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LINKING BIOGEOCHEMISTRY AND MICROBIAL ECOLOGY TO UNDERSTAND THE FATE OF METHANE IN LAKES AND HYDROPOWER RESERVOIRS

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COUPLER LA BIOGÉOCHIMIE À L'ÉCOLOGIE MICROBIENNE POUR COMPRENDRE LE SORT DU MÉTHANE DANS LES LACS ET LES RÉSERVOIRS HYDROÉLECTRIQUES

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DEDICATION

I dedicate this work to my nephew Mateus,
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Te amo, Tequinho!

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RÉSUMÉ

Le méthane (CH_4) est un puissant gaz à effet de serre avec un potentiel de réchauffement 34 fois plus élevé que le dioxyde de carbone. Les lacs et les réservoirs d'eau douce sont des sources importantes de CH_4 vers l'atmosphère, mais une large fraction du CH_4 produit dans ces écosystèmes est oxydée par des bactéries oxydatives du méthane (BOM), et n'atteint jamais l'atmosphère. Malgré l'importance bien connue de l'oxydation du CH_4 dans l'atténuation des émissions de CH_4 vers l'atmosphère, beaucoup moins de connaissances existent quant à comment l'environnement et les traits de la communauté BOM interagissent pour déterminer le sort du CH_4 dans les lacs et réservoirs. Pour aider à résoudre cette problématique, cette thèse étudie les liens entre l'environnement, les BOM (du niveau cellulaire à la communauté), et l'oxydation du CH_4 dans les lacs et dans un complexe hydroélectrique.

Le chapitre I examine le couplage entre la structure de la communauté BOM et les taux d'oxydation du CH_4 dans la colonne d'eau de six lacs tempérés d'eau douce. Les résultats montrent une séparation verticale de niche au sein de la communauté BOM, principalement modulée par la concentration en oxygène. Les approches de modélisation suggèrent que les facteurs environnementaux et la composition de la communauté BOM influencent tous les deux les taux estivaux d'oxydation du CH_4 dans les lacs, avec un effet indépendant de l'abondance des BOM-Gamma.

Le chapitre II examine comment le CH_4 et l'abondance, la composition, et l'activité de la communauté BOM changent dans l'eau d'amont en aval d'un complexe hydroélectrique tropical. Les changements de la communauté BOM le long du système rivière-réservoir reflètent un tri environnemental des taxons et une interruption de la connectivité hydrologique. Le pic de BOM se trouvait dans l'hypolimnion hypoxique dans le bassin principal du réservoir, où l'oxydation microbienne du CH_4 déconnecte le CH_4 en profondeur des émissions de CH_4 vers l'atmosphère. L'oxydation du CH_4 dans la rivière en aval était accompagnée d'un changement rapide de la communauté, avec une croissance préférentielle des BOM-Alpha, permettant la réduction des émissions de CH_4 également dans les eaux en aval du barrage.

Le chapitre III montre que, bien que généralement présentes en faibles abondances dans les lacs, les cellules BOM, particulièrement au sein des BOM-Gamma, sont plus grandes et plus actives que le reste de la communauté bactérienne. À hauts ratios molaires CH_4 : O_2, les BOM ont un avantage écologique par rapport aux bactéries hétérotrophes, métabolisant une plus grande quantité totale de carbone. À l'échelle d'un lac, l'importance relative de la méthanotrophie dépend de la concentration en carbone organique dissout (COD) et de la morphométrie, à travers leurs effets sur la stratification et l'oxygénation de la colonne d'eau. Dans les lacs riches en COD, la méthanotrophie peut être le processus principal de transformation du carbone, remettant en question notre compréhension actuelle de la boucle microbienne.

Dans l'ensemble, les résultats des trois chapitres montrent que le sort du CH_4 dans les lacs et complexes hydroélectriques dépend des caractéristiques environnementales et microbiennes. L'environnement contrôle les conditions de base pour que l'oxydation du CH_4 se produise, et façonne aussi la composition, l'abondance, et l'activité des BOM, ce qui peut à son tour affecter le sort du CH_4 dû à des différences intrinsèques de niche, de métabolisme, et de taille de cellule. Différents taxons de BOM peuvent être responsables de taux d'oxydation locaux dépendamment des conditions environnementales, et les caractéristiques métaboliques des BOM influencent l'importance de la méthanotrophie dans le cycle du carbone à l'échelle de l'écosystème et dans le potentiel du CH_4 à supporter les réseaux trophiques dans les lacs.

Mots-clés : méthanotrophe, bactérie, méthane, lac, hydroélectricité.

ABSTRACT

Methane (CH_4) is a potent greenhouse gas, with a global warming potential approximately 34 times greater than carbon dioxide. Lakes and freshwater reservoirs are important sources of CH_4 to the atmosphere, but a large fraction of the CH_4 produced in these ecosystems is oxidized by methane-oxidizing bacteria (MOB) and never evades to the atmosphere. Despite the well-known importance of CH_4 oxidation in mitigating CH_4 emissions to the atmosphere, much less is known about how environment and MOB microbial features interact to determine the fate of CH_4 in lakes and reservoirs. To help overcome this problem, this thesis investigates the links between environment, MOB (from the community to the cell level), and CH_4 oxidation in lakes and a hydropower complex.

Chapter I examines the coupling between MOB community structure and CH_4 oxidation rates along the water column of six freshwater temperate lakes. The results show that there is a consistent vertical niche separation within the MOB community, mostly driven by the oxygen concentration. Modelling approaches suggested that both environmental factors and MOB community composition influence summer CH_4 oxidation rates in lakes with an independent effect of Gamma-MOB abundance.

Chapter II investigates how CH_4 and MOB community abundance, composition, and activity change from upstream to downstream waters in a tropical hydropower complex. MOB community shifts along the river-reservoir system reflected environmental sorting of taxa and an interrupted hydrologic connectivity. MOB peaked in the hypoxic hypolimnion at the main basin of the reservoir, where microbial CH_4 oxidation disconnected the deep CH_4 from CH_4 emissions to the atmosphere. CH_4 oxidation in the downstream river was accompanied by a rapid community shift with preferential growth of Alpha-MOB, enabling mitigation of CH_4 emissions also in waters below the dam.

Chapter III shows that despite generally present in low abundance in lakes, MOB cells, particularly within Gamma-MOB, are larger and more active than the bulk bacteria. At high CH_4:O_2 molar ratios, MOB have an ecological advantage over heterotrophic bacteria, processing a higher total amount of carbon. At the lake-level, the relative importance of methanotrophy depends on lake dissolved organic carbon

(DOC) concentration and morphometry, through their effects on the stratification and oxygenation of the water column. In DOC-rich lakes, methanotrophy can be the main process of bacterial carbon transformation, challenging our current understanding of the microbial loop in lakes.

Taken together, the results from the three chapters show that the fate of CH_4 in lakes and hydropower complexes depends on both environmental and microbial characteristics. While the environment controls the basic conditions for CH_4 oxidation to occur, it also shapes MOB composition, abundance, and activity which in turn can affect the fate of CH_4 due to intrinsic differences in niches, metabolism, and cell size. Different MOB taxa may be responsible for local CH_4 oxidation rates depending on the environmental conditions, and MOB metabolic characteristics influence the importance of methanotrophy to the ecosystem scale carbon cycling and potential CH_4 support to food webs in lakes.

Keywords: methanotroph, bacteria, methane, lake, hydropower.

INTRODUCTION

0.1 Context

0.1.1 The CH_4 cycle and the role of CH_4 oxidation in lakes and hydropower reservoirs

Methane (CH_4) is a potent greenhouse gas (GHG), with a global warming potential roughly 34 times greater than carbon dioxide (CO_2) over a 100 years' perspective (IPCC 2013). Despite the relatively small global area covered by freshwaters (about 3% of Earth's land surface; Downing et al. 2006), these ecosystems are important environmental sources of CH_4 to the atmosphere, emitting roughly 103 Tg of CH_4 $year^{-1}$ or 25% of the land GHG sink (Bastviken *et al.*, 2011). Biogenic CH_4 is mostly produced by methanogenic archaea during the final step of anaerobic organic matter degradation when inorganic oxidants such as sulfate, nitrate, or ferric iron are depleted (Conrad, 2005, 2009). In lakes, methanogenesis is the dominant degradation process in anoxic sediments and waters, making these environments hotspots of CH_4 production (Bastviken *et al.*, 2004 ; Conrad, 2009). In addition, CH_4 can be produced in oxic waters contributing to the overall CH_4 production and emission from lakes (Bogard *et al.*, 2014 ; Donis *et al.*, 2017 ; Grossart *et al.*, 2011 ; Günthel *et al.*, 2019). Different pathways for CH_4 production in oxic waters have been proposed, including CH_4 production in anoxic microhabitats such as in the gut of organisms (Oremland, 1979) or in sinking particles (Karl et Tilbrook, 1994), CH_4 release during the cleavage of dissolved compounds by bacteria (Repeta *et al.*, 2016 ; Yao *et al.*, 2016), and CH_4 production by algae (Lenhart *et al.*, 2016) and cyanobacteria (Bižić *et al.*, 2020) as a byproduct of photosynthesis.

Of the total CH_4 produced within lakes, a substantial but variable portion is not emitted to the atmosphere as it is microbially oxidized into CO_2 and microbial biomass (Bastviken *et al.*, 2002, 2008 ; Conrad, 2009). For instance, in temperate lakes, the extent of CH_4 oxidation can vary from negligible to near complete (Bastviken *et al.*, 2008 ; Thottathil *et al.*, 2018) and in the tropical permanently stratified Lake Kivu, between 51 and 89% of the upward flux of CH_4 is oxidized across seasons (Morana *et al.*, 2015). Similarly, during water column overturn, CH_4 oxidation removes roughly 94% of the stored CH_4 in Lake Nojiri (Japan) (Utsumi et al. 1998), 83-88% in a polyhumic boreal lake (Kankaala et al. 2007b), and 95-98% of the CH_4 stored annually in Lake Rotsee (Zimmermann *et al.*, 2019). Therefore, microbial CH_4 oxidation may vary among systems and the physical structure of the water column, but certainly plays a central role in the regulation of CH_4 emissions from freshwaters.

Likewise, in hydropower reservoirs, a large fraction of the CH_4 produced can be microbially oxidized, strongly mitigating the carbon footprint of such impoundments. The damming of rivers often leads to increased CH_4 production due to flooding of terrestrial organic carbon, increase in water residence time, and creation of anoxic conditions in the bottom of reservoirs (Tremblay *et al.*, 2005). However, pelagic CH_4 oxidation has been shown to reduce CH_4 emissions by more than 85% in reservoir-river systems (Guérin et Abril, 2007). In addition to diffusive CH_4 emissions at the surface of reservoirs, hydropower complexes can emit significant amounts of CH_4 in the downstream river below the dam since the water intake for the turbines is often located in CH_4-rich hypolimnia. Downstream CH_4 emissions are particularly important in strongly stratified reservoirs, where stable conditions and slow gas exchange across the thermocline allows microbial CH_4 oxidation of most of the upward CH_4 flux (e.g. Borges et al. 2011; Itoh et al. 2015). Due to the drastic change in pressure from the hypolimnion to the river below the dam, most of he CH_4 emitted downstream is released through degassing right after the turbines, while another

portion is emitted by diffusion at the surface of the downstream river (Guérin *et al.*, 2006 ; Soued et Prairie, 2020). However, evidence from stable isotopes and mass balance has shown that microbial CH_4 oxidation in downstream waters mitigate diffusive CH_4 emissions below the dam, further reducing the carbon footprint of hydropower complexes (Abril *et al.*, 2005 ; Guérin et Abril, 2007 ; Kemenes *et al.*, 2007 ; Soued et Prairie, 2020).

0.1.2 Significance of aerobic and anaerobic CH_4 oxidation in freshwaters

The microbial oxidation of CH_4 consists of aerobic and anaerobic processes. While a fraction of the CH_4 produced in lakes can be anaerobically oxidized within anoxic sediments or anoxic waters (e.g. Eller et al. 2005; Schubert et al. 2010, 2011), more often most CH_4 is consumed aerobically by methane-oxidizing bacteria (MOB or methanotrophic bacteria) as it reaches the oxygenated sediments or water column (Carini *et al.*, 2005 ; Frenzel *et al.*, 1990 ; Pasche *et al.*, 2011 ; Zigah *et al.*, 2015). This efficient aerobic CH_4 filter is clear from profiles in stratified water columns where CH_4 concentration accumulates in the anoxic hypolimnia but drastically reduces (often 2-3 orders of magnitude) as it approaches the oxic-anoxic interface. These changes are followed by mirrored carbon stable isotopic signatures of CH_4 ($\delta^{13}CH_4$), which remain very negative and stable within the anoxic layer but quickly become less negative in the presence of oxygen (Morana *et al.*, 2015 ; Schubert *et al.*, 2010 ; Thottathil *et al.*, 2018). Such enrichment in the $\delta^{13}CH_4$ indicates the extent of CH_4 oxidation since the methane monooxygenase enzyme (MMO), which oxidizes CH_4 into methanol in the first step of aerobic CH_4 oxidation, preferentially uptakes $^{12}CH_4$, leaving the remaining CH_4 pool enriched in $^{13}CH_4$.

Differently from the oceans, in freshwaters, the significance of aerobic CH_4 oxidation is often greater than that of the anaerobic process because freshwaters are generally depleted in sulfate which is the main electron acceptor in the anaerobic oxidation of CH_4 (Bastviken, 2009 ; Conrad, 2007). However, recent evidence for CH_4 oxidation

in the absence of oxygen by traditional aerobic methanotrophic bacteria has challenged the current knowledge on microbial processes and players in the oxidation of CH_4 in freshwaters (Blees *et al.*, 2014 ; Milucka *et al.*, 2015 ; Oswald *et al.*, 2015, 2016b, 2016a ; Rissanen *et al.*, 2018). Different pathways have been proposed to explain the activity of aerobic methanotrophic bacteria in virtually anoxic conditions, which include cryptic photosynthetic oxygen production at the bottom of the oxycline (Oswald *et al.*, 2015 ; Savvichev *et al.*, 2019), use of alternative electron acceptors such as metal oxides (Oswald *et al.*, 2016b) and the ability to perform anaerobic respiration by denitrification (Kits *et al.*, 2015b ; Oswald *et al.*, 2017 ; Skennerton *et al.*, 2015). Still, the relative importance of such pathways across lakes is unknown.

0.1.3 Phylogenetic and metabolic diversity of methanotrophs

Methanotrophs use CH_4 as source of carbon and energy and can use O_2 (aerobic) or other electron acceptors (anaerobic) to oxidize CH_4. Anaerobic methanotrophs are archaea belonging to three clades (ANME-1, 2, and 3) related to the Orders Methanosarcinales and Methanomicrobiales (Borrel *et al.*, 2011). They oxidize CH_4 by performing 'reversed' methanogenesis in consortia with sulfate-reducing Deltaproteobacteria or using nitrate as electron acceptor without a bacterial partner (ANME-2d) (Timmers *et al.*, 2017). Like methanogenic archaea, they posses the *mcrA* (methyl coenzyme M reductase A) gene, which is also an essential and diagnostic gene of methanogenesis (Hallam *et al.*, 2003). In addition, anaerobic CH_4 oxidation coupled to iron and manganese reduction by an uncultivated marine benthic group-D has been detected in marine seep sediments (Beal *et al.*, 2009), but the process and identity of microbial players are still unclear.

In contrast, all known aerobic methanotrophs are bacteria. Methane-oxidizing bacteria (MOB or methanotrophic bacteria) are a subset of methylotrophs (i.e. aerobic bacteria that utilize one-C compounds more reduced than formic acids) (Hanson et Hanson, 1996). The presence of methane monooxygenase enzymes (MMO) encoded

by *pmoCAB* genes is a defining characteristic of MOB and confers to these bacteria the ability of oxidizing methane to methanol. MOB comprise a polyphyletic group of bacteria distributed in the Proteobacteria, Verrucomicrobia, and candidate NC10 phyla (Dunfield *et al.*, 2007 ; Ettwig *et al.*, 2010 ; Hanson et Hanson, 1996) (Table 0.1). MOB belonging to the NC10 candidate phylum (*C*a. Methylomirabilis oxyfera) are a particular case, as they produce oxygen internally from nitrite to perform aerobic CH_4 oxidation under anoxic conditions (Ettwig *et al.*, 2010). Due to this unique metabolism of *Ca.* Methylomirabilis oxyfera and related taxa, these microorganisms are also classified as anaerobic methanotrophs in the literature. Although MOB comprise a relatively restricted group of taxa (approx. 60 cultivated species, but an unknown number of uncultivated species, Knief (2015)), they are widespread and ecologically diverse, are present in terrestrial and aquatic habitats and grow optimally in a wide range of temperature, pH, and salinity (Hanson et Hanson, 1996 ; Knief, 2015 ; Semrau *et al.*, 2010 ; Trotsenko et Khmelenina, 2005).

The mechanisms behind the rise of methanotrophy in different prokaryotic clades is still under debate. Within the Proteobacteria, some studies support vertical descent based on phylogenetic congruence between 16S rRNA and *pmoA* sequence analyses (Knief, 2015 ; Stein *et al.*, 2012), while others found evidence for an important role of horizontal gene transfer in the spread of methanotrophy (Khadka *et al.*, 2018 ; Osborne et Haritos, 2018).

Table 0.1. Taxonomic distribution of known methanotrophic bacterial taxa (Kalyuzhnaya *et al.*, 2019 ; Knief, 2015).

Phylum	Class	Order	Family	Genera
Proteobacteria	Gammaproteobacteria	Methylococcales		
	Alphaproteobacteria		Methylocystaceae	*Methylocystis*
				Methylosinus
			Beijerinckiaceae	*Methylocella*
				Methylocapsa
				Methyloferula
Verrucomicrobia				*Methylacidiphilum*
				Methylacidimicrobium
NC10 (candidate phylum)		Methylomirabilales		*Ca.* Methylomirabilis oxyfera

0.1.4 Metabolic and ecological diversity within Proteobacterial MOB

Within Proteobacteria, methanotrophs are distributed in the classes Gammaproteobacteria (Gamma-MOB, also known as Type I and X MOB) and Alphaproteobacteria (Alpha-MOB, also known as Type II MOB; Hanson and Hanson 1996; Bowman 2006). In lakes, the Proteobacterial MOB taxa are commonly the most abundant and active methanotrophs (e.g. Blees et al., 2014b; Carini et al., 2005; Crevecoeur et al., 2015; Oswald et al., 2015) while Verrucomicrobia MOB are typically found in acidic environments such as volcanic soils and hot springs (Dunfield *et al.*, 2007 ; Op den Camp *et al.*, 2009 ; van Teeseling *et al.*, 2014) and the general importance of NC10 in lakes is less known but has been demonstrated in lake

sediments and in a subtropical reservoir (Deutzmann *et al.*, 2014 ; Kojima *et al.*, 2014).

Gamma- and Alpha-MOB differ in their phylogenetic affiliation and in morphological, physiological, and metabolic traits (Hanson et Hanson, 1996 ; Knief, 2015 ; Semrau *et al.*, 2010). Major morphological differences are the arrangement of internal membranes and major phospholipids fatty acids (PLFAs), with most Alpha-MOB showing signature PLFAs with 18 carbons, while signature PLFAs of Gamma-MOB have 16 carbons (Bodelier *et al.*, 2009 ; Bowman *et al.*, 1991). These signature PLFAs not only distinguish MOB types but are also MOB-specific, being useful markers of these organisms in the environment (Bodelier *et al.*, 2009 ; Boschker et Middelburg, 2002). A distinctive feature between Gamma- and Alpha-MOB is their pathways for C assimilation (Knief 2015). The ribulose monophosphate (RuMP) pathway characteristic of Gamma-MOB has a higher C conversion efficiency than the serine pathway characteristic of Alpha-MOB. While the RuMP pathway produces a 3-C intermediate compound from formaldehyde (which is the central intermediate in anabolism and catabolism in methanotrophs), the serine pathway produces a 2-C compound from formaldehyde and additionally utilizes CO_2 in a longer and less energetically efficient pathway (Hanson et Hanson, 1996 ; Trotsenko et Murrell, 2008). This metabolic difference suggests that Gamma-MOB and Alpha-MOB may show different growth rates in the environment and thus that microbial physiology may be an important factor to consider in lake CH_4 cycling, as demonstrated for C cycling in soils (Allison *et al.*, 2010 ; Wieder *et al.*, 2013).

Ecophysiologically, Gamma- and Alpha-MOB seem to differ mainly regarding optimal temperatures and CH_4, O_2, and inorganic nitrogen concentrations, but the literature is often contradictory. Experiment-based studies have shown that Gamma-MOB outcompete Alpha-MOB under relatively low CH_4 concentrations, while Alpha-MOB is favored under higher CH_4, lower O_2, and higher temperatures (Amaral

et Knowles, 1995 ; Börjesson *et al.*, 2004 ; Hanson et Hanson, 1996 ; He *et al.*, 2012). However, Alpha-MOB strains have also been shown to be active at trace atmospheric levels of CH_4 due to the possession of two pMMO isozymes with different CH_4 oxidation kinetics (Baani et Liesack, 2008 ; Dunfield *et al.*, 1999). Another physiological difference between Proteobacterial MOB groups is the ability of Alpha-MOB to fix nitrogen (N). Due to this ability, when N compounds are limiting, it is expected that Alpha-MOB dominates over Gamma-MOB (Hanson et Hanson, 1996). However, it has been shown that N-fixing genes may be present in many MOB taxa within Gammaproteobacteria as well (Heylen *et al.*, 2016 ; Knief, 2015).

The diversity of traits within MOB has been related to different life strategies between MOB groups and to a selective advantage of taxa under different environmental conditions (Ho *et al.*, 2013). This suggets that the taxonomic composition of MOB communities may change largely along environmental gradients and that such variations could translate into differences in CH_4 oxidation rates within and across ecosystems. Similarly, due to metabolic differences, changes in the taxonomic composition of MOB communities may lead to variations in the fraction of the CH_4-derived C that is assimilated into biomass and thus becomes available to higher trophic levels.

