al. 2018) as well as actual measurements of DF in boreal lakes (Kuuppo-Leinikki & Salonen 1992; Nõges & Kangro 2005).

Another important feature of boreal lakes is that they are covered in ice during the winter. As temperature drops, both the lake chemistry and physics change. Temperature stratification is inverted (Figure 1) and once covered in ice, light decreases, letting heterotrophic metabolism dominate. These changes drive an increase in dissolved nitrogen (DN) and organic carbon (DOC) and a decrease in dissolved oxygen (DO) (Gammons, Henne et al. 2014, Hampton, Galloway et al. 2017, Powers, Labou et al. 2017, Kalinowska, Napiórkowska-Krzebietke et al. 2019). When anoxia is reached, methanogenic activity can be detected (Gammons, Henne et al. 2014), potentially leading to methane accumulation under the ice and significant emissions at ice-off (Bellido et al. 2009; Sepulveda-Jauregui et al. 2015; Denfeld et al. 2018; Jansen et al. 2019). If the ice cover favorizes anoxic conditions, crack in the ice coupled with intermittent light often allows a thin layer of oxic water to subside. In those conditions it was demonstrated that methanotrophs can consume CH_4 (Samad and Bertilsson 2017). However, it is not clear to what extent, as in some lakes no methane oxidation was detected, and factor controlling the oxidation of methane under ice remain mostly unknow (Denfeld et al. 2018). Even if oxygen is a known factor influencing CH_4 oxidation rates, it does not help understanding

the variability observed in oxic layer of frozen lakes. A recent study highlighted the potential role of P concentration (Denfeld et al. 2016). But it gave no clear evidence supporting this hypothesis.

Finally, little is known about the methanotrophs distribution and ecology in boreal lakes. Some recent studies suggest that the O_2 and CH_4 counter gradients are responsible for niche partitioning of alphaproteobacterial methane oxidizers (α-MO) and gammaproteobacterial methane oxidizers (γ-MO) and underline how this partitioning might be essential for predicting the efficiency of the CH_4 biofilter (Mayr et al. 2020b; Reis et al. 2020; Rissanen et al. 2020). Other environmental parameters, like phosphorus, light or temperature, have been suspected to influence the composition of the methanotrophic guild (Thottathil et al. 2018; Yang et al. 2020; Zhou et al. 2020). However, the impact of those environmental parameters on the methanotrophic communities is still unclear and often seems contradictory (Ho et al. 2013). Furthermore, most studies on freshwater MO neglect the potential importance of less abundant MO like anaerobic MO and *Verrucomicrobia* (Ho et al. 2013; Knief 2015; Crevecoeur et al. 2017, 2019a; Reis et al. 2020). Finally, existing studies focus on one or a small number of lakes in a limited geographic area (Tsutsumi et al. 2011; Crevecoeur et al. 2017; Oswald et al. 2017; Samad & Bertilsson 2017; Graf et al. 2018) or look only at the top layer of the studied water bodies (Crevecoeur et al. 2019a). This incomplete overview restrains the identification of factors that could be used for global estimations of the abundance and distribution of methanotrophs. CH_4 biofilter efficiency estimations vary from 30-99%, and can be partly explained by environmental parameters (Frenzel et al. 1990; Kankaala et al. 2006; Bastviken et al. 2008; Mayr et al. 2020a), but differential affinity for methane of different taxa likely plays are role too (Mayr et al. 2020b). Therefore, a better knowledge of the diversity and distribution of methanotrophic communities is essential to understanding the biological mechanisms behind the dynamic methane equilibrium and eventually predicting possible future changes in the functioning of the CH_4 biofilter (Wagg et al. 2019).

1.5 Objectives

Among the many unanswered questions about the carbon cycle in boreal lakes my thesis focused on two processes with a potential to limit the future GHGs emissions:

- Pelagic methanotrophy (articles II and III). In stratified lakes, the methanotrophic hotspot is at the chemocline rather than in the sediment, as the oxic/anoxic interphase is in the water column. The first hypothesis was that the composition of methanotrophic (MO) community is shaped by the environment. The goal was to determine key variables that would help to understand better what taxa of methanotrophs dominate (or not) in different environments. Some emphasis was also put on less abundant MO as those are often disregarded in studies and little is known of their interactions with the environment. The second hypothesis was that P plays an important role in the methane oxidation potential of ice-covered lakes. Previous works revealed a link between the ability to oxidize CH_4 and P concentration. The project aimed to experimentally test that link and assess how the methanotrophs and general microbial community were affected by P addition.
- Dark carbon fixation (article IV). DF is a forgotten process, likely to become more and more relevant as lakes are expected to get darker with time. Based on previous records of DF in one lake in Estonia (Nõges & Kangro 2005) and genomic data suggesting the presence of potential ChLithO (Taipale et al. 2009; Peura et al. 2018) the hypothesis we wanted to test was that DF is a common process in stratified boreal lakes. A secondary objective was to identify ChLithO responsible for DF in boreal lakes as well as the major pathways they rely on.

2. Methods

2.1 So much in common

This thesis is mostly based on field data and experiments. Part of the data is presented in paper I, which also includes data collected during several field campaigns anterior to this thesis. The study of environmental preferences of methanotrophs (paper II) was based on data presented in paper I, including data collected both during this thesis and in previous campaigns. Data were collected specifically for this thesis during three distinct field campaigns across Sweden (Figure 2). The first field campaign was performed during the 2017/2018 winter and provided genomic and environmental data presented in the paper I. It also provided the samples used for the experiments presented in paper II and some of the data were used for the analysis presented in paper III. The second field campaign in summer 2018 included the collection of more genomics and environmental data presented in paper I. Beside sample collection, ^{14}C incorporation assays were performed to detect and quantify the presence of DF. Those data, combined with the results of the last field campaign (summer 2019), are the source material for paper IV. For each lake, a full oxygen and temperature profile was performed. Additional variables, like pH, redox potential (ORP) and salinity were also recorded depending on the probes available at the time of sampling. Samples for genomic analysis were collected by filtering water on 0.22 µm filters and frozen until DNA extraction and sequencing. Additional samples were collected for nutrient analysis (e.g., phosphorus, DN, DOC, Fe) and dissolved gas quantification (i.e., CH_4 and CO_2). Details of sampling

procedures and analysis can be found in the material and methods sections of papers I-IV.

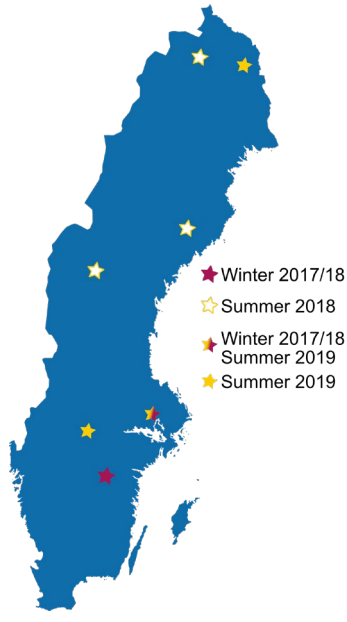

Figure 2: approximate location of field sites. Each star indicates one "location" were several lakes (2-7) were sampled.

2.2 Winter sampling and incubations

Between March and April 2018, we sampled 12 lakes around Uppsala and Linköping. All those lakes were covered with 30-40 cm of ice at the time of the sampling. The seven lakes in Uppsala region were studied in a previous study addressing the impact of winter conditions on CH_4 oxidation (Denfeld et al. 2016). The lakes around Linköping were scouted by members of the Department of Thematic Studies of Linköping University. Besides the samples described above, 4.5 l of water were collected 10 cm below the ice. Those 4.5 l were used to perform experimental methane oxidation essays. Five replicates of 80 ml with and without the addition of an aqueous phosphate solution (final concentration of ~500 µg/l) were prepared for each lake. The incubation vessels were sealed and flushed with high purity

carbon-free synthetic air. After that, 1.8 ml of 99% CH_4 was added to the headspace and incubation was carried out in the dark at 4°C for about two months. During the essay, methane concentration in the headspaces was monitored. The oxidation rates were calculated as the decay (λ) constant according to the following equation

$$C_t = C_0 e^{-\lambda t}$$

2.3 Summer sampling and ^{14}C essays

During summers 2018 and 2019, 18 lakes were sampled and tested for the presence of active DF. Small lakes in boreal forest are less likely to by mixed by wind and therefore more likely to be stratified (Klaus et al. 2021). Based on satellite images, we selected lakes with a diameter below 150m. On-site, we located the deepest point and checked for oxygen stratification. If the bottom of the lake was anoxic, a full profile was performed, and three depths were selected. The first depth was selected to represent the condition in the euphotic epilimnion, the second to represent the chemocline were O_2 concentration decrease sharply. Finally, the third one was set at the very top of the hypolimnion. Samples were collected at each depth for nutrient and DNA analysis. Water was also collected for carbon incorporation measurements using ^{14}C labelled $NaCO_3$. For that purpose, water from each depth was transferred in 9 serum bottles. After the addition of 1µCi of $Na^{14}CO_3$ in each bottle, they were randomly assigned to 3 treatments. For the dark treatment, bottles were put in an opaque incubating chamber, for the light treatment in a transparent chamber, and for the control treatment, bottles were spiked with 0.5 ml of 50% glutaraldehyde and put in an opaque chamber. All incubations were carried out for 24h, *in situ* at a depth of the origin of each sample.

Back in the lab incorporation activity was measured by scintillation counting, and raw CO_2 fixation rates (RwF) were calculated with the following equation:

$$\mathrm{RwF} = \frac{\mathrm{IC} * \ \mathrm{DIC\ x\ 1.06}}{\mathrm{Ta}}$$

IC is the measured incorporated C value, Ta the total activity added to each essay and DIC is the total dissolved inorganic carbon concentration at the incubation/sampling site. The value 1.06 is the correction factor for isotopic fractionation between ^{12}C and ^{14}C. Total primary production (PP) was calculated as the difference between the light treatment and the killed control treatment, photosynthetic carbon fixation (PF) was calculated as the difference between the mean RwF in the light treatment and the dark treatment, and DF as the difference between the mean RwF of the dark treatment and the killed control treatment.

$$PP = RwF^{\text{light treatment}} - RwF^{\text{killed control}}$$
$$PF = RwF^{\text{light treatment}} - RwF^{\text{dark treatment}}$$
$$DF = RwF^{\text{dark treatment}} - RwF^{\text{killed control}}$$

In all cases, t-tests were performed to control that the differences between the two treatments were significant. If the p-value was above 0.05, the difference between the treatments was assumed to be null.

2.4 From DNA to data

Except for the samples collected at the end of the methane and phosphorus incubation (section 2.2), all samples were processed similarly. Details can be found in papers I, III and IV. In short, samples were prepared using the standard procedure for Illumina libraries, and sequenced on the NovaSeq6000-platform at the Science for Life Laboratory (Uppsala University, Sweden). Raw data processing was performed as described in paper I. Then Kaiju with default parameters (Menzel et al. 2016) was used to taxonomically classify the trimmed but unassembled shotgun data.

At the end of the winter-phosphorus incubation, 40 ml were filtered through 0.22 µm filters and DNA was extracted to perform "barcode" sequencing of 16S rRNA genes as described in Sinclair et al. (2015). Sequences were analysed and classified into OTUs using Mothur (Schloss et al. 2009).

For the paper IV ChLithO were examined by looking for taxa for which read-based abundances correlated with DF. We then looked in the literature if any of the correlating families was known to harbor ChLithO potential.

We also considered the following taxa which were previously detected in boreal lakes and assumed chemolithoautotrophic: *Ferrovaceae*, *Nitrospiraceae* and *Hyphomicrobiaceae* (Taipale et al. 2011; Peura et al. 2018). We then looked for gene linked to carbon fixation and iron metabolism in MAGs assigned two particularly promising families : *Ferrovaceae* and *Gallionellaceae*

All subsequent steps and analyses were carried in R (R Core Team 2020).

3. Results and discussion

3.1 Methane oxidation: a winter story

The incubations performed for paper II allowed to show a causal link between methane oxidation (MOx) and phosphorus. First, we confirmed the correlation observed by Denfeld (2016) between P concentration in lakes and MOx rates. Indeed, the MOx rates observed in the non-P-amended incubations correlated with the P concentrations measured in the lakes that provided the water for the essay. But more interestingly, we showed experimentally that increased P concentration can positively impact MOx rates (Figure 3). While not systematic and sometimes not significant, P addition clearly had an impact on MOx rate. Interestingly, that impact appears to be very variable even among incubation bottles from one single lake. This suggests that while significant, the effect of P addition is not the only factor triggering an increase in MOx rate. As both CH_4 and O_2 were the same in all incubations these alone cannot explain the variation. It is therefore tempting to assume that a change in the microbial population could be responsible for variation in the changes observed when P was added to the media. A small difference between the community of the two bottles could yield different dynamics. Indeed, the combined presence of abundant P, CH_4 and O_2 was likely to support fast-growing organisms. In such conditions, a small difference in the initial community could rapidly see different populations dominate the community.

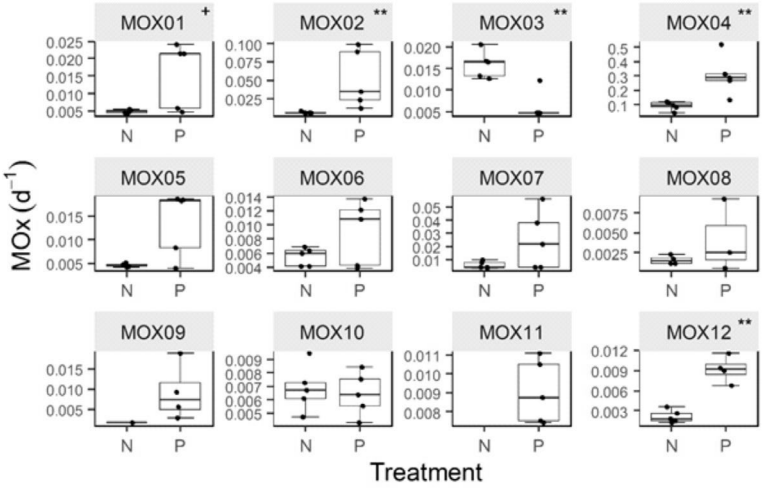

Figure 3: Comparison of methane oxidation rate in non-amended (N) and P-amended (P) incubations. MOx stands for methane oxidation, MOX## is the reference number of each lake. Lower and upper boundaries represent 25th and 75th percentiles, and the error bar correspond to 1.5* the inter-quartile range. Middle lines are indicating the median. Symbols on the right upper corner of the panels represent the p-value ranges based on Two-sided Wilcoxon rank-sum test (0 - 0.001 = '***'; 0.001 - 0.01 = '**'; 0.01 - 0.05 = '*'; 0.05 - 0.1 = '+'; 0.1 - 1.0 = "No symbol"). Note that the y-axis scale is adjusted for the values observed for each lake (Sawakuchi et al, 2021)

Unfortunately, no change was visible in the methanotroph community. Based on the 16S rRNA gene sequencing data, it was impossible to distinguish the methanotrophic communities found in amended cultures from the ones found in the controls. This suggest that the MO community composition was most likely selected by other factors than P. The most probable culprit are the other variables that were manipulated to be similar in all incubations, i.e., high CH_4 and O_2 as well as temperature. This assumption is supported as a high relative abundance of *Methylococcaceae* was also observed in previous experiments where enrichment was expected (Denfeld et al. 2016; Samad & Bertilsson 2017). Furthermore, the MO communities at the end of the experiment were highly dominated by a few taxa closely related to known γ-MO psychrophilic methanotrophs, that have been noted as dominant in several cold environments and boreal lakes (Rissanen et al. 2018; Crevecoeur et al. 2019b).

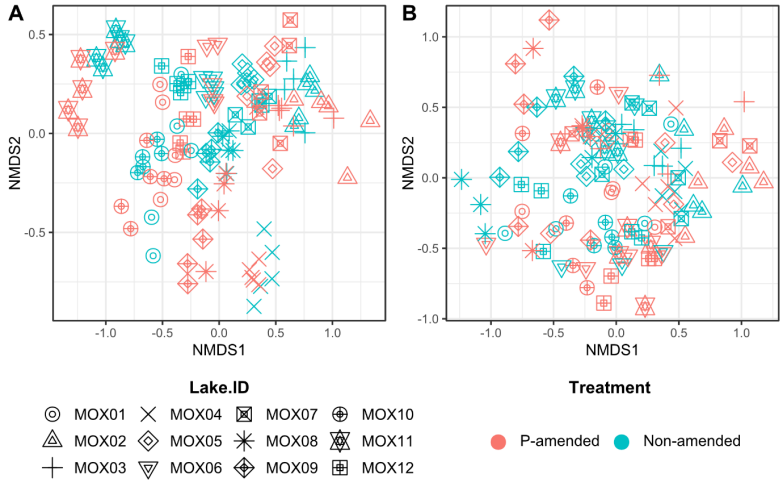

Figure 4: Beta diversity for total bacterial and methanotrophic community. Visualization of Beta diversity based on non-metric multidimensional scaling (NMDS) for total bacterial (A) and methanotrophic (B) communities. Note how for each community there are two different groups for each lake in the panel A but not in panel B. (Sawakuchi et al. 2021)

Despite this lack of difference in the methanotrophs, P addition influenced the total community (Figure 4). We, therefore, argue that a systemic effect of P on the total population influenced MOx rate. A P fuelled increase in heterotrophic activity could benefit MO by producing growth factors (Iguchi et al. 2011; Stock et al. 2013). Another possibility is that MOx potential is limited by other factors when P concentration increased. In that case, heterotrophs could play a role in detoxifying formaldehyde, a toxic intermediate compound of methane oxidation (Wilkinson et al. 1974) or by accelerating recycling of other key nutrients like N (Piontek et al. 2011; Mühlenbruch et al. 2018). This would be in line with observations of high production of EPS or formaldehyde by MO in unbalanced conditions (e.g., high CH_4 and high P concentration but low N concentration) (Malashenko et al. 2001; Wilshusen et al. 2004; Khadem et al. 2012). In that scenario, P would directly impact the MO, but a cooperation would be necessary to overcome lack of other nutrients or accumulation of toxic by-products, leading to an uncoupling of bacterial production and activity (Vrede et al.

1999; DeBruyn et al. 2004; Zheng et al. 2013). This type of system would also fit in a more general ecological perceptive. Addition of P is expected to increase the overall productivity of the system. Highly productive systems often rely on grazing to keep the system "young" and productive (Charles-Dominique et al. 2015). Addition of P could, hence, not only support extra primary production, but also a quick turnover of the biomass produced, allowing the system to stay "young" and highly productive. This would also be in line with the hypothesis that γ-MO are competitive strategists which can overgrow their competitors when CH_4 is abundant (see paper III).

3.2 Methanotrophs who's where

Based on our dataset covering multiple lakes and locations (paper III), gammaproteobacterial methanotrophs (γ-MO) generally dominated the methanotrophic communities throughout the water columns. A few samples were nevertheless dominated by alphaproteobacterial methanotrophs (α-MO). It appeared that α-MO were dominating in the conditions where CH_4 was limiting and O_2 abundant, whereas γ-MO were associated with high CH_4 concentration, with no clear impact of O_2. Furthermore, γ-MO were the dominant taxa when the MO community was important, while α-MO would only dominate in samples with low proportion of MO. We, therefore, concluded that γ-MO, particularly *Candidatus* Methylumidiphilus, are fast-growing, highly competitive organisms, dominating when conditions are favorable. This generally assumes low substrate affinity. On the other hand, the α-MO seems to have a high CH_4 affinity and slow growth rate, allowing them to dominate the MO community when substrate is scarce. It could also, as previously suggested (Ho et al. 2013), be phrased in a more classical ecology way, presenting the α-MO as stress-tolerant and γ-MO as competitive type.

Besides CH_4 and O_2, several other variables were considered as potentially influencing the MO community structure. But despite literature suggesting that several variables can inhibit or enhance CH_4 oxidation (Rudd et al. 1976; Bédard & Knowles 1989; Murase & Sugimoto 2005; Milucka et al. 2015; Guggenheim et al. 2020), we could not see those effects reflected in the MO community structure. It seems then likely that the regulation is site specific and depends on the specific conditions prevailing in each lake.

Thus, our comparison across lakes might hide the importance of each of these parameters in individual lakes or even in lake compartments.

Among less abundant taxa, relative abundances of methane oxidizing archaea (MOA) and MO belonging to the bacterial phylum *NC10* were strongly correlated across all lake compartments. The strong correlation between the abundance of the two taxa suggests a cooperative interaction between them. This hypothesis is supported by the physiology of those two taxa. Indeed, the most abundant of them, *Ca.* Methanoperedens uses NO_3 as an electron acceptor and releases NO_2 that can potentially be used by *Ca*. Methylomirabilis. Both taxa were also correlated with CH_4 concentration regardless of the lake compartment, including oxic water. This suggest that the presence and abundances of *Ca.* Methanoperedens and *Ca*. Methylomirabilis in oxic water were not random nor accidental (e.g, a product of mixing, or a sequencing artifact). As both of those taxa are known anaerobes, it was surprising that our data suggests that they might be not only be cooperating, but also potentially active in oxic waters. Previous studies showed that they are at least oxygen tolerant (Guerrero-Cruz et al. 2018), but in our case it is not clear if they strive in oxic condition or rely on anoxic micro-niche inside particles (Schramm et al. 1999; Lehto et al. 2014).

3.3 What happens in the dark.

The main goal of that project leading to paper IV was to check if dark carbon fixation (DF) is a common feature in stratified boreal lakes. It appears that this is indeed the case. We could detect DF in all but one of the lakes that were tested (Figure 5). The only lake in which we could not detect DF was harboring a community of potential ChLithO very similar in its composition to the ones of lakes were DF was detected. So, while we cannot exclude that this lake never experiences DF, it seems unlikely. Furthermore, this "negative" lake was sampled a few day short of two month after the ice off, which is in line with the time necessary for stratification in boreal lakes (Mammarella et al. 2018). It is therefore reasonable to think that the ChLithO community may not have had the time to settle. Similarly, lakes sampled around Kiruna (Ki1 and Ki2), which were also sampled about two months after ice-off displayed low DF rates.

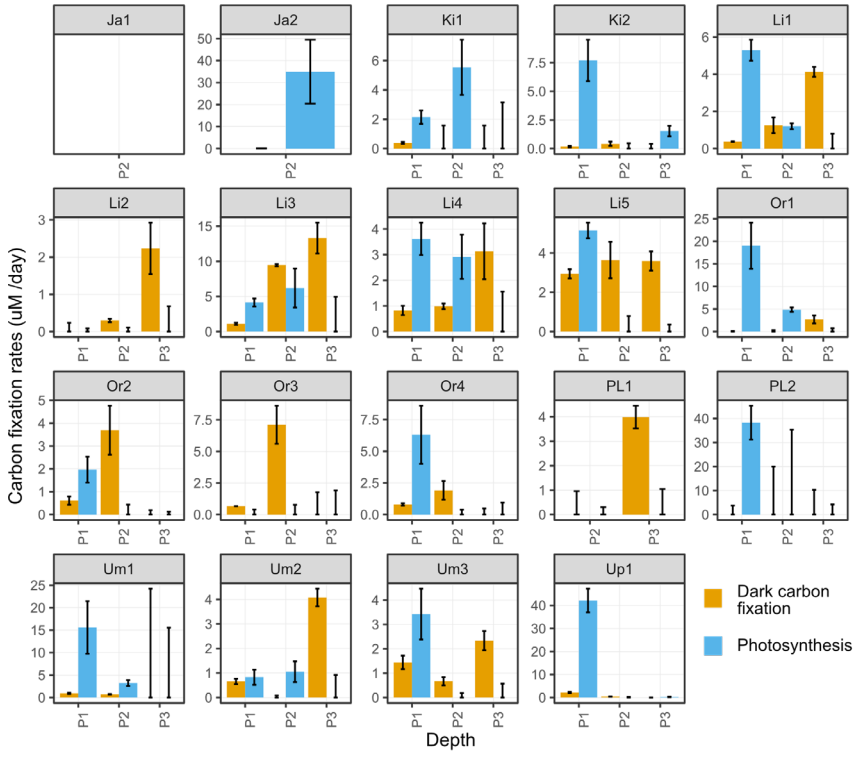

Figure 5: Carbon fixation rates. Bars represent the mean values of biological replicates of carbon fixation rates measured using ^{14}C incubation essay. Values presented are net values, that is, the differences between dark essays and the killed controls for the DF rates and between the rates measured in light and the DF for photosynthesis. P1 is euphotic epilimnion, P2 is microoxic metalimnion and P3 is upper hypolimnion.

DF was mostly associated with lake compartments with low O_2 concentration (Figure 5). However, it was not clear how O_2 rates influence DF rates, as no correlation with redox potential could be observed. But the presence of DF at the chemocline is what is generally observed in stratified lakes (Camacho et al. 2001; Zopfi et al. 2001; Casamayor et al. 2008). The fact that higher rates were generally found in the hypolimnion (i.e., where O_2 levels were below detection limit) was unexpected and it is not clear if this is the product of fully anaerobic ChLithO or organisms striving when O_2 level is very low. The second option would be relevant as we observed that

DF is strongly associated with Fe concentration. Fe ChLithO, like *Gallionellaceae* or *Ferrovaceae*, use Fe^{2+} to fuel DF and, thus, tend to prefer very low O_2 concentration to avoid competition with chemical oxidation of Fe^{2+}. But this possibility is hindered by the fact that Fe^{2+} was high even in the oxygen rich epilimnion, suggesting that oxygen might not limit Fe^{2+} availability in boreal lakes.

DF rates were also strongly correlated with DOC and CO_2. As correlation does not allow to determine causality, it is not sufficient to tell if this indicates a preference of ChLithO for high DOC and CO_2. It would make sense that DF is favored by high DOC, which can be associated with darker water, and potentially lower competition for resources with phototrophs. It would also make sense that high CO_2, ChLithO carbon source, favors higher DF rates. But the measurement of the light absorbance at 420 nm (α420) tells us a different story. A420 is a more direct measurement of the color of water, and is normally correlated with DOC. This was the case in our study, but α420 did not correlate with DF, hinting that DF rates were covarying with non-colored DOC. It seems then likely that the DOC observed was not a necessary condition of DF, but its product. This would in turn explain the high CO_2 concentration associated with DF, as this fresh source of DOC could fuel heterotrophic respiration (Wickland et al. 2007). This would be in line with several studies reporting production of exudates by ChLithO (Schnaitman & Lundgren 1965; Borichewski 1967; Ñancucheo & Johnson 2010; Emerson et al. 2013), but more in depth analysis will be needed to confirm this hypothesis.

Based on the relative abundance of taxa with the potential for DF, as well as literature, we selected a set of taxonomical families that harbor a potential for DF. The analysis of the abundance of these families suggested that a diverse group of taxa might play a role in DF, including methanogens. Part of this diverse community seems to be ubiquitous and predict well the DF rates, with a few taxa (e.g., *Gallionellaceae* or *Chromatiaceae*) seemingly the potential main drivers of DF. Other taxa, like *Ferrovaceae*, seems to have a significant role in DF only in certain condition. This uneven role of taxa like *Ferrovaceae* could be triggered by environmental conditions, like pH, affecting certain taxa more than others.

4. Conclusion and perspectives

4.1 What's up doc?

To sum up, the results of the study of methane oxidation in winter condition (paper II) showed an overall positive response of CH_4 oxidation to phosphate addition. We concluded that when CH_4 and O_2 are not limiting factors, CH_4 oxidation in lakes may be constrained by P availability. We could not clearly identify how phosphate impacted the methanotrophs responsible for methane oxidation, but our observations suggest that this could be the product of a systemic effect. In such, heterotrophic microbes would boost MO activity by providing key vitamins, recycling key nutrient like N, or consuming toxic by-products produced during methane oxidation.

The study on the diversity of methane oxidizing communities (paper III) was the first large-scale analysis of methanotrophic communities from oxygen stratified lakes spanning from Europe to North America. It confirmed the importance of O_2 and CH_4 in shaping the methanotrophic communities and suggested that one variable cannot explain the diversity and distribution of the methanotrophs across the lakes. Instead, we suggested that the diversity and distribution of freshwater methanotrophs are regulated by lake-specific factors. We also confirmed that many of the results gained from analyzing a limited number of lakes are relevant for freshwater bodies above 50°N. Further, we suggested that the ability to consume CH_4 at a low concentration is probably a key element in discriminating between the dominance of α-MO and γ-MO.

The work presented in paper IV demonstrated that DF is widely spread in stratified boreal lakes and that it may be associated with iron cycling. It also

suggests that DF is driven by a diverse set of microbes, some ubiquitous and some more lake specific.

Overall, my work investigated several microbial pathways that limit GHGs emissions from lakes. As emission are the net balance between production and consumption of GHGs, we show that this balance is more complex than previously thought. So far, the consensus has been that GHGs emissions from boreal lakes will increase. But predicting the future of methane and CO_2 might not be as straightforward as expected. The work presented in this thesis highlights several understudied microbial guilds with a potential to mitigating some effects of changes to come.