0.1.5 Regulation of aerobic CH_4 oxidation rates in lakes

Aerobic methane oxidation (MOx) is a highly variable process among and within lakes (e.g. Utsumi et al. 1998; Bastviken et al. 2008; Blees et al. 2014a; Martinez-Cruz et al. 2015). Individual studies collectively indicate that a complex interaction of chemical, physical, and biological factors influence aerobic CH_4 oxidation, and this complex regulation makes MOx rates highly variable both spatially and temporally. Besides the concentrations of substrates (CH_4 and O_2), light, inorganic nitrogen, phosphorus, and grazing have been shown to influence the abundance and activity of MOB in the environment (Devlin *et al.*, 2015 ; Hanson et Hanson, 1996 ; Kankaala *et*

al., 2007a ; Lofton *et al.*, 2014 ; Martinez-Cruz *et al.*, 2015 ; Murase et Sugimoto, 2005 ; Oswald *et al.*, 2015 ; Rudd *et al.*, 1976 ; Semrau *et al.*, 2010 ; Veraart *et al.*, 2015). In experimental studies, CH_4 concentrations seem to primarily regulate MOx rates (Lofton *et al.*, 2014 ; Sundh *et al.*, 2005), however, a direct effect of CH_4 concentration on MOx or MOB activity is not always observed (Crevecoeur *et al.*, 2017 ; Steinle *et al.*, 2017). Highest methanotrophic activity and abundances in lakes are usually observed at low oxygen concentrations at oxic-anoxic interfaces (e.g. Lidstrom and Somers 1984; Kankaala et al. 2006; Schubert et al. 2010; Blees et al. 2014a). The mechanism behind the higher abundance and activity of MOB under low O_2 concentrations is not clear, but it may be related to the fact that lower O_2 concentrations are coincident with higher CH_4 concentrations in the water column (Harrits et Hanson, 1980 ; Lidstrom et Somers, 1984), the ability to use electron acceptors other than O_2 by some of these organisms (Kits *et al.*, 2015b ; Oswald *et al.*, 2016a, 2017 ; Skennerton *et al.*, 2015), or inhibition at high O_2 concentrations (Thottathil *et al.*, 2019). However, although at lower rates, CH_4 oxidation also occurs under high O_2 and low CH_4 concentrations in shallower waters (Kankaala *et al.*, 2006 ; Thottathil *et al.*, 2019 ; Zigah *et al.*, 2015).

Light has been shown to influence MOx in lakes in different ways. While some studies found evidence for light inhibition of MOB activity (Dumestre *et al.*, 1999b ; Murase et Sugimoto, 2005 ; Thottathil *et al.*, 2018), others found higher MOx rates under light conditions suggesting that MOx is coupled to light-dependent photosynthetic oxygen production at the bottom of the oxycline in lakes (Oswald *et al.*, 2015 ; Savvichev *et al.*, 2019). Top-down control seems to be another important factor regulating MOx rates as shown by microcosms and whole-lake experiments that grazing by protists and zooplankton can affect the amount of CH_4 consumed by MOB (Devlin *et al.*, 2015 ; Kankaala *et al.*, 2007a).

Community composition may not influence metabolic responses in widely distributed metabolic processes such as heterotrophic bacterial production or respiration (Comte et del Giorgio, 2011), but a more direct link between taxonomic composition and metabolism may exist in highly specialized processes that are more narrowly distributed across taxa (Levine *et al.*, 2011 ; Schimel, 1995). For instance, in soils, Levine et al. (2011) found that higher methanotrophic diversity is related to stability in CH_4 consumption and suggested that conserving methanotroph diversity could help mitigate atmospheric CH_4 concentrations. Judd et al. (2016) found that methanotrophic activity and community composition co-varied in grassland soils suggesting a strong potential for community composition effect on CH_4 uptake. These studies indicate that microbial community structure may also influence MOx rates in lakes and freshwater reserovirs and may play a role in the complex regulation of CH_4 oxidation at the ecosystem level.

0.1.6 Hydrologic connectivity of microbial communities and the effect of the damming of rivers

The importance of hydrologic connectivity in shaping the structure of freshwater bacterioplankton has been shown for whole communities (Niño-García *et al.*, 2016 ; Ruiz-González *et al.*, 2013, 2015a) and specifically for MOB assemblages across boreal lakes and rivers (Crevecoeur *et al.*, 2019). The relative position in the aquatic network, hydrologic connections, and local water residence time are known to regulate the role of immigration and species sorting in the structure of aquatic bacterial communities (Lindström *et al.*, 2005, 2006 ; Logue et Lindström, 2010 ; Read *et al.*, 2015). Dams create a discontinuity in rivers that can cause large changes between upstream, reservoir, and downstream segments such as differences in local water residence time, physical structure of the water column, temperature, and many others that affect both connectivity of communities and species sorting. The changes caused by dams have been shown to alter the structure of inflowing river bacterioplankton communities (Dumestre et al., 2001; Ruiz-González et al., 2015b,

2013) and to rearrange them among water column layers due to thermal stratification within reservoirs (Dumestre *et al.*, 1999a). This microbial shifts may be particularly important to methanotrophic taxa, given that the damming of rivers cause displacement of carbon processes over space and time with, for example, anoxia in and increased CH_4 concentrations in reservoirs (Prairie *et al.*, 2017 ; Tremblay *et al.*, 2005). Such changes have the potential to trigger shifts in the total abundance of MOB taxa as well as the establishment of distinct MOB communities and associated activity between zones within hydropower complexes. Changes in MOB community size and structure may have important implications to the role of MOB taxa in mitigating CH_4 emissions and thus the carbon footprint of hydropower impoundments.

0.1.7 The role of methanotrophy in lake carbon cycling

During CH_4 oxidation, methanotrophs produce carbon dioxide (CO_2) and bacterial biomass from CH_4, being this the sole source of metabolic energy and structural carbon (C) to obligate methanotrophs (Hanson and Hanson 1996). Given that a substantial fraction of the CH_4 produced in lakes is oxidized by MOB, methanotrophy is a potentially important pathway of C transformation and of biomass and CO_2 production in lakes.

Indeed, Bastviken et al. (2003) showed that methanotrophy can represent a notable pathway of C incorporation into microbial biomass in boreal lakes, particularly during the winter when it corresponded from 3 to 120% of heterotrophic bacterial production. By assuming a MOB growth efficiency between 10 and 40%, Kankaala et al. (2013) estimated that MOB production can contribute to a major part of the total pelagic C mobilization in small humic lakes where high CH_4 concentrations accumulate in the hypolimnion during summer. In the tropical meromictic Lake Kivu, Morana et al. (2015) determined that methanotrophic production was equivalent to 16-60% of average primary production and MOB contributed significantly to the microbial biomass at the oxycline. Collectively, these studies indicate that

methanotrophy is an important pathway of biomass production, comparable in some cases to the total C fixation by heterotrophic bacteria and primary production, and a substantial potential C source to consumers in lakes. In addition, there is widespread stable isotopic and biomarkers evidence of CH_4-derived C incorporation into aquatic food webs (Grey, 2016 ; Jones et Grey, 2011 and references therein). However, the mechanisms controlling the relative importance of methanotrophy at the lake level are still to be determined.

Besides the factors controlling CH_4 oxidation rates, the importance of methanotrophy at the lake level may depend also on metabolic features that may render ecological advantage of MOB relative to the bulk dissolved organic carbon (DOC)-consuming bacteria. These features include cell size and activity, turnover rates (rate of cell replacement), total biomass, and efficiency in producing cellular C from the CH_4 consumed (i.e. MOB growth efficiency). Consequently, determining these characteristics of MOB at *in situ* conditions is crucial to understanding their biogeochemical and ecological roles in the environment.

0.2 Thesis objectives

The general objective of this thesis is to better understand how the fate of CH_4 in lakes and hydropower reservoirs is influenced by the interplay between the environment and methanotrophic composition, activity, and metabolism. More specifically, the research presented here aims at assessing the ecological mechanisms influencing the oxidation of CH_4 in lakes and hydropower complexes and the importance of methanotrophy at the lake level. This thesis is divided in three chapters (Figure 0.1) that address the following specific objectives:

Chapter I: assess the environmental drivers of the abundance of Proteobacterial MOB groups in lakes and determine how their abundances relate to CH_4 oxidation rates along and across water columns.

Chapter II: determine how CH_4 and MOB community abundance, composition, and activity change from upstream to downstream waters in a tropical hydropower complex and assess whether CH_4 oxidation dynamics are accompanied by microbial shifts in this system.

Chapter III: determine the relative importance of methanotrophy to the total bacterial carbon cycling in lakes and identify the metabolic and environmental factors driving the importance of methanotrophy at the lake level.

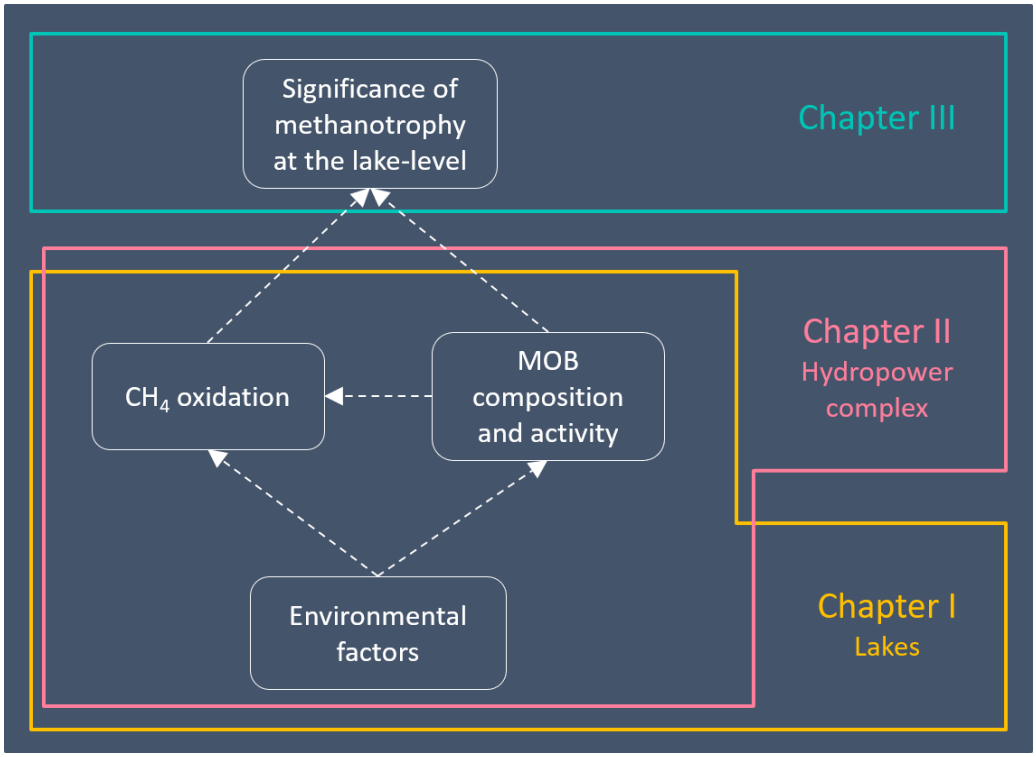

Figure 0.1. Conceptual diagram showing the structure of this thesis.

0.3 General approach

To address the objectives outlined above, a combination of approaches that encompasses biogeochemical and microbial measurements was used. These include field sampling, laboratory incubations, carbon stable isotopic measurements, microscopic identification, enumeration and size determination of cells, DNA-RNA sequencing, and modelling.

In chapter I, CH_4 oxidation rates were measured in laboratory incubations using unamended lake water from different depths of six temperate lakes located in the Laurentians region of Québec. The lakes were chosen to cover large environmental gradients in CH_4, O_2, DOC, size, maximum depth, and others. *In situ* and experimental Proteobacterial MOB composition and abundance were determined through microscopic cell identification and enumeration by catalyzed reporter deposition-fluorescent in situ hybridization (CARD-FISH). The absolute cell abundance of Proteobacterial MOB groups provided by CARD-FISH allowed the utilization of modelling approaches to assess the environmental drivers of MOB taxa and their individual role in the bulk CH_4 oxidation rates.

In chapter II, MOB and CH_4 dynamics were studied in the tropical hydropower complex Batang Ai (Borneo island, Malaysia). Two field campaigns were performed, in which samples from different locations from upstream to downstream waters and from different depths within the reservoir were taken for the determination of CH_4 concentration and carbon stable isotopic signature, DNA-RNA sequencing, and CARD-FISH. The DNA-RNA sequencing provided a fine characterization (at the amplicon sequence variant-ASV level) of potentially active MOB assemblages and enabled the tracking of MOB taxa along the river-reservoir system. In contrast, the CARD-FISH allowed cell enumeration and the detection of rapid MOB cell growth that could not be inferred from the sequencing data.

Finally, in chapter III, laboratory incubations, microscopy, and lake profiles performed in the same lakes of chapter I in Québec were used to investigate the importance of methanotrophy as a pathway of carbon transformation in lakes. CARD-FISH and DAPI (4',6-diamidino-2-phenylindole) staining and microscopic visualization were used to measure heterotrophic and methanotrophic microbial cell abundance, size, and biomass. Rates of heterotrophic and methanotrophic metabolisms were determined in incubations and the importance of methanotrophy at the lake level was estimated based on lake profiles taken across the summer period and a model developed in a parallel study (Thottathil *et al.*, 2019).

CHAPTER I

NICHE SEPARATION WITHIN AEROBIC METHANOTROPHIC BACTERIA ACROSS LAKES AND ITS LINKS TO METHANE OXIDATION RATES

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N.B. References cited in this chapter are presented at the end of the thesis.

1.1 Abstract

Lake methane (CH_4) emissions are largely controlled by aerobic methane-oxidizing bacteria (MOB) which mostly belong to the classes Alpha- and Gammaproteobacteria (Alpha- and Gamma-MOB). Despite the known metabolic and ecological differences between the two MOB groups, their main environmental drivers and their relative contribution to CH_4 oxidation rates across lakes remain unknown. Here, we quantified the two MOB groups through CARD-FISH along the water column of six temperate lakes and during incubations in which we measured ambient CH_4 oxidation rates. We found a clear niche separation of Alpha- and Gamma-MOB across lake water columns, which is mostly driven by oxygen concentration. Gamma-MOB appears to dominate methanotrophy throughout the water column, but Alpha-MOB may also be an important player particularly in well-oxygenated bottom waters. The inclusion of Gamma-MOB cell abundance improved environmental models of CH_4 oxidation rate, explaining part of the variation that could not be explained by environmental factors alone. Altogether, our results show that MOB composition is linked to CH_4 oxidation rates in lakes and that information on the MOB community can help predict CH_4 oxidation rates and thus emissions from lakes.

1.2 Introduction

Freshwater lakes are disproportionally large sources of methane (CH_4) – a highly potent greenhouse gas – to the atmosphere, emitting roughly 71.6 Tg CH_4 per year despite their small global area (Bastviken *et al.*, 2011 ; Downing *et al.*, 2006 ; Kirschke *et al.*, 2013). To a large extent, lake CH_4 emissions are controlled by the activity of aerobic methane-oxidizing bacteria (MOB), which oxidize a substantial, but variable portion of the total CH_4 produced before it is emitted (Bastviken *et al.*, 2008 ; Morana *et al.*, 2015 ; Thottathil *et al.*, 2018 ; Utsumi *et al.*, 1998). CH_4

oxidation rates in lakes have been shown to vary widely depending on water column stratification (Kankaala *et al.*, 2007b ; Morana *et al.*, 2015), the ratio between area of deep and epilimnetic sediments (Bastviken *et al.*, 2008), light (Dumestre et al., 1999; Murase and Sugimoto, 2005; Oswald et al., 2015), and dissolved organic carbon (DOC) concentration (Thottathil *et al.*, 2018), all of which affect the availability of substrates (CH_4 and oxygen, O_2) or other putative drivers of MOB abundance and activity. However, it remains unknown how these drivers affect the different MOB groups or how MOB community composition is related to CH_4 oxidation rates across lakes.

Lake methanotrophic bacteria are mostly distributed between the classes Alphaproteobacteria (also known as Type II, hereafter Alpha-MOB) and Gammaproteobacteria (also known as Type I, hereafter Gamma-MOB) (Bowman, 2006 ; Hanson et Hanson, 1996). Alpha- and Gamma-MOB are known to differ in many metabolic and ecological traits (Hanson et Hanson, 1996 ; Knief, 2015 ; Semrau *et al.*, 2010), such as different carbon conversion efficiencies of the C1 assimilation pathways (Kalyuzhnaya *et al.*, 2015 ; Trotsenko et Murrell, 2008), the possession of two particulate methane monooxygenases (pMMOs) isozymes within Alpha-MOB (Baani et Liesack, 2008), the ability of Gamma-MOB to oxidize CH_4 in virtually anoxic environments (Blees *et al.*, 2014 ; Oswald *et al.*, 2016b ; Rissanen *et al.*, 2018), and different life strategies (Ho *et al.*, 2013). Yet, the environmental factors that drive the abundance and activity of each MOB group remain poorly constrained, limiting our understanding on the relative role of these microorganisms in the oxidation of CH_4 under natural conditions. For example, some studies have detected the presence or activity of MOB groups under highly variable CH_4 and O_2 levels (Amaral et Knowles, 1995 ; Biderre-Petit *et al.*, 2011 ; Crevecoeur *et al.*, 2015 ; Graef *et al.*, 2011 ; Zigah *et al.*, 2015), while others have found a strong temperature effect on MOB composition (Börjesson *et al.*, 2004 ; He *et al.*, 2012 ; Knoblauch *et al.*, 2008) or no discernable pattern (Urmann *et al.*, 2009).

Highest methanotrophic activity in lakes is normally encountered at oxic-anoxic interfaces (Kankaala *et al.*, 2006 ; Lidstrom et Somers, 1984 ; Morana *et al.*, 2015 ; Reim *et al.*, 2012 ; Schubert *et al.*, 2010), but CH_4 oxidation rates are often detected throughout the water column, even in surface waters with low CH_4 and high O_2 concentrations (Kankaala *et al.*, 2006 ; Thottathil *et al.*, 2018 ; Zigah *et al.*, 2015). This suggests metabolic versatility within taxa or activity of MOB taxa with different environmental preferences along the water column. If MOB groups partition niches or activity, then they may have different contributions to overall CH_4 oxidation rates throughout the water column. Although studies have reported a link between the taxonomic composition of methanotrophs and CH_4 oxidation in soils (Bodelier *et al.*, 2013 ; Judd *et al.*, 2016 ; Levine *et al.*, 2011 ; Nazaries *et al.*, 2013), no study has evaluated the extent to which vertical changes in the abundances of MOB groups are related to CH_4 oxidation rates in freshwater aquatic systems. Recent studies have suggested Gamma-MOB activity throughout the water column of lakes (Oswald *et al.*, 2016b ; Rissanen *et al.*, 2018), but as both proteobacterial MOB groups are often encountered in these ecosystems, it is not clear how resources and habitat are partitioned between the two groups or how the overall CH_4 oxidation rate is linked to the abundances of the two players. Moreover, given the large metabolic diversity within proteobacterial MOB, the inclusion of microbial information among the predictors of CH_4 oxidation rates might improve our ability to understand or even predict methanotrophy in lakes.

In this study, we examined the coupling between MOB community structure and activity (i.e. CH_4 oxidation rates) along the water column of six freshwater temperate lakes, which differed in their concentrations of CH_4, O_2 and other environmental variables. More specifically, we investigated the drivers of the abundance of each MOB group and the relationship between MOB groups and CH_4 oxidation rates. We combined microscopic quantification of Alpha- and Gamma-MOB abundances along the water column of each lake with laboratory incubations performed with water from

different depths to measure CH_4 oxidation rates and abundance changes of the two MOB groups. Incubations were performed with unamended lake water (i.e. *in situ* CH_4 and O_2 concentrations) and at three different temperatures to explore the effect of CH_4 and O_2 levels and temperature on MOB composition and activity. Alpha- and Gamma-MOB cells were microscopically identified and quantified using catalysed reporter deposition-fluorescence in situ hybridization (CARD-FISH). We hypothesized that environmental gradients among and within lakes influence the abundance of the two MOB groups, which in turn relates to changes in CH_4 oxidation rates along the water column. We further expected that including MOB abundance data would improve CH_4 oxidation models and help increase our understanding of how environmental and biotic factors influence CH_4 dynamics in lakes.

1.3 Methods

1.3.1 Study lakes and sampling

This study was conducted in six environmentally different lakes in the Laurentians region of Quebec, Canada (Table 1.1) during summer stratification (June to October) in 2016. We measured vertical profiles of environmental conditions and of the *in situ* abundances of proteobacterial MOB groups and conducted laboratory experiments to measure MOB activity. Profiles of temperature, pH, and O_2 were taken at the deepest site of each lake with a YSI multiparameter probe (Yellow Springs Instruments, OH, USA). Concentrations and isotopic signatures of dissolved CH_4 were measured at every meter by the headspace technique followed by gas chromatography (GC-8A/GC-2014; Shimadzu, Kyoto, Japan) and stable isotope analysis (G2201-i, Picarro, CA, USA). Water samples for incubation experiments were collected from the surface and other two depths (range 3–12 m) in lakes Morency, Croche, Cromwell, and Geai, and from the surface and bottom (range 3–7.5 m) in lakes en Coeur and Triton. Sampling depths were determined based on O_2 profiles in order to cover a

large range of CH_4 and O_2 concentrations. Samples were collected by pumping water into acid-rinsed collapsible bags, which were allowed to overflow and closed bubble-free. The collapsible bags were kept cold in the dark for 2–4 h until further processing in the laboratory.

1.3.2 Nutrients, chlorophyll-a, and organic carbon concentrations

Total phosphorus (TP), total nitrogen (TN), chlorophyll-a (chl-a), DOC, and coloured dissolved organic matter (cDOM) concentrations were measured from water samples at each lake and sampled depth. TP was measured using potassium persulfate digestion followed by ammonium molybdate colorimetric method (Wetzel et Likens, 2000); TN was measured using a continuous flow analyser (ALPKEM Flow Solution 3100) using an alkaline persulfate digestion method, coupled with a cadmium reactor (Patton et Kryskalla, 2003); and chl-a was analysed using a standard ethanol extraction protocol (Lorenzen, 1967). Water samples for determination of DOC and cDOM concentrations were filtered through a 0.45-µm filter (Sarstedt AG & Co, Germany). The concentration of DOC was measured with an Aurora 1030W TOC Analyser using the persulfate oxidation method; and cDOM was determined at 440 nm using a spectrophotometric absorbance scan (Ultrospec 3100pro spectrophotometer) (Cuthbert et del Giorgio, 1992).

Table 1.1. General characteristics of study lakes.

Lake	Area (km^2)	Max. depth (m)	Sampling depths (m)*	pH	TP (µg L^{-1})	TN (mg L^{-1})	Chl-*a* (µg L^{-1})	DOC (mg L^{-1})	cDOM (m^{-1})
Morency	0.23	20.0	0	7.4	5.0	0.23	1.4	3.4	0.69
			8	7.41	7.5	0.32	6.6	2.8	-
			12	6.67	12.7	0.36	1.4	2.6	-
Croche	0.179	11.4	0	6.08	5.5	0.18	1.4	4.1	0.92
			6	5.6	13.5	0.3	21.7	4.7	1.26
			6.5	5.46	16.2	0.27	15.3	4.2	1.38
Cromwell	0.102	9.8	0	5.79	9.6	0.28	2.5	5.2	1.38
			3	5.67	13.5	0.28	5.8	4.3	1.72
			5	5.26	25.2	0.52	24.0	4.3	1.95
Geai	0.008	8.3	0	5.79	13.5	0.32	2.4	8.2	4.26
			3	5.37	25.0	0.32	9.2	10.9	7.36
			6	6.1	50.4	0.40	29.9	10.8	9.55
en Coeur	0.44	8.3	0	7.44	6.9	0.25	1.6	4.2	0.46
			7.5	6.01	29.1	0.43	16.1	4.0	1.26
Triton	0.017	4.3	0	6.03	10.3	0.29	2.26	4.6	1.04
			3	6.07	17.9	0.32	4.45	3.7	1.61

*Depth of sampling for MOB *in situ* community and incubations. TP: total phosphorus; TN: total nitrogen; Chl-*a*: chlorophyll-a; DOC: dissolved organic carbon; cDOM: Absorbance at 440 nm.

1.3.3 CH_4 oxidation rates

Experimental incubations for the determination of CH_4 oxidation rates were conducted as explained in a parallel study (Thottathil *et al.*, 2019). Briefly, thirty 500 ml-flasks equipped with O_2 optodes (Fibox 3 system; PreSens, Regensburg, Germany) per depth were filled with unamended water, allowed to overflow, sealed with silicone stoppers without headspace, and incubated under different temperatures (5 or 10, 20, and 25 °C; 10 flasks per temperature treatment) using dark circulating water baths for 3 to 8 days. Intermediate temperatures were approximately the *in situ* temperature for each sampled depth and therefore worked as controls for the temperature treatments. The concentration of CH_4 and stable isotopic signature ($\delta^{13}CH_4$) was measured three to five times during each incubation by measuring duplicate flasks and using the headspace technique, gas chromatography, and stable isotope analysis. Due to enzymatic fractionation against ^{13}C-CH_4 during CH_4

oxidation, the increase in $\delta^{13}CH_4$ values in the remaining CH_4 pool during incubations was used to confirm that the reported decreases in CH_4 concentration reflected CH_4 oxidation. Because the incubation followed near perfect first-order kinetics, instantaneous CH_4 oxidation rates were calculated by differentiation of the first-order CH_4 depletion curve at each time point. Samples for MOB enumeration were also taken at the end of each incubation series.

1.3.4 Alpha- and Gamma-MOB cell enumeration by CARD-FISH

The abundances of Alpha- and Gamma-MOB were determined *in situ* and at the end of incubations in duplicates or triplicates by catalysed reporter deposition-fluorescence in situ hybridization (CARD-FISH), using probes for aerobic methanotrophic bacteria belonging to Alpha- (Mα450) and Gammaproteobacteria (Mγ84 and Mγ705) (Supporting Information Table S1.1) (Eller *et al.*, 2001). 40 ml of samples were fixed with buffered paraformaldehyde (PFA, 1% final concentration) and kept refrigerated at 4 °C before filtration; 2–6 ml of samples were filtered through 0.2 μm polycarbonate filters (Millipore GTTP, 25 mm), and filters were frozen until analysis. The volumes filtered were determined based on tests and microscopic verification with 40,6-diamidino-2-phenylindole (DAPI) staining to optimize the volume filtered without overlapping cells. Prior to hybridization, cells were attached with 0.1% agarose and permeabilized with lysozyme and achromopeptidase. Hybridization was performed overnight at 35 °C with 40% formamide hybridization buffer. Hybridized cells were quantified under an automated epifluorescence microscope under 630× magnification counting on average 46 (24–55, min-max) quality-checked fields of view. Cell counts of Alpha- and Gamma-MOB as well as those of total prokaryotic cells (DAPI staining) were determined using the ACME tool3 software (Zeder, 2014). Only CARD-FISH signals that were also detected in the DAPI channel were considered as MOB bacterial cells. We used the abundance of each MOB group *in situ* and at the end of each incubation

experiment to evaluate how they relate to environmental factors, to each other, and to the measured CH_4 oxidation rates.

1.3.5 Statistical Methods

We used paired t-tests to detect differences between the abundance of MOB groups within depths and ANOVA and Tukey-Kramer HSD to detect differences within MOB groups across depths. To assess the effect of environmental factors on the abundance of each MOB group, we used Generalized Additive Models (GAMs) which allowed us to mix linear model terms with a smoothed and flexible model term. The flexibility of GAMs was preferred over linear models because of the strong non-linearity in the response of CH_4 oxidation to O_2 (Thottathil *et al.*, 2019). To evaluate the contribution of MOB cell abundance in predicting CH_4 oxidation rates together with environmental variables, generalized regression with adaptive elastic net variable selection and AICc validation was used. Statistical analyses were performed in JMP software (JMP. SAS Institute Inc., 2019) and R 3.6.0 (The R Core Team, 2019). GAMs were performed with the R package mgcv 1.8.28 (Wood, 2019) and GAMs figures were produced with the package mgcViz 0.1.4 (Fasiolo *et al.*, 2019). Other figures were plotted with the R packages plotly (Plotly Technologies Inc., 2015) and ggplot2 3.1.0 (Wickham, 2016).

1.4 Results

1.4.1 Vertical variations in temperature, O_2, CH_4, and MOB community

All lakes, except Triton, were thermally stratified during sampling with dissolved O_2 concentrations decreasing sharply below the oxycline (Fig. 1.1A–E). Conversely, CH_4 concentrations increased steeply below the oxycline and varied up to four orders of magnitude across depths and lakes. In the shallowest lake (Triton), the water column was mixed and fully oxygenated with slight and gradual decrease in O_2 and

increase in CH_4 concentrations from surface to bottom waters (Fig. 1.1F). Total MOB abundance *in situ* ranged from 1.0×10^3 to 2.2×10^5 cells mL^{-1} across lakes and depths and represented between 0.1% and 2.3% of total bacterial cell abundance. In all stratified lakes, the MOB community dominance shifted from the top to the bottom of water columns (Fig. 1.1A–E): Alpha-MOB were more abundant than Gamma-MOB in the surface (paired t-test, $t = 1.99$, d.f. = 70, $p < 0.0001$) and in intermediate depths (paired t-test, $t = 2.01$, d.f. = 46, $p = 0.002$), while Gamma-MOB dominated in deep waters (paired t-test, $t = 1.99$, d. f. = 70, $p = 0.004$) (Fig. 1.2A). However, in Triton Alpha-MOB cells were more abundant than Gamma-MOB cells in both surface and bottom waters although differences were not statistically significant ($p > 0.05$; Figs. 1.1F and 1.2B). In general, both MOB types were more abundant in the deeper waters than surface or intermediate waters, but Gamma-MOB abundance showed a much more pronounced increase than Alpha-MOB towards the deeper waters in the stratified lakes (Fig. 1.2A).

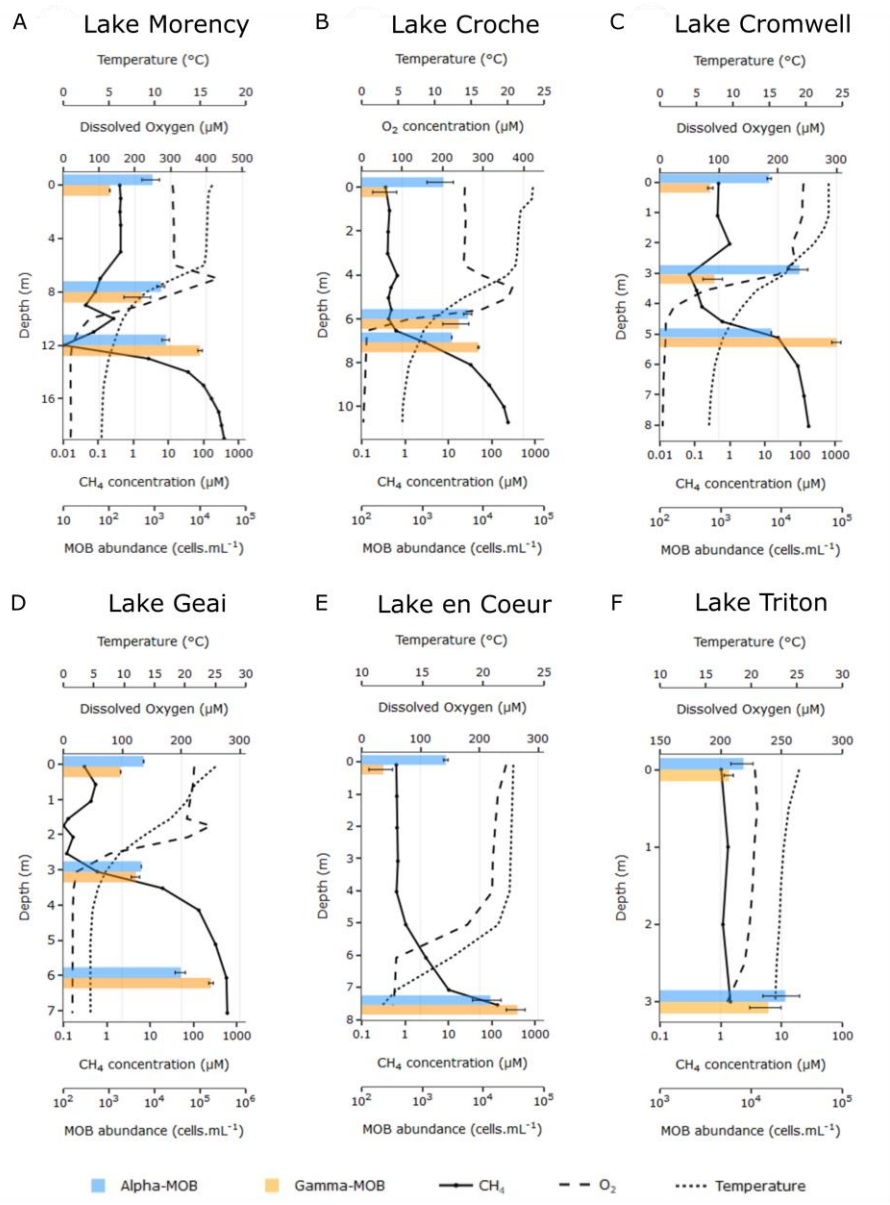

Figure 1.1. Vertical profiles of temperature, O_2, CH_4, and MOB cell abundance in the water column of study lakes. Alpha-MOB (first horizontal bar, in blue) and Gamma-MOB (second horizontal bar, in orange) cell abundances were enumerated by CARD-FISH in duplicates or triplicates using specific probes. Note log scale in the axes of CH_4 concentration and MOB cell abundance. Bars for MOB abundance indicate mean and whiskers indicate standard deviations.

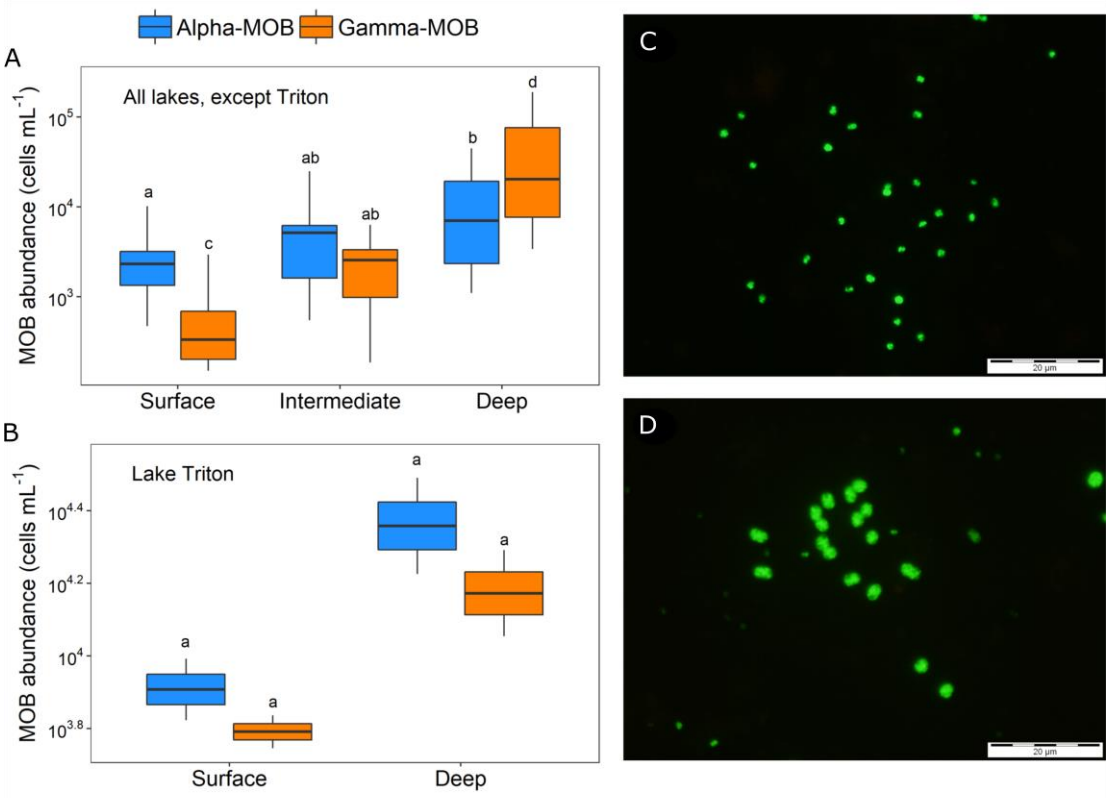

Figure 1.2. Vertical differences between Alpha-MOB and Gamma-MOB cell abundances across the water column of study lakes. Alpha-MOB (first, in blue) and Gamma-MOB (second, in orange) absolute cell abundance (cells mL^{-1}) *in situ* in (A) stratified lakes (all, except Triton) (Alpha-MOB: N = 35; Gamma-MOB: N = 32) and in (B) Triton, the only lake with well-oxygenated deep waters (Alpha-MOB: N = 4; Gamma-MOB: N = 4). C) Alpha-MOB and D) Gamma-MOB cells under the microscope visualized by CARD-FISH. Boxplots represent median, first and third quartiles (hinges), and 1.5 x interquartile range (whiskers). Levels not connected by same letter have significantly different means ($p < 0.05$; paired t-tests were used to detect differences between MOB groups within depths, and ANOVA and Tukey–Kramer HSD were used to detect differences within MOB groups across depths). 'Intermediate' refers to the intermediate depths sampled in the four deeper lakes and 'Deep' refers to the deepest layer sampled in each lake (as shown in Fig. 1.1). Note log scale in the y axes of A and B. Whole scale bars in C and D represent 20 µm.

1.4.2 CH_4 oxidation rates and MOB dynamics during incubations

CH_4 oxidation was detected in every sampled lake and depth (Fig. 1.3A). Decreases in CH_4 concentration through time were highly log-linear, a clear indication of first-order kinetics (minimum $R^2 = 0.92$). CH_4 oxidation rates varied widely from 0.004 to 65.7 μmol CH_4 L^{-1} d^{-1} across lakes, depths, and incubation temperatures, with the highest CH_4 oxidation rates in each lake happening in deep waters (Fig. 1.3A). In Morency, Cromwell and Geai, highest rates were associated with an increase in the proportion of Gamma-MOB in the community; i.e. there was an increase in the ratio between Gamma- and Alpha-MOB abundances (Gamma:Alpha-MOB) during the incubation with deep waters (Fig. 1.3B). In addition, the highest CH_4 oxidation rates across lakes were observed in the deep waters of Geai and Cromwell where the largest increases in the Gamma:Alpha-MOB ratio were also observed (Fig. 1.3B). In these incubations, Gamma-MOB cell abundance increased by up to 66% in Cromwell and up to 300% in Geai (Supporting Information Figs. S1.3c and S1.3d). In contrast, in en Coeur and Triton, there was a decrease in the Gamma:Alpha-MOB ratio during incubations, even though CH_4 oxidation rates were comparatively high in these lakes (Fig. 1.3B). In summary, in the well-stratified lakes, highest CH_4 oxidation rates were observed where Gamma-MOB were initially dominant and were generally coincident with an increase in the Gamma:Alpha-MOB ratio, while in the lakes with weak or no stratification CH_4 oxidation rates were coincident with a decrease in the Gamma:Alpha-MOB ratio. We did not find consistent effect of temperature on MOB community composition as both MOB groups showed variable response to temperature (Supporting Information Figs. S1.1–S1.3). However, in the deep waters of Geai, where the highest Gamma-MOB abundance was observed (7×10^5 cells mL^{-1}), temperature and Gamma-MOB abundance were inversely related (Supporting Information Fig. S1.3d). In general, the most abundant MOB group *in situ* maintained its higher abundance relative to the other during incubations independent of the temperature treatment (Supporting Information Figs. S1.1–S1.3).

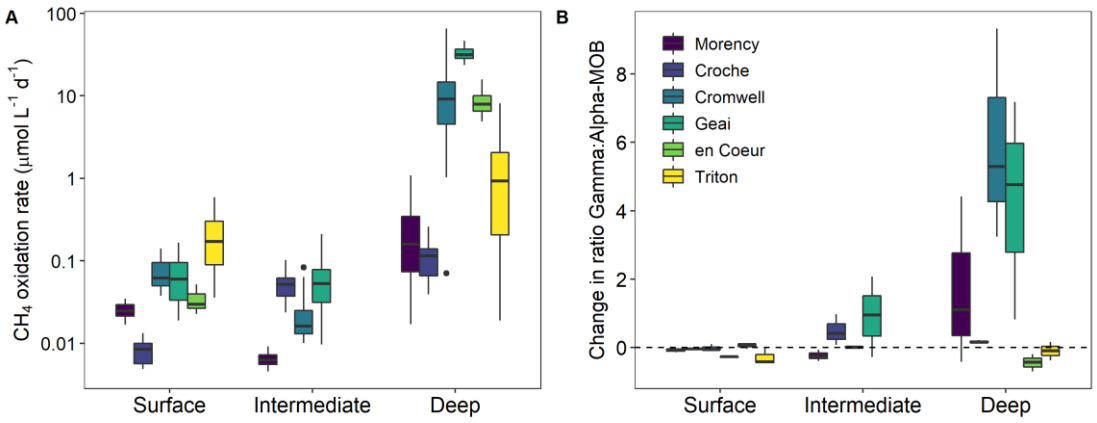

Figure 1.3. CH_4 oxidation rates and MOB dynamics in incubations in each studied lake (ordered from deepest to shallowest as shown in the legend). A) CH_4 oxidation rates B) Change in the ratio between the cell abundance of Gamma-MOB and Alpha-MOB at the end of incubations relative to *in situ* ratio (final ratio – *in situ* ratio). Plotted data include all CH_4 oxidation rates and MOB cell abundances measured across incubations, pooling together incubations carried out at different temperatures. Boxplots represent median, first and third quartiles (hinges), and 1.5 x interquartile range (whiskers). Dashed line in B indicates no change in the Gamma:Alpha-MOB ratio. 'Intermediate' refers to the intermediate depths sampled in the four deeper lakes and 'Deep' refers to the deepest layer sampled in each lake (as shown in Fig. 1.1).

1.4.3 Drivers of the abundance of MOB groups

Because of potential non-linearities between environmental factors and MOB dynamics, we examined their relationships using generalized additive models (GAMs). Besides CH_4, O_2, and temperature, we also included cDOM as a proxy for light (Thrane *et al.*, 2014) and DOC concentration because of previous findings on the importance of these factors to methanotrophic activity (Dumestre et al., 1999; Murase and Sugimoto, 2005; Oswald et al., 2015; Thottathil et al., 2018). We included both *in situ* and experimental data (taken at the end of incubations) in our models, since the outcomes were the same as when we considered only *in situ* MOB abundances. For DOC and cDOM concentrations, which were measured only *in situ*,

we assumed that they did not change significantly throughout the incubation experiments given the dark conditions and the duration of incubations (max. 8 days), which could have led to a maximum DOC loss of 0.4 mg L^{-1} assuming a DOC decay constant of 0.05 d^{-1} (Guillemette et del Giorgio, 2011). CH_4 ($\log_{10}$ CH_4), temperature, DOC ($\log_{10}$ DOC), and cDOM ($\log_{10}$ cDOM) were used as linear variables, but dissolved O_2 concentration was used as a smoothed variable because of our observation that the residuals of linear models predicting Alpha-MOB cell abundance had a clear quadratic relationship with O_2 residuals (details not shown). We found that Alpha-MOB abundance was predicted well by CH_4 ($p < 0.0001$), O_2 ($p < 0.0001$), and cDOM ($p = 0.006$) ($R^2_{adj} = 0.69$, $n = 82$, GAM; Supporting Information Table S1.2; Fig. 1.4A–C), while temperature ($p = 0.82$) and DOC ($p = 0.48$) were not significant predictors. O_2 showed a quasi bell-shaped relationship with Alpha-MOB, with highest cell abundance around 150 µM O_2 and declining on either side of this 'optimum' O_2 but increasing again close to 0 (Fig. 1.4A). CH_4 and cDOM were strongly and positively related to Alpha-MOB abundance (Fig. 1.4B, C). Differently, Gamma-MOB cell abundance was predicted well by O_2 ($p < 0.0001$; Fig. 1.4D) and CH_4 ($p < 0.0001$; Fig. 1.4E) only ($R^2_{adj} = 0.81$, $n = 95$, GAM; Supporting Information Table S1.2), while the other parameters were not significant (temperature: $p = 0.99$; DOC: $p = 0.08$; cDOM: $p = 0.1$). O_2 had a strong negative effect on Gamma-MOB abundance (Fig. 1.4D), while CH_4 had a strong positive effect on it (Fig. 1.4E).