4.2 Why it matters

As both CH_4 and CO_2 are important GHGs, better understanding of the mechanisms responsible for their natural emissions is critical. Even if global warming is mainly caused by human production of these gases, the natural cycle of carbon is also important as it has a buffering effect, and its disruption could lead to a series of cascading GHGs releases, increasing the threat of irreversible and dramatic climate changes (Lenton et al. 2019). Lakes are generally GHGs sources, and it is expected that their GHGs emissions increase as temperature rises. Microbes will play a major role in defining the future emissions from lakes and this work focused on several microbial processes that have been neglected. The results showed that some mechanisms expected to increase GHG emissions could be, at least partially, balanced by understudied processes. This shows that microbes have a strong role in the resilience of aquatic ecosystems. This new knowledge could also play an important role in developing prediction tools for the future of boreal lakes. Our results also bring a new light on the microbes with important ecological roles. Both, methanotrophs and ChLithO are indeed able to turn inorganic carbon into a source of biomass and energy available for the food web (Molari et al. 2013; van Duinen et al. 2013).

More specifically, the potential role of P on MOx highlighted in paper II is of particular importance, as eutrophication is a widespread issue. It is expected to increase CH_4 production in the hypolimnion. In paper II, we showed that this negative effect could, at least partially, be balanced by the

positive effect the P on MOx rates during winter. This could also offer a base to better predict the winter and ice-off emissions from boreal lakes.

Paper III highlighted the importance of α-MO as a key player in limiting CH_4 emission from lakes. Low but above saturation levels of CH_4 are often measured in surface waters. So, while efficient to remove the bulk of CH_4, γ-MO might not be able to stop emissions from lakes. Our work suggests that to better understand the future emissions, a particular attention should be given to the ecology of α-MO. Paper III identified *Candidatus* Methylumidiphilus as a major component of the methane oxidizer guild. This is also of particular interest as it shows that a recently described bacteria could be of major importance. Furthermore, its apparent ability to present a significant proportion of populations under high CH_4 could be of interest for the industry. Indeed, several projects are trying to produce biomass and/or value added chemicals using methane as a carbon source (Kalyuzhnaya et al. 2013; Guerrero-Cruz et al. 2021).

The work presented in paper IV shows that DF could be an important component in the food web of boreal lakes and fill a knowledge gap regarding energy mobilization. Our work shows that this largely ignored guild of microbes could be of major importance in understanding the ecology of boreal lakes. Furthermore, the fact that DF in boreal lakes may be driven by oxidation of iron is of particular interest as browning of lake is partially caused by iron (Weyhenmeyer et al. 2014; Xiao & Riise 2021). Our results suggest that if browning of water might decrease photosynthesis, DF could, at least partially, balance the reduction in primary production.

4.3 What's next?

This work raised several questions that would deserve further investigation. One key aspect that is often dismissed is what happens under ice when lakes are frozen. Several reviews show that there is a renewed interest in winter limnology (Bertilsson et al. 2013; Hampton et al. 2015; Powers & Hampton 2016). They all agree that there is still a huge gap in knowledge for many functional and structural features of frozen water bodies (Kalinowska et al. 2019). Recent works suggest that there is an increase of the microbial community richness during the ice-on period (Butler et al. 2019) and that activity during winter remains important (Gammons et al. 2014; Garcia 2016; Rue et al. 2019). This activity can impact the overall greenhouse gas

emissions from lakes (Bellido et al. 2009; Sepulveda-Jauregui et al. 2015) and also the survival of macro organisms (White et al. 2008). With all this in mind, it would be particularly exciting to extend the work done for paper II and to combine it with what was accomplished in paper IV.

It would be of great interest to better determine how the relationship between the total microbial community and methanotrophs is influencing MOx rates. It would also be important to see if this positive effect of P also exists when CH_4 concentration is lower, and what would be the role of α-MO in that context. Similarly, another key question would be to test the effect of P in different oxygen conditions. It is indeed likely that O_2 varies throughout the winter as ice and snow cover more or less limit the penetration of light and oxygen into the top water, which can impact primary production and methane cycling (Garcia et al. 2019). With that in mind a time series-based study of the MO community would be particularly interesting.

The role of α-MO would also deserve more attention, and not only in winter conditions. It would be of particular interest to see if they can lower the CH_4 concentration below saturation, and how their affinity for methane would be affected by changing condition in boreal lakes. It would also be of interest to see what role they play when γ-MO dominate. This would be of particular interest in winter condition, where CH_4 can reach high concentrations in the top water, and emission are potentially high. We observed that at high CH_4 and O_2 concentrations, as well as in low temperature, γ-MO dominated, but it is not clear if α-MO might play a role in consuming residual CH_4 which could be of relevance for estimating GHG emissions from boreal lakes. Similarly, the role of less abundant methanotrophs should be considered more carefully. Our data suggest that while in low abundance, anaerobic MO could be active throughout the water column, but we could not tell what proportion of methane oxidation is performed by those less abundant taxa. Finally, it was striking to see that the most dominant taxa when methanotrophs were abundant was *Candidatus* Methylumidiphilus, a recently described taxa. It would be a great interest to investigate its biogeography. Is *Candidatus* Methylumidiphilus dominance specific to boreal lakes or is it a common occurrence in all latitudes? It could also be of interest to test if *Candidatus* Methylumidiphilus is indeed a fast-grower, or if it just accumulates over time. Its sensitivity to temperature could also be a key question, as this could be critical to the resilience of MO community as temperature increases. To answer several of these questions,

it could be of interest to isolate *Ca.* Methylumidiphilus as this would allow easier *in vitro* manipulations. Finally, *Candidatus* Methylumidiphilus isolation could also present an interest for the industry as several projects are trying to use methane and methanotrophs as a source of biomass and valuable bioproducts (Kalyuzhnaya et al. 2013; Guerrero-Cruz et al. 2021).

The fact that DF appears to be prevalent in boreal lakes also opens a lot of new questions. Our data suggest that a big part of their production may be released as DOC and this needs confirmation. Similarly, the observation of DF in the euphotic zone would need better scrutiny. If it could be a measurement artifact, the presence of Fe^{2+} in the oxic top water suggests that ChLithO could actually be active in the epilimnion. Overall, paper IV suggests that iron could be of utter importance in boreal lakes and invite for more in depth study of its role and chemistry in the environment. Finally, to have a better sense of the actual importance of DF, it will be critical to assess the variation of DF rates throughout the year. Mixing, ice cover, and iron photooxidation are only a few of the parameters that could influence DF and are expected to vary with the seasons.

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5. Popular science summary

Lakes are a home for a multitude of microbes. These microbes rely on a huge diversity of biochemical process to survive and multiply. Two major needs to be fulfilled are to get carbon and energy. Carbon is the source material of most biologically relevant substances. It is widely available in many forms, but most microbes can only handle a specific source of carbon. Microbes will often use one source of carbon as "food" and release another form as waste, that will be a source for other microbes. Such chains are a critical part of any ecosystem. These processes are not only important for providing the building material for microbial cells, but also to provide the energy necessary to live. Indeed, when carbon substances, like sugars, are consumed by microbes, they can release energy. But at each step of the chain, the amount of energy decreases until the moment where no more energy is available. If oxygen is available, the last step of the chain will be CO_2.

During the last decade, CO_2 acquired a bit of a bad reputation as it is the main cause of the climate change. CO_2 indeed plays a key role in regulating the climate on Earth. And accumulation of it leads to an increase in the global temperature. Luckily, the production of CO_2 by living organism does not lead to a dead end. Carbon, like all element necessary to life, have been recycled, allowing life to keep going for about 4 billion years. Many organisms can turn that CO_2 back into energy rich substances, starting a new cycle. To do so they need an alternative energy source. The most common and best-known source of energy to turn CO_2 back into energy rich biomass (e.g., sugars) is sun light. This is photosynthesis. It can be performed by a wide array of organism, including plants and microbes. But in the early days of life, this key process did not exist, and microbes had to rely on other energy sources to gather enough energy to live. Microbes able to do that are called **chemolithoautotrophs**. Their energy source was chemical reaction between

unstable substances. This instability comes from the fact that certain compounds hold more electrons than they should, while other have less than they need. When they meet, the surplus of electron(s) is transferred to the element lacking some, releasing energy in the process. Such reactions are called redox reactions for reduction (getting more electrons) and oxidation (losing electrons). The name oxidation sounds a lot like oxygen for a reason: oxygen is really good at taking electrons. A good example of this is rust. In the presence of oxygen, iron will lose some of its electrons turning into rust. Rust is still made of iron, but in an electron deprived form. This reaction between iron and oxygen is one of the many chemical reactions that microbes have be exploiting during early days of life to gather the energy they need. Such way of life still exists and provides carbon and energy to ecosystems where the light is absent, like deep in the ocean, under the ice sheet of Antarctica or in caves. The reason why **chemolithoautotrophs** do not strive in the light is oxygen. If there is too much of it, electron rich compounds lose them rapidly to oxygen, and are useless for microbes. That is why we often find **chemolithoautotrophs** where ecosystem rich in oxygen meet ecosystem poor in oxygen, and rich in electron rich compounds. Such condition can be found in many small boreal lakes. As they heat up in early summer, the water density changes, creating layers that will not mix. In the bottom layer microbes use all oxygen leaving it anoxic (that is, oxygen free), whereas in the top layer, the contact with the atmosphere and active photosynthesis maintain oxygen levels high. In between those layers we find the ideal conditions to find microbes using chemical redox reactions to get the necessary energy to transform CO_2 into energy rich carbon compounds (e.g. sugars). Surprisingly, despite clearly showing ideal condition for **chemolithoautotrophs** it was not clear if they were indeed present, and more critically, active in Swedish lakes. During my thesis, I performed experiments to measure the activity of those light independent carbon fixers. I tested 18 lakes all over Sweden and discovered that such mechanism is indeed common. The results of my research suggest that **chemolithoautotrophs** striving in boreal lakes use iron based chemical reactions. This is particularly interesting because it has been observed that lakes are getting darker as a consequence of increased human activity and climate change. This darkening is worrisome as with less light lakes would be less likely to trap CO_2 with photosynthesis. But as part of the darkening is caused by iron rich compounds, it is possible that this expected decrease

in photosynthesis could be at least partly compensated by the activity of **chemolithoautotrophs.**

CO_2 is the final product of the carbon feeding chain when oxygen is available. But what happens if none is available, like at the bottom of boreal lakes? In the absence of oxygen microbes get somewhat creative and while some still end up releasing CO_2, some also start to release hydrogen. Hydrogen is so desperate to share its only electron that it can react with CO_2 with a little help from specialized microbes. Those microbes, called methanogens, can take CO_2 and hydrogen and produce methane (CH_4). In the process they get the carbon and energy they need. A major downside of this process, on a human perspective, is that CH_4 is a very powerful greenhouse gas. CH_4 is up to 80 time more efficient in trapping heat than CO_2. But life is all about recycling, and what is one organism´s waste is substrate for another. Methane is a very energy rich compound, and therefor valuable source of C and energy for some microbes. Consequently, most of it is consumed before it can reach the surface. But most of it is not all of it. Overall, lakes are net emitters of methane. One situation where major amounts of methane are released is during ice-off. During winter, most of the lake can get anoxic and accumulate methane that is released when the ice breaks. But the amount of methane released at the ice-off varies a lot from lake to lake. It is not clear why, but a recent study suggested that phosphorus could play a role in the ability to consume methane in winter conditions. To test that hypothesis, we ran an experiment to test the effect of phosphorus on the ability of microbes to consume methane. We compared the methane consumption in two sets of incubations with lake water, methane, and oxygen. The only difference between the two set was that we added phosphorus in one. Based on that experiment we could confirm that higher phosphorus content helps microbes consume methane. But direct study of the microbes did not tell us what the mechanisms behind the impact of phosphorus on the microbial usage of methane are. These results are interesting as an increase in phosphorus in lakes is generally expected to also increase methane emissions. Here we showed that things are might not be that straightforward. Even if phosphorus might help to produce more methane, it could also help to consume it, and hopefully have an overall neutral effect on the net emissions from lakes.

Another question that we wanted to answer regarding methane consuming microbes was to know what kind of methanotrophs are the most

important in boreal lakes and if this hierarchy is influenced by the chemical composition of the lakes. Based on the analysis of the DNA of the microbes, we could tell that some are the main methane consumers only when there is a lot of methane available while others can live with very little methane. These frugal microbes generally dominate the community in the top layer of lakes where there is only little CH_4 left. Those microbes are very interesting as they might use methane leftovers from microbes living in lower layers where methane is abundant. This is very important, because if those methane leftovers are not consumed, they end up in the atmosphere where they can trap more heat and accelerate the climate change.

My different projects help us better understand what happens in lakes and might help modeling the future emissions from boreal lakes. Lakes will probably keep emitting greenhouse gases but knowing if those emissions will stay stable or increase is of vital importance in predicting the future of our climate. The new knowledge brought by this thesis cannot fully answer that question, but it tells us that boreal lakes might be more resilient than expected. More work will be necessary to have a clear picture of the future of boreal lakes, but I hope that this work will bring useful knowledge to the scientific community working on those questions, and hopefully inspire new research projects.

Populärvetenskaplig sammanfattning

Sjöar är ett hem för en stor mångfald av mikroorganismer som är beroende av olika biokemiska processer för att leva. Två grundläggande behov som måste vara uppfyllda är att det finns kolföreningar och energi. Alla biologiska ämnena byggs upp av kol och finns i många former. De flesta mikroorganismerna kan bara utnyttja vissa kolföreningar som "mat" som sedan släpps ut i andra former som avfall, som i sin tur kommer att vara ”mat” för andra mikroorganismer. Sådana omvandlingskedjor är en del av alla ekosystems processer. Dessa omvandlingar är viktiga både för att ge mikroorganismer byggmaterial och energi. När mikroorganismer bryter ner kolföreningarna, till exempel socker, frigörs energi. Vid varje steg i kedjan minskar energimängden och koldioxid avges tills ingen mer energi finns tillgänglig.

Under det senaste decenniet har koldioxid fått dåligt rykte eftersom den är den främsta orsaken till klimatförändringen. Ökad koncentration av koldioxid i atmosfären leder till en ökning av temperaturen. Lyckligtvis leder produktionen av koldioxid från levande organismer inte till en återvändsgränd. Kol, liksom alla livsnödvändiga ämnen, har återvunnits och cirkulerats så att livet har kunnat pågå i närmare 4 miljarder år. Många organismer kan omvandla koldioxid till energirika ämnen. För att göra det behövs en energikälla. Den mest betydelsefulla processen är fotosyntesen, där koldioxid omvandlas till socker som kan bygga upp energirik biomassa. Fotosyntes utförs av växter och vissa mikroorganismer. Vid livets början på jorden existerade inte fotosyntesen och mikroorganismerna förlitade sig på andra energikällor än solen. Mikroorganismer som kan göra det kallas kemolitoautotrofer. Deras energikälla är kemiska reaktioner mellan instabila ämnen. Denna instabilitet beror på att vissa föreningar har ett överskott av elektroner, medan andra har ett underskott. När sådana föreningar möts

överförs överskottet av elektroner till föreningen som har underskott, vilket frigör energi. Denna reaktion kallas redoxreaktion, där det ämne som reduceras får fler elektroner och det som oxideras förlorar elektroner. Begreppet oxidation för tankarna till syre av en anledning. Syre är nämligen riktigt bra på att ta emot elektroner. Ett bra exempel är rost. Järn som är i kontakt med syre tappar elektroner och omvandlas till rost. Rost är fortfarande uppbyggt av järn, men i en elektronberövad form. Det som händer mellan järn och syre är en av många kemiska reaktioner som mikroorganismer kunde använda under livets första tid på jorden. Denna process som genererar kolföreningar och energi till ekosystem där solljuset inte når och mörker råder, som i djupt nere i havet, under isen på Antarktis, i berggrunden och i grottor. Anledningen till att kemolitoautotrofer undviker ljus är syret. Finns för mycket av syre, oxideras elektronrika ämnen och blir värdelösa för kemolitoautotrofer. Det är därför vi ofta hittar kemolitoautotrofer där miljöer som är rika på syre möter miljöer som är fattiga på syre och rika på elektronrika ämnen. Sådana miljöer finns i många små boreala sjöar. När sjöarna värms upp på försommaren ändras vattentätheten och skapar skikt mellan vattenvolymer som inte blandas. I den nedre vattenvolymen använder mikroberna allt syre tills det blir helt blir syrefritt, anoxiskt, medan den övre vattenvolymen som har kontakt med atmosfären och dessutom fotosyntes behåller höga syrenivåer. I gränszonen finns perfekta förutsättningarna för mikroorganismer som använder kemiska redoxreaktioner för att få energi och samtidigt omvandlar koldioxid till energirika kolföreningar. Trots att det varit känt att denna lämplig miljö för kemolitoautotrofer finns i boreala sjöar, var det tidigare inte undersökt om de verkligen fanns där, och mer kritiskt, om dom var aktiva. I min avhandling utförde jag experiment för att mäta aktiviteten hos dessa ljusoberoende och mörkerlevande mikroorganismer. Jag undersökte 18 sjöar i Sverige och fann att processen är vanlig. Resultaten tyder på att kemolitoautotroferna använder järnbaserade kemiska reaktioner. Detta är särskilt intressant eftersom vattnet i boreala sjöar håller på att bli mörkare som en följd av ökad mänsklig aktivitet och klimatförändringar. Detta är oroande. När solljuset når mindre djupt innebär det att sjöarnas fotosyntes minskar och därmed sjöarnas förmåga att binda koldioxid. Men eftersom en del av mörkningen orsakas av järnrika substanser är det möjligt att minskning av fotosyntesen delvis kan kompenseras av kemolitoautotrofernas aktivitet.

Koldioxid är slutprodukten av nedbrytning när det finns syre. Men vad händer när det inte finns något syre , som på bottnar av boreala sjöar? Mikroorganismer hanterar syrefria miljöer på lite olika sätt. Medan vissa fortfarande släpper ut koldioxid, börjar andra frigöra väte. Väte delar nämligen gärna sin enda elektron och kan reagera med koldioxid med hjälp av vissa mikroorganismer. Dessa mikroorganismer, så kallade metanogener, tar upp koldioxid och väte och producerar metan. Processen ger de kolföreningar och energi mikroorganismerna behöver. En baksida är att det bildas metan som är en mycket kraftfull växthusgas. Den är upp till 80 gånger effektivare för att fånga upp värme än koldioxid. Men livet handlar om återvinning, och det som är avfall från en organism är födokälla för en annan. Metan är ett mycket energirikt substrat och därför en värdefull kol- och energikälla för vissa mikroorganismer. Det mesta av metan som bildas konsumeras därför innan det når sjöytan. Men en del av metanet släpps ut. Speciellt under våren när isen smälter frigörs stora mängder eftersom större delen av sjön blir syrefri under vintern och metan då ackumuleras under isen. Mängden metan som släpps ut när isen smälter varierar mycket mellan olika sjöar. Det är inte klarlagt varför, men en studie föreslog att fosfor kan spela en viktig roll för mikroorganismernas förmåga att bryta ner metan under vinterförhållanden. För att testa den hypotesen utförde vi ett experiment där vi undersökte effekten av fosfor. Vi jämförde metanförbrukningen i ett kontrollerat experiment där vi inkuberade sjövatten, metan och syre. Vi hade två varianter, en kontroll och en där vi tillsatte fosfor. Experimentet bekräftade att högre fosforhalt hjälper mikroorganismer att bryta ner metan. Vår studie förklarar dock inte vilka mekanismer ligger bakom påverkan av fosfor. Resultat är intressanta eftersom en ökning av fosfor i sjöar förväntas öka metanutsläppen. Vi visar med vår undersökning att slutresultat kan vara mer komplicerad. Även om fosfor kan bidra till att producera mer metan, kan det samtidigt också hjälpa att konsumera det och möjligen resultera i att nettoutsläppen från sjöar inte ökar.

En annan fråga som intresserade oss var att ta reda på vilken typ av mikroorganismer som livnär sig på metan som är de viktigaste och om sjöarnas kemiska sammansättning påverkar vilken typ som dominerar. Vår analys av mikrobernas DNA visar att vissa mikroorganismer är viktiga metankonsumenterna när det finns mycket metan medan andra när det är lite metan. De senare dominerar i allmänhet samhället i det översta lagret av sjöar där det bara finns lite metan kvar. Dessa mikroorganismer är mycket

intressanta eftersom de kan använda metanrester från mikroorganismer som lever i lägre metanrika lager. Detta är viktigt, för de metanrester som inte bryts ner hamnar i atmosfären och förstärker växthuseffekten.

Mina projekt hjälper oss att bättre förstå vad som händer i sjöar och kan hjälpa till att beräkna storleken på utsläpp av växthusgaser från dessa sjöar i framtiden. Troligtvis kommer sjöarna att fortsätta släppa ut växthusgaser, men vetskap kunskap om dessa utsläpp kommer att förbli stabila eller öka är viktigt för att kunna beräkna hur framtidens klimat kan komma att bli. De nya kunskaperna i min avhandling svarar inte på alla frågor, men den visar att boreala sjöar kan vara mer motståndskraftiga än vi tidigare trott när klimatet ändras. Mer arbete kommer att krävas för att få en bättre bild av vad som sker och kommer att ske, men min förhoppning är att mitt arbete bidrar med kunskaper och inspirerar till fortsatt forskning.

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I also got the chance to get invited to work on a very fun winter project. It has been a real pleasure to work with you, Henrique, and I hope we will have more opportunities to collaborate. Last, but not least, I want to underline how much I owe to the people who helped me in the field. Working long hours in all kinds of weather with a stranger is challenging. So, thank you for your patience and hard work. Victoria, the time I spent with you was truly inspiring and I hope we will get to meet again some time. Plus, you got me to finally to read Harry Potter. That is great, because now I can nag Potter heads with firsthand knowledge.

Finally thank you, reader, for taking the time to read my work. Specially you, the members of the committee and opponent, who are taking the time to evaluate and challenge my work. I look forward to meeting you and discussing my results with you.

If you feel like you should have been mentioned, please consider that I'm writing this late at night after a few long weeks of work. I might have forgotten you right now, but it doesn't mean that I don't think of you from time to time.

I

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DATA DESCRIPTOR

Comprehensive dataset of shotgun metagenomes from oxygen stratified freshwater lakes and ponds

Moritz Buck[1]✉, Sarahi L. Garcia[2,3], Leyden Fernandez[3], Gaëtan Martin[4], Gustavo A. Martinez-Rodriguez[5], Jatta Saarenheimo[6], Jakob Zopfi[7], Stefan Bertilsson[1] & Sari Peura[4]✉

Stratified lakes and ponds featuring steep oxygen gradients are significant net sources of greenhouse gases and hotspots in the carbon cycle. Despite their significant biogeochemical roles, the microbial communities, especially in the oxygen depleted compartments, are poorly known. Here, we present a comprehensive dataset including 267 shotgun metagenomes from 41 stratified lakes and ponds mainly located in the boreal and subarctic regions, but also including one tropical reservoir and one temperate lake. For most lakes and ponds, the data includes a vertical sample set spanning from the oxic surface to the anoxic bottom layer. The majority of the samples were collected during the open water period, but also a total of 29 samples were collected from under the ice. In addition to the metagenomic sequences, the dataset includes environmental variables for the samples, such as oxygen, nutrient and organic carbon concentrations. The dataset is ideal for further exploring the microbial taxonomic and functional diversity in freshwater environments and potential climate change impacts on the functioning of these ecosystems.

Background & Summary

Stratified lakes are a typical feature of the northern landscapes and are also significant sources of greenhouse gas (GHG) emissions[1]. These lakes largely reside in regions critically impacted by climate change[2] and the future contribution of these lakes to climate change via GHG emissions is dependent on the microorganisms inhabiting their waters[3,4]. In this regard, organisms residing in the anoxic compartment of these waters are of particular interest, as many of the more potent GHGs are produced under such conditions[1]. However, our knowledge regarding the diversity and functioning of these microbial communities is still sparse, and only a few studies have addressed the ecology and functional features of microorganisms in anoxic lake compartments[5–9]. To address this gap in knowledge, we have collected a set of 267 samples from 41 waterbodies including thermally stratified lakes and ponds from boreal and subarctic regions, as well as a depth profile of a tropical reservoir in Puerto Rico and a time series of depth-resolved samples from a temperate and seasonally stratifying eutrophic lake in Switzerland (Fig. 1, Table 1). For the majority of the lakes, samples were available from across the water column, including the oxic epilimnion, the oxygen transition zone (metalimnion) and the deep anoxic hypolimnion (Auxillary Table S1)[10]. Additionally, diverse environmental factors were analysed for all samples, including but not limited to, oxygen, nutrients and organic carbon concentrations (Auxillary Table S1)[10]. For all samples, metagenomes were sequenced using deep shotgun sequencing on the Illumina NovaSeq platform at the Science for Life Laboratory (Uppsala University, Uppsala, Sweden). Additionally, for two of the waterbodies (Alinen Mustajärvi and Lomtjärnan), genomes from single cells were amplified and sequenced, specifically targeting poorly known community members, such as members of the lineage *Chlorobia* and candidate phyla radiation.

[1]Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences, Uppsala, Sweden. [2]Department of Ecology, Environment and Plant Sciences, Science for Life Laboratory, Stockholm University, Stockholm, Sweden. [3]Department of Ecology and Genetics, Limnology, Uppsala University, Uppsala, Sweden. [4]Department of Forest Mycology and Plant Pathology, Science for Life Laboratory, Swedish University of Agricultural Sciences, Uppsala, Sweden. [5]Agroenvironmental Science Department, University of Puerto Rico, Mayaguez Campus, San Juan, Puerto Rico, USA. [6]Department of Biological and Environmental Science, University of Jyväskylä, Jyväskylä, Finland. [7]Department of Environmental Sciences, Aquatic and Stable Isotope Biogeochemistry, University of Basel, Basel, Switzerland. ✉e-mail: moritz.buck@slu.se; sari.peura@slu.se

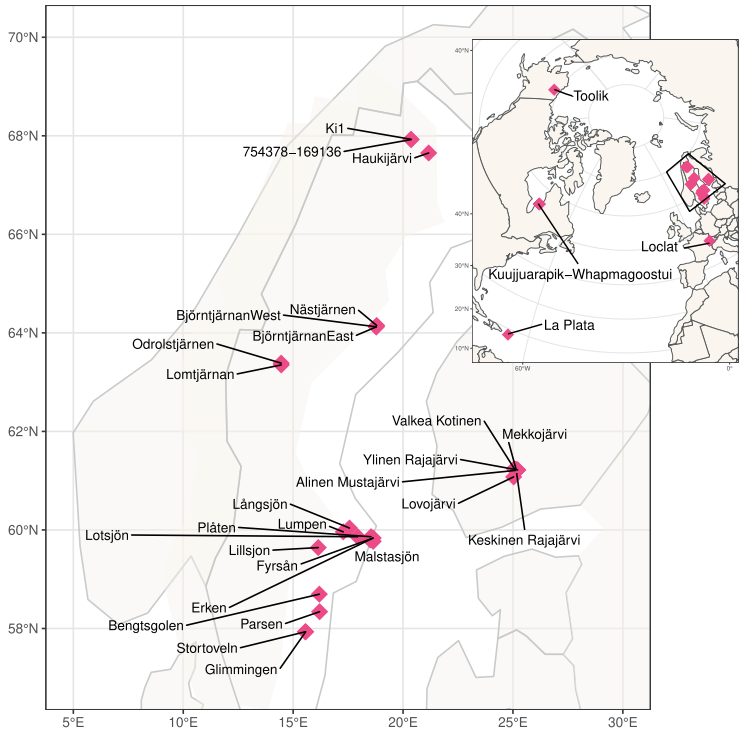

Fig. 1 Global distribution of sampling sites. Large Scandinavian map represents the squared region in the insert.

Our goal was to collect a comprehensive dataset that would allow broad analyses of the functioning of the microbial communities in oxygen stratified lakes with emphasis on lakes representing high carbon concentration but with variable environmental conditions with regards to nutrient concentrations, trophic state and other lake features. Based on this data, it is possible to describe the taxonomic identities and genome-encoded functional traits of the predominant microbes across boreal and subarctic lakes and ponds. The dataset represents a major asset for advancing our understanding of the biogeochemical and ecological functioning of these key environments and their present and possible future roles in elemental cycles. The dataset as such can be used, for example, for assessing general metabolic potential of the total lake communities. Further, we have assembled and binned the data into 12665 metagenome-assembled genomes (MAGs), which were further clustered into 3640 metagenomic Operational Taxonomic Units (mOTUs; species level genome clusters). Of these mOTUs, 328 are classified down to species level, while 2141, 878, 220, and 65 could only be assigned to genus, family, order, and class level, respectively. This dataset can, thus, be used for the exploration of population genomes, to study individual community members, and for deeper genome characterization of poorly known members of the resident communities, including their metabolic potentials.

Methods

Sample collection. The 267 samples were collected between 2009 and 2018 from 41 locations expanding from the subarctic region to the tropics (Fig. 1, Auxillary Table S1)[10] and processed using the same analytical pipeline (Fig. 2). The majority of the samples were collected using a depth-discrete Limnos tube-sampler (Limnos, Poland), with the exception of the samples from La Plata reservoir (Puerto Rico), which were collected using horizontal Van Dorn sampler (5 L capacity) and samples from Lake Loclat, which were collected using a deployed PVC-inlet connected to a peristaltic pump via tubing. Of all the lakes, 29 were sampled during the open water season and the majority of the lakes were sampled once. For 12 of the lakes only surface samples taken during the ice-covered period in winter were available, and one of the Swedish lakes (Lake Lomtjärnan) was sampled twice during the ice-covered period. Moreover, a total of 5 samples (one depth profile) from the time series of the Swiss lake (Loclat) were taken from under the ice. Time series samples were taken for Lake Loclat (seven time points, Auxillary Table S1)[10] and for Lake Mekkojärvi (22 time points, see Saarenheimo *et al.*[11] for details). For most lakes and ponds, samples were collected from multiple depths, including samples from the oxic surface layer

site name	country	latitude	longitude	max depth sampled	number of samples	sampling years
SAS B1_2	Canada	55.23	−77.7	0.45	3	2014; 2017
SAS B4	Canada	55.22	−77.7	NA	2	2014; 2017
SAS C2_4	Canada	55.23	−77.7	0.75	3	2014; 2017
SAS C5	Canada	55.23	−77.7	NA	2	2014; 2017
SAS F5	Canada	55.23	−77.69	2.35	1	2014; 2017
SAS G1	Canada	55.23	−77.7	0.15	2	2014; 2017
SAS H1	Canada	55.23	−77.7	NA	1	2014; 2017
SAS I1_2	Canada	55.23	−77.7	0.35	2	2014; 2017
SAS I3	Canada	55.23	−77.7	NA	2	2014; 2017
SAS2A	Canada	55.23	−77.7	2.25	4	2014; 2017
SAS2B	Canada	55.23	−77.7	2.05	4	2014; 2017
SAS2C	Canada	55.23	−77.7	1.85	4	2014; 2017
SAS2D	Canada	55.23	−77.69	2.35	4	2014; 2017
Alinen Mustajärvi	Finland	61.21	25.11	6.56	42	2014-2015;2018
Keskinen Rajajärvi	Finland	61.22	25.22	10	5	2015
Lovojärvi	Finland	61.08	25.03	13.9	7	2015
Mekkojärvi	Finland	61.23	25.14	3.6	51	2011–2015
Valkea Kotinen	Finland	61.24	25.06	4.5	4	2015
Ylinen Rajajärvi	Finland	61.22	25.21	5	4	2015
La Plata	Puerto Rico	18.33	−66.24	15	8	2018
Bengtsgolen	Sweden	67.92	20.37	5.5	4	2018
Björntjärnan	Sweden	58.7	16.19	0.2	1	2018
Erken	Sweden	64.12	18.78	8	8	2018
Fyrsån	Sweden	59.84	18.64	20	12	2018
Glimmingen	Sweden	59.8	18.51	0.1	1	2018
Haukijärvi	Sweden	57.93	15.57	0.4	1	2018
Kiruna 754378–169136	Sweden	67.65	21.17	3.1	4	2018
Kiruna Ki1	Sweden	67.93	20.36	2.7	4	2018
Långsjön	Sweden	59.64	16.14	0.2	1	2018
Lillsjon	Sweden	63.35	14.46	3.5	21	2018
Lomtjärnan	Sweden	59.86	17.94	0.1	1	2016–2018
Lotsjön	Sweden	59.96	17.28	0.1	1	2018
Lumpen	Sweden	60.04	17.56	0.1	1	2018
Malstasjön	Sweden	59.77	18.64	0.1	1	2018
Nästjärnen	Sweden	64.15	18.8	5	4	2018
Odrolstjärnen	Sweden	63.39	14.46	4.4	2	2018
Parsen	Sweden	58.34	16.2	0.2	1	2018
Plåten	Sweden	59.86	18.54	0.1	1	2018
Stortoveln	Sweden	57.93	15.55	0.2	1	2018
Loclat	Switzerland	47.01	6.59	9	44	2008–2009
Toolik	USA	68.63	−149.6	NA	4	2017

Table 1. Location, maximum depth, number of samples and sampling years for all of the lakes included to the dataset.

(epilimnion), the layer with steepest change in oxygen concentration and temperature (metalimnion) and from the layer where oxygen levels were below the detection limit (hypolimnion). The exception to this were the 12 Swedish lakes sampled during ice-covered period, and five shallow ponds in Canada, for which only one sample from the oxic surface layer was taken (see Auxillary Table S1)[10].

From two of the lakes, Lake Lomtjärnan in Sweden and Lake Alinen Mustajärvi in Finland, samples were collected also for single cell sorting. From both locations samples were preserved in glycerol-TE (gly-TE) and from Lomtjärnan samples were preserved also using phosphate buffered saline (PBS). For both preservants, the samples were flash frozen in liquid nitrogen after first incubating for 1 minute at ambient temperature.

Simultaneous to collection of the DNA samples, also samples for environmental variables were taken. Variables included temperature, pH, conductivity, oxygen, total and dissolved nutrients (P and N species), gases (CO_2 or dissolved inorganic carbon and methane (CH_4)), total or dissolved organic carbon, iron, sulfate and chlorophyll a (Auxillary Table S1 and Auxillary Table S2[10] for the methods). As the samples were collected during multiple years and by different research groups, there was some variation for the procedures between the different sampling occasions, leading to variation in the final set of environmental data across the samples.

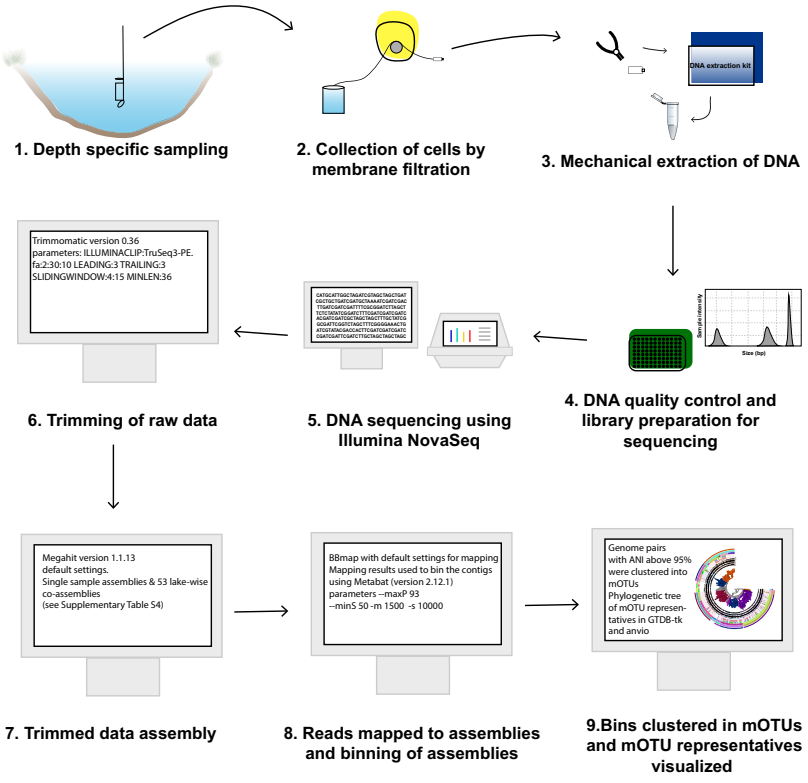

Fig. 2 Overview of the workflow from sample collection to mOTUs.

DNA extraction and metagenome sequencing. Most of the DNA samples were collected on 0.2 µm Sterivex filters (Millipore), except for the time-series samples collected from Loclat, which were collected by vacuum filtration onto 47 mm polycarbonate membrane filters with 0.2 µm pore size, and time series samples from Finnish Lake Mekkojärvi, for which the water for DNA extraction was collected from epilimnion (0–0.5 m), metalimnion (0.5–1 m) and hypolimnion (1–3 m) and pooled samples from each stratum were stored in 100 ml plastic containers and frozen at −20 °C and eventually freeze-dried (Alpha 1–4 LD plus, Christ). For all filter samples, water was filtered until the filter clogged. All filters were stored frozen (−20 to −80 °C) until the extraction of DNA. For all samples, DNA was extracted using PowerSoil DNA extraction kit (MoBio, Carlsbad, CA, USA) following the manufacturer's instructions and the DNA concentrations were measured using Qubit dsDNA HS kit (Thermo Fisher Scientific Inc.).

Sequencing libraries were prepared from 10 or 20 ng of DNA using the ThruPLEX DNA-seq Prep Kit according to the manufacturer's preparation guide. Briefly, the DNA was fragmented using a Covaris E220 system, aiming at 400 bp fragments. The ends of the fragments were end-repaired and stem-loop adapters were ligated to the 5′ ends of the fragments. The 3′ end of the stem loop were subsequently extended to close the nick. Finally, the fragments were amplified and unique index sequences were introduced using 7 cycles of PCR followed by purification using AMPure XP beads (Beckman Coulter).

The quality of the libraries was evaluated using the Agilent Fragment Analyzer system and the DNF-910-kit. The adapter-ligated fragments were quantified by qPCR using the Library quantification kit for Illumina (KAPA Biosystems/Roche) on a CFX384Touch instrument (BioRad) prior to cluster generation and sequencing.

The sequencing libraries were pooled and subjected to cluster generation and paired-end sequencing with 150 bp read length S2/S4 flow-cells and the NovaSeq 6000 system (Illumina Inc.) using the v1 chemistry according to the manufacturer's protocols. Negative controls were included to the sequencing as well as 1% of PhiX control library as a positive control.

Base calling was done on the instrument by RTA (v3.3.3, 3.3.5, 3.4.4) and the resulting.bcl files were demultiplexed and converted to fastq format with tools provided by Illumina Inc., allowing for one mismatch in the index

sequence. Additional statistics on sequence quality were compiled with an in-house script from the fastq-files, RTA and CASAVA output files. Sequencing was performed by the SNP&SEQ Technology Platform in Uppsala, Sweden.

Single-cell sorting and DNA amplification. All Gly-TE cryopreserved samples were thawed and diluted in 1 xPBS if needed while all plates with PBS were UV-treated with a dose of 2 J prior to sorting. Samples collected from both lakes were sorted, and then screened for organisms belonging to candidate phyla radiation. Samples collected from Lake Lomtjärnan were additionally subjected to sorting based on autofluorescence to identify and sequence cells belonging to lineage *Chlorobia*.

For obtaining SAGs from representatives of the candidate phyla radiation (CPR), samples were first stained with 1 x SYBR Green I for approximate 30 minutes. Subsequent single cell sorting was performed with a MoFlo Astrios EQ (Beckman Coulter, USA) cell sorter using a 488 nm laser for excitation, 70 µm nozzle, sheath pressure of 60 psi and 0.1 µm sterile filtered 1x PBS as sheath fluid. Individual cells were deposited into empty 384-well plates (Biorad, CA USA) UVed at 2 Joules using a CyCloneTM robotic arm and the most stringent single cell sort settings (single mode, 0.5 drop envelope). Green fluorescence (488–530/40) was used as trigger and sort decisions were made based on combined gates of 488–530/40 Height log vs 488–530/40 Area log and 488–530/40 Height log vs SSC with increasing side scatter divided up in three different regions. Flow sorting data was interpreted and displayed using the associated software Summit v 6.3.1. Next, individual cells were subject to lysis, neutralization and whole genome amplification using MDA based on the protocol and workflow described by Rinke *et al.*[12] but with several modifications. Reagent mastermixes were added using the MANTIS liquid dispenser (Formulatrix) and the LV or HV silicone chips. The lysozyme, D2 buffer, stop solution and MDA-mastermix were each dispensed with its own chip. Most MDA-reactions were run using the phi29 from ThermoFisher but a few were run with a more heat-stable phi29, EquiPhi also provided by ThermoFisher. The MDA reaction was carried out in a total volume of 5.2 µl. Thawed, sorted cells were first pre-treated with 400 nl/well of 12 U/µl of Ready-Lyse™ Lysozyme Solution (R1804M, Lucigen) at room temperature for 15 minutes before adding 400 nl Qiagen lysis buffer D2 followed by incubation at 95 °C for 10 seconds and 10 minutes on ice. Reactions were neutralized by adding 400 nl Qiagen Stop solution. Four µl of MDA mix containing 1x reaction buffer, 0.4 mM dNTP, 0.05 mM exonuclease-resistant Hexamers, 10 mM DTT, 1.7 U phi29 DNA polymerase (ThermoFisher Scientific) and 0.5 µM Syto13 was added to a final reaction volume of 5.2 µl. All reagents except SYTO13 were UV decontaminated at 2 Joules in a UV crosslinker. The whole genome amplification was run at 30 °C for 7 or 10 h followed by an inactivation step at 65 °C for 5 min. The reaction was monitored in real time by detection of SYTO13 fluorescence every 15 minutes using a FLUOstar® Omega plate reader (BMG Labtech, Germany) or a qPCR instrument. The EquiPhi protocol was run as previously described for ThermoFisher phi29 with the following exceptions; the EquiPhi polymerase was added in 1U/reaction, reaction buffer included with the polymerase was used and the reaction was carried out at 45 °C. The single amplified genome (SAG) DNA was stored at −20 °C until further PCR screening, library preparation and Illumina sequencing.

The CPR SAGs were screened using the bacterial PCR primers targeting the 16 S rRNA gene, Bact_341 F and Bact_805 R[13]. The reactions were run in a LightCycler 480 PCR machine (ROCHE, MA USA) in 10 µl and a final concentration of 1 x LightCycler480 SYBR Green I Master mix, 0.25 µM of each primer and 2 µl of 60 to 80 times diluted SAGs. Following a 3 min denaturation at 95 °C, targets were amplified for 40 cycles of 95 °C for 10 s, 55 °C for 20 s, 72 °C for 30 s and a final 10 min extension at 72 °C followed by melting curve analysis. The products were purified using the NucleoSpin Gel and PCR clean-up purification kit (Macherey-Nagel, Germany), quantified using the Quant-iT TM PicoGreen® dsDNA assay kit (Invitrogen, MA USA) in a FLUOstar® Omega microplate reader (BMG Labtech, Germany) and submitted for identification by Sanger sequencing at Eurofin Genomics. All SAGs were further screened using the newly designed primers targeting the phylum Parcubacteria 684F-OD1 (3′ GTAGKRRTRAAATSCGTT 5′) and 784 R (5′ TAMNVGGGTATCTAATCC -3′). These primers target with good specificity 67% of Parcubacteria in the SILVA database[14]. Parcu-PCR was run at 3 min at 95 °C, 40 cycles of 95 °C for 10 s, 55 °C for 20 s, 72 °C for 30 s and a final 10 min extension at 72 °C followed by melting curve analysis. The products were purified using the NucleoSpin Gel and PCR clean-up purification kit (Macherey-Nagel, Germany), quantified using the Quant-iT TM PicoGreen® dsDNA assay kit (Invitrogen, MA USA) in a FLUOstar® Omega microplate reader (BMG Labtech, Germany) and submitted for identification by Sanger sequencing at Eurofin Genomics.

To recover *Chlorobia* single amplified genomes, sorting was done in 2016 on a MoFlo™ Astrios EQ sorter (Beckman Coulter, USA) using a 488 and 532 nm laser for excitation, a 70 µm nozzle, a sheath pressure of 60 psi, and 0.1 µm filtered 1x PBS as sheath fluid. An ND filter ND = 1 and the masks M1 and M2 were used. The trigger channel was set to the forward scatter (FSC) at a threshold of 0.025% and sort regions were defined on autofluorescence using laser 532 nm and band pass filters 710/45 and 664/22. Three populations were sorted based on differences in autofluorescence signals. The sort mode was set to single cell with a drop envelope of 0.5. The target populations were sorted at approximately 400 events per second into 96-well plates containing 1 µl 1x PBS per well with either 1 or 10 cells (positive control) deposited. A few wells remained empty (no cell sorted) were kept as negative controls. Sorted plates were stored frozen at −80 °C.

The subsequent whole genome amplification was performed in 2018 using the REPLI-g Single Cell kit (QIAGEN) following the instructions provided by the manufacturer but with total reaction volume reduced to 12.5 µl. The denaturation reagent D2, stop solution, water, and reagent tubes and strips were UV-treated at 2.5 J. The lysis was changed slightly to 10 min at 65 °C, followed by 5 min on ice before adding the stop solution. To the master mix containing water, reaction buffer, and the DNA the polymerase we added SYTO 13 (Invitrogen) at a final concentration of 0.5 µM. The amplification was performed at 30 °C for 8 hours in a plate reader with fluorescence readings every 15 min. The reaction was stopped by incubating it for 5 min at 65 °C. The plate was stored for less than a week at −20 °C. Amplified DNA was mixed thoroughly by pipetting up

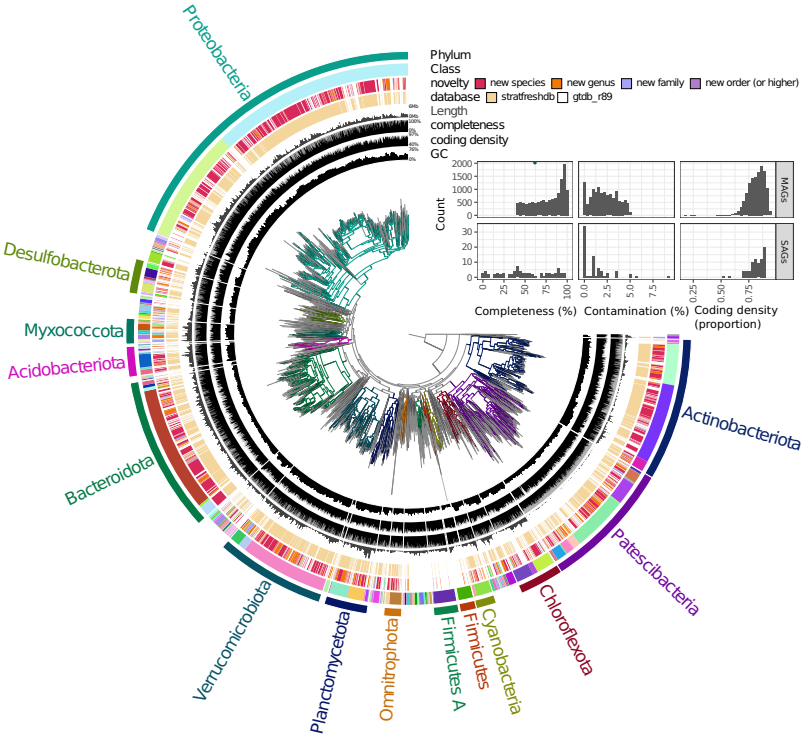

Fig. 3 Bacterial diversity of the stratfreshDB[27]. The insert illustrates the quality of the MAGs and SAGs included in the tree. Interactive version of the tree with more information available at https://anvi-server.org/moritzbuck/bacterial_diversity_of_the_stratfreshdb.

and down 20 times before diluting it 50x and 100x in nuclease-free water. The DNA was screened for bacterial 16 S rRNA applying the primers Bact_341 F (5′- CCTACGGGNGGCWGCAG- 3′) and Bact_805 R (5′-GACTACHVGGGTATCTAATCC-3′)[13] using the LightCycler® 480 SYBR Green I Master (Roche) kit. The PCR mix contained 1.5 µl diluted amplified DNA, 1x the LightCycler® 480 SYBR Green I Master mix, 0.25 µM of each primer, and nuclease-free water in a total reaction volume of 10 µl. The PCR cycling (5 min at 95 °C, followed by 40 cycles of 10 sec at 95 °C, 20 sec at 60 °C, 30 sec at 72 °C) was followed by meltcurve analysis on the LightCycler® 480 Instrument (Roche). DNA of confirmed *Chlorobia* was sent to sequencing as outlined below.

Library preparation and Illumina sequencing of the single cells. For the CPR-targeted analysis, Illumina libraries were prepared from sixty SAGs mainly selected from the screening procedure in a PCR-free workflow using the sparQ DNA Frag & Library Prep Kit (Quantabio) and IDT for Illumina TruSeq UD Indexes (Illumina). Libraries were prepared from 50–250 ng of MDA-products in 25% of the recommended reaction volumes according to manufacturer's instructions. The MDA-products were fragmented for 7 minutes (5 minutes for 4 samples) without using the DNA Frag Enhancer Solution. Library insert sizes were determined using Bioanalyzer High Sensitivity DNA Kit (Agilent). Each library was quantified using the KAPA Library Quantification kit (Roche) in 5 µl reaction volumes in a 384-well plate run on LightCycler 480 (Roche) to allow equimolar pooling before sequencing on Illumina HiSeqX v2.5 PE 2 × 150 bp including negative and positive (PhiX) controls.

For the *Chlorobia*-targeted sequencing, amplified DNA from 23 SAGs were quantified individually with Qubit dsDNA HS assay kit (ThermoFisher Scientific) and diluted to 0.2 ng/ul in nuclease free water. Sequencing libraries were prepared with Nextera XT DNA Library Preparation Kit and combinatorial combinations of molecular identifiers in the Nextera XT Index Kit (Illumina, CA USA) according to manufacturer's instructions. Libraries with an average length of 1200 bp were quantified with Qubit dsDNA HS assay kit to allow pooling of equal amounts of the libraries based on mass. The libraries were sequenced on an Illumina MiSeq v3 PE 2 × 300 bp including negative and positive (PhiX) controls.

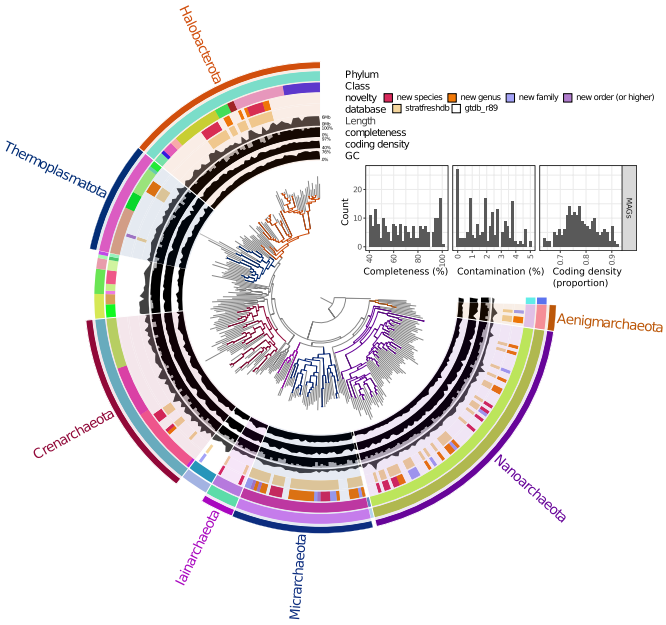

Fig. 4 Archaeal diversity of the stratfreshDB[27]. The insert illustrates the quality of the MAGs included in the tree. Interactive version of the tree with more information available at https://anvi-server.org/moritzbuck/archaeal_diversity_of_the_stratfreshdb.

Data processing of the metagenome and single cell sequences. The metagenome sequencing resulted in a total of ~10^7 paired-end reads of length 2 × 150 bp, amounting to a total of total 3 Tbp. The raw data was trimmed using Trimmomatic (version 0.36; parameters: ILLUMINACLIP:TruSeq 3-PE.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36)[15] (Auxillary Table S3)[10]. The trimmed data was assembled using Megahit (version 1.1.13)[16] with default settings. Two types of assemblies were done, single sample assemblies for all the samples individually and a total of 53, mainly lake-wise, co-assemblies (see Auxillary Table S4)[10], some samples of the Canadian ponds have also been coassembled with previously sequenced libraries of the same sample (see Auxillary Table S5)[10]. The relevant quality controlled reads were mapped to all the assemblies using BBmap[17] with default settings and the mapping results were used to bin the contigs using Metabat (version 2.12.1, parameters --maxP 93 --minS 50 -m 1500 -s 10000)[18]. Genes of obtained bins were predicted and annotated using Prokka (version 1.13.3)[19] using standard parameters except for the bin containing all the unbinned contigs where the –metagenome flag was used. Single-cell libraries were processed similarly to the metagenomes, but without the binning step, and using the single-cell variant of the SPAdes[20] assembler instead of Megahit.

Prokaryotic completeness and redundancy of all bins from Metabat and for all assembled single cells were computed using CheckM (version 1.0.13)[21] (Auxillary Tables S6 and S7 for MAGs and SAGs, respectively)[10]. Average Nucleotide Identity (ANI) for all bin-pairs was computed with fastANI (version 1.3)[22]. The bins were clustered into metagenomic Operational Taxonomic Units (mOTUs) starting with 40% complete genomes with less than 5% contamination. Genome pairs with ANI above 95% were clustered into connected components. Additionally, less complete genomes were recruited to the mOTU if its ANI similarity was above 95%. Bins were taxonomically annotated in a two-step process. GTDB-Tk (version 102 with database release 89)[23] was used first with default settings. Using this classification an lca database for SourMASH (version 1.0)[24] was made. This database as well as one based on the GTDB release 89 was then used with SourMASH's lca classifier for a second round of classification of bins that were not annotated with GTDB-tk (Auxillary Table S8)[10].

The taxonomic diversity of the bacterial (Fig. 3) and archaeal (Fig. 4) mOTUs, respectively, were visualized in a tree format. The trees were computed using GTDB-tk with one representative MAG per mOTU of the stratfreshDB, and one random representative genome per family of the GTDB. Trees were visualized using anvi'o[25].

Data Records

All sequences are deposited to the European Nucleotide Archive (ENA, mirrored to SRA, and accessible at the NCBI) under the project number PRJEB38681[26]. Statistics for reads, metagenome assemblies, high-quality bins (e.g. MAGs), and SAGs can be found in Auxillary Table S3, S2, S4, and S5[10], respectively. For the MAGs the accession numbers are found in Auxillary Table S3 and the for the SAGs in Auxillary Table S7[10]. Additional tables and information can be found under the https://doi.org/10.17044/scilifelab.13005311.v2[27].

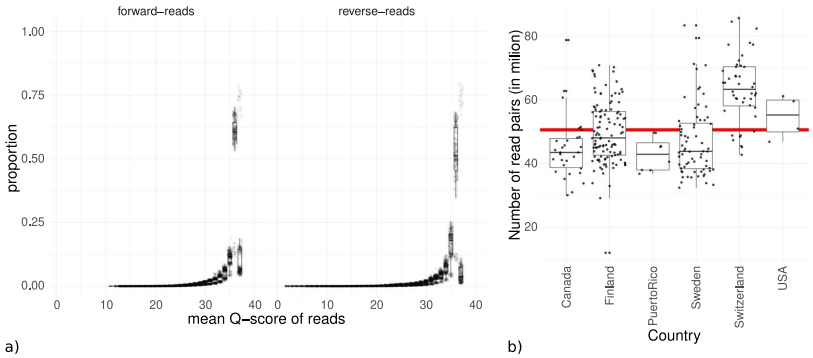

Fig. 5 Overview of quality of metagenomic libraries. (**a**) boxplot summarizing read-quality histograms of all libraries, each dot represents the proportion of reads of a specific library with a mean phred-score of that value. (**b**) boxplots of metagenome depths across regions, each dot represents the number of read pairs for a specific library. The readline is the mean number of pairs for all libraries. Full data available in Auxillary Data S9[10].

Type of data	Number	Total size (Gbases)
Libraries (samples)	346	2800
Assembled metagenomes	341	105
Total number of bins	~500000	98
Number of high quality metagenome assembled genomes (MAGs; >70% complete, <5% contaminated) from the total number of bins	8554	26
Number of moderate quality metagenome assembled genomes (MAGs; >40% complete, <5% contaminated) from the total number of bins	4487	7
Single-amplified genomes (SAGs)	83	0.1

Table 2. Summary of all genomic data.

Technical Validation

The Q scores for the raw reads were calculated prior to any further processing using the MultiQC[25] wrapper for FastQC[28] (Fig. 5). The observed quality distribution showed that most of the reads had Q scores Q30 or higher indicating libraries with low error rates and without serious abnormalities (Fig. 5a). Number of reads per sequenced library was relatively even also indicating no quality problems (Fig. 5b). Full data of the quality reports can be found in Auxillary Data S9[10], including machine-readable quantitative data, the summarized data of the figure and HTML-report. The quality of the MAGs and SAGs was computed with CheckM[21] (Figs. 3 and 4, Auxillary Tables S6 and S7[10]). The MAGs and SAGs were deemed as high quality if the completeness was >70%, and contamination was <5% and moderate quality if the completeness was >40%, and contamination was <5%. Using these thresholds, the data had about 8500 high quality and 4500 moderate quality MAGs (Table 2).

Usage Notes

The full metagenome samples can be accessed at ENA[26]. Additionally, all bins and SAGs as well as subsets of reads for all samples (1 M reads per sample) can be accessed at https://export.uppmax.uu.se/uppstore2018116/stratfreshdb/ using the paths in the files available at https://doi.org/10.17044/scilifelab.13005311.v2[27] for navigation.

Code availability

Code used to process the data is available at https://github.com/moritzbuck/metasssnake, code for the computing of mOTUs is available at https://github.com/moritzbuck/mOTUlizer and some additional scripts, particularly the script used for the submission of the data and summary of the quality reports is available at https://github.com/moritzbuck/0023_anoxicencyclo.

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Author contributions

S.P., S.B., M.B. and S.L.G. conceived the project idea and secured funding for realizing the sequencing. All authors contributed to the collection of the samples. M.B. conducted and coordinated the bioinformatic processing of the metagenomic data. L.F., G.M., G.M.R., J.S. and J.Z. have provided considerable number of samples and data. S.P. drafted the manuscript with significant inputs and contributions from S.B., M.B. and S.L.G., and assistance of all the other authors. All authors have read and approved the final version of the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Correspondence and requests for materials should be addressed to M.B. or S.P.