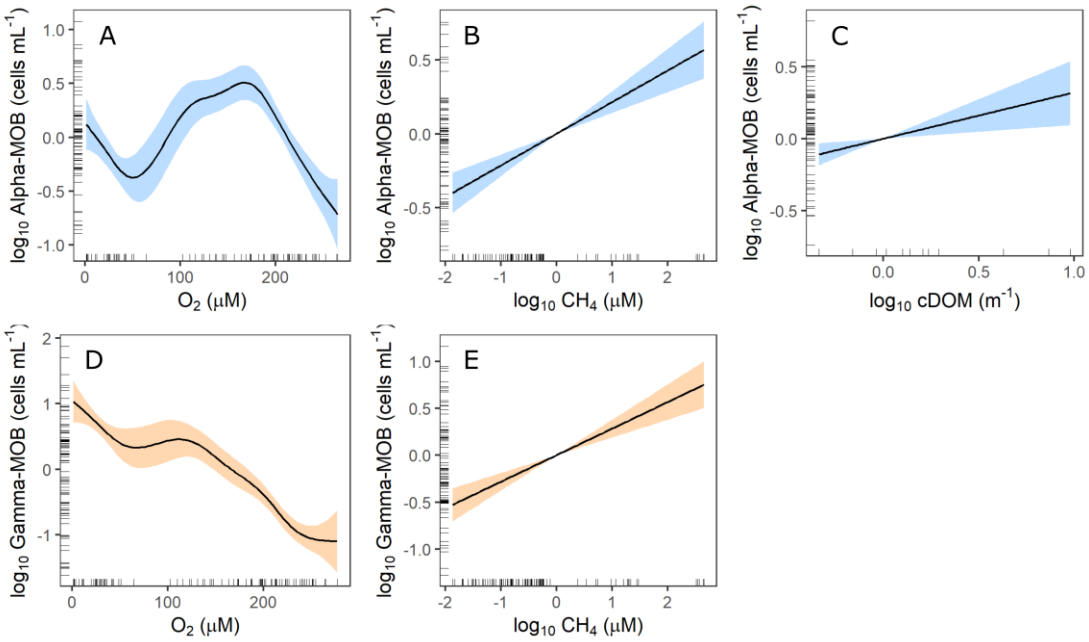

Figure 1.4. Partial effect plots (GAMs) of significant environmental predictors of each MOB group abundance (cells mL^{-1}). Significant predictors of Alpha-MOB were (A) O_2, (B) CH_4, and (C) cDOM, and of Gamma-MOB were (D) O_2 and (E) CH_4. Shaded areas mean 95% confidence intervals and marginal rug lines represent data distribution.

1.4.4 Relationship between MOB groups and CH_4 oxidation rates

Combining incubation experiments and MOB abundances *in situ*, the abundances of both MOB groups were well correlated with CH_4 oxidation rates. However, the correlation with Gamma-MOB was much stronger ($R^2 = 0.65$, $p < 0.0001$; Fig. 1.5B) than that with Alpha-MOB ($R^2 = 0.33$, $p < 0.0001$; Fig. 1.5A). As the MOB community was vertically structured along the water column (Figs 1.1 and 1.2), we evaluated whether there was also a vertical structuring of the contribution of each group to the overall CH_4 oxidation rates. We found that both MOB groups correlated well with CH_4 oxidation rates in every layer of the water column, but that Gamma-MOB showed stronger (R^2) and more statistically significant (p values) correlations

with the rates in every layer, including in the surface where they were less abundant than Alpha-MOB (Figs. 1.5C and D). In addition, an increase in both Gamma- and Alpha-MOB cell abundance seems to have a larger effect on CH_4 oxidation rates in deep waters than in the other layers, as indicated by the higher slope between the MOB abundances and rates in the deep than in the other layers (Fig. 1.5C and D).

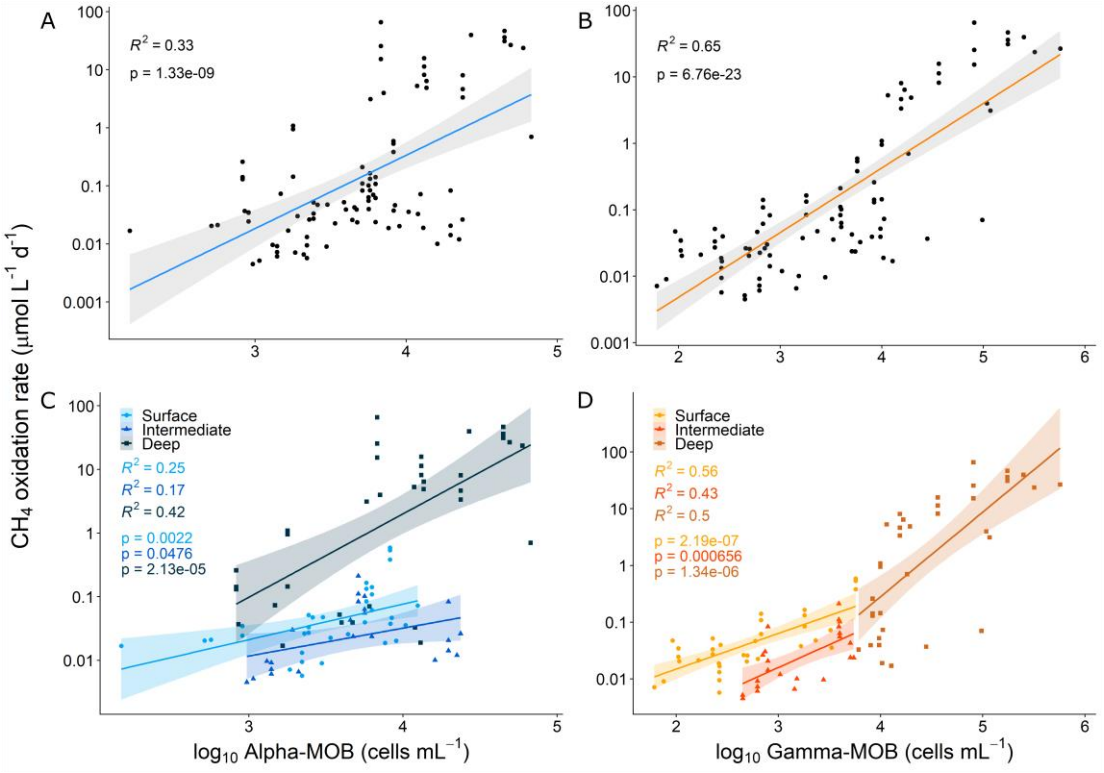

Figure 1.5. Relationship between MOB groups and CH_4 oxidation rates in the water column of studied lakes. A-B) Linear regressions between measured CH_4 oxidation rates (y axes) and MOB cell abundances (x axes) across all lakes and C-D) distinguishing between water column layers (circles: surface, triangles: intermediate, and squares: deep). Plotted data include samples from the six studied lakes. Note log-scale in y axes.

1.4.5 Contribution of MOB to the prediction of CH_4 oxidation rate

CH_4 oxidation rates could be strongly predicted by environmental variables, namely CH_4 concentration, temperature, and O_2 concentration (R^2 =0.90, $p < 0.0001$, n = 277, generalized regression, adaptive elastic net with AICc validation; Supporting Information Fig. S1.4). With such high R^2, the addition of other variables can only marginally increase the model fit. In the subset of observations where we had MOB abundances (initial and end of incubations; n = 94), including Gamma-MOB abundance explained 20% of the remaining variation (R^2 = 0.92, $p < 0.0001$; Fig. 1.6A), even though the model including microbial information had only one-third of the sample size of the environmental model. In contrast, while Alpha-MOB cell abundance was selected as a significant predictor of CH_4 oxidation rates, its addition to the environmental model did not increase its prediction power (R^2 = 0.90, $p < 0.0001$; details not shown). To determine whether Gamma-MOB abundance explained an additional amount of the variance in CH_4 oxidation rates than environmental factors alone, we fitted a linear regression between the residuals of the environmental model (which has temperature, CH_4, and O_2 as predictors) and the residuals of the model that predicts Gamma-MOB abundance based on the same environmental variables (as in a partial leverage plot). We found that these residuals were significantly correlated, and that Gamma-MOB abundance explains alone 23% of the variance in CH_4 oxidation rates (R^2 = 0.23, $p < 0.0001$, n = 94; Fig. 1.6B). More importantly, the effect size (log–log slope) associated with Gamma-MOB (0.43) suggests that, all environmental variables remaining constant, a doubling in Gamma-MOB abundance would increase the CH_4 oxidation rate by 34.7%. As a comparison, this is equivalent to the expected effect of a 3.6 °C increase in temperature assuming a Q_{10} of 2.4 for CH_4 oxidation (Thottathil *et al.*, 2019).

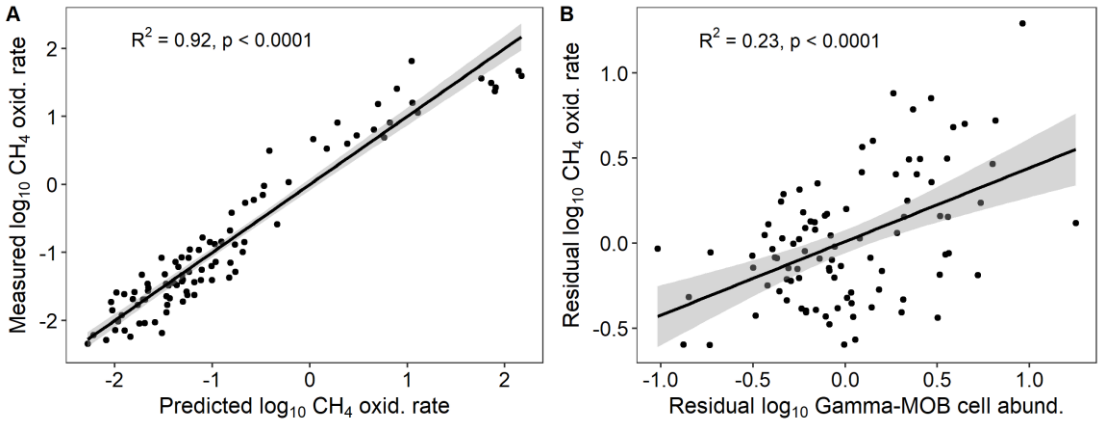

Figure 1.6. Effect of the addition of Gamma-MOB cell abundance to environmental model predicting CH_4 oxidation rate. A) Measured by predicted plot of the model of CH_4 oxidation rate using environmental variables and Gamma-MOB cell abundance as predictors. B) Linear regression between the residuals of the model of Gamma-MOB cell abundance using temperature, CH_4 concentration and square of O_2 concentration as predictors and the residuals of the environmental model of CH_4 oxidation rates.

1.5 Discussion

Our results provide evidence that the two major groups of methanotrophic bacteria in freshwater lakes (Alpha- and Gamma-MOB) show niche separation in temperate lake water columns which is mostly driven by O_2 concentration. Changes in the ratio of MOB groups during incubations with deep waters indicated that lower O_2 concentrations in well-stratified lakes favour the growth and activity of Gamma-MOB while higher O_2 concentrations in weakly or non-stratified lakes favour the growth and activity of Alpha-MOB. However, when all lakes are pooled together, Gamma-MOB seems to dominate methanotrophic activity across lakes and layers of the water column. Thus, our analysis lends strong support to the claim that Gamma-MOB generally dominates methanotrophic activity in lakes (Oswald *et al.*, 2016b ;

Rissanen *et al.*, 2018) but also provides insight into the contribution of Alpha-MOB. In addition, we demonstrate that microbial composition contributes to the regulation of CH_4 oxidation rates and that quantitative microbial data can help predict variations in lake methanotrophy.

1.5.1 Niche separation of MOB groups in lakes is mostly driven by O_2

We found evidence of a clear vertical stratification of the two proteobacterial MOB groups across lakes despite the wide range in environmental characteristics (Table 1.1). In the stratified lakes, the surface and intermediate waters were dominated by Alpha-MOB, whereas Gamma-MOB was dominant in deep waters (Fig. 1.1). These results are consistent with observations from the permanently stratified Lake Kivu where phospholipids fatty acids indicated dominance of Alpha-MOB in surface waters and dominance of Gamma-MOB in deep waters (Zigah *et al.*, 2015). However, in the well-oxygenated water column of shallow Triton, Alpha-MOB was more abundant than Gamma-MOB including in bottom waters. Accordingly, our GAMs results suggest that O_2, rather than CH_4 or temperature, drives the distribution of MOB groups in the water columns, with O_2 showing a non-linear relationship to Alpha-MOB with highest cell abundance around 150 µM (Fig. 1.4A) but a strong negative relationship to Gamma-MOB (Fig. 1.4D). Different studies have reported presence of Gamma-MOB under seemingly anoxic conditions in lakes (Biderre-Petit *et al.*, 2011 ; Blees *et al.*, 2014 ; Oswald *et al.*, 2016b), but the mechanisms underlying a preference for microaerophilic conditions despite their aerobic nature remain uncertain. These could include the utilization of cryptic photosynthetically produced O_2 (Oswald *et al.*, 2015 ; Savvichev *et al.*, 2019), the use of alternative electron acceptors such as metal oxides (Oswald *et al.*, 2016b), the ability to perform anaerobic respiration by denitrification (Kits *et al.*, 2015a, 2015b ; Oswald *et al.*, 2017 ; Skennerton *et al.*, 2015), or the ability to perform fermentation (Kalyuzhnaya *et al.*, 2013).

Still, although the GAMs showed that both MOB groups are positively correlated with CH_4 concentrations, the slope of the linear relationship between CH_4 and Alpha-MOB abundance (0.21) was lower than that between CH_4 and Gamma-MOB abundance (0.28; Supporting Information Table S1.2), suggesting a higher affinity for CH_4 of Alpha-MOB. Also, in Triton, where Alpha-MOB were more abundant than Gamma-MOB including in deeper waters, CH_4 concentrations in the deep were much lower than that in the deep waters of the stratified lakes (Fig. 1.1). The possession of genes encoding a high-affinity particulate methane monooxygenase enzyme (pMMO2) has been shown for a strain of *Methylocystis* which allows these members of the Alpha-MOB to oxidize CH_4 even at atmospheric CH_4 levels (Baani et Liesack, 2008). If this is the case for the Alpha-MOB present in our studied lakes, a higher affinity for CH_4 combined to the preference for or tolerance to higher O_2 levels could together explain the higher abundance of Alpha-MOB over Gamma-MOB in upper layers of the water columns.

We found that cDOM is also a significant predictor of Alpha-MOB abundance but not of Gamma-MOB. The removal of O_2 from the model for Gamma-MOB abundance lead to the inclusion of DOC and cDOM as significant predictors ($p < 0.0001$; details not shown), suggesting that organic carbon concentrations may affect Gamma-MOB abundance indirectly through effects on O_2 concentration such as increased respiration rates. On the other hand, the effect of cDOM on Alpha-MOB abundance is unlikely caused by its correlation with O_2 as both were selected simultaneously as predictors of Alpha-MOB. The positive effect of cDOM on Alpha-MOB may indicate light inhibition in clear water lakes (Dumestre et al., 1999; Murase and Sugimoto, 2005), but the weaker correlation of CH_4 oxidation rates with Alpha-MOB than with Gamma-MOB in surface waters incubated in the dark does not support this idea (Fig. 1.5C and D). Alternatively, as cDOM is a proxy of humic substances and thus of terrestrial organic matter (Cuthbert et del Giorgio, 1992), its relationship with Alpha-MOB abundance may indicate that a fraction of Alpha-MOB

cells in high cDOM lakes is originated from terrestrial environments. Accordingly, it has been shown that most Alpha-MOB taxa present in lakes are also found in soils, while Gamma-MOB taxa are mostly detected in lakes but not in soils (Crevecoeur *et al.*, 2019).

1.5.2 Both MOB groups contribute to overall CH_4 oxidation, but Gamma-MOB dominate methanotrophy in lakes

The significant correlations between both MOB groups and CH_4 oxidation rates in our study suggest that both Alpha- and Gamma-MOB are important players in the overall methanotrophic potential in lakes. The higher slopes of our linear regressions in deep waters (Fig. 1.5C and D) indicate the highest activity of both MOB groups in the deeper layers of the water column, which is in agreement with observed highest changes in Gamma:Alpha-MOB ratios in incubations with deep waters (Fig. 1.3B). However, Gamma-MOB abundances showed a stronger relationship with CH_4 oxidation rates than Alpha-MOB in every layer (Fig. 1.5C and D) suggesting that Gamma-MOB contributes more to the methanotrophic activity throughout the water column. Gamma-MOB may have an important role in the oxidation of CH_4 even in the surface waters despite their lower cell abundance as they showed stronger relationship to CH_4 oxidation rates than Alpha-MOB including in this layer. Accordingly, the Gamma-MOB abundance *in situ* showed a stronger positive relationship with $\delta^{13}CH_4$ than Alpha-MOB across lakes surface waters (Supporting Information Fig. S1.5), suggesting that the isotopic enrichment of CH_4 in the surface is mostly due to CH_4 oxidation by Gamma-MOB. A predominance of Gamma-MOB activity regardless of CH_4 and O_2 concentrations has been observed in other lakes (Biderre-Petit *et al.*, 2011 ; Crevecoeur *et al.*, 2017 ; Oswald *et al.*, 2016b ; Rissanen *et al.*, 2018 ; Schubert *et al.*, 2010). The wide range of O_2 and CH_4 concentrations measured in our incubations (1.14–277 μM O_2 and 0.01–455 μM CH_4) also supports a high tolerance to O_2 and CH_4 concentrations within Gamma-MOB, despite optimal conditions at low O_2 and high CH_4 concentrations (Fig. 1.4D and E). Moreover, we

found that in the well-stratified lakes, highest CH_4 oxidation rates were coincident with higher *in situ* Gamma-MOB abundance and often increase in the ratio between Gamma:Alpha-MOB cells during incubations. In contrast, in en Coeur and Triton where thermal stratification was weak or absent, highest CH_4 oxidation rates were coincident with low Gamma:Alpha-MOB ratio. In these lakes, deep waters were much more oxygenated than in the deep layers of other lakes, likely favouring Alpha-MOB activity over Gamma-MOB. Therefore, our results suggest that water column stratification indirectly affects the distribution of MOB groups and their contribution to the overall methanotrophic activity in lakes through its effect on the vertical distribution of O_2.

An overall weaker relationship of Alpha-MOB to CH_4 oxidation rates when compared to Gamma-MOB may be related to the potential soil origin of most Alpha-MOB taxa as previously mentioned (Crevecoeur *et al.*, 2019), which may lead to an inactive state or death of cells in waters. Moreover, Alpha-MOB has been suggested to be commonly dormant in the environment as they can be usually detected with gene markers but are often undetectable when using gene transcript markers (Ho *et al.*, 2013 ; Reim *et al.*, 2012). In addition, the existence of facultative methanotrophy within Alpha-MOB (genus *Methylocella*) (Dedysh *et al.*, 2005) could also reduce the strength of the relationship between Alpha-MOB abundance and CH_4 oxidation rates. The capability of growing on other carbon compounds besides CH_4 seems to be related to the presence of the soluble methane monooxygenase (MMO) enzyme, which is much more catalytically versatile than the pMMO and is widespread among Alpha-MOB but known to be present in only one Gamma-MOB taxon (Koh *et al.*, 1993 ; Theisen et Murrell, 2005).

1.5.3 The addition of Gamma-MOB cell abundance improves models of CH_4 oxidation rate

Information on microbial communities such as the presence of organisms, genes, or genes transcripts is not always well related to the rates of biogeochemical processes (Bier *et al.*, 2015 ; Rocca *et al.*, 2015). In our study, we not only found significant correlations between the cell abundance of the two MOB groups and CH_4 oxidation rates, but also that the addition of specific taxon (Gamma-MOB) abundance to environmental predictors increased the explanatory power of CH_4 oxidation models in lakes. Although the 2% increase in the model performance caused by the addition of Gamma-MOB cell abundance may seem marginal, it represents an important increase (20% of the unexplained variation) since at high R^2 values, small increases in R^2 are proportionally more important than at low R^2 (Prairie, 1996). In addition, Gamma-MOB explained 23% of the variation in CH_4 oxidation rate that could not be explained by environmental predictors alone. This suggests that intrinsic metabolic characteristics of Gamma-MOB explain part of the variation on CH_4 oxidation rates that cannot be explained by environmental factors. Also, our results show that a reduction in Gamma-MOB cell abundance, caused by grazing for example, leads to reduced CH_4 oxidation rates and therefore potentially higher lake CH_4 emissions, consistent with the recent finding that trophic cascade effects can modulate CH_4 efflux from lakes (Devlin *et al.*, 2015). Here, we provide a measure of the extent of this effect and further show that it is largely associated to one specific methanotrophic group, namely Gamma-MOB.

It is also important to consider that, although not evaluated in our study, other existing taxa of methanotrophic bacteria belonging to the Verrucomicrobia and the candidate phyla NC10 could also have contributed to the measured CH_4 oxidation rates in our incubations. However, we expect the role of these taxa in our incubations to be negligible since Verrucomicrobia methanotrophs are acidophilic, growing optimally at pH levels much lower than those found across our lakes (pH 2.0–2.5,

Dunfield et al., 2007), and NC10 methanotrophs aerobically oxidize CH_4 intracellularly under anoxic conditions (Ettwig *et al.*, 2010) while our incubations were performed under oxic conditions.