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RESEARCH ARTICLE
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Special Section:
Winter limnology in a changing world

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

Henrique O. Sawakuchi[1,2], Gaëtan Martin[3], Sari Peura[3], Stefan Bertilsson[4], Jan Karlsson[2], and David Bastviken[1]

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

Abstract Winter methane (CH_4) 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_4 emissions, but despite favorable CH_4 and O_2 concentrations, CH_4 oxidation appears constrained in some lakes for unknown reasons. Here we experimentally test the hypothesis that phosphorus (P) availability is limiting CH_4 oxidation, resulting in differences in ice-out emissions among lakes. We observed a positive relationship between potential CH_4 oxidation and P concentration across 12 studied lakes and found an increase in CH_4 oxidation in response to P amendment, without any parallel change in the methanotrophic community composition. Hence, while an increase in sedimentary CH_4 production and ebullitive emissions may happen with eutrophication, our study indicates that the increase in P associated with eutrophication may also enhance CH_4 oxidation. The increase in CH_4 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_4. This may be an important factor regulating CH_4 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.

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

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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

1. Introduction

Lakes are important global sources of CH_4 to the atmosphere. Climate change, especially in northern lakes, may increase CH_4 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_4 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_4-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_4 is exposed to oxic water for extended periods, which should create favorable conditions for CH_4 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_4 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_4 in sediment is the main CH_4 source in most lakes, except for the limited number of lakes experiencing continuous seepage of CH_4 of geogenic origin or from deep melting permafrost (Walter et al., 2008). Therefore, extensive renewal of under-ice CH_4 bubbles in the late phase of the ice-covered winter period is unlikely in most lakes. Despite this, CH_4-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_4 oxidation in water from ice-covered lakes, while CH_4 oxidation was extensive in other nearby lakes, highlighting that information regarding the factors controlling winter CH_4 oxidation in lakes is still scarce and contradictory (Denfeld et al., 2018). Martinez-Cruz et al. (2015) observed that the winter CH_4 oxidation variability was mainly related to O_2 availability in Alaskan lakes, which is in agreement with previous observations (Bastviken, 2009; Segers, 1998), but cannot explain the range of potential CH_4 oxidation found in oxic surface waters under the ice (Denfeld et al., 2016). It has been suggested that high O_2 concentration may inhibit CH_4 oxidation (Reis et al., 2020), and light is another factor suggested to inhibit CH_4 oxidation (Dumestre et al., 1999; Murase & Sugimoto, 2005). However, a persistent ice cover may both reduce the O_2 and attenuate light below inhibitory levels in the water column. Under such conditions, Denfeld et al. (2016) found that the lakes with extensive CH_4 oxidation in under-ice lake water had higher phosphate (PO_4^{3-}) concentrations than lakes where CH_4 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_4 oxidation and P concentration has been previously observed in soils (Chauhan et al., 2012; Gray et al., 2014).

Clearly, under-ice CH_4 oxidation can be an important constraint for ice-off CH_4 emissions, but for unknown reasons, CH_4 oxidation activity seems to vary widely among lakes. The present study aimed to estimate CH_4 oxidation in lakes covering a range of total P availability and experimentally assess cause-effect relationships between P concentration and CH_4 oxidation to test the hypothesis that P availability favors CH_4 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^2 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_4 oxidation (Denfeld et al., 2016).

Samples (5 ml) for the in-situ concentration of CH_4 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_2) and with 150 µL H_3PO_4 (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_2SO_4 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_{CSH}). TP and PO_4^{3-} 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_4^{3-} solution ($NaH_2PO_4*H_2O$) was added in five of them to increase the PO_4^{3-} 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_4 and CO_2 and standardize the concentration of O_2. After that, 1.8 ml of 99% CH_4 was added to the headspace to reach a CH_4 concentration of 100 μM CH_4 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_4 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_4 oxidation at low temperatures (5°C) require incubations over several weeks before a significant change in CH_4 concentration can be detected. Here, CH_4 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_4 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_4 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_4, and with or without the addition of PO_4^{3-}, and monitored together. Thus, the decrease in CH_4 in the blank vials was due to dilution caused by the addition of synthetic air when sampling. Analysis of CH_4 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^{-7} mmol of CH_4.

To calculate CH_4 oxidation based on the decline in headspace CH_4 over time, we used the decay constant (λ) as the specific rate of methane oxidation (SRMOx) according to the following equation:

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

λ is the decay constant representing the fraction of the CH_4 being oxidized per time unit (day^{-1}; dimensionless), C_t is the concentration at time t (day), and C_0 is the starting concentration after the lag-phase. λ 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^2 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_4 concentration. A lag-phase of 12–23 days was seen in most vials. However, cases without lag-phase or where no CH_4 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_4 oxidation capacity of each vial, was calculated using the time after the lag-phase period.

Our incubations had a large reservoir of O_2 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_2 in the air, leading to an expected O_2 concentration of approximately 13 mg/L in all vials. Assuming that the decrease in O_2 is mainly attributed to DOC degradation we estimated the maximum expected O_2 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_2 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_2 reservoir in our vials. Our incubation over much shorter time (88 days) and at 4°C makes such high mineralization and O_2 consumption unrealistic and a more likely maximum O_2 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_2 reservoir in the vial headspace kept the O_2 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'-CACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNCCTACGGGNGGCWGCAG-5') and 805NR (3'-AGACGTGTGCTCTTCCGATCTGACTACNVGGGTATCTAATCC-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 at 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 Methylacidiphilales, as well as the genera *Methylocystis, Methylosinus, Methylocapsa, Methylocella, Methyloferula, Candidatus Methylomirabilis, 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_4), Total Phosphorus (TP), and in the Water 10 cm Below the Ice

Lake ID	Lakes	DO (mg/L)	DOC (mg/L)	CH_4 (μM)	TP (μg/L)
MOX01[a]	Lumpen	6.7	25.2	0.08	14.9
MOX02[a]	Björklinge-Långsjön	11.8	2.6	1.14	9.9
MOX03[a]	Erken	13.4	4.9	0.12	24.6
MOX04[a]	Malstasjön	3.8	9.9	0.07	74.3
MOX05[a]	Fyrsjön	8.6	6.3	0.07	10.5
MOX06[a]	Plåten	3.0	23.6	0.04	14.7
MOX07[a]	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

[a]Lakes where under-ice CH_4 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 10^6 extra permutations.

3. Results

In-situ CH_4 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_2 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_4 concentrations in the P-amended vials was larger than in the non-amended. The largest change in CH_4 concentration was observed in MOX04 vials where 98.7% and 99.9% of the initial amount of CH_4 were consumed in the non-amended and P-amended vials, respectively, with the lowest concentration reaching 2×10^{-5} mmol of CH_4 (Table S2). For individual lakes, we observed a large variability of SRMOx ranging from 0 (no CH_4 oxidation registered for Lake MOX11) to 0.09 $days^{-1}$ for non-amended vials and from 0.006 to 0.306 $days^{-1}$ for P-amended vials. The overall average fit error based on the R^2 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^{-3} d^{-1} and 3.9–29.4 mmol m^{-3} d^{-1} 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 $days^{-1}$ and 0.014 ± 0.027 $days^{-1}$, 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_4 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 ($R^2 = 0.71$, $p = 0.001$, 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 ($R^2 = 0.50$, $p = 0.022$). 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 ($R^2 = 0.006$, $p = 0.82$).

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.

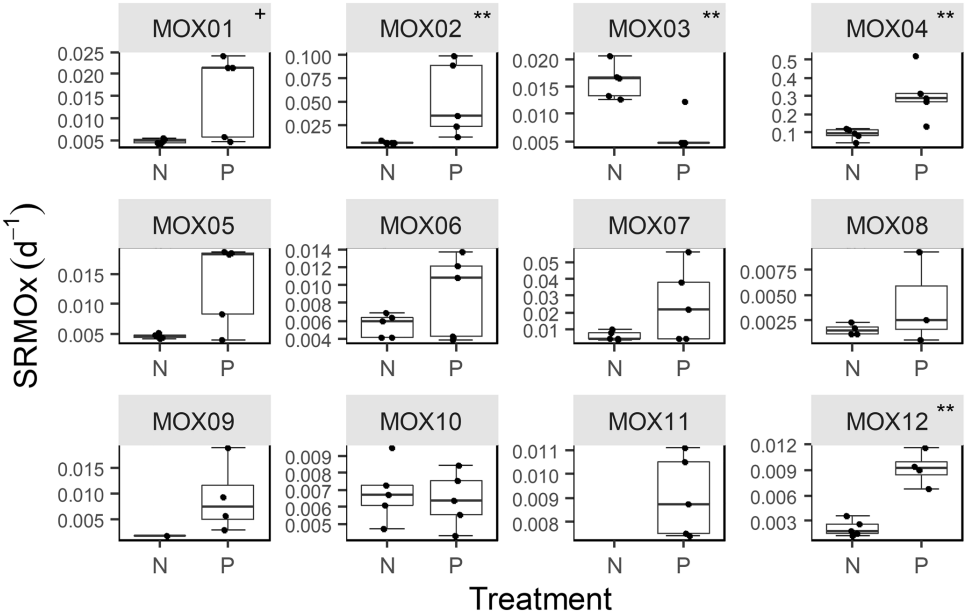

Figure 1. Comparison of the specific methane oxidation rate (SRMOx) observed among non-amended (N) and P-amended (P) vials. CH_4 oxidation was not observed in non-amended vials of MOX11. Note that the *y*-axis scale is adjusted for the values observed for each lake. Dots show the SRMOx values of replicates where CH_4 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.

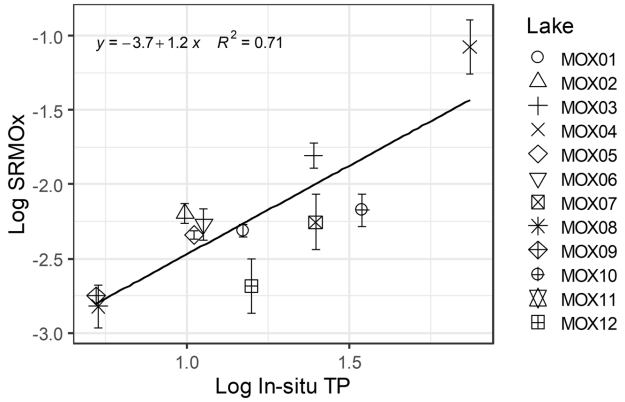

Figure 2. Linear relationship between the log-transformed mean specific rates of methane oxidation (SRMOx) for non-amended vials and in-situ total phosphorus (TP) concentrations. The error bar shows the standard deviation of the mean for the replicates.

Table 2
Similarity of the Most Common Operational Taxonomic Units With Their Closest Isolated Organism and Their Occurrence Across Vials, Treatments, and Lakes

				Occurrence		
OTU	Closest cultivated relative	Identity (%)	Proportion of reads (%)	All vials	Treatment	Lake
otu_0003	*Methylobacter tundripaludum*	98.7	36.0	118/120	24/24	12/12
otu_0004	*Methylovulum psychrotolerans*	96.8	31.3	99/120	24/24	12/12
otu_0010	*Methylovulum psychrotolerans*	99.4	13.3	96/120	23/24	10/12
otu_0014	*Methylobacter tundripaludum*	95.5	7.7	86/120	24/24	12/12
otu_0018	*Methylobacter tundripaludum*	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 ± 0.18 and 0.14 ± 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

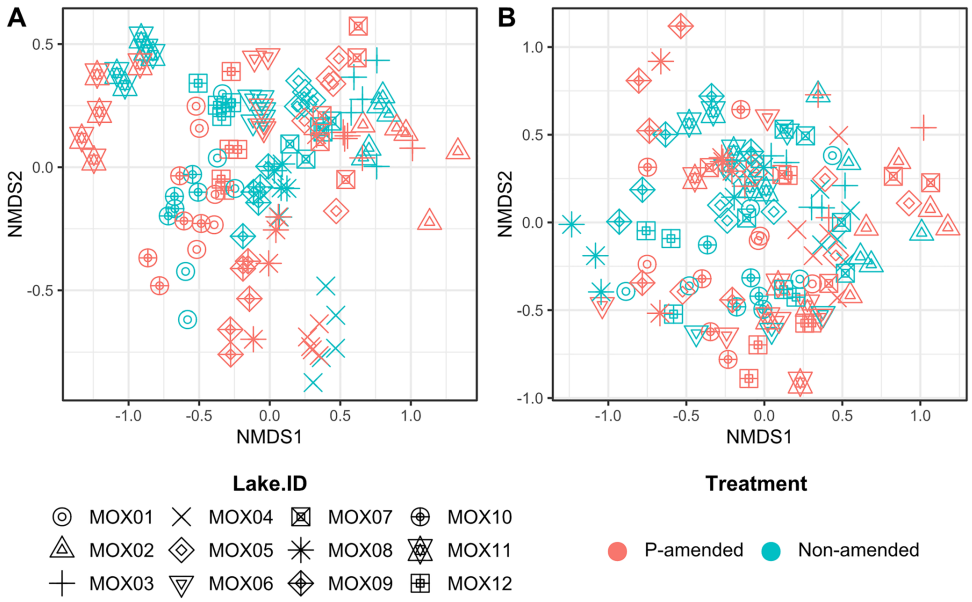

Figure 3. Overall beta-diversity for total bacterial and methanotrophic community. Beta-diversity analysis using non-metric multidimensional scaling (NMDS) for total bacterial (a) and methanotrophic (b) communities.

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 CH_4 and 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 $days^{-1}$ 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 $days^{-1}$ was, in general, higher than what we observed in winter (Bastviken et al., 2008; Thottathil et al., 2019). However, seasonal variation indicating higher 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 mmol m^{-3} d^{-1}) previously observed in incubations at low temperature (i.e., 2°C–5°C) for northern lakes (Martinez-Cruz et al., 2015; Sepulveda-Jauregui et al., 2018; Thottathil et al., 2018).

We found a similar variability in CH_4 oxidation among the lakes previously studied by Denfeld et al. (2016), with increasing CH_4 oxidation from MOX07 (Lötsjön), MOX03 (Erken), and MOX04 (Malstasjön). Variability in CH_4 oxidation among 30 ice-covered lakes was also observed in Alaska, where the causes of such variability were attributed to CH_4 or O_2 limitations (Martinez-Cruz et al., 2015). Thottathil et al. (2018) found a positive correlation between 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 O_2 availability, which agrees with the lack of correlation between DOC and SRMOx in this study. In our experiment, both CH_4 and 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 CH_4 oxidation pointed out by Denfeld et al. (2016). Previously, correlation between 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 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-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 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 CH_4 and 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 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 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 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 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 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 that 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 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 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 O_2 was present in most lakes. Inhibition of CH_4 oxidation by high O_2 concentration have been recently suggested as a factor influencing CH_4 oxidation in lakes (Reis et al., 2020; Thottathil et al., 2019). The O_2 reservoir in the vial headspace kept the 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 O_2 inhibition of CH_4 oxidation among vials could not have been large enough to explain the results. As O_2 levels were high during incubation we cannot exclude O_2 inhibition of CH_4 oxidation. However, maximum CH_4 oxidation observed at high O_2 saturation suggest that O_2 inhibition is not always happening, and that this potential inhibition remain unclear (van Grinsven et al., 2020). Nevertheless, any potential O_2 inhibition did not prevent the P-induced stimulation of the CH_4 oxidation. We also do not discard the possibility that the higher O_2 concentration in our experiment in comparison with in-situ conditions could have stressed the methanotrophic community adapted to low O_2 concentration, limiting their capacity to oxidize CH_4 in the experiment. Further work on how the magnitude of the P-stimulation of CH_4 oxidation is interacting with levels of O_2 or other factors influencing 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 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_4 production in microcosm lake sediment experiments (Sepulveda-Jauregui et al., 2018), and ebullitive CH_4 emissions from mesocosm experiments (Davidson et al., 2018) suggest that despite the higher offset by oxidation, CH_4 emissions could still increase with warming and eutrophication. It is worth highlighting that enhanced CH_4 oxidation can significantly reduce the amount of CH_4 in the water column, reducing the emissions through diffusion. Hence, it is important to note that assessments of CH_4 fluxes from eutrophic systems should be designed to appropriately cover ebullitive emissions of CH_4 to prevent underestimation of the total CH_4 emissions. The P-CH_4 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_4 oxidation also mitigates ebullition by oxidation of CH_4 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_4 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_4 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_4 oxidation with high potential for mitigating CH_4 emissions to the atmosphere. The identification of P as a controlling factor of CH_4 oxidation can improve predictions of ice-off and open water emissions and, thus, annual CH_4 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_4 oxidation. This can result in overall higher annual emissions from lakes, and contribute as a additional CH_4 feedback to global warming (Dean et al., 2018).

5. Conclusion

Based on the overall positive response of CH_4 oxidation to phosphate addition observed in the experiment, we conclude that when CH_4 and O_2 are not limiting factors, CH_4 oxidation in lakes could be constrained by P availability. We found that limited CH_4 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_4 oxidation capacity, especially in winter, when ebullitive emissions are trapped under the ice and subjected to CH_4 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_4 oxidation. We also acknowledge that P may favor CH_4 emissions by increasing eutrophication, which negatively influences O_2 availability due to increased heterotrophic respiration, creating conditions that could limit under ice CH_4 oxidation resulting in higher ice-off emissions. However, despite the seemingly important role P has on winter CH_4 oxidation, its influences on the balance between CH_4 production and CH_4 oxidation needs further investigation across different seasons and lake types, helping understand the regulations of the net CH_4 balance and improve estimates of annual CH_4 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/bioproject/PRJNA638356. All data used in the paper appear as metadata of each sample.

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Supporting Information for

Phosphorus regulation of methane oxidation in water from ice-covered lakes

Henrique O. Sawakuchi[1,2]*, Gaëtan Martin[3], Sari Peura[3], Stefan Bertilsson[4], Jan Karlsson[2], David Bastviken[1]

[1]Department of Thematic Studies – Environmental Change, Linköping University, Linköping, SE-581 83, Sweden;

[2]Climate Impacts Research Centre (CIRC), Department of Ecology and Environmental Science, Umeå University, SE-901 87 Umeå, Sweden;

[3]Department of Forest Mycology and Plant Pathology, Science for Life Laboratory, Swedish University of Agricultural Sciences, Uppsala, Sweden;

[4]Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Contents of this file

Supplementary Figures and Tables.

Figure S1. Representation of the beta-diversity of the total bacterial (a) and methanotroph (b) communities as non-metric multidimensional scaling plots showing the influence of treatments for each lake. Values in brackets are the results of ADONIS analysis (R2 and p-value).

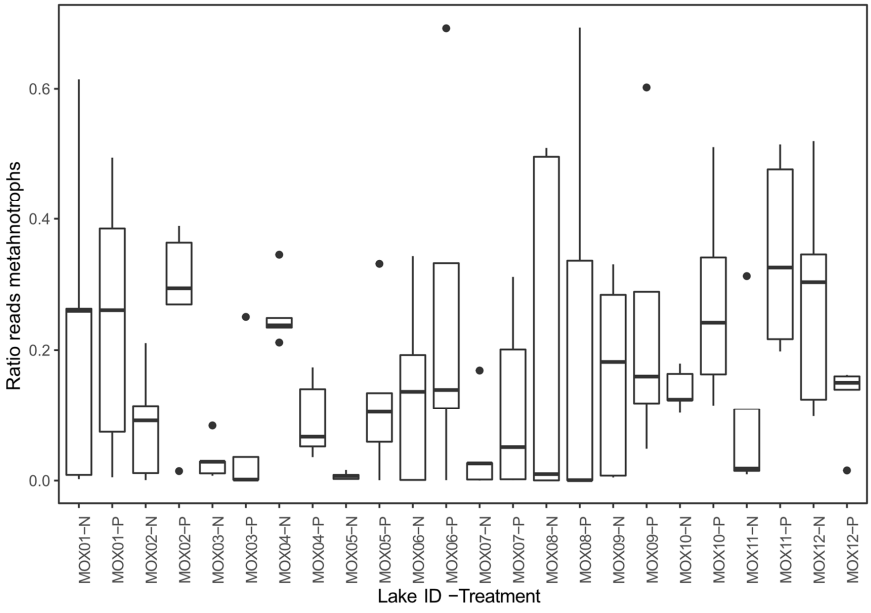

Figure S2. Ratio of reads assigned to methanotroph taxa in each set of replicates. N indicates non-treated samples and P samples amended with phosphorus. The lower, upper, and middle hinges correspond to the 25th, 75th and median percentiles. The upper whisker extends up to the largest value, but no further than 1.5 * the inter-quartile range.

Table S1. General information on studied lakes. In-situ physicochemical parameters measurements were done 10 cm below the ice. Trophic state classification based on total phosphorus concentration according to Carlson (1977).

Lake ID	**Lake name**	**Lat/Long (decimal degree)**	**Lake area (km^2)**	**Temp. (°C)**	**pH**	**EC (µS/cm)**	**Trophic status**
MOX01	Lumpen	59.9612°/17.2810°	0.24	0.9	6.3	57	Mesotrophic
MOX02	Björklinge-Långsjön	60.0388°/17.5638°	2.46	0.6	8	490	Oligotrophic
MOX03	Erken	59.8399°/18.6314°	23.7	0.3[a]	8.1[a]	239[a]	Mesotrophic/Eutrophic
MOX04	Malstasjön	59.7691°/18.6429°	0.24	0.2[a]	7.9[a]	307[a]	Eutrophic
MOX05	Fyrsjön	59.7978°/18.5062°	0.16	0.7[a]	7.8[a]	203[a]	Oligotrophic
MOX06	Plåten	59.8627°/18.5425°	0.02	0.7[a]	6.7[a]	93[a]	Mesotrophic
MOX07	Lötsjön	59.8623°/17.9391°	0.58	0.4[a]	7.9[a]	201[a]	Mesotrophic/Eutrophic
MOX08	Glimmingen	57.9337°/15.5725°	1.74	1.6	5.6	63	Oligotrophic
MOX09	Stortoveln	57.9331°/15.5518°	0.2	1	9.2	62	Oligotrophic
MOX10	Bengtsgolen	58.6962°/16.1911°	0.03	0.7	6.8	69	Eutrophic
MOX11	Lillsjön	58.6590°/16.1438°	0.02	1.5	4.6	56	Mesotrophic
MOX12	Parsen	58.3404°/16.2039°	0.14	1.1	6.7	75	Mesotrophic

[a]Data from Denfeld et al. (2016).

Table S2. Summary of the total amount of CH_4 (mmol) observed during the incubation, showing the minimum, maximum, average, and standard deviation of CH_4 amounts at the start (Co), end (Cf), and difference from start and end (d), observed in five replicates of each treatment.

Lake	Treat.	$C_{0\ min}$	$C_{0\ max}$	$C_{0\ avg}$	$C_{0\ sd}$	$C_{f\ min}$	$C_{f\ max}$	$C_{f\ avg}$	$C_{f\ sd}$	d_{min}	d_{max}	d_{avg}	d_{sd}	d (%)
MOX01	N	0.072	0.075	0.073	0.001	0.049	0.053	0.052	0.001	0.020	0.023	0.022	0.022	29.5
	P	0.074	0.077	0.075	0.001	0.014	0.056	0.031	0.018	0.020	0.062	0.044	0.044	58.7
MOX02	N	0.075	0.077	0.076	0.001	0.043	0.052	0.049	0.003	0.024	0.033	0.027	0.027	35.8
	P	0.059	0.062	0.060	0.001	0.000	0.031	0.010	0.011	0.031	0.060	0.050	0.050	83.9
MOX03	N	0.067	0.074	0.072	0.003	0.019	0.032	0.026	0.005	0.040	0.054	0.045	0.045	63.2
	P	0.072	0.077	0.075	0.002	0.034	0.055	0.050	0.008	0.019	0.041	0.025	0.025	33.3
MOX04	N	0.072	0.073	0.072	0.000	0.00003	0.004	0.001	0.002	0.067	0.073	0.071	0.071	98.7
	P	0.069	0.074	0.072	0.002	0.00002	0.00004	0.00003	0.000005	0.069	0.074	0.072	0.072	99.9
MOX05	N	0.070	0.072	0.072	0.001	0.050	0.052	0.051	0.001	0.020	0.023	0.021	0.021	28.8
	P	0.073	0.075	0.073	0.001	0.019	0.053	0.031	0.013	0.021	0.054	0.043	0.043	58.6
MOX06	N	0.072	0.075	0.074	0.001	0.043	0.054	0.049	0.004	0.019	0.029	0.025	0.025	34.0
	P	0.073	0.076	0.075	0.001	0.029	0.054	0.040	0.010	0.021	0.047	0.034	0.034	46.1
MOX07	N	0.073	0.078	0.075	0.002	0.036	0.055	0.048	0.007	0.020	0.038	0.027	0.027	35.7
	P	0.070	0.074	0.072	0.001	0.001	0.053	0.026	0.022	0.017	0.071	0.045	0.045	63.2
MOX08	N	0.089	0.099	0.093	0.004	0.083	0.090	0.086	0.003	0.004	0.009	0.006	0.006	6.7
	P	0.091	0.099	0.095	0.003	0.057	0.091	0.082	0.013	0.005	0.043	0.013	0.013	13.4
MOX09	N	0.083	0.093	0.086	0.003	0.080	0.087	0.083	0.002	0.002	0.006	0.003	0.003	4.0
	P	0.074	0.090	0.084	0.005	0.034	0.079	0.063	0.017	0.000	0.052	0.021	0.021	24.6
MOX10	N	0.075	0.092	0.081	0.007	0.047	0.052	0.049	0.002	0.023	0.040	0.032	0.032	38.7
	P	0.076	0.100	0.087	0.008	0.041	0.073	0.055	0.012	0.012	0.043	0.032	0.032	36.8
MOX11	N	0.086	0.089	0.088	0.001	0.082	0.085	0.084	0.001	0.004	0.005	0.004	0.004	5.1
	P	0.085	0.091	0.088	0.002	0.038	0.056	0.047	0.006	0.033	0.049	0.041	0.041	46.6
MOX12	N	0.084	0.103	0.094	0.008	0.077	0.095	0.085	0.007	0.006	0.012	0.009	0.009	9.1
	P	0.087	0.094	0.090	0.003	0.049	0.090	0.062	0.015	0.000	0.043	0.028	0.028	30.9

Table S3. Mean and standard deviation specific rate of methane oxidation (SRMOx (d^{-1})) for P-amended and non-amendment incubations observed for each lake, and the number of replicates (n) where CH_4 oxidation was observed. A total of 5 vials were incubated per treatment. For each vial, the SRMOx was calculated using the change in concentration observed during the course of each incubation stage, discarding the lag-phase period, when it happened, see the methods section for more details. Two-sided Wilcoxon rank-sum test was used to assess the difference among treatments (P-amended and non-amendment) for each lake. Note that the reported p-values did not account for a potential increase in familywise error rates.

Lake ID	Non-amended			P-amended			Wilcoxon	
	Mean	sd	n	Mean	sd	n	W	*p*-value
MOX01	0.005	0.0005	5	0.015	0.010	5	3	0.056
MOX02	0.006	0.001	5	0.052	0.040	5	0	0.008
MOX03	0.016	0.003	5	0.006	0.003	5	25	0.008
MOX04	0.090	0.031	5	0.306	0.141	5	0	0.008
MOX05	0.005	0.0003	5	0.014	0.007	5	5	0.151
MOX06	0.005	0.001	5	0.009	0.005	5	8	0.421
MOX07	0.006	0.003	5	0.025	0.022	5	7	0.310
MOX08	0.002	0.001	4	0.004	0.005	3	4	0.629
MOX09	0.002	0.000	1	0.009	0.007	4	0	0.400
MOX10	0.007	0.002	5	0.006	0.002	5	14	0.841
MOX11	0.000	0.000	0	0.009	0.002	5	-	-
MOX12	0.002	0.001	5	0.009	0.002	4	0	0.016
All lakes	**0.014**	**0.027**	**50**	**0.041**	**0.094**	**56**	**877**	**<0.001**

Table S4. Methane oxidation rate (mmol m^{-3} d^{-1}) for non-amendment and P-amended incubations observed for each lake, and the number of replicates (n) where CH_4 oxidation was observed.

Lake ID	Non-amended			P-amended		
	Mean	sd	n	Mean	sd	n
MOX01	3.66	0.33	5	8.01	3.20	5
MOX02	4.84	0.53	5	9.40	1.35	5
MOX03	8.39	0.90	5	4.35	1.39	5
MOX04	13.46	1.86	5	29.40	5.10	5
MOX05	5.47	3.77	5	7.19	2.32	5
MOX06	4.31	0.66	5	5.71	1.92	5
MOX07	4.60	1.32	5	8.15	3.95	5
MOX08	1.90	0.44	4	3.90	3.19	3
MOX09	1.98	0.00	1	7.30	3.49	4
MOX10	5.97	1.84	5	5.61	0.83	5
MOX11	-	-	0	7.09	0.70	5
MOX12	2.52	1.05	5	7.79	1.51	4
All lakes	**5.19**	**3.18**	**50**	**8.66**	**6.44**	**56**

Table S5: Number of reads in each sample after trimming and clustering before rarefaction. Rep stands for replicate, N for non-amended samples and P for samples amended with phosphorus.

Lake ID	Rep N1	Rep N2	Rep N3	Rep N4	Rep N5	Rep P1	Rep P2	Rep P3	Rep P4	Rep P5	Mean N	Mean P
MOX01	51783	76519	59449	78836	75713	66775	79481	81813	62150	72660	68460	73099
MOX02	61244	82223	70612	69494	73973	85573	80970	73751	77893	65001	71509	76193
MOX03	71382	78828	77750	76599	87088	80411	71663	74745	63406	74638	78329	75325
MOX04	81291	91131	77703	74870	62189	73621	75275	72348	63938	73195	77437	70094
MOX05	85458	72550	71309	85194	55335	61456	67739	80475	72290	70662	73969	67993
MOX06	67260	69516	67741	71207	66510	67402	62511	69362	66203	78289	68447	68379
MOX07	68289	78340	51894	79479	66936	82211	82372	82565	90357	73680	68988	79687
MOX08	84484	79041	79294	59331	81172	92750	65510	71558	58279	69233	76664	73084
MOX09	78260	79317	95009	81357	87535	67872	72908	70906	74491	78800	84296	75419
MOX10	84122	77174	100994	86147	88218	89763	87237	60340	91135	87960	87331	84109
MOX11	77650	74336	80034	84806	83599	94679	74450	85117	76227	43452	80085	76254
MOX12	82760	82250	83458	90446	92713	70292	81960	82556	85811	78753	86325	82014

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III

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Candidatus Methylumidiphilus Drives Peaks in Methanotrophic Relative Abundance in Stratified Lakes and Ponds Across Northern Landscapes

Gaëtan Martin[1], Antti J. Rissanen[2], Sarahi L. Garcia[3], Maliheh Mehrshad[4], Moritz Buck[4] and Sari Peura[1]**

[1] Department of Forest Mycology and Plant Pathology, Science for Life Laboratory, Swedish University of Agricultural Sciences, Uppsala, Sweden, [2] Faculty of Engineering and Natural Sciences, Tampere University, Tampere, Finland, [3] Department of Ecology, Environment and Plant Sciences, Science for Life Laboratory, Stockholm University, Stockholm, Sweden, [4] Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences, Uppsala, Sweden

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***Correspondence:**
Gaëtan Martin
gaetan.microbio@gmail.com
Sari Peura
sari.peura@slu.se

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Boreal lakes and ponds produce two-thirds of the total natural methane emissions above the latitude of 50° North. These lake emissions are regulated by methanotrophs which can oxidize up to 99% of the methane produced in the sediments and the water column. Despite their importance, the diversity and distribution of the methanotrophs in lakes are still poorly understood. Here, we used shotgun metagenomic data to explore the diversity and distribution of methanotrophs in 40 oxygen-stratified water bodies in boreal and subarctic areas in Europe and North America. In our data, gammaproteobacterial methanotrophs (order *Methylococcales*) generally dominated the methanotrophic communities throughout the water columns. A recently discovered lineage of *Methylococcales*, *Candidatus* Methylumidiphilus, was present in all the studied water bodies and dominated the methanotrophic community in lakes with a high relative abundance of methanotrophs. Alphaproteobacterial methanotrophs were the second most abundant group of methanotrophs. In the top layer of the lakes, characterized by low CH_4 concentration, their abundance could surpass that of the gammaproteobacterial methanotrophs. These results support the theory that the alphaproteobacterial methanotrophs have a high affinity for CH_4 and can be considered stress-tolerant strategists. In contrast, the gammaproteobacterial methanotrophs are competitive strategists. In addition, relative abundances of anaerobic methanotrophs, *Candidatus* Methanoperedenaceae and *Candidatus* Methylomirabilis, were strongly correlated, suggesting possible co-metabolism. Our data also suggest that these anaerobic methanotrophs could be active even in the oxic layers. In non-metric multidimensional scaling, alpha- and gammaproteobacterial methanotrophs formed separate clusters based on their abundances in the samples, except for the gammaproteobacterial *Candidatus* Methylumidiphilus, which was separated from these two clusters. This may reflect similarities in the niche and environmental requirements of the different genera within alpha- and gammaproteobacterial methanotrophs. Our study

confirms the importance of O_2 and CH_4 in shaping the methanotrophic communities and suggests that one variable cannot explain the diversity and distribution of the methanotrophs across lakes. Instead, we suggest that the diversity and distribution of freshwater methanotrophs are regulated by lake-specific factors.

Keywords: methanotroph, greenhouse gas, metagenomics, thaw ponds, microbial diversity, lakes

INTRODUCTION

Whereas anthropogenic carbon dioxide (CO_2) has been the most important greenhouse gas (GHG) since the early days of the industrial era, most recent estimates indicate that methane (CH_4) has been responsible for a quarter of cumulative radiative forcing for CO_2, CH_4, and nitrous oxide (Etminan et al., 2016). Several studies suggest that lakes and ponds are the dominant and underestimated sources of natural CH_4 emissions at high northern latitudes (in boreal and arctic areas) (Bastviken et al., 2011; Wik et al., 2016a,b). Furthermore, the physics and biology of lakes are all expected to change globally due to direct human activities and climate change, which might lead to increased CH_4 emissions (Tranvik et al., 2009; Wik et al., 2016b). For this reason, it is of utmost importance to gather more information on the organisms and processes behind the CH_4 emissions.

CH_4 emissions from lakes are a net balance between methane production by methanogens and consumption by methane oxidizers [methanotrophs (MO)]. According to the estimates, MO can consume between 30 and 99% of CH_4 produced in the sediments and the water column before it reaches the atmosphere (Frenzel et al., 1990; Kankaala et al., 2006; Bastviken et al., 2008; Mayr et al., 2020b). The extent of emissions depends on the efficiency of the methanotrophic biofilter and environmental conditions, such as mixing patterns, ebullition, and trophic state of the lakes (Kankaala et al., 2007; Bellido et al., 2009; Yang et al., 2019). Increased temperature and eutrophication are expected to surge the CH_4 production in lakes (Sepulveda-Jauregui et al., 2018; Zhou et al., 2020). However, those could also improve the efficiency of the methanotrophic biofilter (Davidson et al., 2015; Denfeld et al., 2018; de Jong et al., 2018). A better knowledge of the diversity and distribution of methanotrophic communities is essential for understanding the biological mechanisms behind the dynamic methane equilibrium and eventually predicting possible future changes in the functioning of the CH_4 biofilter (Wagg et al., 2019).

Oxygen-stratified lakes are hotspots for CH_4 oxidizing bacteria. Known methanotrophs inhabit and are active throughout the water column but are typically most abundant in the metalimnion (Sundh et al., 2005; Pimenov et al., 2010; Samad and Bertilsson, 2017; Rissanen et al., 2018; Reis et al., 2020). The metalimnion is characterized by decreasing oxygen and temperature and increasing nutrient and CH_4 concentrations (**Figure 1**). Thus, layers low in oxygen and high in CH_4 are considered hubs for CH_4 oxidation (Bastviken et al., 2004; Pimenov et al., 2010; Blees et al., 2014). In those conditions, as well as in oxygen saturated water, CH_4 oxidation is considered to be performed mainly by aerobic methanotrophs belonging to alpha- and gammaproteobacteria (Taipale et al., 2011; Tsutsumi et al., 2011; Blees et al., 2014; Biderre-Petit et al., 2019; Reis et al., 2020). Methane oxidizers of the recently discovered acidophilic genus *Methylacidiphilum* in the phylum Verrucomicrobia (V-MO) (Camp et al., 2009) are also using O_2 as an electron acceptor but are associated with extreme environments (Sharp et al., 2014; van Teeseling et al., 2014; Schmitz et al., 2021). The taxa involved in CH_4 oxidation in anoxic environments include Archaea (ANME archaea, referred to as MOA in the following text) (Valentine, 2002) and Bacteria belonging to genus *Candidatus* Methylomirabilis (in the phylum NC10, referred to as NC10-MO in the following text) (Raghoebarsing et al., 2006). These taxa use alternative electron acceptors, such as SO_4^{2-} and $NO_3^-NO_2^-$ instead of O_2 (Valentine, 2002; Beal et al., 2009; Wu et al., 2011; Oswald et al., 2017). Furthermore, recent studies suggest that some gammaproteobacterial methane oxidizers (γ-MO) have the potential for fermentation (Kalyuzhnaya et al., 2013; Gilman et al., 2017) and anaerobic respiration (Kits et al., 2015; Oswald et al., 2016; Zheng et al., 2020). The importance of MO as a methane biofilter in anoxic freshwaters is still unclear (Reed et al., 2017). However, it is known that anaerobic MO can consume large quantities of CH_4 and represent a substantial portion of the microbial biomass in the anoxic layer of the lakes (hypolimnion) when conditions are favorable (Graf et al., 2018).

Some recent studies suggest that the O_2 and CH_4 counter gradients are responsible for niche partitioning of alphaproteobacterial methane oxidizers (α-MO) and γ-MO and underline how this partitioning might be essential for predicting the efficiency of the CH_4 biofilter (Mayr et al., 2020c; Reis et al., 2020; Rissanen et al., 2020). Apart from CH_4 and O_2, it is necessary to include other physicochemical parameters that potentially influence CH_4 oxidation. Indeed, factors such as light (Rissanen et al., 2018; Thottathil et al., 2018), phosphorus (Denfeld et al., 2018; Zhou et al., 2020), community richness (Ho et al., 2014), temperature (Yang et al., 2019), and different forms of nitrogen (Bodelier and Laanbroek, 2004) have been shown to influence CH_4 oxidation rates. However, the impact of those environmental parameters on the methanotrophic communities is still unclear and often seems contradictory (Ho et al., 2013). Furthermore, most studies on freshwater MO neglect the potential importance of anaerobic MO and Verrucomicrobia as they are less abundant (Ho et al., 2013; Knief, 2015; Crevecoeur et al., 2017, 2019; Reis et al., 2020). As the role of rare microorganisms is still poorly understood (Galand et al., 2009), there is a pressing need to include those into the analyses for a complete understanding of the functioning and interactions in the methanotrophic community. So far, the abundance of rare MO has been associated with the expression and detection

of the genes associated with the CH_4 oxidation (Crevecoeur et al., 2017), while the diversity of the MO communities may be correlated with oxidation rates (Bodelier et al., 2013). Rare taxa can also serve as a seed for when conditions change (Graf et al., 2018; Mayr et al., 2020b) and should therefore be considered. Also, methodological issues should be taken into account as most previous studies on methanotrophs have been done using PCR-based methods looking into the diversity of 16S rRNA or *pmoA* genes (Crevecoeur et al., 2017, 2019; Rissanen et al., 2018; Mayr et al., 2020c). While being a well-established method in microbial ecology, it introduces biases to the data, especially in primer mismatches and problems related to coverage of especially new and poorly known taxa (Bourne et al., 2001; Wang et al., 2017). The shotgun metagenomic approaches are not bias free either, but they avoid the primer bias associated with amplicon sequencing. Studies looking into the ecology of methanotrophs using shotgun metagenomics are still rare (Rissanen et al., 2018; Mayr et al., 2020b). Last but not least, all known previous studies focus on one or a small number of lakes in a limited geographic area (Tsutsumi et al., 2011; Crevecoeur et al., 2017; Oswald et al., 2017; Samad and Bertilsson, 2017; Graf et al., 2018; Mayr et al., 2020c; Rissanen et al., 2020) or look only at the top layer of the studied water bodies (Crevecoeur et al., 2019), restraining the identification of factors that could be used for global estimations of the abundance and distribution of methanotrophs.

The main aims of our study were to (i) study the taxonomic patterns of MO in stratified lakes and ponds situated above 50° N of latitudes, (ii) test if environmental parameters can explain the distribution of MO groups in those water bodies, and (iii) confirm the general dominance of ɣ-MO throughout the water columns of boreal lakes and subarctic thaw ponds. To achieve these aims, we used a shotgun metagenomic dataset of 208 samples from 28 oxygen-stratified lakes and 12 permafrost thaw ponds from boreal and subarctic areas in both Europe and North America. Thus, we offer a novel insight into the diversity and distribution of methanotrophs, including rare methanotrophic taxa. While most of the studied lakes are located in Scandinavia, the addition of North American thaw ponds in the data expands our approach both geographically and functionally. Our study is based on metagenomic shotgun data and considers the importance of stratification patterns of lakes and ponds with a high concentration of dissolved organic matter (DOC).

MATERIALS AND METHODS

We obtained 208 metagenomes from four countries, covering the subarctic and boreal regions. The samples are a part of a project aiming to study microbial diversity in anoxic freshwater environments. The full details of the sample collection, sample analyses, sequencing, and data processing are provided in Buck et al. (2021). In short, for all lakes, samples were collected for both metagenome analysis and measurements of environmental parameters. For most of the lakes and ponds, the samples were taken from multiple depths, including samples from all three layers of the stratified water bodies (**Figure 1**). DNA was extracted from all the samples using DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Libraries were prepared with ThruPLEX DNA-seq Prep Kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. The protocol includes a short PCR step (seven cycles) using random primers during which sample-specific indexes are added to the samples. The shotgun sequencing of all samples was conducted at the Science for Life Laboratory (Uppsala University, Sweden) on Illumina NovaSeq6000-platform. The measured parameters varied between lakes, and for the analyses here, we selected those that were available for at least half of the samples (temperature, pH, dissolved CH_4, O_2, CO_2, NH_4, NO_3, PO_4, SO_4, and Fe). For the following analyses, the samples were assigned to a layer (i.e., epi-, meta-, or hypolimnion) based on the oxygen and temperature profiles of the lakes as follows: (1) samples with O_2 concentration above 2 mg/l were classified as epilimnion, (2) samples with temperature around 4°C and O_2 close to 0 mg/l were classified as hypolimnion, and (3) samples from areas between the epilimnion and hypolimnion with a sharp change in oxygen and temperature were classified as metalimnion (**Figure 1**).

For the analyses of the methanotrophic community, we used trimmed but unassembled shotgun data, which was taxonomically classified using Kaiju with default parameters (Menzel et al., 2016) with the NCBI nr-database including eukaryotes and the fungi of JGIs 1,000 fungi project (Grigoriev et al., 2014). Kaiju is a classifier with high sensitivity and precision based on finding maximum (in-)exact matches on the protein level using the Burrows-Wheeler transform. This enabled us to detect the rare members of the community that have too low abundance to be assembled and would thus be disregarded in the analyses of assembled data (**Supplementary Methods 1**). The community composition was additionally analyzed using 16S rRNA reads parsed out from the shotgun data, and the community composition was compared with the Kaiju data as described in **Supplementary Methods and Results**.

All further analyses were done using R version 4.0.2 (R Core Team, 2020). After removal of all the reads assigned to Eukaryotes, the Kaiju data were rarefied to 90% of the number of reads in the sample with the lowest read count ($1.2*10^6$) in the whole dataset. We picked a value lower than the number of reads in the smallest sample to have a random subsampling for all of the samples. Rarefaction was performed using the phyloseq package in R (McMurdie and Holmes, 2013) with set.seed (1) used to initialize repeatable random subsampling. Following rarefaction, all taxa with less than 25 reads in the subsampled data were removed from the taxa table.

The abundance of methanotrophic taxa was calculated as the sum of all reads attributed to each individual taxon (e.g., MO or α-MO) divided by the sum of all reads in the sample after rarefaction. Hence, the calculated abundances are relative abundance throughout this study. The dominance of a taxon was calculated as the sum of all reads attributed to the taxon divided by the total of reads attributed to MO in the sample after rarefaction. The included taxa were the following ones: α-MO (all the bacteria in the following genera: *Methylocystis*, *Methylosinus*,

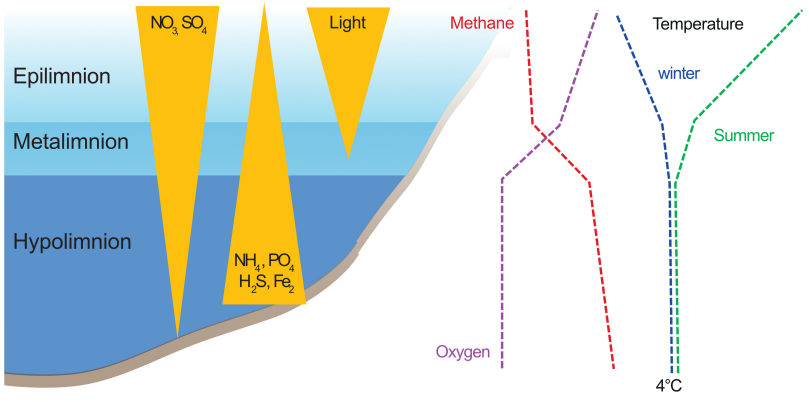

FIGURE 1 | Schematic representation of a stratified lake. During stratification, lake water column is divided into three distinct layers. Whereas the stability and depth of these layers depend on several parameters, such as temperature, salinity, color, morphology of the lake, climate, etc.; the different layers tend to share a few common features. The surface layer, *epilimnion*, is rich in oxygen while at the lake bottom, in the hypolimnion, anaerobic conditions prevail. Between these two layers, a thin but distinct layer develops, where temperature and oxygen change drastically, this is the *metalimnion*. The condition in the hypolimnion are favorable for reduced compounds, such as CH_4, NH_4, or H_2S, whereas in the epilimnion, conditions favor oxidation. Concentration curves of reduced and oxidized compounds often cross each other in the metalimnion (e.g., oxygen and methane). During winter, when the lake freezes, the surface water gets colder (i.e., below 4°C) and the temperatures of the layers are inverted, with the warmer layer being the hypolimnion. The depths of each of the layers are not the same during winter and summer stratification.

Methylocapsa, *Methylocella*, and *Methyloferula*), γ-MO (all the bacteria in the order *Methylococcales*), NC10-MO [all the bacteria in genus *Ca.* Methylomirabilis)], MOA (ANME – archaea and *Ca.* Methanoperedenaceae), and V-MO (all the bacteria in the order *Methylacidiphilales*).

Non-metric multidimensional scaling (NMDS) projection and permutational multivariate analysis of variance (PERMANOVA) were performed using Bray–Curtis distance matrix and 1,000 permutations with the phyloseq package (McMurdie and Holmes, 2013). Partial least squares (PLS) regression was performed using the mixOmics package (Rohart et al., 2017) with the classic regression mode, including two components. Environmental variables were used as an observable variable (X) and the relative abundances of MO groups and their dominances were considered as predicted variables (Y). Pairwise correlations (Spearman) and Pairwise Wilcoxon rank sum tests were also performed with their p-values corrected using the Bonferroni method. The metagenomic dataset is available at European Nucleotide Archive (ENA) under accession number PRJEB38681.

RESULTS

Gammaproteobacterial Methanotrophs Dominate the Methanotrophic Communities Throughout the Water Column

The rarefied dataset was composed of $2.56*10^8$ reads and included 2,822 genera. Of those reads, $1.30*10^7$ (5%) were attributed to methanotrophs (MO) and classified into 26 different methanotrophic genera. The abundance of MO varied from 0.5% up to 57.4% of the reads per sample (median at 2.2%). The mean abundance of MO varied significantly between the different layers of lakes (epi-, meta-, and hypolimnion) ($p < 0.05$; **Figure 2A**). Highest median and mean values were found in the hypolimnion and the lowest in the epilimnion. Despite a significantly lower mean value in the metalimnion compared with the hypolimnion ($p < 0.005$), the highest abundance of MO was recorded in a sample from the metalimnion (**Figure 2A**).

Gammaproteobacterial methanotrophs (γ-MO) dominated the MO communities throughout the water columns (**Figure 3** and **Supplementary Figure 1**) with over 50% dominance in 97% of the samples. In the rare occurrences where γ-MO was not the most dominant taxa, they still represented between 30 and 50% of the MO. Peaks in MO abundances were not visible in all lakes, but when existing (e.g., Alinen Mustajärvi, **Figure 3A** and **Supplementary Figure 1**), they were usually located in the metalimnion together with a fast decrease of O_2 and increase of CH_4 concentration. The peaks in the abundance of MO were associated with high dominance of γ-MO, and more specifically, of the newly discovered genus *Candidatus* Methylumidiphilus. The second most abundant group of methanotrophs, α-MO, often represented a significant proportion (i.e., dominance > 20% in 40 samples) of the methanotrophic population (e.g., **Figure 3B**) and in some samples could even dominate over γ-MO (**Supplementary Figure 1**). The high α-MO dominances were typically associated with the oxygenic layer.

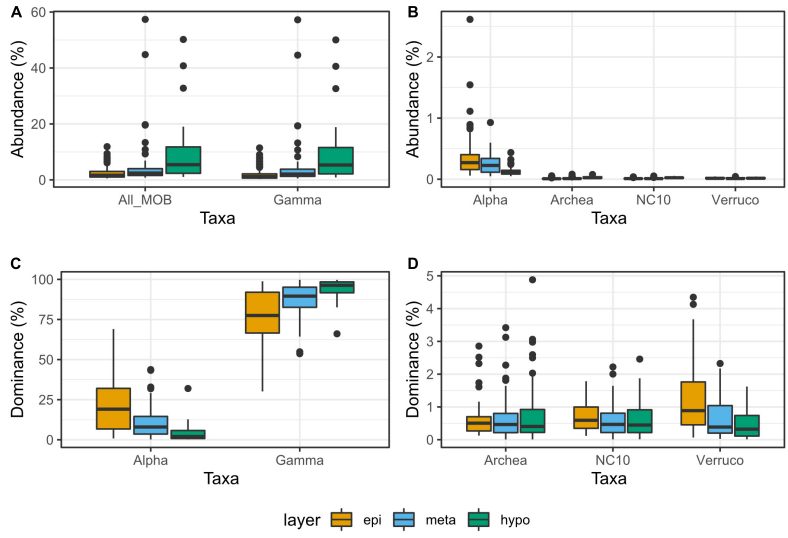

FIGURE 2 | Relative abundances **(A,B)** and dominances **(C,D)** of methane oxidizers (MO) observed in the different layers of the study lakes. Abundances are the proportions of reads attributed to MO in the whole dataset. Dominances are the proportions of reads attributed to a certain MO taxon among all the reads attributed to MO. The MO taxa include: alphaproteobacterial methanotrophs (α-MO), gammaproteobacterial methanotrophs (γ), methanotrophic Archaea (MOA), bacterial phylum NC10 (NC10-MO), and Verrucomicrobial methanotrophs (V-MO). The lower, upper, and middle hinges correspond to the 25th, 75th, and median percentiles. The upper whisker extends up to the largest value, but not further than 1.5* the interquartile range.

The Abundance of *Candidatus* Methylumidiphilus Is Correlated With the Abundance and Dominance of Gammaproteobacterial Methanotrophs

Order *Methylococcales* (i.e., γ-MO) was the most abundant methanotrophic taxon, representing 4.9% of all reads and 94.4% of the reads attributed to MO. This could be up to 57.2% of all reads in a single sample and up to 99.7% of reads attributed to MO (respective medians at 1.9 and 89.6%). Like the total MO community, the abundance of γ-MO increased toward deeper layers ($p < 0.005$) and their highest abundances were observed in metalimnion samples (**Figures 2A,B**). A similar pattern was observed for γ-MO dominance (**Figures 2C,D**). Furthermore, γ-MO dominance was strongly correlated with the total abundance of MO ($\rho = 0.8$). This relation between MO abundance and γ-MO was driven mainly by the abundance of *Candidatus* Methylumidiphilus, correlated with γ-MO abundance and dominance ($\rho = 0.76$ and 0.57). The dominance of γ-MO was not affected by O_2 content or the layer of origin, and γ-MO dominance of over 80% could be observed in all O_2 conditions (**Figure 2C**).

Among γ-MO, *Ca.* Methylumidiphilus accounted for 2.7% of all reads and 53.0% of reads attributed to MO. In individual samples, *Ca.* Methylumidiphilus represented up to 55.7% of all reads, corresponding to up to 97.1% of all MO reads. While *Ca.* Methylumidiphilus was the best represented MO genus in the dataset in regards to the number of samples with this taxon, *Methylobacter* was the most abundant MO in 106 samples (vs. 81 for *Ca.* Methylumidiphilus). Contrary to *Ca.* Methylumidiphilus, the relative abundance of *Methylobacter* was poorly correlated with the total abundance of MO (**Figure 4**). Furthermore, all samples having *Methylobacter* as the most abundant MO had a relatively low total abundance of MO (i.e., less than 20%). Other abundant γ-MO genera in the order of decreasing abundance were *Methylomonas*, *Crenothrix*, and *Methylovulum* (**Supplementary Table 1**).

The second most abundant class among the MO was α-MO, representing 0.2% of all reads across all the samples and 4.59% of the MO reads. The maximum abundance of α-MO was 2.6% of all reads, but α-MO could have a dominance of up to 69.0% of all the MO reads per sample. The highest abundances and dominances of α-MO were recorded in epilimnion samples and all samples but one with α-MO dominance over 20% were originating from oxic water layer (**Figures 2C**, **5A,B**). The mean abundance of α-MO increased significantly from hypo- to metalimnion ($p < 0.005$), whereas the mean abundance between meta- and epilimnion was not significantly different (**Figure 2B**). Furthermore, the mean dominance of α-MO was increasing from the hypolimnion to metalimnion to epilimnion ($p < 0.005$ in all cases, **Figure 2C**). The most abundant α-MO genus was *Methylocystis*, followed by *Methylocapsa*, *Methylocella*, *Methylosinus*, and *Methyloferula* (**Supplementary Table 1**).

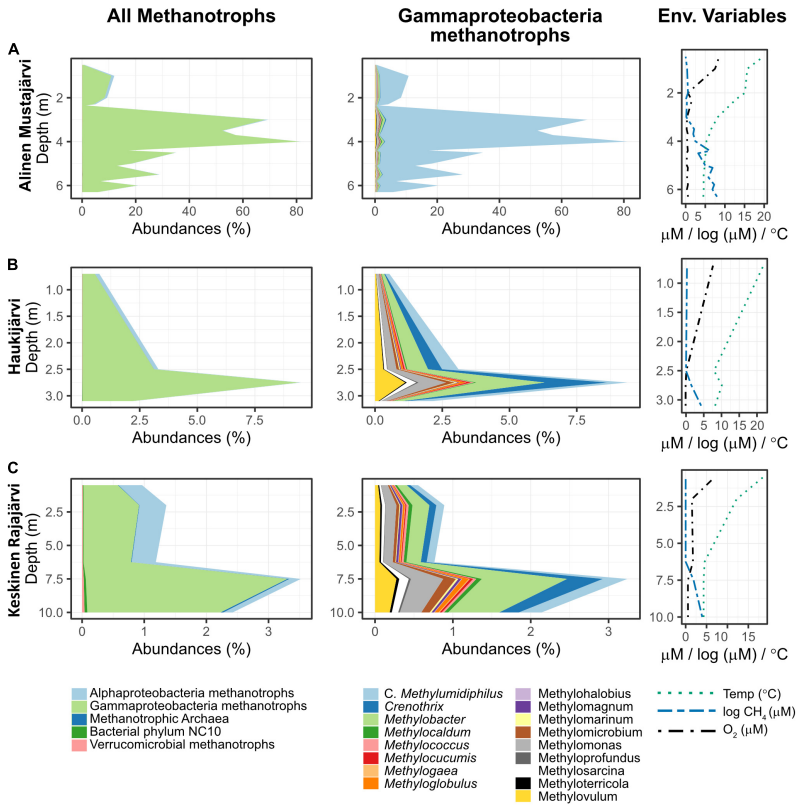

FIGURE 3 | Vertical variation in the relative abundance of methane oxidizers and different genera of gammaproteobacterial methanotrophs, as well as in key environmental variables. Three lakes were selected to represent the general dominance (i.e., higher relative abundances) of gammaproteobacterial methane oxidizers **(A–C)**. Alinen Mustajärvi is an example where *Candidatus* Methylumidiphilus dominates an abundant methanotrophic community **(A)**, whereas in both Haukijärvi and Keskinen Rajajärvi, the most abundant genus in methanotrophic community is *Methylobacter* **(B,C)**. Keskinen Rajajärvi also shows a higher dominance of alphaproteobacterial methanotrophs in the epilimnion **(C)**. Please note the much higher abundance of methanotrophs in Alinen Mustajärvi **(A)**. The profiles for the rest of the lakes that had data for at least three depths are available in the **Supplementary Figure 1**. Note the change in scale of abundance and depth from one lake to the other.

Anaerobic methanotrophic taxa belonging to Archaea (MOA) and bacterial phylum NC10 (NC10-MO) as well as aerobic Verrucomicrobial methanotrophs (V-MO) were each represented by a single genus: *Candidatus* Methanoperedens, *Candidatus* Methylomirabilis, and *Methylacidiphilum*, respectively. They were all detected in all samples. Overall, each of these taxa represented less than 0.02% of the total reads across all samples. Furthermore, none of these MO taxa represented more than 0.1% of all reads or 4.5% of the reads attributed to MO in one sample (**Supplementary Table 1**). They both had their highest mean abundances in the hypolimnion ($p < 0.005$, **Figure 2B**). For MOA, the difference in mean abundance between meta- and epilimnion was significant ($p < 0.05$) but not for NC10-MO. While both the maximum abundances were detected in the hypolimnion, the epi- and metalimnion abundances reached similar levels. The dominance of both MOA and NC10-MO did not vary significantly between the layers (**Figure 2D**). The mean normalized abundance of V-MO did not differ between layers, but its dominance was higher in the epilimnion ($p < 0.005$) than in the meta- and hypolimnion (**Figures 2B,D**).