1.6 Conclusions

Most previous studies on the MOB community dynamics in lake water columns were performed on single systems, restricting general conclusions on lake MOB niches. By sampling in different lakes and distinct layers within the water column covering a large range of CH_4, O_2, DOC, and cDOM concentrations and using an absolute cell quantification method (CARD-FISH), we found a consistent vertical niche separation within the methanotrophic community in lakes which is mostly driven by O_2. We showed that this separation was related, to a large extent, to the inter- and intra-lake variability in CH_4 oxidation rates, with low O_2 concentrations in deep waters of well-stratified lakes favouring Gamma-MOB activity and growth while Alpha-MOB are mostly favoured under higher O_2 concentrations in lakes with well-oxygenated bottom layers. These findings suggest that Alpha-MOB may also be an important player in methanotrophy particularly in lakes with oxygenated bottom waters, despite the dominance of Gamma-MOB activity in well-stratified lakes as indicated by our study and others (Oswald *et al.*, 2016b ; Rissanen *et al.*, 2018). Using a modelling approach, we found that a combination of environmental factors and MOB community composition determines CH_4 oxidation rates in lake water columns with a significant and independent effect of Gamma-MOB cell abundance. This direct link between Gamma-MOB and CH_4 oxidation rates indicates that a reduction in Gamma-MOB abundance – for example, through grazing – leads to a reduction in CH_4 oxidation rates and potentially to an increase in lake CH_4 emissions. In summary, we showed that microbial composition is related to CH_4 oxidation rates in the water column of lakes and that microbial community information can improve our capacity to understand or even predict CH_4 dynamics in lakes.

1.7 Acknowledgements

The authors thank Irene Forn for the assistance during CARD-FISH analyses at the Institut de Ciències del Mar (ICM-CSIC), Barcelona, Spain and Felipe Rust for the help in the production of Figs. 1.1 and 1.5. This study was supported by Natural Sciences and Engineering Research Council of Canada Discovery grants to YTP and is a contribution to UNESCO Chair in Global Environmental Change. We also acknowledge financial support from the Groupe de recherche interuniversitaire en limnologie (GRIL), a strategic cluster of the Fonds derecherche du Québec - Nature et technologies (FRQNT). PCJR is supported by a doctoral scholarship from the Fonds de recherche Nature et technologies du Québec (FRQNT) and SDT was supported by the same scholarship during the sampling and experiments of this study.

1.8 Supplementary information

Table S 1.1 Group specific 16S rRNA target probes used for the detection of Proteobacterial aerobic methanotrophic bacteria. Probes Mγ84 and Mγ705 were used together in order to cover the whole Gamma-MOB group and provide good hybridization signal.

Probe	Target group	Probe sequence
Mα450	Alphaproteobacteria MOB	5'-ATC CAG GTA CCG TCA TTA TC-3'
Mγ84	Gammaproteobacteria MOB	5'-CCA CTC GTC AGC GCC CGA-3'
Mγ705	Gammaproteobacteria MOB	5'-CTG GTG TTC CTT CAG ATC-3'

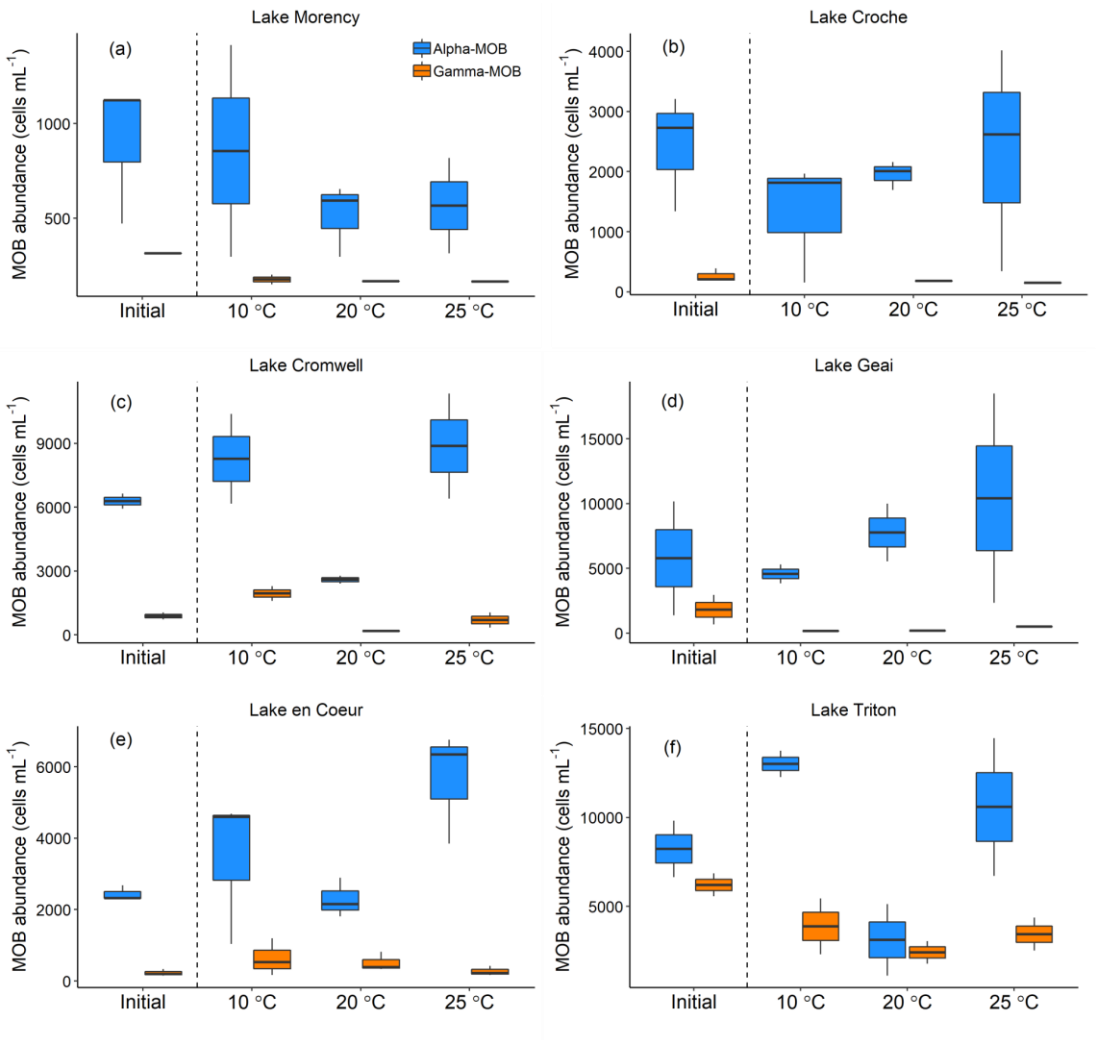

Figure S 1.1. Alpha- (first, in blue) and Gamma-MOB (second, in orange) cell abundance (cells mL^{-1}) measured before ('initial') and after experimental incubations with surface waters at different incubation temperatures. (a) Lake Morency; (b) Lake Croche; (c) Lake Cromwell; (d) Lake Geai; (e) Lake en Coeur; (f) Lake Triton. Boxplots represent median, first and third quartiles (hinges), and 1.5 *x* interquartile range (whiskers). Intermediate temperature is the approx. *in situ* temperature (20 °C).

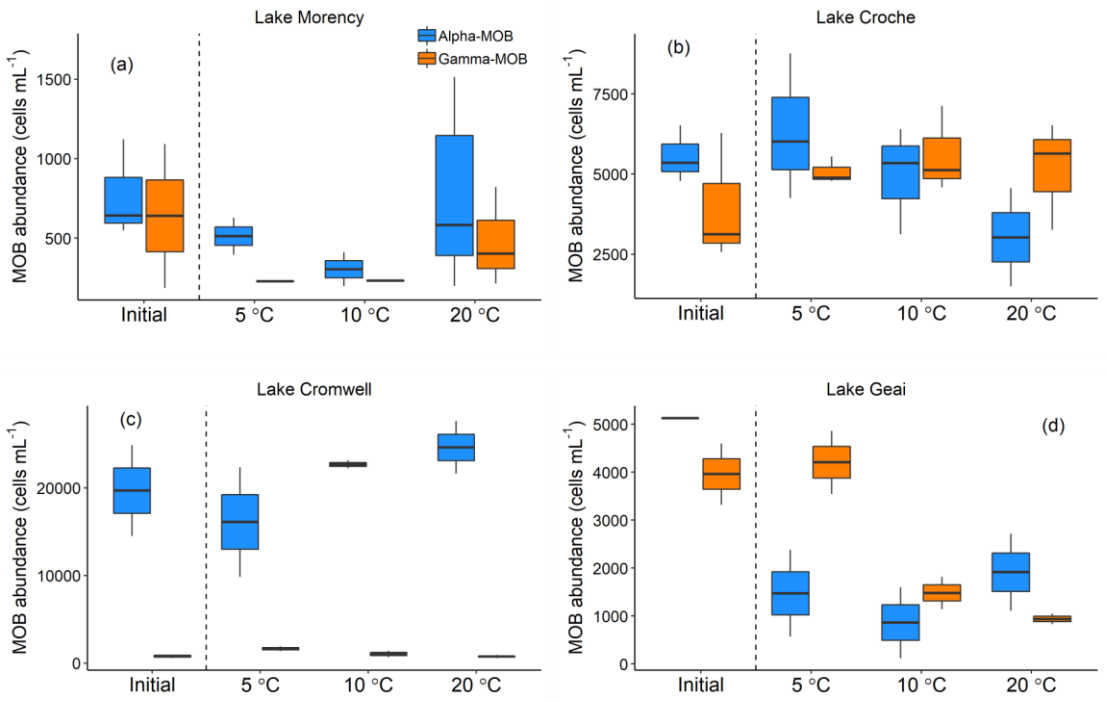

Figure S 1.2. Alpha- (first, in blue) and Gamma-MOB (second, in orange) cell abundance (cells mL^{-1}) measured before ('initial') and after experimental incubations with intermediate waters at different incubation temperatures. (a) Lake Morency; (b) Lake Croche; (c) Lake Cromwell; (d) Lake Geai. Boxplots represent median, first and third quartiles (hinges), and 1.5 *x* interquartile range (whiskers). Intermediate temperature is the approx. *in situ* temperature (10 °C).

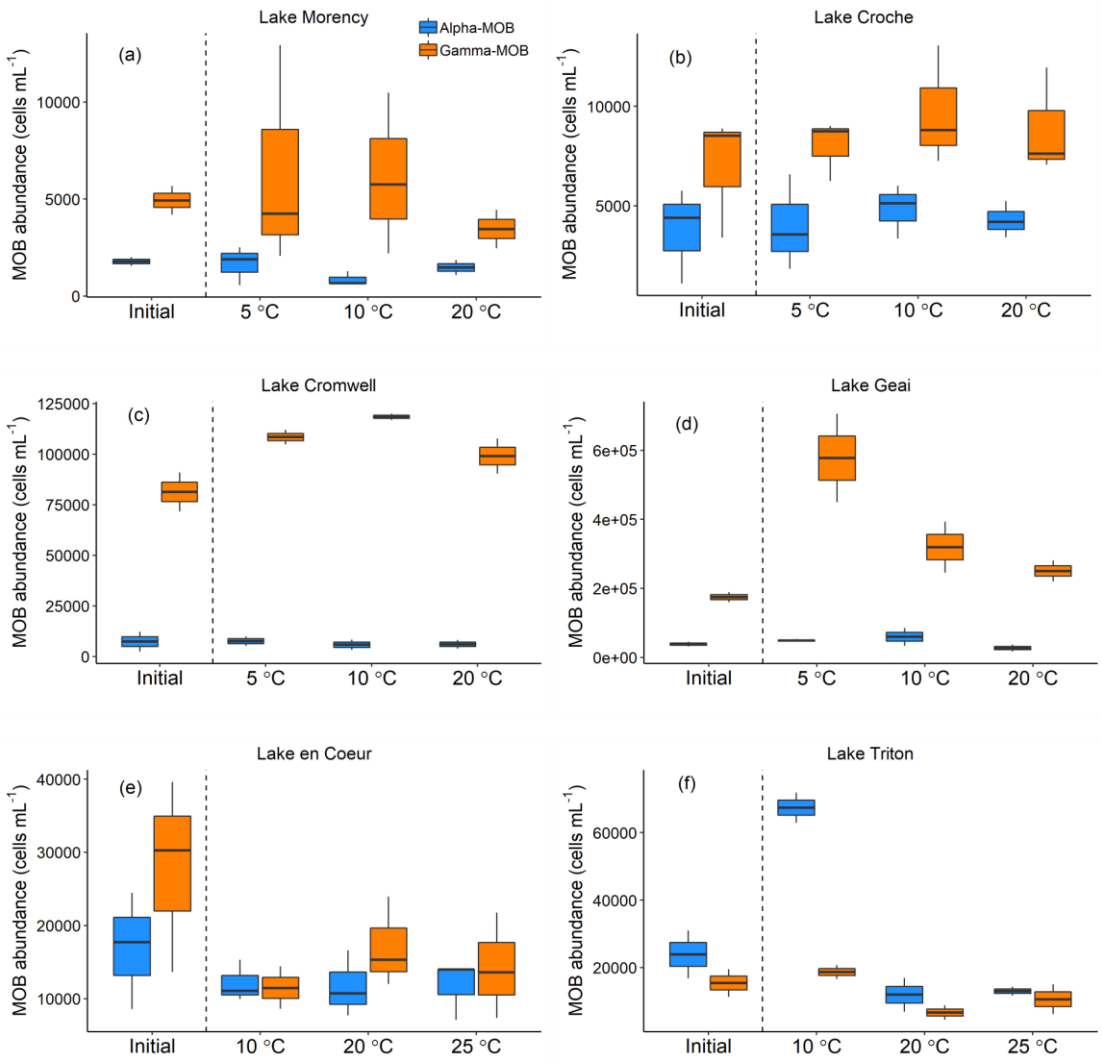

Figure S 1.3. Alpha- (first, in blue) and Gamma-MOB (second, in orange) cell abundance (cells mL^{-1}) measured before ('initial') and after experimental incubations with deep waters at different incubation temperatures. (a) Lake Morency; (b) Lake Croche; (c) Lake Cromwell; (d) Lake Geai; (e) Lake en Coeur; (f) Lake Triton. Boxplots represent median, first and third quartiles (hinges), and 1.5 *x* interquartile range (whiskers). Intermediate temperature is the approx. *in situ* temperature (10 °C for deep lakes (a, b, c, d) and or 20 °C for shallow lakes (e, f)).

Table S 1.2. Summary statistics for generalized additive models (GAM) predicting Alpha-MOB and Gamma-MOB cell abundances (cells mL^{-1}) based on environmental variables. Significant predictors for Alpha-MOB were O_2 (μM), CH_4 (μM), and cDOM (m^{-1}), and for Gamma-MOB were O_2 (μM) and CH_4 (μM). CH_4 and cDOM were used as linear terms whereas O_2 was used as smoothed term.

Alpha-MOB GAM model

Smoothed model term	**edf**	**Ref df**	**F**			**p-value**
$s(O_2)$	5.89	7.02	14.87			7.84e-14
Linear model term	**df**	**F**	**Estimate**	**Std. error**	**t-value**	**p-value**
Intercept			3.75	0.04	86.83	< 2e-16
$l(\log_{10}CH_4)$	1	33.14	0.21	0.03	5.75	1.88e-07
$l(\log_{10}$cDOM)	1	7.76	0.32	0.11	2.78	0.006

Gamma-MOB GAM model

Smoothed model term	**edf**	**Ref df**	**F**			**p-value**
$s(O_2)$	5.05	6.15	37.87			< 2e-16
Linear model term	**df**	**F**	**Estimate**	**Std. error**	**t-value**	**p-value**
Intercept			3.59	0.04	79.49	< 2e-16
$l(\log_{10}CH_4)$	1	34.9	0.28	0.04	5.90	6.44e-08

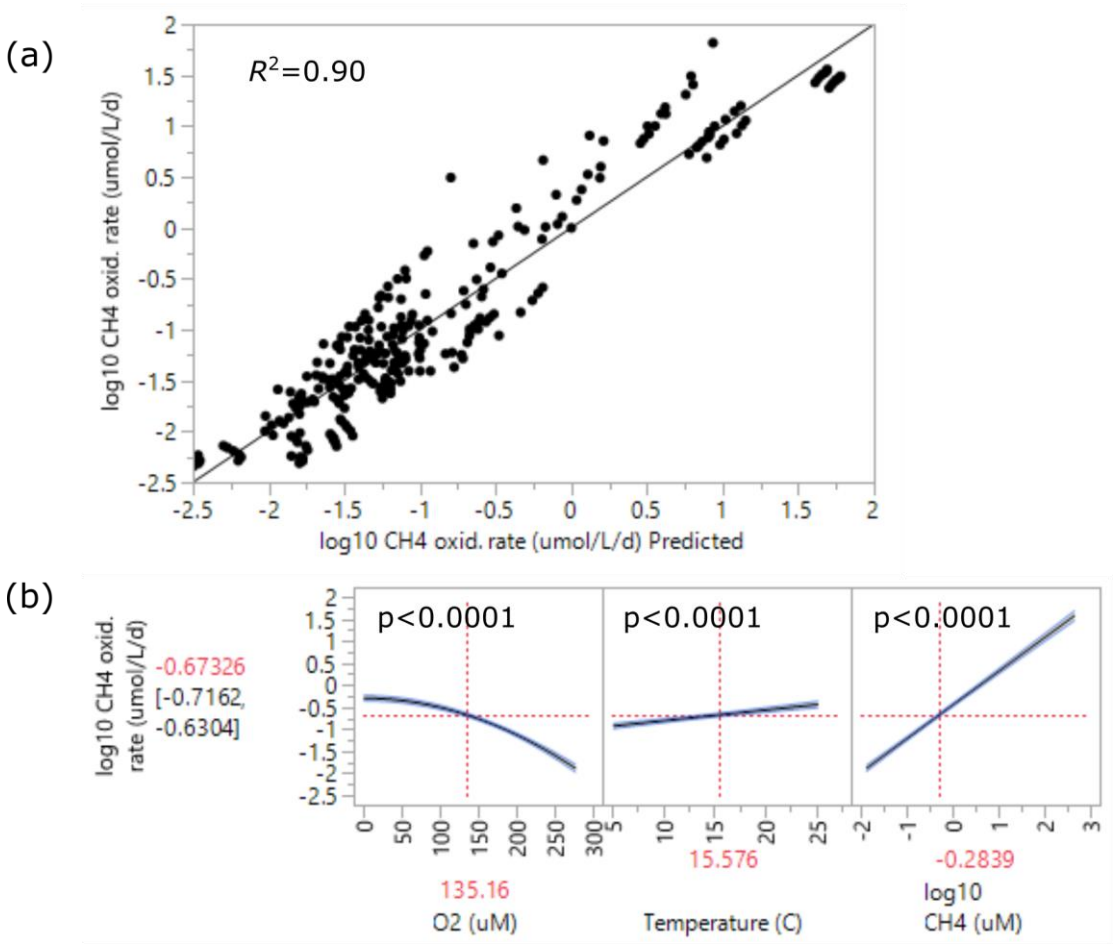

Figure S 1.4. Environmental model of CH_4 oxidation rate predicted by temperature, CH_4, and square O_2 concentrations (R^2 = 0.90, n = 277, RMSE = 0.33, Generalized Regression, Adaptive Elastic Net, AICc validation). (a) Actual by predicted CH_4 oxidation rates. (b) Prediction profilers of each predictor variable of the model showing their individual effects on CH_4 oxidation rate (O_2: $p < 0.0001$, temperature: $p < 0.0001$; CH_4: $p < 0.0001$).

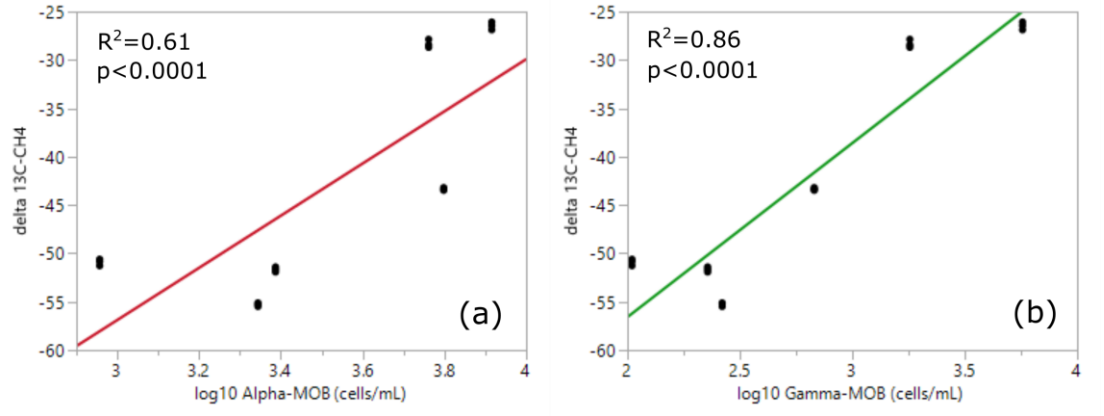

Figure S 1.5. Relationship between *in situ* δ^{13}C-CH_4 and average Alpha-MOB (a) and Gamma-MOB (b) cell abundance (cells mL^{-1}) in the surface waters of studied lakes.

CHAPTER II

RAPID SHIFTS IN METHANOTROPHIC BACTERIAL COMMUNITIES MITIGATE METHANE EMISSIONS FROM A TROPICAL HYDROPOWER RESERVOIR AND ITS DOWNSTREAM RIVER

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N.B. References cited in this chapter are presented at the end of the thesis.

2.1 Abstract

Methane-oxidizing bacteria (MOB) present in the water column mitigate methane (CH_4) emissions from hydropower complexes to the atmosphere. By creating a discontinuity in rivers, dams cause large environmental variations, including in CH_4 and oxygen concentrations, between upstream, reservoir, and downstream segments. Although highest freshwater methanotrophic activity is often detected at low oxygen concentrations, CH_4 oxidation in well-oxygenated downstream rivers below dams has also been reported. Here we combined DNA and RNA high-throughput sequencing with microscopic enumeration (by CARD-FISH) and biogeochemical data to investigate the abundance, composition, and potential activity of MOB taxa from upstream to downstream waters in the tropical hydropower complex Batang Ai (Malaysia). High relative abundance of MOB (up to 61% in 16S rRNA sequences and 19% in cell counts) and enrichment of stable isotopic signatures of CH_4 (up to 0‰) were detected in the hypoxic hypolimnion of the reservoir and in the outflowing downstream river. MOB community shifts along the river-reservoir system reflected environmental sorting of taxa and an interrupted hydrologic connectivity in which downstream MOB communities resembled reservoir's hypolimnetic communities but differed from upstream and surface reservoir communities. In downstream waters, CH_4 oxidation was accompanied by fast cell growth of particular MOB taxa. Our results suggest that rapid shifts in active MOB communities allow the mitigation of CH_4 emissions from different zones of hydropower complexes, including in quickly re-oxygenated rivers downstream of dams.