Correlation Between the Community Structure of Methanotrophs and CH_4 Concentration Varies Between Layers

In the NMDS representation of the samples based on both the composition of the whole microbial communities and

the MO communities (**Figures 5A,B**), samples were grouped based on their oxygen status with hypolimnion samples on one side of the plot and the epilimnion samples in the other. In contrast, the samples from metalimnion were more dispersed across the plot. However, no differences between the layers could be detected in statistical tests, likely due to the large dispersion among the oxic samples. Also, the distribution of the samples seemed to be strongly related to the dominance of α-MO. The NMDS plot based on taxa (**Figure 5C**) also showed that α- and γ-MO were grouped into two different clusters. The γ-MO further subdivided into three distinct clusters of genera (**Supplementary Figure 2**). The first cluster included genera *Methyloprofundus*, *Methylomarinum*, *Methyloglobulus*, *Methylosarcina*, *Crenothrix*, *Methylomicrobium*, *Methylomonas*, *Methylovulum*, *Methylocucumis*, and *Methylobacter*, while *Methylococcus*, *Methylogaea*, *Methylohalobius*, *Methyloterricola*, *Methylomagnum*, and *Methylocaldum*, formed the second one. The last "cluster" was composed of the genus *Ca*. Methylumidiphilus alone. The third cluster (*Ca. Methylumidiphilus*) was the most abundant in each layer, followed by group 1. The difference in the mean abundance between these groups was significant in each layer ($p < 0.005$).

When PLS included MO abundance (data not shown), the location of MO taxa close to the center of the PLS regression graph suggested the absence of correlation between MO abundance and the tested (i.e., environmental) variables. MO abundance was removed for further analysis because of its strong correlation with γ-MO. When relative abundances of the taxa were plotted, explained variances carried by the latent variables (i.e., the component axes 1 and 2) were low (<0.25). The position of the variables on the plot suggested a potential correlation between NC10-MO, MOA, CO_2, CH_4, Fe, PO_4, and CO_2, as well as between α-MO, O_2, and temperature. γ-MO appeared close to the center, suggesting that none of the variables could explain its abundance (**Supplementary Figure 3A**). The PLS plot of MO dominances and environmental variables had latent variables explaining more of the carried variance (0.41 and 0.52 for components 1 and 2, respectively). This plot suggested a correlation between α-MO, O_2, and temperature. Those three variables also seemed to negatively correlate with γ-MO dominance (**Supplementary Figure 3B**). The correlations between the abundances of α-MO (**Supplementary Figure 4**) and dominances of α-MO and γ-MO with O_2 were confirmed by Spearman's correlation ($|\text{rho}| \geq 0.5$). For both MOA and NC10-MO abundances, medium or stronger correlations were confirmed with CH_4, NH_4, temperature, and CO_2 but not with PO_4 nor Fe (**Supplementary Figure 5**).

Whereas CH_4 concentration seemed to have little effect on MO abundances when all samples were considered, the picture changed when we considered its effect in each layer (**Figure 6**). The abundance of MO in the epilimnion showed a positive medium strength correlation with CH_4 but no clear correlation in the meta- or hypolimnion. While weak and not significant, the trend in the hypolimnion was negative. The correlation pattern observed for the γ-MO was the same as for MO in general. The α-MO showed a medium strength negative correlation with CH_4 in the metalimnion. In the other layers, there were no significant correlations, but the trend was systematically negative. We did not detect any correlation between the γ-MO dominance and CH_4 concentration in any of the layers.

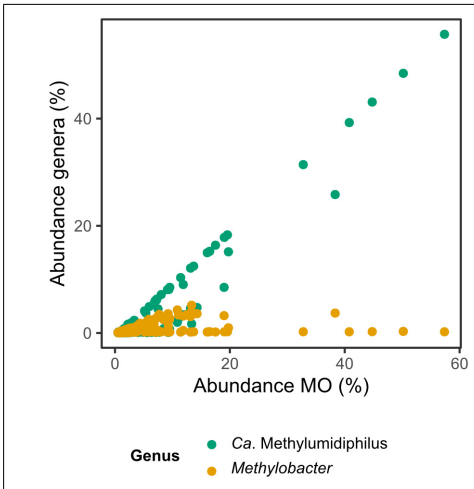

FIGURE 4 | Relative abundances of the most abundant methanotrophic taxa in the function of the total relative abundance of methanotrophs (MO). The two most abundant taxa of MO are plotted against the total abundance of MO. The Spearman's rank correlation coefficient is 0.96 for *Candidatus* Methylumidiphilus and 0.27 for *Methylobacter*. Each sample is represented by two points, one for each genus.

For both MOA and NC10-MO, the correlations calculated with the entire dataset were similar to those calculated with data from each independent layer (**Supplementary Tables 2–4**). Relative abundances of MOA and NC10-MO were also strongly correlated ($\rho = 0.78$). V-MO abundance was only correlated with SO_4 when all samples were considered together and showed medium strength correlation with O_2 concentration in the hypolimnion (**Supplementary Figure 5** and **Supplementary Table 2**).

DISCUSSION

Our multilake and multilayer approach showed that none of the measured environmental variables could predict the abundance or structure of the community, suggesting that the methanotrophs are controlled by lake-specific interactions between the methanotrophic community and environment. However, we did observe some overarching tendencies within the dataset, such as the dominance of γ-MO, especially the genera *Methylobacter* and *Ca*. Methylumidiphilus. In line with previous studies, our results showed a diverse methanotrophic community with variation in the abundance both across and within the different water bodies (Taipale et al., 2011; Oswald et al., 2016; Samad and Bertilsson, 2017; Crevecoeur et al., 2019). Furthermore, the pertinence of our approach based on Kaiju was

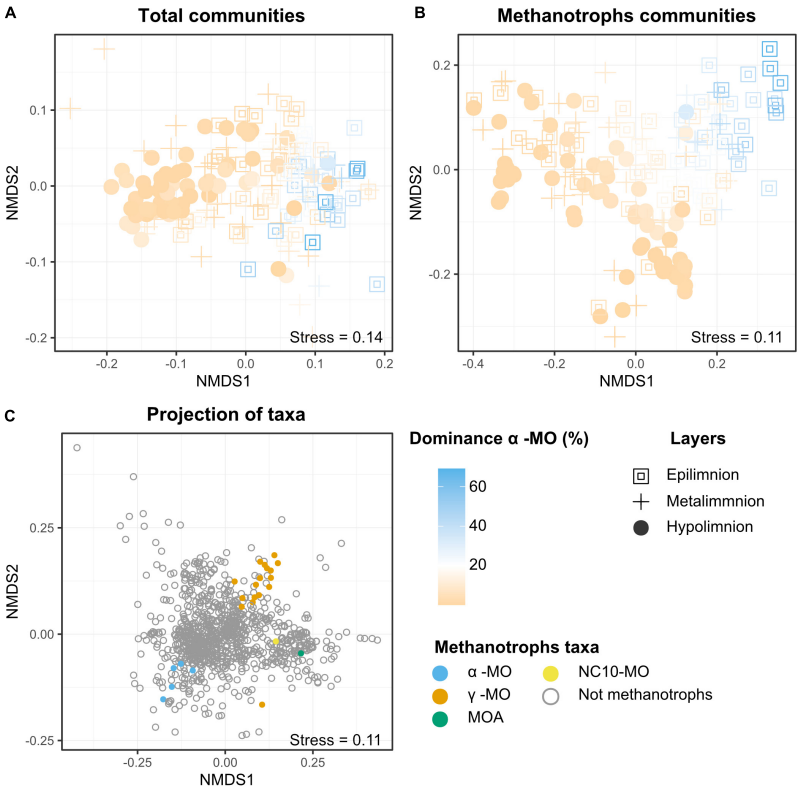

FIGURE 5 | NMDS plots visualizing the composition of the **(A)** total and **(B)** methanotrophic (MO) communities in the samples as well as **(C)** distribution of different taxa. In **(A,B)**, colors represent the dominance of alphaproteobacterial methane oxidizers (i.e., the proportion of reads attributed to α-MO among all the reads attributed to MO) in each of the samples. The non-linear color scale visualizes that, except for one samples, all the samples with more than 20% of the MO communities belonging to the Alphaproteobacteria are grouped and belong to the epilimnion or the metalimnion (blue). Samples with MO communities dominated by Gammaproteobacteria (orange) are spread across the plot, including the area where Alphaproteobacteria dominated. They also include samples from all the three layers. The layers are a proxy for oxygen levels, epilimnion being fully oxic, metalimnion poor in oxygen (>0.2 mg/l), and hypolimnion anoxic. The taxa plot **(C)** shows the distance between the taxa based on their relative abundances in the samples. For readability purpose, only the 900 most abundant taxa are plotted. The MO taxa represented are alphaproteobacterial methanotrophs (α-MO), gammaproteobacterial methane oxidizers (γ), methanotrophic Archaea (MOA), and bacterial phylum N10 (NC10-MO).

confirmed by comparing our results with an alternative method based on 16S rRNA read alignment. The comparison showed high correlation between the two sets of results for the major taxa (**Supplementary Methods and Results**). However, the 16S-based approach was unable to detect the rare methanotrophs.

High Relative Abundance of MO in the Meta- and Hypolimnion Has a Complex Relationship With O_2 and CH_4

The highest relative abundances of methanotrophs were found in the metalimnion, which is in line with previous studies that have found that MO abundance and CH_4 oxidation are often highest in the oxygen transition zone or at the top of the hypolimnion (Kankaala et al., 2006; Tsutsumi et al., 2011; Oswald et al., 2016; Mayr et al., 2020a). However, the highest mean relative abundance of MO was found in the hypolimnion, again in line with previous studies (Oswald et al., 2015; Peura et al., 2015). The peaks in MO abundance and CH_4 oxidation in the metalimnion have been suggested to be due to optimal O_2 concentrations (Oswald et al., 2016; van Grinsven et al., 2020), as too high O_2 levels may be inhibitory for MO activity (Thottathil et al., 2019). One possible explanation for the high MO abundance in the hypolimnion could be higher PO_4 availability in anoxic conditions (Beutel et al., 2008). Indeed, several papers linked methane oxidation rates with P availability (Boiesen et al., 1993;

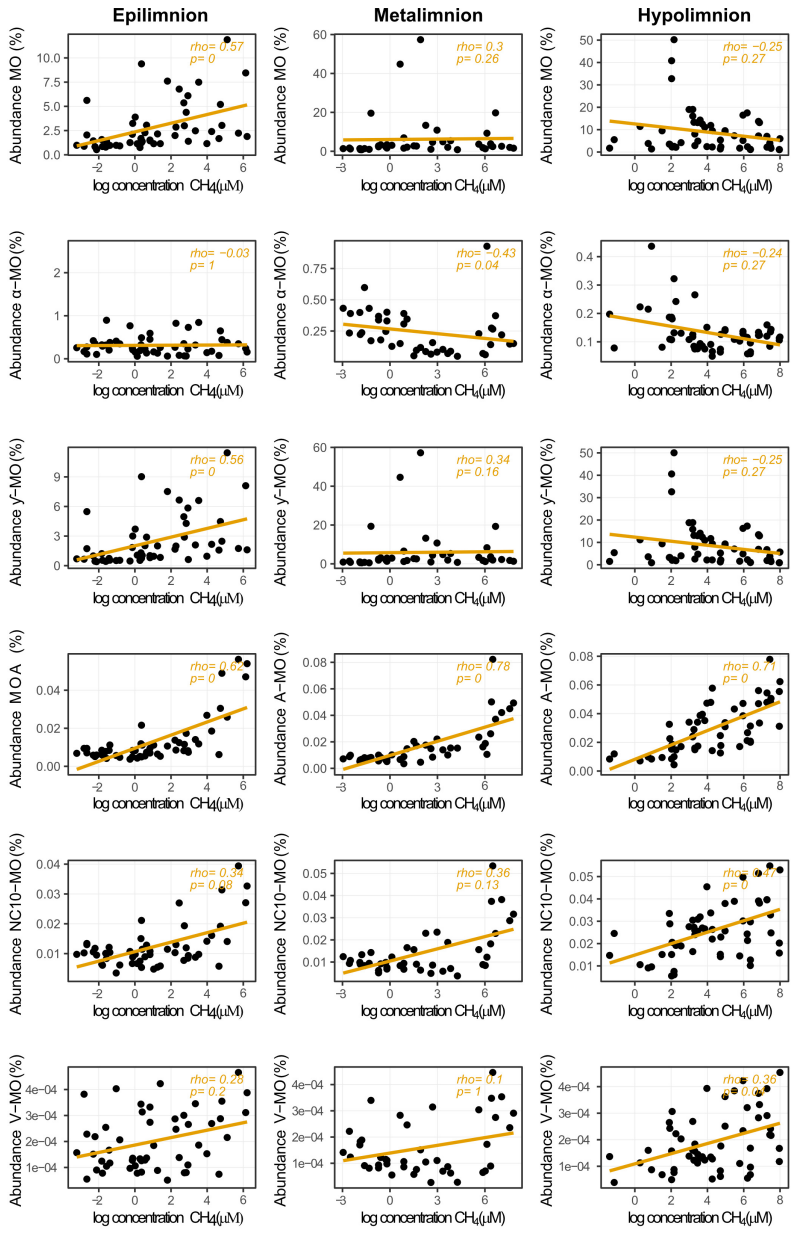

FIGURE 6 | Relationship between relative abundances of methane oxidizers and CH_4 concentrations in the different layers of stratified lakes. The taxa represented are alphaproteobacterial methanotrophs (α-MO), gammaproteobacterial methanotrophs (γ), Archaea (MOA), bacterial phylum NC10 (NC10-MO), Verrucomicrobial methanotrophs (V-MO), and the sum of all methane oxidizers (MO). Rho indicates Spearman's rank correlation coefficients. *p*-values were adjusted with the Bonferroni method and rounded to two digits. Null *p*-values indicate $p \leq 0.005$.

Denfeld et al., 2016). However, our data could not confirm this possibility as no significant correlation between PO_4 concentration and abundance was detected in any of the layers. Another reason for the high abundances of methanotrophs could be higher CH_4 concentrations in anoxic hypolimnion, but based on our data, it does not seem that straightforward as in both meta- and hypolimnion, the highest abundances were observed in samples with lower CH_4 concentrations (4–7 μM). Furthermore, the correlation with CH_4 was not significant in the metalimnion and very weak and negative in the hypolimnion, suggesting that CH_4 is not a limiting factor in those layers. The high MO abundances at low CH_4 concentrations could reflect a rapid turnover of CH_4 by an abundant MO community. Still, the absence of positive correlation between CH_4 and abundance in low oxygen condition suggest that these peaks do not depend on CH_4. In the oxic epilimnion, where CH_4 concentrations were much lower, increases in the relative abundances of MO were observed when CH_4 concentrations were higher. This suggests that in such conditions, CH_4 may be a limiting factor in such conditions (Kankaala et al., 2006).

Methane being a limiting factor only when oxygen is abundant enough to be potentially inhibiting also suggests that electron acceptors could be a limiting factor for CH_4 oxidation in the lower layers of the studied lakes. This limitation in electron acceptors in CH_4-rich waters has been suggested by several studies where experimental addition of O_2 or alternative electron acceptors in anoxic water as well as oxygenic photosynthesis have increased CH_4 oxidation rates (Milucka et al., 2015; Oswald et al., 2015, 2016; van Grinsven et al., 2021). Whereas not necessarily related to abundance, these studies showed that the lack of electron acceptors could limit MO metabolism. While this would explain the lack of correlation between MO abundance and CH_4 in low oxygen conditions and the presence of the highest recorded abundances in low oxygen samples from the metalimnion, it does not help to understand the highest mean abundance in the hypoxic layer of the lakes. These high abundances could simply be related to lower predation in anoxic waters, as zooplankton has been shown to have a strong grazing effect on MO abundance (Devlin et al., 2015). This lack of predation is also suggested by the observation of higher cell counts in the hypolimnion (Oswald et al., 2016). However, as our measurements only reflect relative abundances, augmentation in size of the whole community cannot explain the higher proportion of MO. One explanation for the higher relative abundance could be the sinking of cells following a peak in the upper layer. However, the same impact should be seen for other microbes, diluting the impact on methanotrophs. Methanotrophs could also have an advantage over other microbes as they do not need to compete for energy or carbon sources. While our data, which is based on DNA, cannot tell us if the MO in the hypolimnion are active or just the byproduct of growth in upper layers, several studies have shown that MO, both aerobic and anaerobic, can be active in the hypolimnion (Blees et al., 2014; Mayr et al., 2020b; Reis et al., 2020) and even a bloom of anaerobic MO have been observed in the hypolimnion (Graf et al., 2018). Finally, a peak of abundance in the metalimnion has been associated with stable stratification (Mayr et al., 2020a), suggesting that MO can prevail in favorable conditions. This all would suggest that the high abundance of MO in the hypolimnion is an actively growing population.

CH_4 Affinity Might Define the Relation Between α-Mo and γ-MO

The dominance of γ-MO has been widely reported for freshwaters (Biderre-Petit et al., 2011; Oswald et al., 2016; Rissanen et al., 2018; Chen et al., 2020; Mayr et al., 2020c), as well as higher α-MO dominance in the upper oxic layers (Biderre-Petit et al., 2011; Oswald et al., 2016; Crevecoeur et al., 2019; Mayr et al., 2020a; Reis et al., 2020). The correlation of both α-MO dominance and relative abundance with O_2 concentration, combined with the fact that samples with high α-MO dominance all come from oxic samples, suggests that O_2 is a crucial factor explaining the α-MO abundance and dominance. However, a closer look at our data and the literature suggests that while α-MO have higher abundance and dominance in the epilimnion, they do not appear to be responsible for the increase in MO abundance when CH_4 concentration increases in the epilimnion. Indeed, α-MO is the only taxonomic group that does not increase in abundance with increasing CH_4 concentration. Further, α-MO also seems to have higher abundance and dominance when CH_4 concentrations are low in both the meta- and hypolimnion. This ability to grow in low CH_4 concentration is in line with a well-documented high CH_4 affinity of α-MO (Pratscher et al., 2018), particularly *Methylocystis* (Dunfield et al., 1999; Yimga et al., 2003; Knief and Dunfield, 2005; Baani and Liesack, 2008). This genus has been reported as the most abundant α-MO in an acidic boreal peat bog (Danilova et al., 2016; Esson et al., 2016) and in freshwaters (Biderre-Petit et al., 2011, 2019; Crevecoeur et al., 2019). Another hint indicating that low CH_4 might be more critical than O_2 in favoring α-MO is that whereas all samples with over 20% of α-MO were oxic, not all oxic samples were dominated by α-MO. Thus, several samples from the oxic environment showed the dominance of γ-MO, and while the inhibitory role of O_2 on CH_4 oxidation and its mechanisms are still unclear (Rudd et al., 1976; Thottathil et al., 2019; van Grinsven et al., 2020), it has been demonstrated that γ-MO can strive with high O_2 and high CH_4 (Hernandez et al., 2015; Oswald et al., 2015; Chu et al., 2020; Mayr et al., 2020a). Furthermore, feeding CH_4 to an α-MO-dominated community can shift the dominance toward γ-MO (Knief et al., 2006; Steenbergh et al., 2009). Finally, the strong correlation between γ-MO abundance and dominance with the abundance of MO shows that while γ-MO are dominating the MO communities in most cases, this domination is getting stronger when MO abundance is high. This suggests that γ-MO, particularly *Candidatus* Methylumidiphilus, are fast-growing, highly competitive organisms when conditions are favorable. It, therefore, seems reasonable to see the α-MO community in boreal lakes to have a high CH_4 affinity and slow growth rate, while the γ-MO has a low affinity and a fast growth rate. It could also be phrased in a more classical ecology way presenting the α-MO as stress-tolerant and γ-MO as competitive type, as previously suggested (Ho et al., 2013). Thus, the high α-MO dominance in aerobic samples would result from CH_4 levels

being generally lower in oxic water when the CH_4 biofilter at the oxic-anoxic interface is particularly efficient. But the role of α-MO in limiting CH_4 emission should not be dismissed. Due to the low affinity of γ-MO for CH_4 or methanogenesis in the epilimnion (Bogard and del Giorgio, 2016), surface water tends to be oversaturated in CH_4 (Blees et al., 2015), leading to a release of CH_4 to the atmosphere. In such conditions, high affinity for CH_4 offers not only an interesting niche to exploit but is also a critical mechanism to limit CH_4 emissions.

Both α-MO and γ-MO appear to have specific environmental preferences based on their lifestyle, and all genera within them seem to share similar environmental preferences. However, it is essential to notice that the separation between low CH_4 and high O_2—loving α-MO and high CH_4—loving γ-MO is driven by a very few taxa. In addition, besides the newly discovered *Candidatus* Methylumidiphilus, which might be specific to boreal lakes (see below), in our samples, the dominating genera of both α-MO (*Methylocystis*) and γ-MO (*Methylobacter*, *Methylomonas*, and *Crenothrix*) are the usual suspects for freshwater CH_4 oxidation (Biderre-Petit et al., 2011, 2019; Oswald et al., 2017; Crevecoeur et al., 2019; Mayr et al., 2020a). As all these groups of MO genera seem to share similar ecological preferences, it is tempting to assume that they also share similar preferences for CH_4 and O_2. Yet, it has been shown that within MO, the phylogenetic signal may be stronger for physiological traits associated with optimal growth, such as pH or temperature optimum, rather than for traits related to CH_4 oxidation kinetics (Krause et al., 2014). This suggests that observations on preferences regarding CH_4 concentration might only be relevant for the most abundant α- and γ-MO. The distribution of the other genera would then be explained by a similar preference for other variables due to phylogenetic similarity. This would be in line with our data showing the grouping of γ-MOB in three clusters containing closely related genera (from MO groups 1a and 1b and c, respectively) (Knief, 2015; Frindte et al., 2017; Rissanen et al., 2018).

While we argue that the affinity for CH_4 is a key factor for explaining the niche differentiation between α- and γ-MO, we do not dismiss the importance of other parameters in explaining the distribution and abundance of the genera. It has been shown that at constant CH_4 concentration, O_2 has a selecting effect on γ-MO communities (Hernandez et al., 2015) and other variables like light, metals, or nitrogen compounds have had both inhibiting or enhancing effects on CH_4 oxidation depending on the conditions (Rudd et al., 1976; Bédard and Knowles, 1989; Murase and Sugimoto, 2005; Milucka et al., 2015; Guggenheim et al., 2020). However, we could not detect any selection effect for any of the available variables. Considering that these previous studies have reported several different factors possibly regulating MO community and CH_4 oxidation, and our lack of similar findings, it seems likely that the regulation is lake specific and depends on the specific conditions prevailing in each lake. Thus, our comparison across lakes might hide the importance of each of these parameters in individual lakes or even in lake compartments.

Recently Described *Candidatus* Methylumidiphilus Is Globally Abundant in Boreal Lakes

Among the γ-MO genera, *Candidatus* Methylumidiphilus was the most abundant taxon. This abundance could be overestimated by Kaiju as the database genome of *Candidatus* Methylumidiphilus is relatively large, 6.6 Mb (*Ca*. Methylumidiphilus alinensis, GCA_003242955.1). In comparison, the average for environmental aquatic bacteria is 3.1 Mb (Rodríguez-Gijón et al., 2021). However, the observed high level of dominance seems unlikely to only be due to a methodological bias. The abundances of reads of *Ca*. Methylumidiphilus were up to two orders of magnitude higher than the abundance of the second most-abundant genus. Previously, *Ca*. Methylumidiphilus has been reported only from two boreal lakes in Southern Finland (Rissanen et al., 2018, 2020). Still, here we show that this genus is widely spread across boreal lakes and arctic thaw ponds, both in Europe and North America. Our data show that it is not only commonly found but also often represents an abundant or the most abundant member of the MO population. While *Methylobacter* was the most abundant MO in most samples, it was dominant only when the total abundance of MO was low (i.e., below 15%). This, combined with the strong correlation between *Ca.* Methylumidiphilus and MO abundance suggests that the peaks of abundances observed in certain lakes were driven by this newly described genus. *Ca.* Methylumidiphilus may therefore play an important global role in mitigating the CH_4 emissions from the northern lakes. This could be specific to boreal and arctic lakes as other genera are known to dominate MO community in lakes sampled further south (Oswald et al., 2015, 2017; Graf et al., 2018), but the dominance of unknown OTUs (Mayr et al., 2020a) and general PCR bias makes it possible that *Candidatus* Methylumidiphilus is also present in non-boreal lakes but has escaped detection so far due to these technical problems. In fact, Rissanen et al. (2018) actually noticed that 16S rRNA gene sequences from *Ca*. M. alinensis were assigned as "unclassified Gammaproteobacteria" when using older Silva 119 (released July 24, 2014) and 123 (July 23, 2015) databases, while starting with Silva 128 database they were classified correctly as Methylococcales. This suggests that many previous 16S rRNA amplicon-based studies might have failed to correctly classify this lineage and detect it as a methanotroph. This possibility is also supported by comparing our data with the results of the 16s rRNA-based approach we used as a method validation tool. *Ca.* Methylumidiphilus was absent from the 16s rRNA reference database, whereas the abundance of unknown γ-MO was high (**Supplementary Methods and Results**).

MOA and NC10-MO Are Potential Cooperators Throughout the Water Column

Higher MOA and NC10-MO abundances in the deeper layers, as well as their correlation with variables associated with low

oxygen (CH_4, NH_4, PO_4, and CO_2), were expected as both taxa are known anaerobic CH_4 oxidizers (Ettwig et al., 2010; Haroon et al., 2013; Vaksmaa et al., 2017). However, it might seem surprising to detect them in every sample, including those from oxic waters. Even more striking was that samples from epilimnion showed similar normalized abundances as the anoxic samples with the highest abundances of MOA and NC10-MO. Furthermore, the strong correlation observed between Archaea and NC10 abundance was consistent in every layer as well as the correlation of these two genera with CH_4 concentration. The fact that the abundances of these two anaerobic genera increased significantly when CH_4 concentration was high even in oxic water makes it unlikely that their presence in the epilimnion is accidental. On the contrary, it would suggest that they might be active in oxic water. While both are considered to be anaerobic organisms, they are known to be the least O_2 tolerant (Guerrero-Cruz et al., 2018) and potentially get more active when O_2 is added to anoxic media (Kampman et al., 2018). They might also benefit from the higher concentration of NO_3 in aerobic conditions. A similar kind of activity of an anaerobic organism in oxic environment has been suggested for methanogenic Archaea (Bogard and del Giorgio, 2016). This may be facilitated by anoxic microniches inside particles (Schramm et al., 1999; Lehto et al., 2014). The sharp increase in the abundance of MOA and NC10-MO in samples with CH_4 concentration over 5 μM in the epi- and metalimnion suggest that they need high CH_4 to strive in oxic conditions. However, as abundances of anaerobic methanotrophs at low CH_4 concentrations are higher in anoxic conditions, it seems more likely that the sharp rise observed in the upper layer is related to the inhibitory effect of O_2. Higher CH_4 concentrations could compensate O_2 limited affinity for CH_4 or reflect the presence of more favorable conditions for anoxic organisms (e.g., anoxic microniches). The strong correlation between the abundance of the two taxa suggests a cooperative interaction between them. Indeed, the most abundant of them, *Ca.* Methanoperedens uses NO_3 as an electron acceptor and releases NO_2 that can be used by *Ca.* Methylomirabilis. This possibility is supported by CH_4-fed enrichment that coselected both *Ca.* Methanoperedens and *Ca.* Methylomirabilis when NO_3 was the only electron acceptor provided (Vaksmaa et al., 2017; Gambelli et al., 2018).

CONCLUSION

Our study represents the first large-scale analysis of methanotrophic communities from oxygen-stratified lakes spanning from Europe to North America. While most of our data come from Scandinavian lakes, the presence of North American ponds suggests a similar pattern for this region. With these data, we confirmed that many of the results gained from analyzing a limited number of lakes are relevant for freshwater bodies above 50°N. Furthermore, we suggest that the ability to consume CH_4 at a low concentration is probably a key element in discriminating between the dominance of α-MO and γ-MO. The first appears to be more stress tolerant with a high affinity for CH_4 and low growth speed, while the latter are strong competitors with low affinity and high growth rate.

Consequently, α-MO dominate the communities when MO represent only a small fraction of the microbiome in the surface layer characterized by low CH_4 concentration. When CH_4 is not a limiting resource, γ-MO not only dominate the MO communities but potentially the whole microbiome. The high affinity of α-MO suggests that despite having lower abundances than γ-MO, they could play an important role in consuming CH_4 when concentration are not suitable for fast-growing γ-MO, and α-MO could have a significant role in diminishing the emissions from the recently suggested CH_4 production in the oxic water column (Bogard and del Giorgio, 2016; Günthel et al., 2020; Li et al., 2020). Thus, while representing low abundance organisms, the α-MO could be critical for limiting CH_4 emissions from lakes as CH_4 oversaturation of oxic layer is a widespread phenomenon (Blees et al., 2015; León-Palmero et al., 2020). Among γ, *Candidatus* Methylumidiphilus was found in all lakes and appeared to be the genus responsible for peaks of the relative abundance of MO. Therefore, it is suggested to play an important role in diminishing the CH_4 emissions from the boreal lakes and arctic thaw ponds. Overall, our results significantly improve our knowledge on the diversity and abundance of methanotrophs and strongly suggest that the abundance and diversity of the methanotrophs in any single lake are strongly dependent on specific conditions of that particulate lake. Thus, these communities are controlled by local rather than global factors.

DATA AVAILABILITY STATEMENT

Publicly available datasets were generated for this study. This data can be found here: https://www.ncbi.nlm.nih.gov/bioproject/PRJEB38681.