2.2 Introduction

The damming of rivers for hydropower generation can alter the carbon biogeochemical cycling across the entire river network through increased water

residence time, water column stratification, and anoxic conditions in sediments and bottom waters (Tremblay *et al.*, 2005). These changes can affect the place and time in which carbon is processed and mineralized, ultimately affecting the dynamics of greenhouse gases in these systems (Prairie *et al.*, 2017). For instance, reservoirs are important CH_4 sources to the atmosphere, emitting relevant amounts of CH_4 at a global scale, although current estimates of their significance vary widely (3-14 TgC-CH_4 $year^{-1}$; St. Louis et al. 2000; Barros et al. 2011; Deemer et al. 2016). A better understanding of the processes controlling CH_4 emissions from hydropower reservoirs could contribute to more accurate estimations as well as predictions of future reservoirs' CH_4 emissions.

A particularly important process regulating CH_4 emissions from reservoirs is CH_4 oxidation performed by methane-oxidizing bacteria (MOB, here also referred to as methanotrophs) present in the water column. These bacteria can oxidize a large fraction of the CH_4 produced in freshwaters before it reaches the atmosphere, strongly mitigating the carbon footprint of hydropower impoundments (e.g. Guérin and Abril, 2007; Itoh et al., 2015). MOB comprise an ecologically diverse polyphyletic group of bacteria mostly distributed within the Classes Gammaproteobacteria (Gamma-MOB) and Alphaproteobacteria (Alpha-MOB), but also in the phyla NC10 and Verrucomicrobia (Kalyuzhnaya *et al.*, 2019). These different groups show distinct environmental niches and tolerances, metabolic capacities, and life strategies (Dunfield *et al.*, 2007 ; Ettwig *et al.*, 2010 ; Hanson et Hanson, 1996 ; Ho *et al.*, 2013) that are related to gradients in environmental variables in freshwaters such as O_2 and CH_4 concentrations, temperature, and pH (Borrel et al., 2011; Conrad, 2007; Crevecoeur et al., 2019; Reis et al., 2020b) and that can lead to different contributions to the overall CH_4 oxidation rate (Reis et al. 2020b). Dams constitute a discontinuity in river systems causing large environmental variations between upstream, reservoir, and downstream segments, which have been shown to alter the structure of river bacterioplankton communities (Dumestre et al., 2001; Ruiz-González et al., 2015b,

2013). Similarly, thermal stratification within reservoirs can generate large environmental changes between water column layers leading to a rearrangement of the inflowing bacterial communities (Dumestre et al., 1999b). Such environmental variations could thus trigger the establishment of distinct MOB communities and associated activity between zones within hydropower complexes, thereby influencing the role of MOB taxa in mitigating CH_4 emissions. For instance, it has been shown that while Gamma-MOB dominates overall CH_4 oxidation in temperate lakes (Oswald et al., 2016b; Reis et al., 2020b; Rissanen et al., 2018), Alpha-MOB may be important players particularly in well-oxygenated waters (Reis et al., 2020b; Zigah et al., 2015).

Besides CH_4 emissions from the surface of reservoirs, hydropower turbines often draw water from CH_4-rich hypolimnia, and their CH_4 content is then released either immediately at the outflow (degassing) or at the surface of the downstream river (downstream diffusion). Due to strong water column stratification, these two downstream emission pathways often contribute the most to the greenhouse gas footprint of reservoirs in tropical regions (Abril *et al.*, 2005 ; Guérin *et al.*, 2006 ; Kemenes *et al.*, 2007 ; Soued et Prairie, 2020). Freshwater methanotrophs appear to be most active at oxic-anoxic interfaces, with their activity being reduced at high dissolved O_2 concentrations (Rudd *et al.*, 1974 ; Steinle *et al.*, 2017 ; Thottathil *et al.*, 2019). Therefore, one would expect a decrease in methanotrophic activity due to a rapid re-oxygenation of turbine outflowing waters. Interestingly, however, significant microbial CH_4 oxidation has been detected in rivers downstream of dams by means of CH_4 concentration, flux, and stable isotopes, comprising between 20 and 85% of the CH_4 loss in downstream reaches (Abril *et al.*, 2005 ; Guérin et Abril, 2007 ; Kemenes *et al.*, 2007 ; Soued et Prairie, 2020). However, it remains unknown whether the reported CH_4 oxidation in downstream waters is due to residual activity of MOB transported from the reservoir's hypolimnion, or alternatively, to shifts in methanotrophic communities once these microorganisms are exposed to well-

oxygenated conditions. Knowledge of the dynamics of MOB communities along the river-reservoir continuum can thus provide insights into the ecological mechanisms behind CH_4 oxidation and emissions from hydropower complexes.

Here we examine the dynamics of MOB communities in the tropical Batang Ai hydropower complex throughout its hydrological continuum, from the reservoir inflows to the downstream outflow. We used high-throughput sequencing of the 16S rRNA gene (DNA) and of the 16S rRNA (RNA) to determine the diversity and potential activity of MOB taxa along this hydropower river-reservoir system, as well as catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH) to microscopically quantify the Alpha- and Gamma-MOB groups. We expected that the hydrologic disruption caused by the dam would lead to strong environmental sorting of upstream inflowing MOB taxa and that the change from hypoxic hypolimnetic waters to the rapidly re-oxygenated turbine outflowing waters would trigger the growth and activity of MOB taxa that may be responsible for the oxidation of CH_4 in downstream waters.

2.3 Methods

2.3.1 Study site and sampling

The hydropower complex Batang Ai is located in the Borneo Island, Malaysia (1°09’36”N 111°54’00”E), in a tropical equatorial climate with year-round hot and humid rainy weather. The reservoir was created in 1985, by damming of the Batang Ai river and flooding of un-cleared tropical forest. It has a surface area of 68.4 km^2, a mean depth of 28 m and maximum depth of 72 m, and two major river inflows that flow into two reservoir branches (Fig. 2.1). The water residence time in the reservoir is approx. 11 months, but of only around 1 h in the studied segments of the upstream and downstream rivers. The main basin of the reservoir is permanently stratified with

the thermocline around 12 m depth, the oxycline between 12 and 20 m depth, and anoxic bottom waters. The reservoir is characterized by high water temperatures (31 °C at the surface and 24 °C at the bottom) around the year, and low concentrations of dissolved organic carbon (<1 mg L^{-1} on average), chlorophyll (surface mean: 1.3 µg L^{-1}), total phosphorus (surface mean: 5.9 µg L^{-1}), and total nitrogen (surface mean: 0.1 mg L^{-1}) (Soued et Prairie, 2020). In the main basin of the reservoir, diffusive CH_4 emissions to the atmosphere are moderate, and most CH_4 is emitted downstream of the dam since the water intake for the turbines is located in the highly CH_4-rich hypolimnion (Soued et Prairie, 2020). Nevertheless, CH_4 oxidation in the first 19 km downstream of the dam has been shown to reduce further diffusive CH_4 emissions by roughly 20% (Soued et Prairie, 2020).

Two sampling campaigns were performed, one in April-May 2017 and another in February-March 2018, both showing similar inflow discharge rates, although the official rainy season happens between October and March. In each sampling campaign, samples for CH_4 and microbial communities were collected with a peristaltic pump at 10 different sites and at different depths in the reservoir, covering the horizontal gradient (upstream, reservoir, and downstream sites – one at the power station and another at 2.7 km further downstream) and the vertical stratification within the reservoir (mixed layer and oxic and hypoxic hypolimnion, with oxic and hypoxic being defined as O_2 > and < 2 mg L^{-1}, respectively) (Fig. 2.1). Vertical profiles of temperature, dissolved O_2 concentration, and pH were measured with a YSI multiparameter probe (Yellow Springs Instruments, OH, USA). Sampling depths for microbial communities (Table S2.1) were determined based on the temperature and O_2 profiles in order to cover the thermal stratification layers but also the different O_2 concentrations in the water column, given that the O_2 profiles revealed a variation in the oxygenation of stratified layers from one site to another. To capture the changes in CH_4 and methanotrophic communities from the hypolimnion of the

reservoir until the downstream river, we used the closest available samples to the turbine water intake in both years (2017: site A, depth 20 m; 2018: site I, depth 24 m).

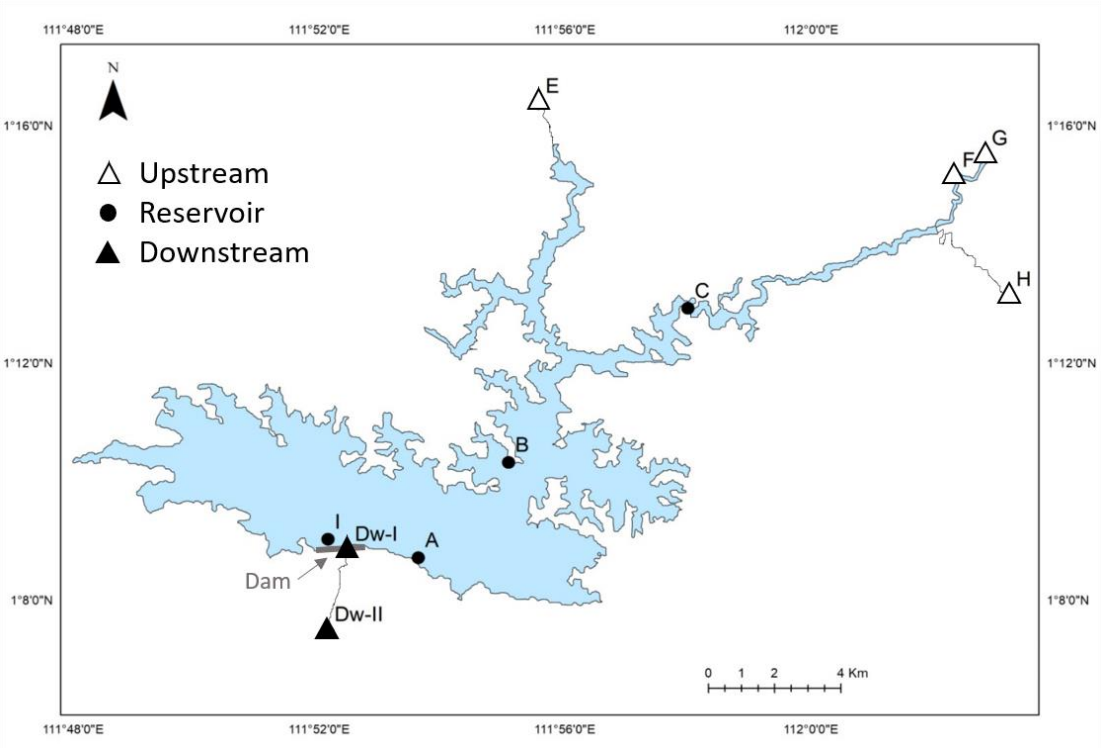

Figure 2.1. Map of Batang Ai hydropower reservoir (Borneo Island, Malaysia) showing sampling locations of this study. Sites A, B, C, and I are located in the reservoir, with site I being the closest to the water intake for the turbines. Sites E, F, G, and H are located upstream of the reservoir. Site Dw-I is located at the power station right after the dam and Dw-II is located at 2.7 Km further downstream. One extra site at 0.6 Km downstream of the dam (not shown) was sampled for CH_4 concentration and stable isotopic signature (see Fig. 2.6). Map created in ArcGIS 10.1.

2.3.2 CH_4 concentration, stable isotopic signature, and downstream CH_4 oxidation

Measurements of dissolved CH_4 concentration and its stable isotopic signature ($\delta^{13}CH_4$) were determined using the headspace technique in 60 mL gas-tight syringes followed by gas chromatography (GC-8A/GC-2014, Shimadzu, Kyoto, Japan) and cavity ring down spectrometry (G2201-i, Picarro Inc., CA, USA). The $\delta^{13}CH_4$

signature is a useful indicator of microbial CH_4 oxidation since the methane monooxygenase enzyme preferentially oxidizes ^{12}C-CH_4 over ^{13}C-CH_4 leaving an enriched $\delta^{13}CH_4$ signature in the remaining CH_4 pool (Coleman *et al.*, 1981).

In the downstream river, we calculated the fraction and amount of CH_4 oxidized based on the evolution of CH_4 concentration and stable isotopic signature from the turbine water intake in the reservoir (considered as the CH_4 source) until 2.7 km downstream of the dam, using model developed in Soued and Prairie (2020).

2.3.3 MOB community composition

We used amplicon sequencing of the 16S rRNA gene (DNA) and of the 16S rRNA (RNA) of 37 water samples to identify and determine the relative abundance of prokaryotes belonging to methane-oxidizing bacteria (MOB) taxa. We then extracted the MOB taxa from the 16S rRNA database to investigate the taxonomic composition of MOB assemblages and community dynamics along the river-reservoir system. By sequencing the DNA, we aimed at evaluating the presence of MOB taxa and their role in the total bacterial community independently of their activity state, while by sequencing the RNA we aimed at assessing the contribution of MOB taxa to the potentially protein synthesizing bacterial community (Blazewicz *et al.*, 2013).

For each sample, between 400 and 700 mL of water were prefiltered through a 30 µm pore size mesh and then filtered through Durapore membrane filters (0.22 µm pore size, GVWP, Millipore). Filters were preserved in RNAlater (Invitrogen), stored at 4 °C overnight, and then stored at -80 °C before nucleic acids extraction. All samples were extracted with PowerWater RNeasy extraction kit (Qiagen) skipping the step for DNA digestion to recover both DNA and RNA from the same filter. PCR amplification of the 16S rRNA gene with primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') was carried out in every sample and samples

with PCR inhibition (detected by the lack of amplification of positive control added into samples) were cleaned with a DNA cleaning kit (Qiagen). After that, half of each sample (25 µL) was digested with a DNase Max kit (Qiagen) to get pure RNA which was verified by the lack of amplification of the 16S rRNA gene by PCR and gel electrophoresis. Finally, cDNA was produced from RNA using a reverse transcription kit (Qiagen). DNA and cDNA 16S amplicons were sequenced in GenomeQuebec following a pair-end approach using Illumina MiSeq platform. The raw Illumina reads have been deposited in NCBI SRA under accession number PRJNA649249.

Sequences were analysed using the dada2 pipeline v1.8 (Callahan *et al.*, 2016) in R v3.5.1 (The R Core Team, 2019) and R Studio (RStudio, 2018). Read quality was inspected using quality profiles and both forward and reverse reads were trimmed to 245 nucleotides. Primers were also removed using trimLeft() function in dada2 (19 nucleotides in forward and 20 in reverse reads). The average number of reads per sample was 77,948 (range 7,399 – 115,142). An Amplicon Sequence Variant (ASV) table was produced following the dada2 pipeline with an additional step to collapse together sequences that are only different in length ('collapseNoMismatch()'), which minimizes false singletons. Taxonomy assignment was performed until the Genus level with SILVA reference database v132 using dada2's 'assignTaxonomy' function. Briefly, the 'assignTaxonomy' function uses a naïve Bayesian classifier to compare the query sequences to sequences with assigned taxonomy in a database and then a bootstrapping approach to assess the confidence estimates for each assignment (Wang *et al.*, 2007). Sequences assigned to Archaea, eukaryotes, or chloroplast were removed. The ASV table (17,050 ASVs) was then rarefied using the R package phyloseq v1.22.3 (McMurdie et Holmes, 2013) at a minimum of 50,000 reads per sample – leading to the loss of two samples – and normalized to proportions so that ASVs abundances are ratios from 0 to 1. The final rarefied dataset contained a total of 14,323 ASVs. The final ASV table was screened for known methanotrophic bacterial taxa: Order Melthylococcales (Class Gammaproteobacteria); genera *Methylocystis*

and *Methylosinus* (Class Alphaproteobacteria, Family Methylocystaceae); genera *Methylocella*, *Methylocapsa*, and *Methyloferula* (Class Alphaproteobacteria, Family Beijerinckiaceae); genera *Methylacidiphilum* and *Methylacidimicrobium* (Phylum Verrucomicrobia); and '*Ca.* Methylomirabilis oxyfera' and putative methanotrophs within the Order Methylomirabilales (Candidate phylum NC10) (Dunfield *et al.*, 2007 ; Ettwig *et al.*, 2010 ; Kalyuzhnaya *et al.*, 2019 ; Knief, 2015). For abundant MOB ASVs that could only be assigned at the Family level, we used the NCBI Nucleotide BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify to which taxa they showed closest percent identity.

2.3.4 Cell abundance of Proteobacterial MOB groups

We determined the absolute abundance of MOB cells belonging to Alpha- and Gammaproteobacteria through catalyzed reporter deposition-fluorescence in situ hybridization (CARD-FISH). Only the subset of samples in which MOB sequences was higher than 1% of total 16S rRNA sequences (DNA or RNA) were analysed (n = 20), which excluded all samples from the surface mixed layer of the reservoir (Table S2.1). Water samples for CARD-FISH were treated and analysed as described in Reis et al. (2020b). Briefly, water samples (40 mL) were fixed overnight with buffered paraformaldehyde (PFA, 1% final concentration) and then 6 to 10 mL of sample were filtered through 0.2 µm polycarbonate filters (Millipore GTTP, 25 mm). Cells were attached with 0.1% agarose and permeabilized with lysozyme and achromopeptidase. Hybridization was carried out with specific probes designed for the detection of Alpha- and Gammaproteobacteria MOB (Table S2.2; Eller et al. 2001) overnight at 35 °C in 40% formamide hybridization buffer. Both CARD-FISH positive and DAPI (4',6-diamidino-2-phenylindole staining) positive cells were quantified under an automated epifluorescence microscope at 630x magnification counting on average 50 (min-max: 36-55) quality-checked fields of view. Cell counts of Alpha- and Gamma-

MOB (CARD-FISH) as well as those of total prokaryotic cells (DAPI) were determined using the ACME tool3 software (Zeder, 2014).

2.3.5 Statistical analyses

Statistical differences between means were tested using Welch's unequal variances t-test on the log data when necessary. Canonical Analyses of Principal Coordinates-CAP (Anderson et Willis, 2003) based on the Bray-Curtis distance of the relative abundances of MOB ASVs in a sample were used to investigate differences in the MOB community structure in DNA and RNA and their environmental drivers across the sampled sites. We included temperature, pH, CH_4, O_2 and dissolved organic carbon (DOC) concentrations as environmental variables in the model because of their reported influence on freshwater MOB community composition or activity (Borrel et al., 2011; Conrad, 2007; Crevecoeur et al., 2019; Reis et al., 2020b; Thottathil et al., 2018). All analyses were performed in R v3.5.1 (The R Core Team, 2019) and R studio (RStudio, 2018) and plots were produced using the packages ggplot2 3.1.0 (Wickham, 2016) and phyloseq v1.22.3 (McMurdie et Holmes, 2013).

2.4 Results

2.4.1 CH_4, $\delta^{13}CH_4$, and O_2 dynamics

CH_4 concentration, CH_4 stable isotopic signature, and dissolved O_2 concentration varied largely both horizontally and vertically in Batang Ai (Figs. 2.2A-C, 2.3). In both sampling campaigns, the highest CH_4 concentration was detected in the anoxic zone of the reservoir's hypolimnion, followed by downstream and upstream waters (Figs. 2.2, 2.3). CH_4 concentration was highly variable within the hypoxic hypolimnion, varying up to three orders of magnitude even within a single profile (Figs. 2.2A, 2.3). This was caused by intense microbial CH_4 oxidation in the hypoxic hypolimnion as indicated by enriched $\delta^{13}CH_4$ values measured in this layer

throughout the system (Fig. 2.2B) and in individual profiles (Fig. 2.3). In contrast, the $\delta^{13}CH_4$ in the oxic hypolimnion and in the surface mixed layer, particularly in 2017, suggests less methanotrophic activity in these layers (Figs. 2.2B, 2.3). O_2 concentrations were highest in upstream waters and in the reservoir mixed layer and decreased steeply with depth in the main basin of the reservoir (Figs. 2.2C, 2.3). Average O_2 concentration in the downstream river was higher than in the hypoxic hypolimnion of the reservoir, demonstrating that hypolimnetic waters get quickly oxygenated once leaving the turbines, despite not being as oxygenated as the upstream river or the surface mixed layer of the reservoir (Fig. 2.2C).

2.4.2 Relative abundance of MOB in DNA and RNA samples and abundance of MOB cells

The relative abundance of total MOB DNA and RNA sequences in the bacterial communities varied widely across samples with an average of 3.7% ± 5.8 (±SD) and 10.4% ± 15.2 of total 16S rRNA sequences in DNA and RNA, respectively. MOB sequences in upstream waters comprised on average 2.5% of the total sequences in DNA and 4.3% of the total sequences in RNA (Figs. 2.2D, E). In the reservoir, the relative abundance of MOB sequences was negligible in the mixed layer but increased in the oxic hypolimnion and reached extremely high values in the hypoxic hypolimnion (up to 27.6% in DNA and 61.2% in RNA), in line with the reduction in CH_4 concentration and enrichment in $\delta^{13}CH_4$ values in this layer observed in the profiles. The downstream river also showed high MOB relative abundances, with MOB sequences accounting for 8.5% ± 3.2 of total 16S rRNA sequences in DNA and 25.4% ± 7 in RNA across campaigns (Figs. 2.2D, E). Across all samples, the relative abundance of MOB sequences based on RNA was on average three times higher than that based on DNA.

The total MOB cell counts assessed by CARD-FISH showed a similar spatial pattern to that of the DNA and RNA data. The highest MOB cell abundance was measured in

the hypoxic hypolimnion in 2017 (median: 6 x 10^4 cells mL^{-1}) followed by the downstream river (median: 4.1 x 10^4 cells mL^{-1}) and upstream sampling sites (median: 3.7 x 10^4 cells mL^{-1}) (Fig. 2.2F). In terms of relative abundances, the highest contribution of MOB cells to total bacterial cells was also detected in the hypoxic hypolimnion of the reservoir, where MOB-hybridized cells made up to 19.2% of total prokaryotic counts (details not shown). The abundance of total MOB cells was weakly but positively correlated to $\delta^{13}CH_4$ ($p=0.01$, $R^2=0.16$; Fig. S2.1A) across the sampled zones in the river-reservoir system, indicating that MOB cell number is related to methanotrophic activity. The relative abundance of MOB 16S rRNA sequences in DNA and RNA, however, were not significantly related to $\delta^{13}CH_4$ ($p>0.05$; Fig. S2.1B, C).

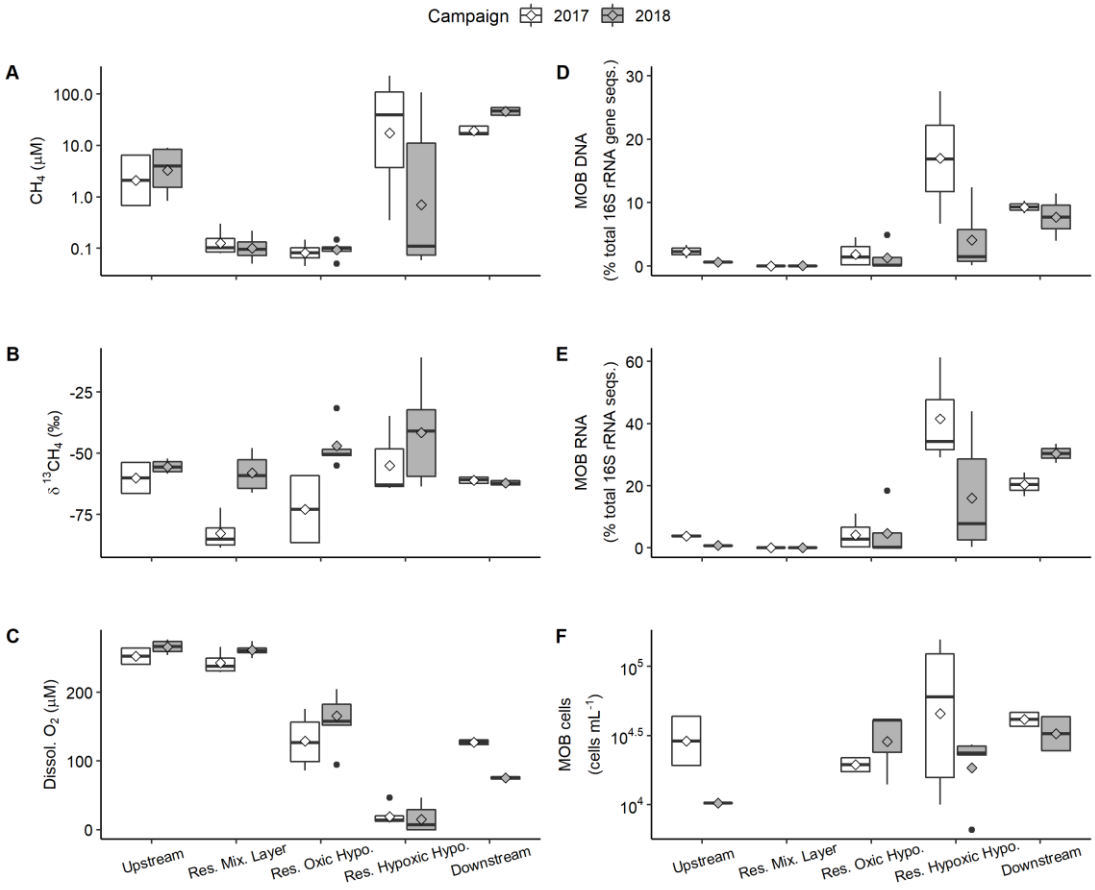

Figure 2.2. CH_4, O_2, and total methane-oxidizing bacteria (MOB) abundance along the Batang Ai river-reservoir system. A) Dissolved CH_4 concentration (n = 36). B) CH_4 stable isotopic signature (n = 32). C) Dissolved O_2 concentration (n = 34). D-E) Abundance of total MOB sequences relative to the total bacterial community (%) in terms of DNA (n = 37) and RNA (n = 37) sequences, respectively. F) Total MOB cell abundance determined by CARD-FISH (n = 20). MOB cells were not determined in the mixed layer of the reservoir since the abundance of MOB 16S rRNA sequences, when detected, was < 0.1% in this layer. Sampling locations are grouped as shown in Fig. 1 with 'Res. Mix. Layer', 'Res. Oxic Hypo.', and 'Res. Hypoxic Hypo.' denoting the surface mixed layer, the oxic layer of the hypolimnion (O_2 > 2 mg L^{-1}), and the hypoxic layer of the hypolimnion (O_2 < 2 mg L^{-1}) within the reservoir. Boxplots represent median, first and third quartiles (hinges), and 1.5 *x* interquartile range (whiskers). Diamonds denote means. Note log scale in y axes of A and F.

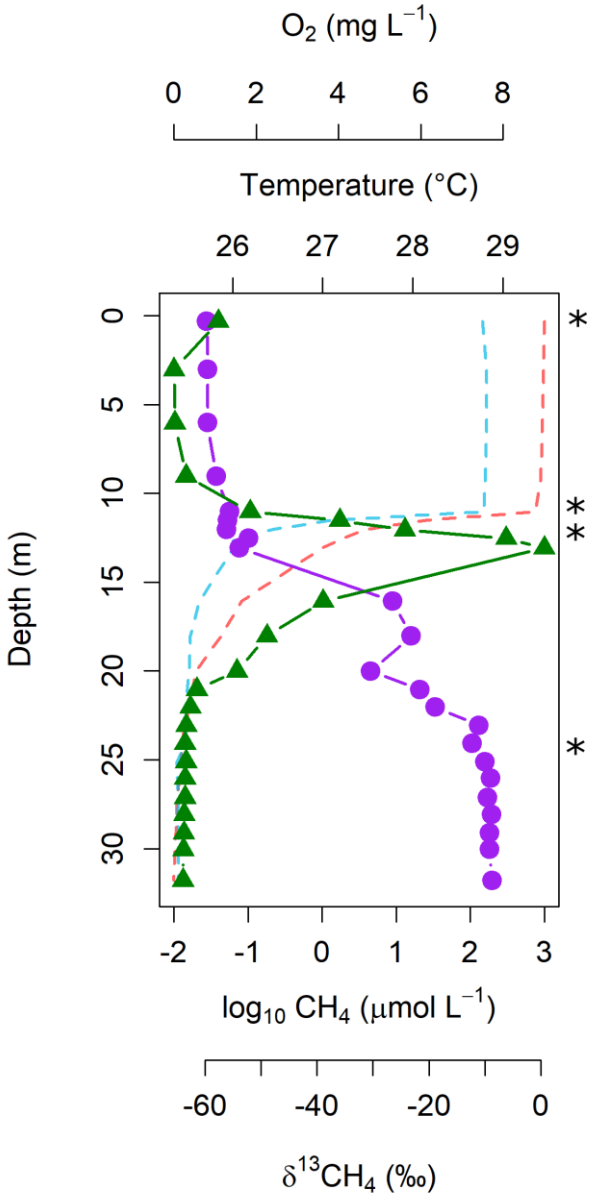

Figure 2.3. Vertical profiles of temperature, O_2 concentration, CH_4 concentration, and CH_4 stable isotopic signature measured in the main basin of the reservoir (site I) in 2018. Dashed red line: water temperature. Dashed blue line: dissolved O_2 concentration. Purple dots and line: CH_4 concentration. Green triangles and line: CH_4 stable isotopic signature. Asterisks show depths where samples for DNA and RNA were taken in this profile.

2.4.3 MOB diversity

A total of 271 amplicon sequence variants (ASVs) belonging to 21 MOB genera were detected across the whole dataset. Of these, 237 ASVs (15 genera) belonged to the Order Methylococcales (Gammaproteobacteria), 9 ASVs (2 genera) belonged to the Family Methylocystaceae (Alphaproteobacteria), 1 ASV (1 genus) belonged to the Family Beijerinckiaceae (Alphaproteobacteria), and 24 ASVs (3 genera) belonged to the putative methanotrophs within the Order Methylomirabilales (candidate phyla NC10) (Fig. S2). No Verrucomicrobia methanotrophs were detected. 48 ASVs within the Family Methylomonaceae (Order Methylococcales, Gammaproteobacteria) and 17 ASVs within the Family Methylococcaceae (Order Methylococcales, Gammaproteobacteria) could not be assigned at the genus level. The unclassified Methylomonaceae ASVs are related to *Methylomonas rubra* (NR_114588.1; 91% identity on average), while the unclassified Methylococcaceae ASVs are related to *Methylococcus capsulatus* (NR_042183.1) and *Methylocaldum marinum* (NR_126189.1; 90% identity on average; NCBI Nucleotide BLAST tool). Within the Gamma-MOB, 24 ASVs belonged to the filamentous methanotrophic bacteria of the genus *Crenothrix* (Stoecker *et al.*, 2006).

MOB sequences were detected in every sampled zone of the river-reservoir system, but in only 6 out of 16 samples from the reservoir surface mixed layer (DNA and RNA; Fig. S2.2). On average, 69.7% of the total MOB DNA sequences belonged to Gammaproteobacteria, 28.3% to Alphaproteobacteria, and 2% to the NC10 candidate phyla across the samples in which MOB were detected (Fig. S2.3). In terms of RNA, the pattern was similar, with Gammaproteobacteria comprising on average 69.9% of the total MOB RNA sequences, followed by Alphaproteobacteria (29.4%) and the NC10 candidate phyla (0.7%, Fig. S2.3).

2.4.4 Dynamics of major MOB groups along the river-reservoir system

MOB community composition changed along the river-reservoir system at the major groups level in terms of both cells and sequences abundance. While average Alpha- and Gamma-MOB cell counts were not significantly different in upstream and reservoir waters ($p>0.05$; Welch's unequal variances t-test on the log data), Alpha-MOB cells were more abundant than Gamma-MOB cells in the downstream river after the dam ($p=0.008$, Welch's unequal variances t-test on the log data; Fig. 2.4A). Also, average Alpha-MOB cell abundance increased from upstream to downstream waters. Both MOB groups showed maximum local cell abundances in the hypoxic hypolimnion of the reservoir, reaching up to 6.1×10^4 and 9.5×10^4 cells mL^{-1} for Alpha- and Gamma-MOB, respectively (Fig. 2.4A).

Both Alpha- and Gamma-MOB 16S rRNA sequences were detected in every zone of the river-reservoir system. However, Gamma-MOB sequences clearly dominated the upstream waters while the relative abundance of Alpha-MOB sequences increased in the reservoir, where both groups showed similar contribution to total MOB sequences (Fig. 2.4B). NC10 sequences were detected at low abundances in upstream waters but reached up to 32% of the total MOB DNA sequences in one specific site at 32 m deep (Fig. 2.4B). In the downstream river, MOB sequences belonged to Alpha- and Gamma-MOB, while NC10 sequences were negligible. In general, the dynamics of the groups were similar in DNA and RNA and between sampling campaigns (Fig. 2.4B).

The relative abundance of 16S rRNA sequences belonging to total, Alpha-, and Gamma-MOB were significantly correlated ($p<0.05$) to the cell counts determined by CARD-FISH (Fig. S2.4). However, sequencing overestimated the abundance of Gamma-MOB cells on average 1.88 times, while it underestimated the Alpha-MOB cell abundance in 20% on average (Fig. S2.4).

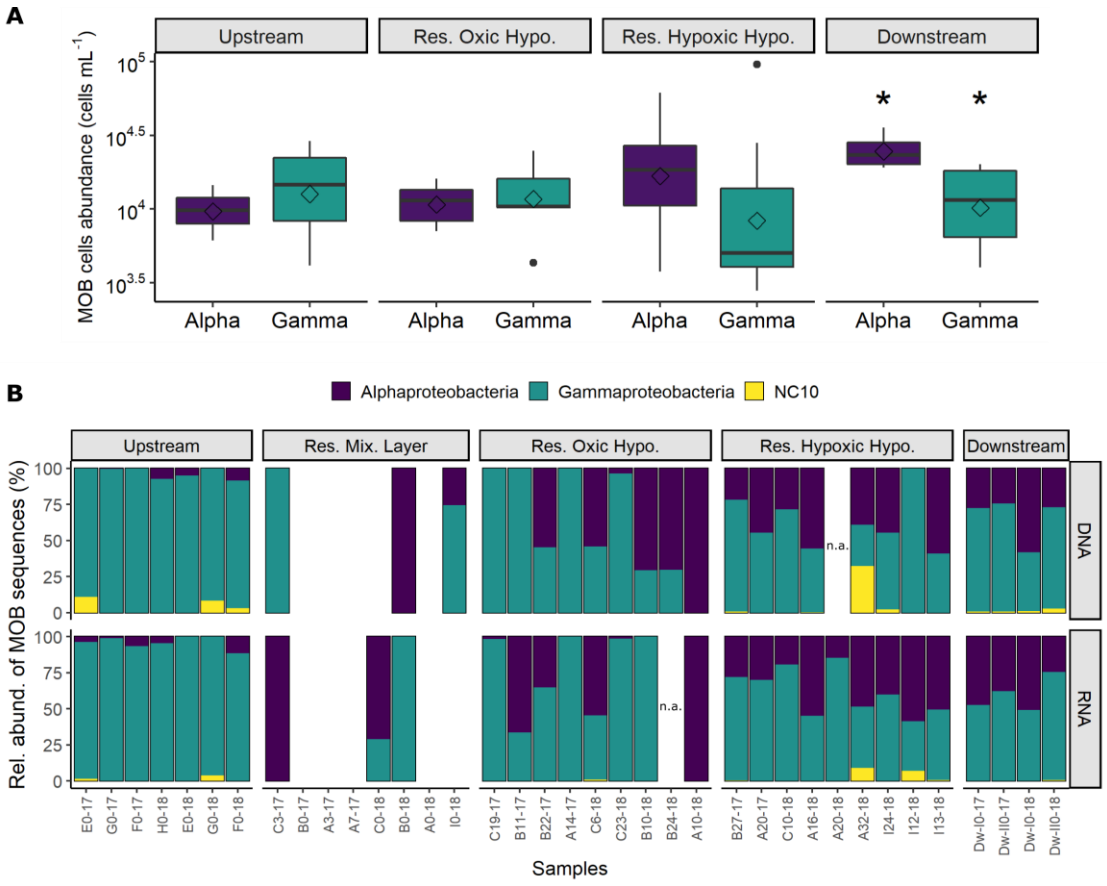

Figure 2.4. Dynamics of major methane-oxidizing bacteria (MOB) groups from upstream to downstream waters in the Batang Ai river-reservoir system. A) Absolute abundances of Alpha-MOB and Gamma-MOB cells determined by CARD-FISH (n = 20). Boxplots represent median, first and third quartiles (hinges), and 1.5 *x* interquartile range (whiskers). Diamonds denote means and stars denote significant difference ($p<0.05$, Welch's unequal variances t-test) between average cell abundance of MOB groups within location. Note log scale in the y axis. Plotted data include both sampling campaigns. B) Relative abundance of DNA and RNA sequences belonging to major taxonomic MOB groups (sum of all MOB sequences in a sample = 1). Each bar represents a sample taken at a different campaign, site, and depth (n = 37). In the x axis, the letters indicate the site code as in Fig. 1, followed by two numbers indicating the sampling depth and the sampling campaign (2017 or 2018), respectively. Empty slots mean that MOB were not detected (Res. Mix. Layer) or that the sample was lost during rarefaction (n.a.; Res. Oxic Hypo. and Res. Hypoxic Hypo.). Res. Mix. Layer: reservoir's mixed layer; Res. Oxic Hypo.: reservoir's oxic hypolimnion; Res. Hypoxic Hypo.: reservoir's hypoxic hypolimnion.

2.4.5 Environmental drivers and connectivity of MOB communities

We performed Canonical Analysis of Principal Coordinates (CAP) based on the DNA and RNA profiles of MOB communities to investigate the similarity between MOB communities from different locations within the river-reservoir system and their links to environmental drivers (Fig. 2.5). Temperature, pH, CH_4 and O_2 concentrations significantly predicted patterns in MOB community structure across the studied system ($p=0.001$), but only explained a limited portion of the variation (Fig. 2.5), suggesting that unmeasured factors might also contribute to the reported patterns in MOB assemblages. Dissolved organic carbon (DOC) concentration was not a significant ($p>0.05$) factor despite the potential role of DOC in MOB activity and community structure (Reis et al., 2020b; Thottathil et al., 2018). This lack of effect of DOC is likely due to the absence of a gradient in DOC concentration, which is constantly very low (mean 0.9 mg L^{-1} ± 0.2 SD) in the reservoir (Soued et Prairie, 2020). Overall, patterns were similar between DNA, RNA, and both sampling campaigns, and MOB communities from the same location often grouped together (Fig. 2.5A, B). MOB communities from the upstream and reservoir mixed layer correlated positively with temperature, O_2, and pH while the oxic hypolimnion communities were spread across the CAP space. Downstream communities and hypoxic hypolimnetic communities grouped closely together reflecting the position of the water intake for the turbines and correlated with CH_4 concentration. In addition, the number of MOB ASVs increased along the continuum, reaching the highest taxonomic richness in the MOB communities in the downstream river (Fig. 2.5A, B).

To check whether the observed changes in MOB communities along the river-reservoir continuum resulted from taxa replacement as opposed to changes in the abundances of existing taxa, we categorized each ASV depending on the location where they were first detected (as in Ruiz-González et al. 2015a). We observed that in most locations, a large fraction of the ASVs was already detected in the upstream

sites, making up >50% of the MOB DNA sequences even in the farthest downstream site sampled (Fig. 2.5C). Besides these 'upstream' ASVs, downstream MOB communities contained a large fraction of ASVs deriving from both the oxic and the hypoxic hypolimnion, but only a negligible proportion of sequences belonging to ASVs first detected in the surface mixed layer of the reservoir (Fig. 2.5C). 'Mixed layer' unique ASVs were detected in 2018 and belonged to Gamma-MOB (Ca. *Methylospira*, *Methylocaldum*, and an unclassified genus; details not shown). Few MOB ASVs unique to the downstream sites (i.e. not present in the reservoir or in upstream waters) were detected (7 in 2017 and 27 in 2018), but they accounted for a small fraction of the total MOB sequences (Fig. 2.5C). In both years, these 'downstream' MOB ASVs belonged to Gamma-MOB and NC10 (*Methylomonas*, *Methylosoma*, candidatus *Methylospira, Methylomagnum*, and *Methyloparacoccus* from Gamma-MOB, and unclassified genera from both groups; details not shown).

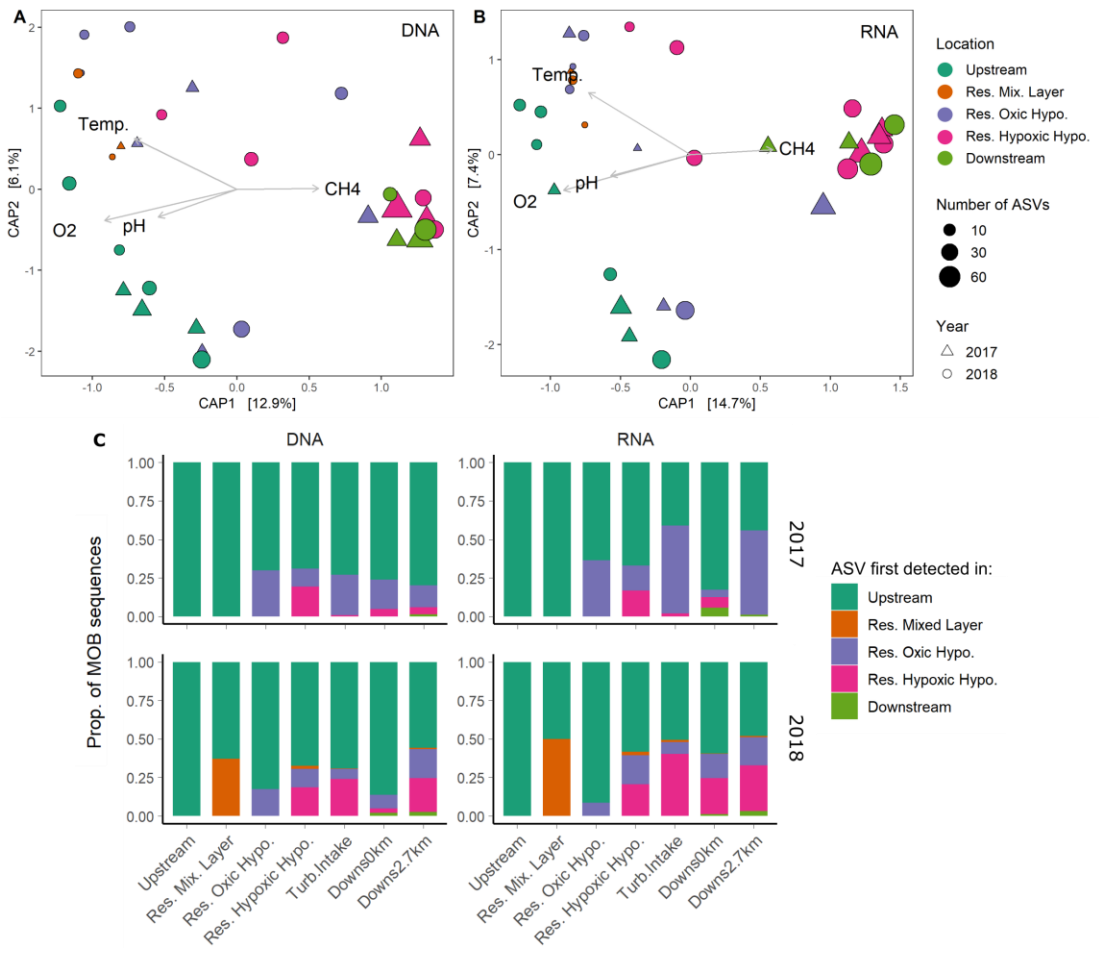

Figure 2.5. Dynamics of methane-oxidizing bacteria (MOB) communities along the river-reservoir system. A-B) Canonical Analysis of Principal Coordinates (CAP) based on Bray-Curtis distance of the DNA (p=0.001) and RNA (p=0.001) MOB community profiles, respectively. 32 samples that did not contain missing values were used in both DNA and RNA CAP analyses. C) Proportion of DNA and RNA MOB sequences associated to MOB ASVs categorized depending on the location of the river-reservoir system where they were first detected. Values are expressed as a fraction of the total MOB sequences for the different sampled sites and campaigns. Res. Mix. Layer: reservoir's mixed layer; Res. Oxic Hypo.: reservoir's oxic hypolimnion; Res. Hypoxic Hypo.: reservoir's hypoxic hypolimnion; Turb. Intake: closest available sample to the turbine water intake (2017: site A, depth 20 m; 2018: site I, depth 24 m); Downs0km: Downstream site at the power station; Downs2.7km: Downstream site at 2.7 km further downstream.