AUTHOR CONTRIBUTIONS

GM, AR, MB, SG, and SP designed the study. GM carried out data analyses with help from MM. GM was responsible for data interpretation with regular input of AR, SG, and SP. All authors discussed the results and conclusion. GM led manuscript writing. All authors participated with substantial comments and edits of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.669937/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary material

Supplementary Table S1: **Summary of relative abundances and dominances of all methane oxidizer (MO) genera present in our data set**. Abundances are the proportions of reads attributed to MO in the whole data set. Dominances are the proportions of reads attributed to a certain MO taxon among all the reads attributed to MO.

Taxa	Mean abundance	Median abundance	Max abundance	Mean dominance	Median dominance	Max dominance
Methylumidiphilus	2.678	0.281	55.724	25.583	12.911	97.140
Methylobacter	0.691	0.344	5.177	19.147	20.523	45.208
Methylomonas	0.369	0.224	3.222	11.582	11.394	51.166
Crenothrix	0.265	0.120	2.332	7.156	7.079	33.503
Methylovulum	0.221	0.104	1.441	5.756	5.772	20.021
Methylomicrobium	0.089	0.054	0.607	2.741	2.898	7.178
Methylosarcina	0.088	0.057	0.469	2.838	2.776	7.136
Methylocystis	0.085	0.055	0.618	4.507	2.905	25.940
Methylocaldum	0.074	0.045	0.948	2.164	2.084	8.347
Methyloglobulus	0.053	0.030	0.330	1.605	1.709	3.711
Methylocapsa	0.051	0.029	0.729	2.637	1.478	19.249
Methyloterricola	0.050	0.024	1.047	1.200	1.124	3.759
Methylomagnum	0.047	0.028	0.565	1.412	1.259	8.119
Methylocella	0.044	0.021	1.393	2.164	1.066	36.773
Methylococcus	0.042	0.027	0.376	1.479	1.114	14.908
Methylocucumis	0.037	0.017	0.288	1.000	0.992	4.722
Methylosinus	0.034	0.024	0.178	1.694	1.205	9.582
Methylomarinum	0.024	0.015	0.137	0.797	0.809	1.695
Methanoperedens	0.020	0.015	0.082	0.881	0.589	4.880
Methyloferula	0.018	0.012	0.101	0.971	0.565	7.276
Methyloprofundus	0.018	0.010	0.105	0.550	0.556	1.504
Methylomirabilis	0.016	0.013	0.055	0.685	0.610	2.460
Methylacidiphilum	0.016	0.012	0.047	0.773	0.605	4.348
Methylogaea	0.011	0.008	0.115	0.362	0.353	0.982
Methylohalobius	0.008	0.007	0.170	0.316	0.280	1.420

Supplementary Table S2: Spearman's rank correlation coefficients (rho) between the abundances and environmental variables in the epilimnion. All p-values (p) were corrected using the Bonferroni method.

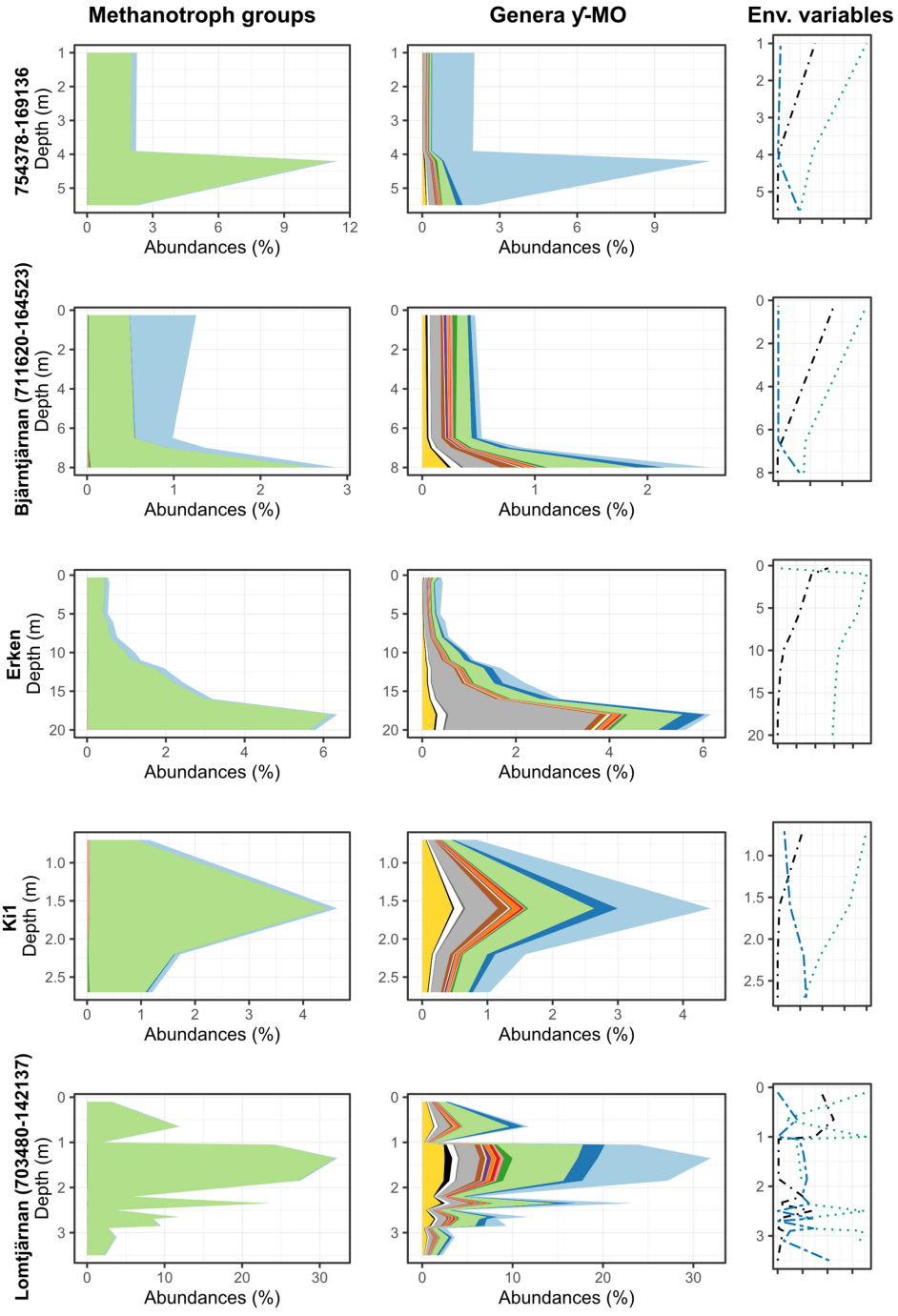
	MO		**α-MO**		**γ-MO**		**MOA**		**NC10-MO**		**V-MO**	
	rho	**p**	**rho**	**p**	**rho**	**p**	**rho**	**p**	**rho**	**p**	**rho**	**p**
O_2	-0.37	0.19	0.13	1.00	-0.47	0.01	-0.36	0.28	-0.39	0.11	-0.16	1.00
Temperature	-0.34	0.35	0.29	1.00	-0.42	0.03	-0.29	1.00	-0.45	0.01	-0.06	1.00
CH_4	0.57	0.00	-0.03	1.00	0.56	0.00	0.62	0.00	0.34	0.77	0.28	1.00
CO_2	0.32	1.00	-0.40	0.20	0.36	0.49	0.28	1.00	0.34	0.85	-0.04	1.00
pH	-0.08	1.00	-0.70	0.00	0.09	1.00	-0.21	1.00	0.02	1.00	-0.20	1.00
NH_4	0.25	1.00	-0.34	1.00	0.25	1.00	0.23	1.00	0.28	1.00	-0.09	1.00
NO_3	-0.28	1.00	-0.06	1.00	-0.41	0.68	-0.56	0.02	-0.47	0.21	-0.62	0.00
PO_4	0.18	1.00	0.37	1.00	0.08	1.00	-0.07	1.00	0.10	1.00	0.15	1.00
SO_4	-0.02	1.00	-0.47	0.19	0.03	1.00	-0.22	1.00	-0.07	1.00	-0.58	0.01
Fe	-0.10	1.00	0.15	1.00	-0.15	1.00	0.17	1.00	-0.03	1.00	0.02	1.00

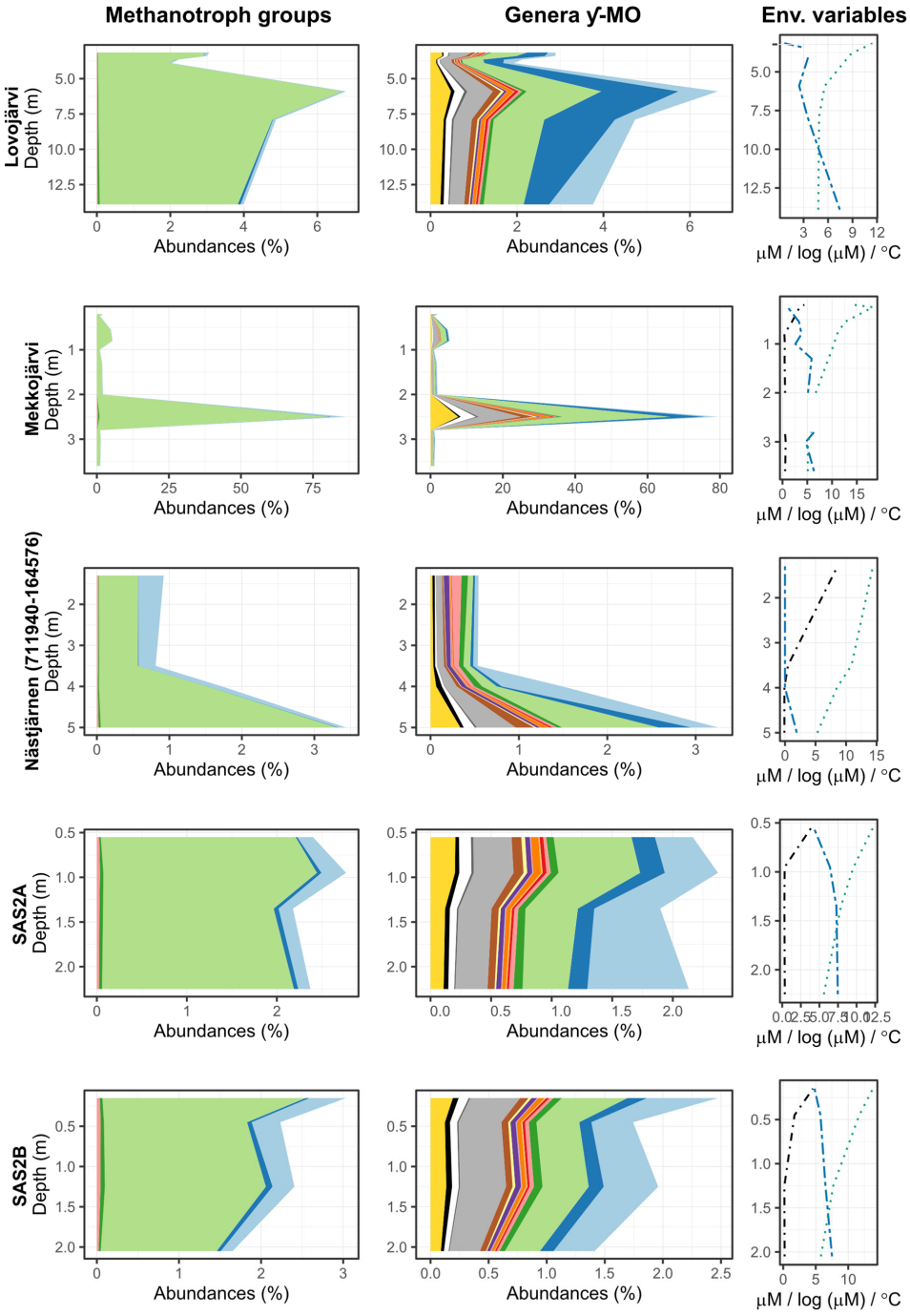
Supplementary Table S3: Spearman's rank correlation coefficients (rho) between the abundances and environmental variables in the metalimnion. All p-values (p) were corrected using the Bonferroni method.

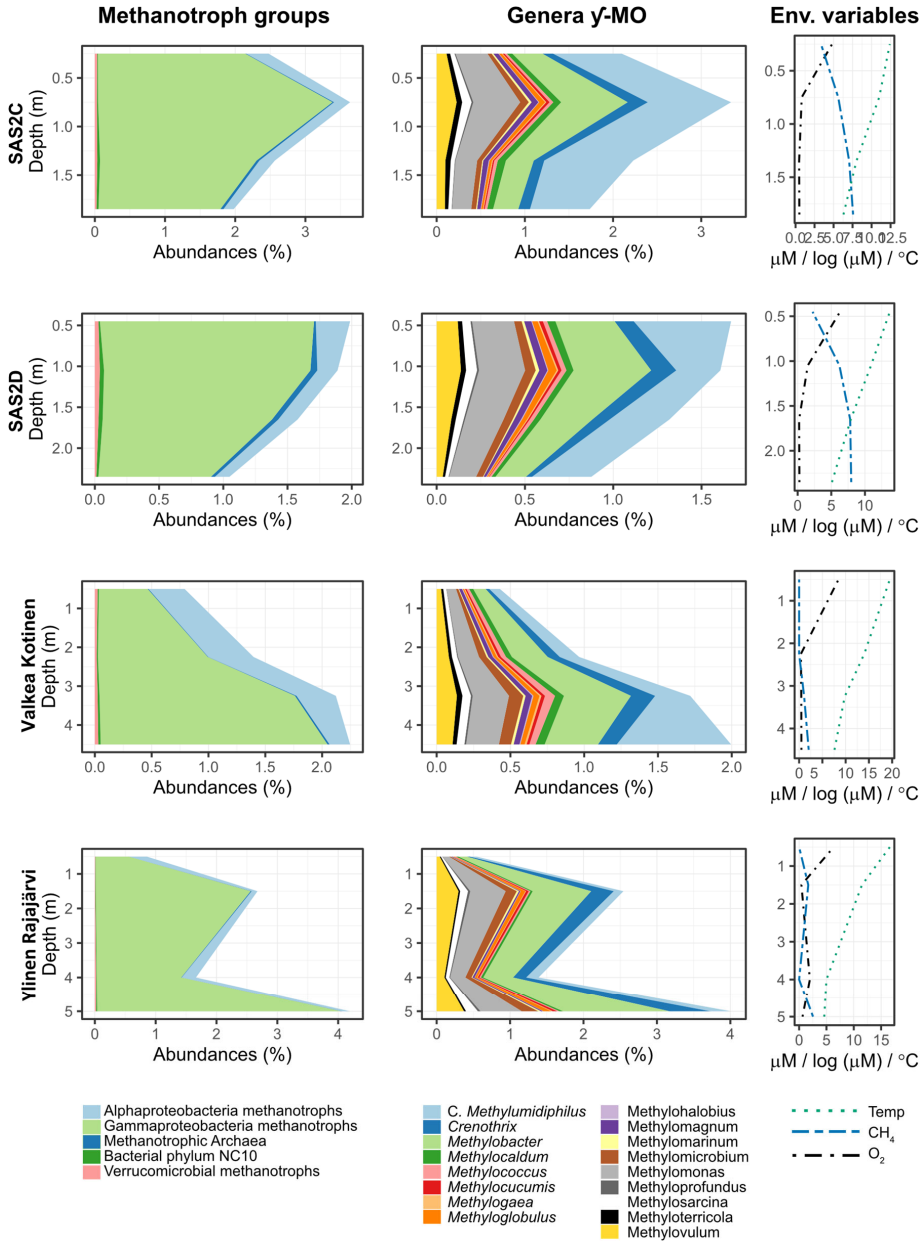
	MO		**α-MO**		**γ-MO**		**MOA**		**NC10-MO**		**V-MO**	
	rho	**p**	**rho**	**p**	**rho**	**p**	**rho**	**p**	**rho**	**p**	**rho**	**p**
O2	-0.12	1	0.32	1.00	-0.16	1	-0.24	1.00	-0.16	1.00	-0.15	1.00
Temperature	-0.02	1	0.26	1.00	-0.04	1	-0.36	0.98	-0.25	1.00	-0.15	1.00
CH4	0.30	1	-0.43	0.25	0.34	1	0.78	0.00	0.36	1.00	0.10	1.00
CO2	0.31	1	-0.26	1.00	0.33	1	0.75	0.00	0.55	0.01	0.19	1.00
pH	-0.27	1	-0.37	1.00	-0.27	1	0.50	0.74	0.54	0.37	-0.13	1.00
NH4	0.15	1	-0.36	1.00	0.16	1	0.58	0.02	0.42	0.75	0.06	1.00
NO3	-0.17	1	-0.29	1.00	-0.14	1	-0.44	0.60	-0.55	0.06	-0.58	0.02
PO4	-0.21	1	0.02	1.00	-0.21	1	-0.01	1.00	-0.29	1.00	-0.22	1.00
SO4	-0.06	1	-0.44	0.27	-0.01	1	0.12	1.00	-0.15	1.00	-0.60	0.00
Fe	-0.40	1	-0.09	1.00	-0.38	1	0.36	1.00	-0.06	1.00	-0.07	1.00

Supplementary Table S4: Spearman's rank correlation coefficients (rho) between the abundances and environmental variables in the hypolimnion. All p-values (p) were corrected using the Bonferroni method.

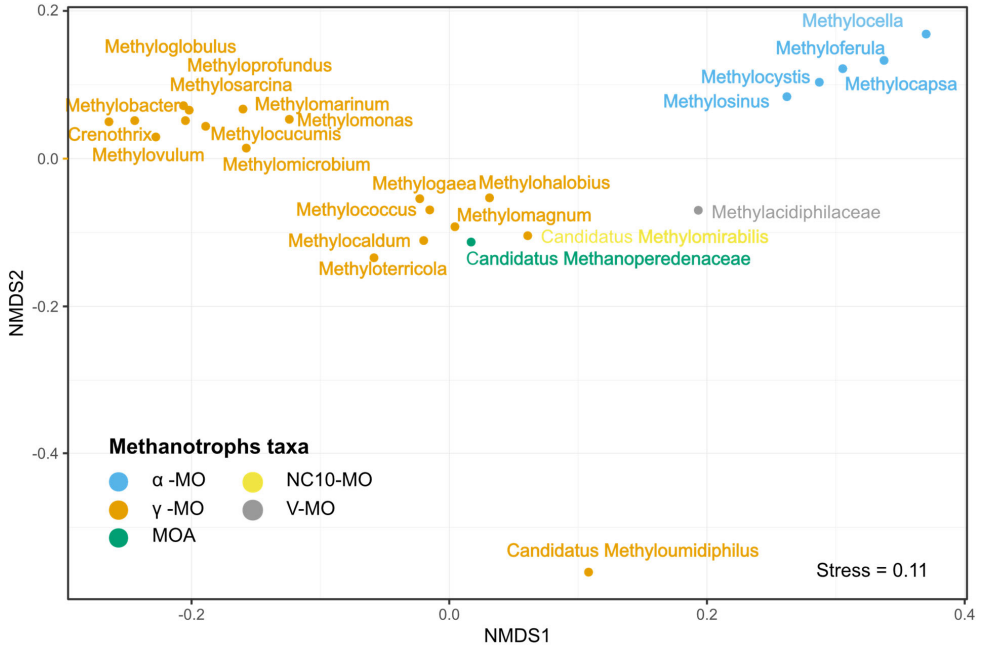
	MO		**α-MO**		**γ-MO**		**MOA**		**NC10-MO**		**V-MO**	
	rho	**p**	**rho**	**p**	**rho**	**p**	**rho**	**p**	**rho**	**p**	**rho**	**p**
O2	0.14	1.00	0.03	1.00	0.12	1.00	0.40	0.15	0.32	0.91	0.52	0.00
Temperature	-0.37	0.35	0.28	1.00	-0.35	0.51	-0.58	0.00	-0.57	0.00	-0.32	1.00
CH4	-0.25	1.00	-0.24	1.00	-0.25	1.00	0.71	0.00	0.47	0.02	0.36	0.42
CO2	-0.39	0.20	-0.34	0.64	-0.37	0.29	0.42	0.08	0.15	1.00	-0.08	1.00
pH	0.26	1.00	-0.64	0.02	0.30	1.00	0.07	1.00	0.11	1.00	-0.28	1.00
NH4	0.02	1.00	-0.32	1.00	0.04	1.00	0.58	0.00	0.51	0.02	0.29	1.00
NO3	0.29	1.00	0.16	1.00	0.29	1.00	-0.40	0.41	-0.18	1.00	0.02	1.00
PO4	-0.13	1.00	0.30	1.00	-0.15	1.00	-0.10	1.00	0.08	1.00	0.32	1.00
SO4	0.07	1.00	-0.36	0.46	0.09	1.00	-0.25	1.00	-0.15	1.00	-0.46	0.03
Fe	-0.39	0.64	-0.15	1.00	-0.40	0.57	0.17	1.00	0.02	1.00	-0.04	1.00


Methanotroph groups
Genera γ-MO
Env. variables
754378-169136
Depth (m)
Bjärntjärnan (711620-164523)
Depth (m)
Erken
Depth (m)
Ki1
Depth (m)
Lomtjärnan (703480-142137)
Depth (m)
Abundances (%)

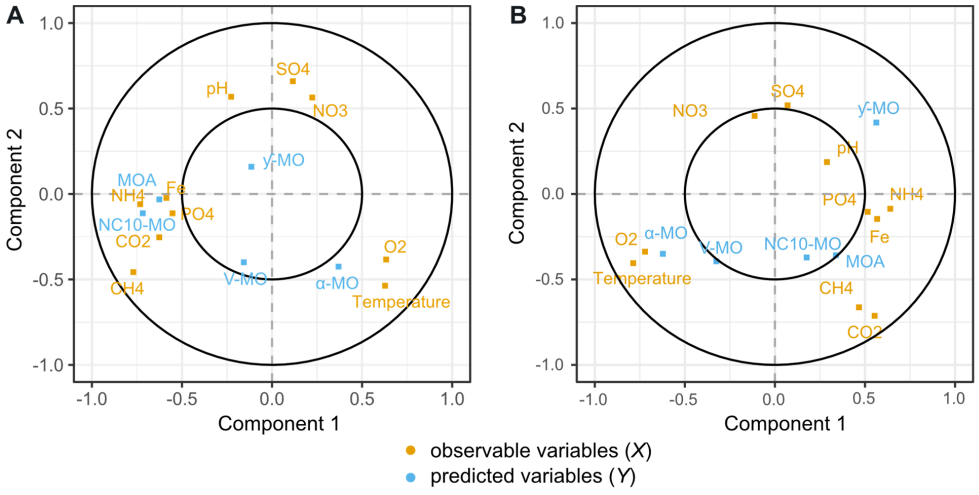

Methanotroph groups
Genera γ-MO
Env. variables
Lovojärvi
Depth (m)
Mekkojärvi
Depth (m)
Nästjärnen (711940-164576)
Depth (m)
SAS2A
Depth (m)
SAS2B
Depth (m)
Abundances (%)
µM / log (µM) / °C


Methanotroph groups
Genera γ-MO
Env. variables
SAS2C
SAS2D
Valkea Kotinen
Ylinen Rajajärvi
Depth (m)
Abundances (%)
µM / log (µM) / °C
Alphaproteobacteria methanotrophs
Gammaproteobacteria methanotrophs
Methanotrophic Archaea
Bacterial phylum NC10
Verrucomicrobial methanotrophs
C. Methylumidiphilus
Crenothrix
Methylobacter
Methylocaldum
Methylococcus
Methylocucumis
Methylogaea
Methyloglobulus
Methylohalobius
Methylomagnum
Methylomarinum
Methylomicrobium
Methylomonas
Methyloprofundus
Methylosarcina
Methyloterricola
Methylovulum
Temp
CH4
O2

Supplementary Figure S1. Vertical variation in the relative abundances of methane oxidizers (MO) (left column) and different genera of gammaproteobacterial methanotrophs (middle column), as well as in key environmental (Env.) variables (CH_4 and O_2 concentration and temperature; right column). Profiles are presented for all the lakes that have data collected from at least three depths. Note the change in the scale of abundance and depth from one lake to another.

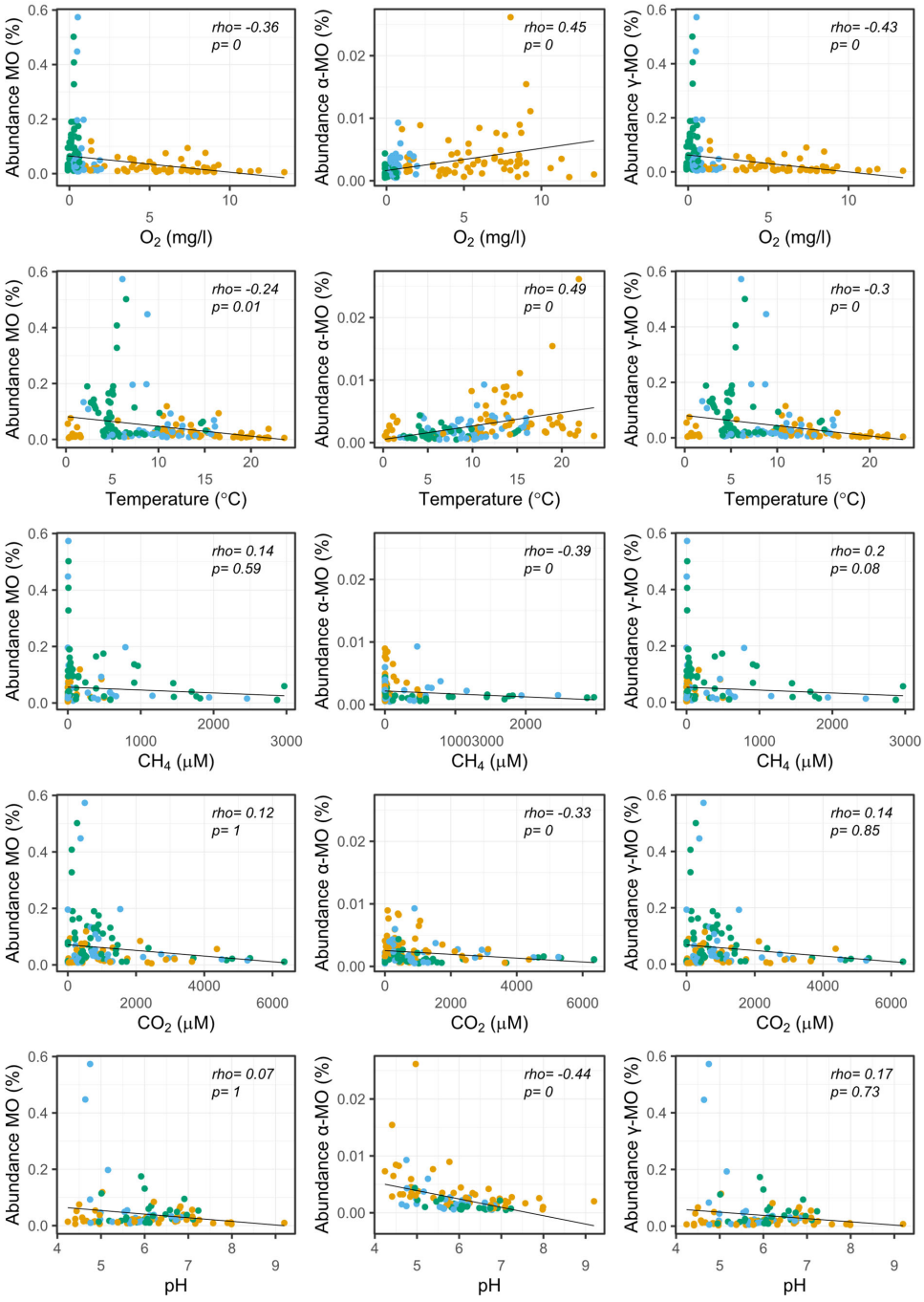


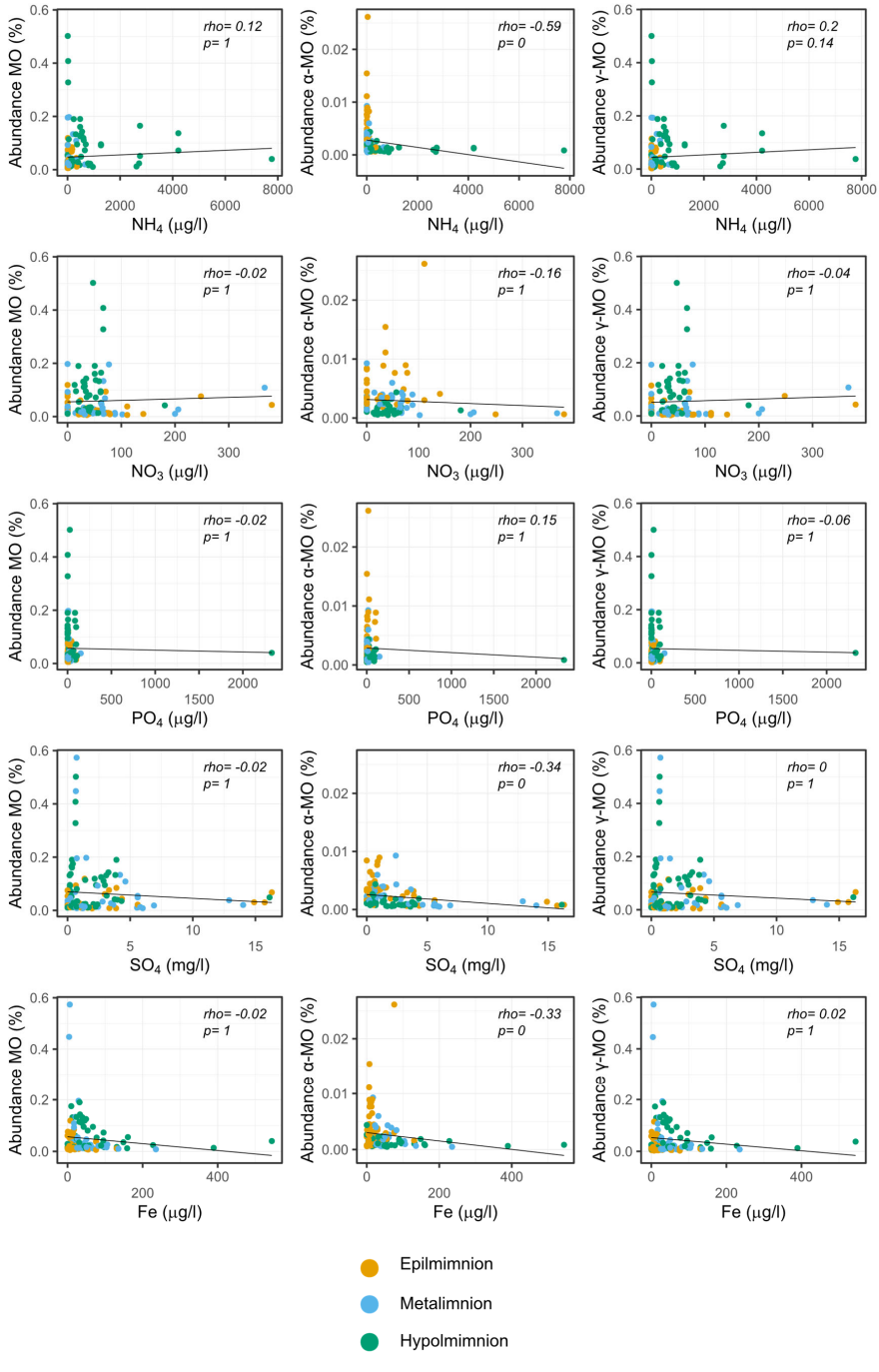
Supplementary Figure S2. **Non-metric multidimensional scaling plot (NMDS) of the methanotrophic (MO) genera.** NMDS representing Bray-Curtis dissimilarities between MO taxa based on their relative abundances in each of the samples. The Bray-Curtis dissimilarities matrix was calculated for the abundances of MO genus only. Abbreviations for the taxonomic groups are: alphaproteobacterial methanotrophs (α-MO), gammaproteobacterial methanotrophs (γ-MO), archaeal methanotrophs (MOA), bacterial phylum NC10 (NC10-MO), and Verrucomicrobial methanotrophs (V-MO).



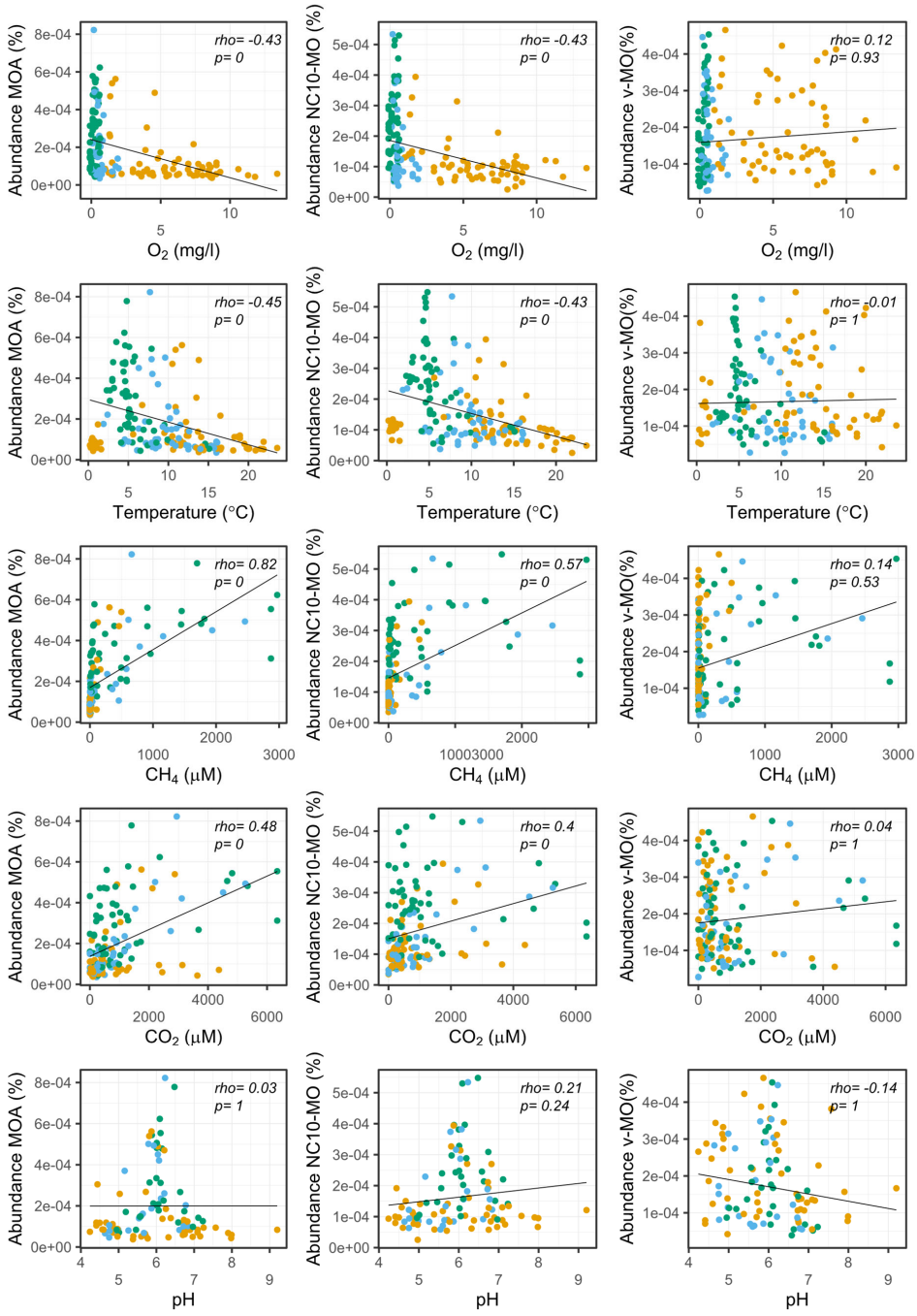
Supplementary Figure S3. Correlation plot of the Partial Least Squares (PLS) regression analysis to evaluate the potential influence of environmental variables on (A) the relative abundance and (B) the dominance of methane oxidizers (MO). Components axis represent latent variables (i.e. a linear combinations of the original observed variables). Position of the observed variables (*X*) indicates how much each *X* variables explains the component axis. Positions of the predicted variables (*Y)* indicate how much of each *Y* variables variance is carried by the latent variable. A variable (*X* or *Y*) that stands right on an axis is only explains (*X)*/ is explained (*Y*) by that latent variable. The distance to the axis indicates the strength of the correlation between the variables (*X or Y*) and the latent variables (component 1 or 2). Consequently, the angles between variables indicate if they are correlated to each other. A null or 180° angle suggest a strong correlation whereas a 90° angle excludes the possibility of a correlation. The strength of the actual correlation can be attenuated by the distance to the center and by the explained variances carried by each component axis. Explained variances carried by the latent variables were 0.25 (comp1) and 0.16 (comp 2) for *X* and 0.41 (comp1) and 0.52 (comp 2) for *Y*.

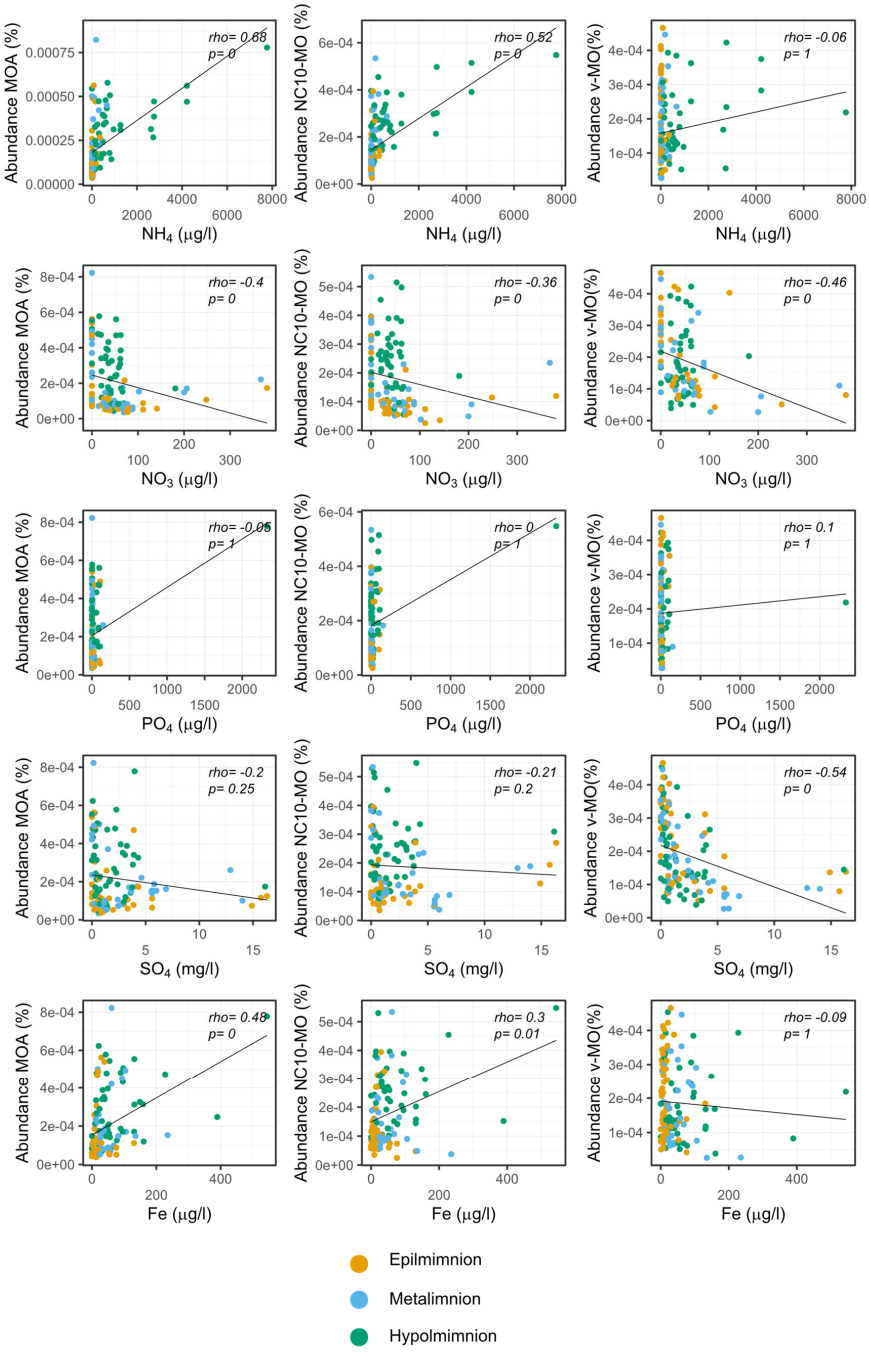
Abundances are the proportions of reads attributed to MO in the whole set of reads. Dominances are the proportion of reads attributed to a certain MO taxon among all the reads attributed to MO. The MO taxa included to the analysis are gammaproteobacterial methane oxidizers (γ-MO), alphaproteobacterial methanotrophs (α-MO), methanotrophic Archaea (MOA), bacterial phylum NC10 (NC10-MO), and Verrucomicrobial methanotrophs (V-MO).


Abundance MO (%)
Abundance α-MO (%)
Abundance γ-MO (%)
rho= -0.36
p= 0
rho= 0.45
p= 0
rho= -0.43
p= 0
O2 (mg/l)
rho= -0.24
p= 0.01
rho= 0.49
p= 0
rho= -0.3
p= 0
Temperature (°C)
rho= 0.14
p= 0.59
rho= -0.39
p= 0
rho= 0.2
p= 0.08
CH4 (µM)
rho= 0.12
p= 1
rho= -0.33
p= 0
rho= 0.14
p= 0.85
CO2 (µM)
rho= 0.07
p= 1
rho= -0.44
p= 0
rho= 0.17
p= 0.73
pH


Abundance MO (%)
rho= 0.12
p= 1
NH4 (µg/l)
Abundance α-MO (%)
rho= -0.59
p= 0
NH4 (µg/l)
Abundance γ-MO (%)
rho= 0.2
p= 0.14
NH4 (µg/l)
Abundance MO (%)
rho= -0.02
p= 1
NO3 (µg/l)
Abundance α-MO (%)
rho= -0.16
p= 1
NO3 (µg/l)
Abundance γ-MO (%)
rho= -0.04
p= 1
NO3 (µg/l)
Abundance MO (%)
rho= -0.02
p= 1
PO4 (µg/l)
Abundance α-MO (%)
rho= 0.15
p= 1
PO4 (µg/l)
Abundance γ-MO (%)
rho= -0.06
p= 1
PO4 (µg/l)
Abundance MO (%)
rho= -0.02
p= 1
SO4 (mg/l)
Abundance α-MO (%)
rho= -0.34
p= 0
SO4 (mg/l)
Abundance γ-MO (%)
rho= 0
p= 1
SO4 (mg/l)
Abundance MO (%)
rho= -0.02
p= 1
Fe (µg/l)
Abundance α-MO (%)
rho= -0.33
p= 0
Fe (µg/l)
Abundance γ-MO (%)
rho= 0.02
p= 1
Fe (µg/l)
Epilmimnion
Metalimnion
Hypolmimnion

Supplementary Figure S4. Relationships between the environmental variables and the relative abundances of the most abundant methane oxidizers (MO). The plots include the alphaproteobacterial methanotrophs (α-MO), gammaproteobacterial methane oxidizers (y-MO) and the total methanotrophic community (MO). Rho indicates Spearman's rank correlation coefficients. P-values were adjusted for multiple comparisons using the Bonferroni method. Null p-values indicate $p \leq 0.005$.


Abundance MOA (%)
Abundance NC10-MO (%)
Abundance v-MO(%)
rho= -0.43
p= 0
rho= 0.12
p= 0.93
O2 (mg/l)
rho= -0.45
p= 0
rho= -0.01
p= 1
Temperature (°C)
rho= 0.82
p= 0
rho= 0.57
p= 0
rho= 0.14
p= 0.53
CH4 (µM)
rho= 0.48
p= 0
rho= 0.4
p= 0
rho= 0.04
p= 1
CO2 (µM)
rho= 0.03
p= 1
rho= 0.21
p= 0.24
rho= -0.14
p= 1
pH


Abundance MOA (%)
Abundance NC10-MO (%)
Abundance v-MO(%)
rho= 0.68
p= 0
rho= 0.52
p= 0
rho= -0.06
p= 1
NH4 (µg/l)
rho= -0.4
p= 0
rho= -0.36
p= 0
rho= -0.46
p= 0
NO3 (µg/l)
rho= -0.05
p= 1
rho= 0
p= 1
rho= 0.1
p= 1
PO4 (µg/l)
rho= -0.2
p= 0.25
rho= -0.21
p= 0.2
rho= -0.54
p= 0
SO4 (mg/l)
rho= 0.48
p= 0
rho= 0.3
p= 0.01
rho= -0.09
p= 1
Fe (µg/l)
Epilimnion
Metalimnion
Hypolimnion

Supplementary Figure S5. The relationships between the environmental variables and the relative abundances of the rare methane oxidizers. The plots include archaeal methanotrophs (MOA), bacterial phylum NC10 (NC10-MO), and verrucomicrobial methanotrophs (V-MO). Rho indicates Spearman's rank correlation coefficients. P-values were adjusted for multiple comparisons using the Bonferroni method and rounded to two digits. Null p-values indicate $p \leq 0.005$.

Supplementary Methods and Results: Comparison of relative abundances obtained with alternative method

Methods

To confirm the results obtained with Kaiju, we also extracted 16S rRNA genes from the metagenomes. For this purpose, a subset of 10 million read was separated for each of metagenomic samples. Reads affiliated to ribosomal RNA genes (16S/18S) were detected in these subsets using SSU-ALIGN software (Nawrocki, 2009). Putative prokaryotic 16S rRNA sequences were compared against the SILVA reference database (release 132SSUParc) using BLAST. Taxonomic affiliations of the reads were assigned based on their closest hit if the read was ≥ 90bp, at the similarity threshold of ≥ 90. Relative abundances calculated with the Kaiju data were then compared with the relative abundances of putative prokaryotic 16S rRNA sequences assigned for the same taxa. The SILVA database does not have a 16S rRNA sequence of *Ca.* Methylumidiphilus, thus it is frequently assigned as unclassified y-MO (Rissanen et al., 2018). For the analyses here, we used unclassified y-MO as an indicator for *Ca.* Methylumidiphilus.

Results

A total of 1166193 reads were assigned to putative prokaryotic 16S rRNA sequences. Number of assigned reads for the samples ranged from 246 to 11637, with mean and median values of 5399 and 5416 respectively. Among these reads, 63597 (5.5 %) were attributed to MO. The relative abundances of MOs in the samples varied from 0.1 % to 46 %.

Relative abundances of total MO and y-MO were strongly correlated between the Kaiju and 16S rRNA gene read data sets (rho >0.8) and values were in the same order of magnitude. It also appeared that the relation between the two data sets changed depending on what taxa dominated the MO community (Supplementary Figure SM1A and SM1B). This indicates that while very different in their approach (Kaiju is protein-level classifier, whereas the validation methods is based on ribosomal RNA genes) the two methods give similar results. The difference in slope for samples depending on their most dominant y-MO suggests, that Kaiju could overestimate MO in samples dominated by *Ca.* Methylumidiphilus or underestimate them in samples dominated by Methylobacter. Or alternatively, the 16S rRNA method was under- and overestimating these, respectively. For Kaiju the estimates could be affected by the relatively large size of *Ca.*

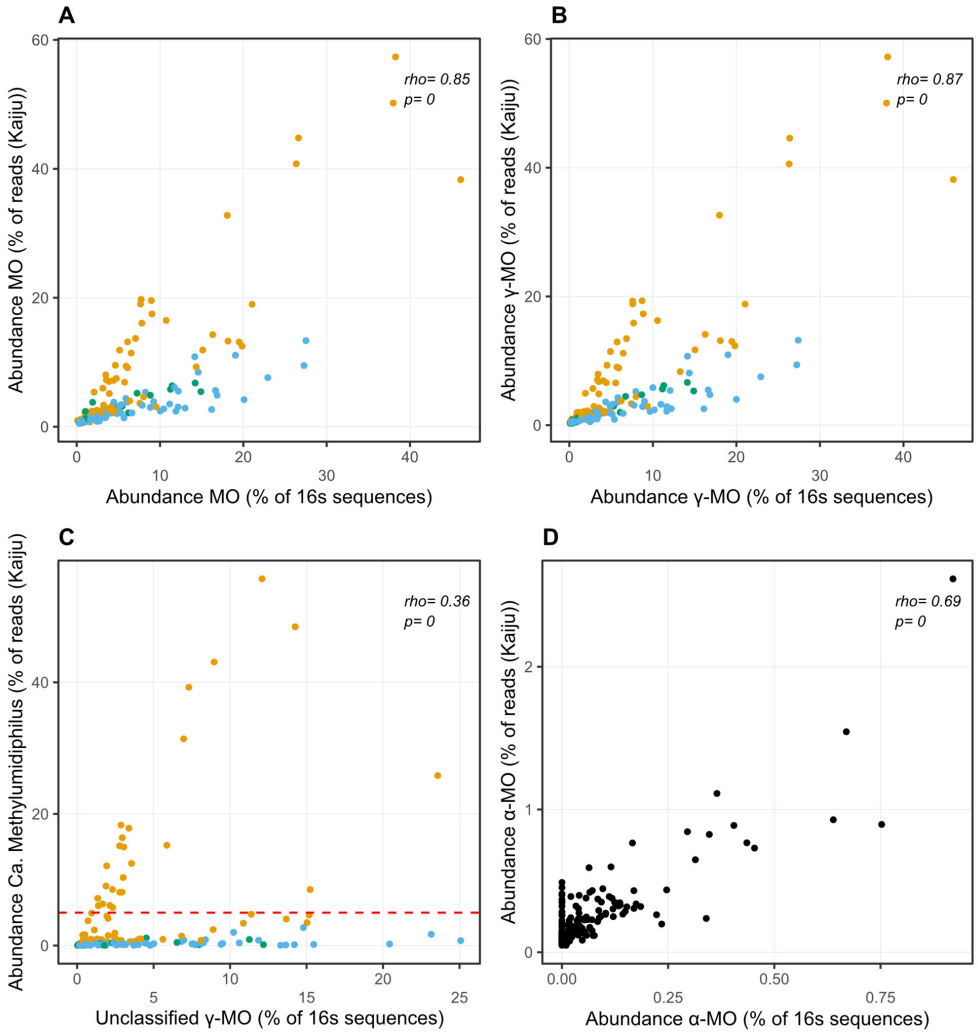
Methylumidiphilus genome (6.6 Mb). However, since there is no reference for *Ca.* Methylumidiphilus in the Silva database, this is the natural cause of lack of observations and the algorithm is likely to dismiss some reads that otherwise would have been counted as y-MO. However, interestingly not all samples dominated by *Ca.* Methylumidiphilus showed signs of a potential overestimation by Kaiju. This suggests that the over/under estimation observed could be the result a combination of the two explanations or that other variables could be at play. Despite this difference, the two methods clearly yield comparable results when it comes to the total abundances of MO and y-MO. Importantly, despite the lack of *Ca.* Methylumidiphilus 16S rRNA sequence in the database, the 16S rRNA gene approach confirmed that the highest relative abundances of MO are recorded in samples dominated by *Ca.* Methylumidiphilus.

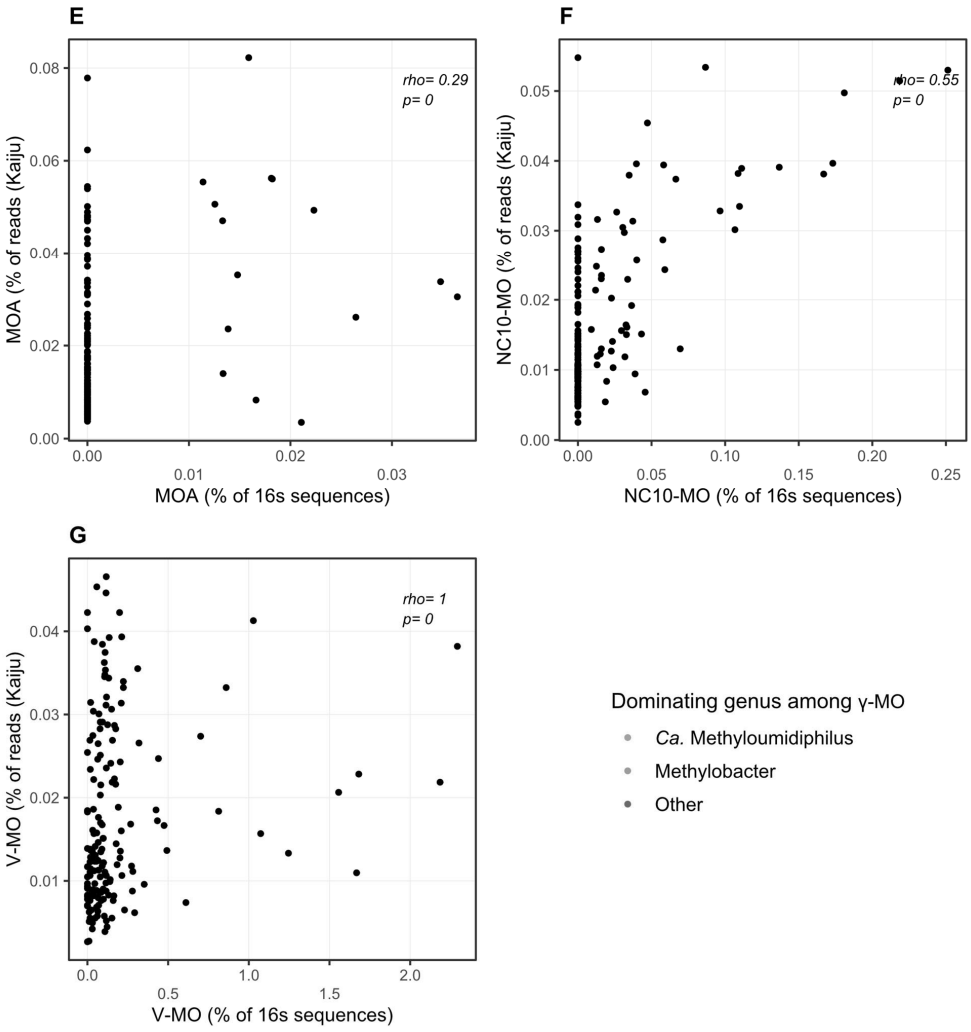
As stated, 16S rRNA gene sequences of *Ca.* Methylumidiphilus were absent in the Silva database at the time of this analysis. *Methylospira* and *Methyloterricola*, which are the closest relatives of relative of *Ca.* Methylumidiphilus, were respectively in very low abundances or not detected in the samples, suggesting that 16S rRNA gene reads of Ca. Methylumidiphilus were not taxonomically assigned to its two closest genera. When we compared the abundance of *Ca*. Methylumidiphilus obtained with Kaiju to the abundances 16S rRNA sequences of unclassified y-MO, the overall correlation was low (rho = 0.36), but this relation changed a lot depending on most the dominant y-MO and the abundance of *Ca.* Methylumidiphilus. When the relative abundance of *Ca.* Methylumidiphilus was above 5 %, its correlation (rho) with the 16S rRNA gene-based abundances of uncultivated y-MO raised to 0.75 (Supplementary Figure SM1C). This suggest that some of putative prokaryotic 16S rRNA sequences detected by SSU-ALIGN were very probably from *Ca*. Methylumidiphilus. This suggests that out hypothesis, that *Ca*. Methylumidiphilus might be a common MO that has escaped detection so far, is correct.

For α-MO the Spearman's rank correlation coefficient between the two 16S rRNA gene and Kaiju datasets was 0.69. This correlation value was strongly influenced by the samples where no α-MO were detected within the 16S rRNA sequences. If those 61 samples were removed from the data set, the Spearman's rank correlation coefficient was 0.78 (Supplementary Figure SM1D). As for MO and y-MO, this indicates that both methods yield comparable results. Using the 16S rRNA gene approach, MOA and NC10-MO were not detected in most samples (152 and 115 respectively) and the correlations between the results of the two methods were poor (Supplementary figure SM1E and SM1F). Finally, no clear pattern was observed for V-MO (Supplementary figure SM1G).

The absence of low abundance MOs in many of the samples when the 16S rRNA gene method is used, suggests that this approach is more likely to miss low abundance taxa, due to the fact that the 16S rRNA gene presents only a fraction of the whole genome. Thus, whole genome based methods, such as Kaiju, detecting the whole genome are superior for the studying of the rare taxa. This is exemplified by the fact that for the 16S rRNA gene method only 1166193 reads were assigned to putative prokaryotic 16S rRNA sequences with most samples including less than 5500 reads that matched to the 16S rRNA gene. On the other hands the kaiju data set was

composed of 2.56 * 10^8 reads (1230562 reads per sample). In conclusion, the limited number of reads assigned to 16S rRNA gene combined with the absence of *Ca*. Methylumidiphilus strongly supports our approach of using Kaiju. Furthermore, we showed that for the taxa with abundances high enough to be detected by the 16S rRNA approach, both the protein-level classifier and the ribosomal RNA gene reads affiliation gives comparable results.





Supplementary Figure SM1. Correlations between the relative abundances of MO taxa based on Kaiju and SSU-ALIGN (Putative prokaryotic 16S rRNA sequences) assignments. Each panel (A-G) represent the correlation between relative abundances calculated for the same taxon but based on a different method of reads assignment. Abbreviations for the taxonomic groups are: alphaproteobacterial methanotrophs (α-MO), gammaproteobacterial methanotrophs (γ-MO), archaeal methanotrophs (MOA), bacterial phylum NC10 (NC10-MO), and Verrucomicrobial methanotrophs (V-MO). MO corresponds to the total methanotrophic community.

Color mapping was added in panels A, B and C to show which γ-MO genus dominated samples that included γ-MO. The dominating genera were determined using the Kaiju dataset. In panel C, the red dashed line marks a threshold at 5% of *Ca.* Methylumidiphilus. This threshold was used to calculate an approximate rho value for the set of samples dominated by *Ca.* Methylumidiphilus (the samples aligned on the left side of the plot). When only samples with *Ca.* Methylumidiphilus abundance above 5% are considered, the rho value is 0.75.

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Greenhouse gas (GHG) emissions from boreal lakes are the net balance between production and consumption of those gases. The consensus has been that GHG emissions from boreal lakes will increase with climate change. The work presented in this thesis highlights several understudied microbial processes with a potential to mitigating some effects of changes to come.

Gaëtan Martin received is graduate education at the Department of Forest Mycology and Plant Pathology, SLU, Uppsala. He received his M.Sc. in Biogeosciences at the University of Neuchâtel, Neuchâtel, Switzerland.

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