2.4.6 CH_4 oxidation and MOB cells dynamics in downstream waters

Based on the DNA and RNA compositional data it seemed that downstream MOB communities were merely hypolimnetic communities transported through the turbine tunnels (Fig. 2.5A, B). However, the CARD-FISH count data indicated a shift in the cell abundance of the two proteobacterial MOB groups from the hypolimnion to the downstream river, leading to significantly higher abundances of Alpha-MOB in the outflowing river waters (Fig. 2.4A). We thus investigated whether this shift was correlated to CH_4 oxidation in the downstream river by following the CH_4 concentration and stable isotopic signature, O_2 concentration, and Alpha- and Gamma-MOB cell abundance from the water intake for the turbines in the hypolimnion of the reservoir until 2.7 km downstream from the dam (Fig. 2.6). The reduction in CH_4 concentration and simultaneous increase in stable isotopic signature of the remaining CH_4 indicated microbial CH_4 oxidation within downstream waters in both years (Fig. 2.6A, B). The calculated fraction of CH_4 oxidized (*F*ox) within the river segment was higher in 2017 than in 2018 (up to 12% in 2017; Fig. 2.6C), but the absolute amount of CH_4 oxidized was higher in 2018 than in 2017 (Fig. 2.6D) because the CH_4 concentration at the turbine water intake was much higher in 2018 than in 2017 (82 and 52 µM, respectively; Fig. 2.6A). O_2 concentration in the water was higher in 2017 than in 2018 both before and after its passage through the turbines (Fig. 2.6E). The cell abundances of both MOB groups decreased between the turbines' intake in the reservoir and the power station (0 km) in 2017 (Fig. 2.6F), which could be caused by death of cells but also by dilution since the turbines draw water from a wide range of depths. However, once in the downstream river, Alpha-MOB cell abundance quickly increased within the approx. 1 h interval along the studied river segment (flow rate of ~1 m s^{-1}, unpubl. data) in 2017 and 2018 (Fig. 2.6F). In both years, Alpha-MOB clearly dominated over Gamma-MOB at 2.7 km, where highest *F*ox and absolute amount of CH_4 oxidized were also observed, suggesting that the oxidation of CH_4 in downstream waters is mostly due to the

activity of Alpha-MOB taxa. A higher role of Alpha-MOB over Gamma-MOB in downstream waters is supported by a strong correlation between the amount of CH_4 oxidized in each segment of the river and Alpha-MOB cells (R^2=0.98, p=0.005) and the lack of correlation with Gamma-MOB cells (R^2=0.04, p=0.78) (Fig S2.5), although only four data points are available. Microscopic visualization also revealed that Alpha-MOB cells occurred often in pairs, potentially indicating that cells were actively dividing in this segment of the downstream river (Fig. S2.6).

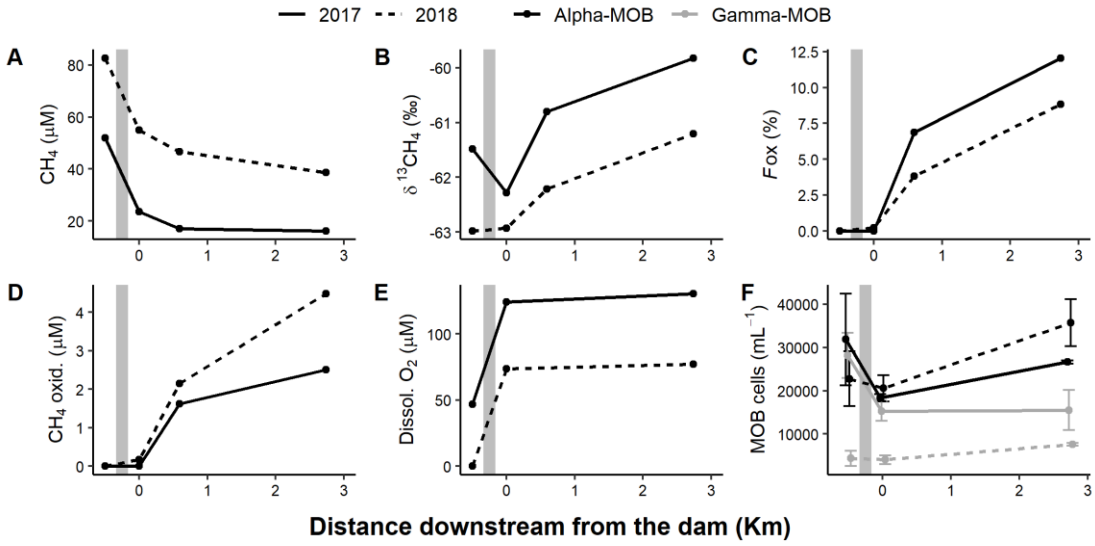

Figure 2.6. CH_4 oxidation and MOB cell dynamics along the sampled sites from the turbines' water intake (located in the hypolimnion of the reservoir) until 2.7 km downstream from the dam (the dam is indicated by the gray vertical bar). A) Dissolved CH_4 concentration. B) CH_4 stable isotopic signature. C) Cumulative fraction of CH_4 oxidized (*F*ox) calculated from the concentration and isotopic signature of CH_4 at each point using the turbine water intake as CH_4 source. D) Cumulative amount of CH_4 oxidized, calculated from *F*ox and CH_4 concentration. E) Dissolved O_2 concentration. F) Alpha- and Gamma-MOB cell abundances enumerated by CARD-FISH. The abundance of MOB cells entering the turbines was determined in the closest available sample to the turbine water intake (2017: site A, depth 20 m; 2018: site I, depth 24 m).

2.5 Discussion

Despite the central role of MOB in regulating CH_4 emissions from hydropower reservoirs, very few studies have investigated MOB communities in these systems (Dumestre et al., 1999; Itoh et al., 2015). In particular, although the importance of CH_4 oxidation has also been recognized in downstream waters, no study has explored how MOB communities respond to drastic environmental changes from the hypolimnia of reservoirs to rapidly re-oxygenated turbine outflowing waters. This is of special interest in lower latitudes, given that tropical hydropower reservoirs are often strongly stratified, reducing the importance of CH_4 diffusive fluxes through the reservoir surface and making downstream CH_4 emissions particularly important (Guérin *et al.*, 2006 ; Soued et Prairie, 2020). Here we show that in the narrow band of environmental conditions that are optimal for CH_4 oxidation to occur, MOB abundance and potential activity can reach very high levels (up to 27% and 61% of the total bacterial DNA and RNA sequences, respectively). This spatial confinement of abundant and highly active MOB communities in the hypoxic zone of the hypolimnion of Batang Ai, which creates a CH_4 concentration minimum in the oxic hypolimnion, imposes a biogeochemical disconnection between the hypolimnetic CH_4 and the reservoir surface CH_4 emissions. We also show that MOB taxa in Batang Ai are to a large extent sourced from upstream waters, but that environmental gradients generated by the dam allow the local establishment of different and specific MOB communities in the different zones of the hydropower complex. In addition, by microscopically quantifying MOB cells, we show that fast cell growth of mostly Alpha-MOB taxa in downstream waters happens simultaneously with CH_4 oxidation in well-oxygenated downstream waters.

2.5.1 High total MOB DNA and RNA sequences in Batang Ai reservoir

We found extremely high relative abundances of MOB 16S rRNA sequences in DNA and RNA in the hypoxic hypolimnion of the tropical reservoir Batang Ai (up to 27% and 61% of total bacterial DNA and RNA sequences, respectively), and these belonged to methanotrophic taxa in the Gammaproteobacteria and Alphaproteobacteria classes. NC10 putative methanotrophs were only abundant in our deepest sample in anoxic waters within the reservoir (32 m) where they accounted for almost one third of the total MOB DNA sequences. Similarly, NC10 putative methanotrophs have been found to constitute a significant portion of the active methanotrophic community in anoxic zones of lakes and reservoirs (Deutzmann *et al.*, 2014 ; Kojima *et al.*, 2014). The peak in total MOB abundances in hypoxic waters observed in our study is in line with previous studies reporting highest MOB abundances at oxic-anoxic interfaces in other lakes and reservoirs (Dumestre et al., 1999; Kojima et al., 2014; Mayr et al., 2019; Reis et al., 2020b). Comparatively high relative abundances of MOB sequences have been detected in permafrost thaw ponds (maximum 27% of the 16S rRNA sequences; Crevecoeur *et al.*, 2015), temperate lakes (11-26% of total 16S rRNA gene sequences; Mayr *et al.*, 2019), a subtropical reservoir (maximum ~30% of total 16S rRNA gene sequences; Kojima *et al.*, 2014), and a tropical hydropower reservoir (up to 24% of DGGE-obtained sequences; Dumestre *et al.*, 2001). Regarding MOB RNA sequences, our values (up to 61%) represent the highest reported values of MOB abundance in freshwaters to our knowledge and suggests that CH_4 defines a clear microbial niche in the hypolimnion of the Batang Ai reservoir. Indeed, the high MOB abundance in the hypoxic hypolimnion, together with the decrease in CH_4 concentration and enrichment of $\delta^{13}CH_4$ (Fig. 2.3), indicates intense methanotrophic activity and that most of the upward flux of CH_4 is oxidized by MOB in the hypoxic zone of the reservoir, as observed in other permanently stratified lakes and reservoirs (*e.g.* Borges *et al.*, 2011 ; Itoh *et al.*, 2015).

In general, the patterns observed in the DNA and RNA MOB sequences were quite similar indicating that the present MOB taxa are potentially active across the sampled zones in Batang Ai. An exception to this tight coupling between DNA and RNA was observed in the reservoir mixed layer, where DNA and RNA patterns were particularly variable (Fig. 2.4B) probably due to an inactive state of cells and the very low relative abundances of total MOB 16S rRNA sequences in this layer (<0.1% of total 16S rRNA gene sequences) (Fig. 2.2D, E).

2.5.2 Hydrologic connectivity of MOB communities along the system

Downstream bacterial communities showed high proportions of MOB sequences and cells, and MOB communities from downstream sites clustered very closely together with those from the hypoxic hypolimnion, differing largely from upstream and mixed layer assemblages (Fig. 2.5A, B). By tracing the origin of ASVs along the studied system, we observed that a large fraction of the downstream MOB communities comprised taxa first detected in both oxic and hypoxic hypolimnetic sites. In 2018, the fraction of MOB ASVs from the hypoxic hypolimnion in the downstream river was higher than in 2017, while MOB ASVs first detected in the oxic hypolimnion were more abundant in the downstream river in 2017 than in 2018. This difference in the source of downstream MOB taxa is likely related to the variation in the reservoir's water level, which affects the water withdrawal depth. Only a few unique downstream MOB ASVs were detected, showing that although downstream conditions allow for the establishment of new MOB taxa, downstream MOB communities are almost entirely dominated by taxa dispersed from upstream reservoir sites. Moreover, although all sampled locations harbored a large fraction of taxa originating in the rivers flowing into the reservoir, most MOB ASVs specific to the mixed layer did not reach downstream waters. This reflects the discontinuity in surface water flow caused by the stratification within the reservoir and the position of the turbines' water intake. These unique ASVs from the mixed layer were only

detected in 2018, while in 2017 all the MOB ASVs detected in the mixed layer were also detected in the upstream sites. Interestingly, upstream riverine waters were much warmer in 2017 (30.6 °C) than in 2018 (26.1 °C) at 1 m depth, suggesting that inflowing upstream waters mixed well with the reservoir's mixed layer (average temperature 31 °C) in 2017 but likely sank below it while entering the reservoir in 2018. This supports the larger connectivity between the upstream and the mixed layer communities in 2017 than in 2018 shown by the analysis of the origin of MOB ASVs.

The importance of hydrologic connectivity in shaping the structure of freshwater bacterioplankton has been shown for whole communities (Niño-García *et al.*, 2016 ; Ruiz-González *et al.*, 2013, 2015a) and specifically for MOB assemblages across boreal lakes and rivers (Crevecoeur *et al.*, 2019). Here we show that although reservoir MOB communities are sourced to a large extent from upstream rivers, the specific environmental conditions and the permanent stratification generated within the reservoir cause the establishment of MOB communities and taxa specific to each reservoir zone. By disconnecting surface waters but connecting the hypolimnion of the reservoir to the river downstream, the damming of the Batang Ai river creates a break in the connectivity between upstream and downstream MOB assemblages and a drastic change in oxygen conditions between the hypolimnion of the reservoir and the downstream river. Yet, rapid shifts in the MOB community in the first few kilometers downstream of the dam enabled continued CH_4 oxidation in the river downstream.

2.5.3 Rapid MOB growth in the downstream river

Based solely on our sequencing results, CH_4 oxidation in the downstream river below the Batang Ai dam is apparently the result of the residual activity of MOB hypolimnetic communities since they are similar in composition (Fig. 2.5A, B), with very few new MOB ASVs detected in the downstream reach (Fig. 2.5C). However, the enumeration of MOB cells by CARD-FISH revealed a rapid growth of particularly Alpha-MOB cells within the downstream river. These Alpha-MOB cells

belonged to taxa that were already present in the hypolimnion of the reservoir, given that none of the unique 'downstream' ASVs belonged to Alpha-MOB, but were possibly favored under the downstream environmental conditions such as both high CH_4 and higher O_2 concentrations (Reis et al., 2020b).

The measured increase in MOB cell numbers represents an extremely rapid response of methanotrophic taxa to changes in the environment rather than just a passive dispersal of hypolimnetic communities. It also represents an extremely fast cell growth rate given the short time interval between the power station (0 km) and 2.7 km further downstream (approx. 1 h, flow rate of ~1 m s^{-1}, unpubl. data). Under the high CH_4 and oxygenated conditions in the downstream river, Alpha-MOB cells showed a surprisingly short average doubling time of 1.7 h, while Gamma-MOB showed a longer but also short average doubling time of 2.3 h. These are in the low range of estimated prokaryotic doubling times in nature – which can vary from minutes to days (Gibson *et al.*, 2018) – and of methanotrophic bacteria doubling times in pure cultures (Baani et Liesack, 2008 ; Puri *et al.*, 2015 ; Sundstrom et Criddle, 2015), suggesting that the downstream river meets ideal growth conditions to some MOB taxa, particularly within the Alpha-MOB group.

To verify whether such fast MOB growth observed by the CARD-FISH enumeration was metabolically possible, we estimated the increase in MOB cell abundance in the downstream segment based on the amount of CH_4 oxidized, the average MOB cell volume (calculated from the area of the DAPI subset of FISH positive cells in this dataset), an assumed MOB growth efficiency of 10%, and a bacterial cell carbon content of 63 fgC μm^{-3} (Fagerbakke *et al.*, 1996) (see details in Suppl. Info.). We found that the total MOB cell increase measured by CARD-FISH was lower than the calculated MOB cell increase (on average 33% lower). We performed sensitivity tests by decreasing the assumed MOB growth efficiency to 5% or increasing the cell carbon content to 126 fgC μm^{-3}, and in both scenarios the measured MOB cell

increase by CARD-FISH could be fully accounted by the amount of CH_4 oxidized within the reach. Interestingly, the total amount of carbon fixed by microbial CH_4 oxidation in the studied river segment varied between 210 and 306 mgC m^{-2} d^{-1} between the years, which is in the upper range of carbon fixation by primary production in tropical rivers generally reported (10-200 mgC m^{-2} d^{-1}; Davies et al. 2008). This suggests that CH_4 is likely an important basal carbon source to the food web of the oligotrophic (chlorophyll-a: 1.7 ± 0.8 µg L^{-1}) river downstream of the Batang Ai dam.

2.5.4 The importance of combining microscopy and sequencing data for the study of MOB communities

In the present study, the sequencing data allowed tracking changes in MOB communities and individual ASVs along the river-reservoir system, enabling the observation of spatial changes in the taxonomic composition and connectivity of the studied communities. However, a link between community shift and CH_4 oxidation in the downstream river could only be determined due to the microscopic cell enumeration, since the sequencing data did not allow the detection of fast quantitative differences between the source hypolimnetic communities and downstream communities, which looked similar based on the sequencing data. Therefore, our study demonstrates the importance of combining high-resolution sequencing data with microscopic quantitative data for attaining a more complete understanding of MOB dynamics and activity in the environment.

2.6 Conclusion

Our study of the MOB dynamics in a tropical hydropower complex contributes to better understanding the mechanisms by which these bacteria play a central role in modulating the carbon footprint of aquatic ecosystems and, in particular, of river-reservoir systems. By combining biogeochemical data, DNA and RNA sequencing,

and cell enumeration, we found that CH_4 dynamics are accompanied by shifts in the total abundance and structure of MOB communities along the Batang Ai river-reservoir system. In the main basin of the reservoir, where diffusive CH_4 emissions are only moderate (Soued et Prairie, 2020), MOB sequences, cells, and activity peak in the hypoxic hypolimnion (up to 61% of 16S rRNA sequences and enriched $\delta^{13}CH_4$), disconnecting the hypolimnetic CH_4 from surface CH_4 emissions to the atmosphere. While most of the MOB taxa present across the system is dispersed from upstream sites, downstream MOB communities resemble reservoir's hypolimnetic communities but differ largely from upstream and mixed layer communities due to the position of the water intake for the turbines. When CH_4-rich hypolimnetic water is released to the downstream river, the re-oxygenation seems to promote the activity and fast growth of particularly Alpha-MOB taxa. Therefore, although downstream emissions constitute a major pathway of CH_4 emissions in Batang Ai (Soued and Prairie 2020) and in other hydropower complexes, rapid shifts in the MOB community enable continued microbial CH_4 oxidation in the downstream river, thereby contributing to lowering the carbon footprint of these systems.

2.7 Acknowledgements

The authors thank Karen Lee Suan Ping, Jenny Choo Cheng Yi, Amar Ma'aruf Bin Ismawi and Gerald Tawie Anak Thomas for their logistic support and great help in the field. We are also grateful to Jenny Choo Cheng Yi for her help in the molecular laboratory. This study was supported by Natural Sciences and Engineering Research Council of Canada Discovery grants and Sarawak Energy Berhad and is a contribution to UNESCO Chair in Global Environmental Change. We also acknowledge financial support from the Groupe de recherche interuniversitaire en limnologie (GRIL), a strategic cluster of the Fonds de recherche du Québec - Nature et technologies (FRQNT). PCJR was supported by a merit doctoral scholarship from the Fonds de recherche du Québec - Nature et technologies (FRQNT).

2.8 Supplementary Information

Table S 2.1. Details of the samples for MOB community characterization analyzed in this study. Site codes and location as shown in Fig. 1.

Site	Sampling year	Location	Depth (m)	DNA and RNA	CARD-FISH
E	2017	Upstream	0	x	x
G	2017	Upstream	0	x	x
F	2017	Upstream	0	x	x
C	2017	Res. Mix. Layer	3	x	
B	2017	Res. Mix. Layer	0	x	
A	2017	Res. Mix. Layer	3	x	
A	2017	Res. Mix. Layer	7	x	
C	2017	Res. Oxic Hypo.	19	x	x
B	2017	Res. Oxic Hypo.	11	x	
B	2017	Res. Oxic Hypo.	22	x	x
A	2017	Res. Oxic Hypo.	14	x	
B	2017	Res. Hypoxic Hypo.	27	x	x
A	2017	Res. Hypoxic Hypo.	20	x	x
Dw-I	2017	Downstream	0	x	x
Dw-II	2017	Downstream	0	x	x
H	2018	Upstream	0	x	x
E	2018	Upstream	0	x	
G	2018	Upstream	0	x	
F	2018	Upstream	0	x	
C	2018	Res. Mix. Layer	0	x	
B	2018	Res. Mix. Layer	0	x	
A	2018	Res. Mix. Layer	0	x	
I	2018	Res. Mix. Layer	0	x	
C	2018	Res. Oxic Hypo.	6	x	
C	2018	Res. Oxic Hypo.	23	x	x
B	2018	Res. Oxic Hypo.	10	x	
B	2018	Res. Oxic Hypo.	24	x	x
A	2018	Res. Oxic Hypo.	10	x	
C	2018	Res. Hypoxic Hypo.	10	x	x
A	2018	Res. Hypoxic Hypo.	16	x	x
A	2018	Res. Hypoxic Hypo.	20	x	x
A	2018	Res. Hypoxic Hypo.	32	x	x
I	2018	Res. Hypoxic Hypo.	24	x	x
I	2018	Res. Hypoxic Hypo.	12	x	
I	2018	Res. Hypoxic Hypo.	13	x	x
Dw-I	2018	Downstream	0	x	x
Dw-II	2018	Downstream	0	x	x

Table S 2.2. Group specific 16S rRNA targeted probes used for the detection of Proteobacterial methane-oxidizing bacteria. Probes Mγ84 and Mγ705 were used together as suggested in Eller et al. (2001).

Probe	**Target group**	**Probe sequence**
Mα450	Alpha-MOB	5'-ATC CAG GTA CCG TCA TTA TC-3'
Mγ84	Gamma-MOB	5'-CCA CTC GTC AGC GCC CGA-3'
Mγ705	Gamma-MOB	5'-CTG GTG TTC CTT CAG ATC-3'

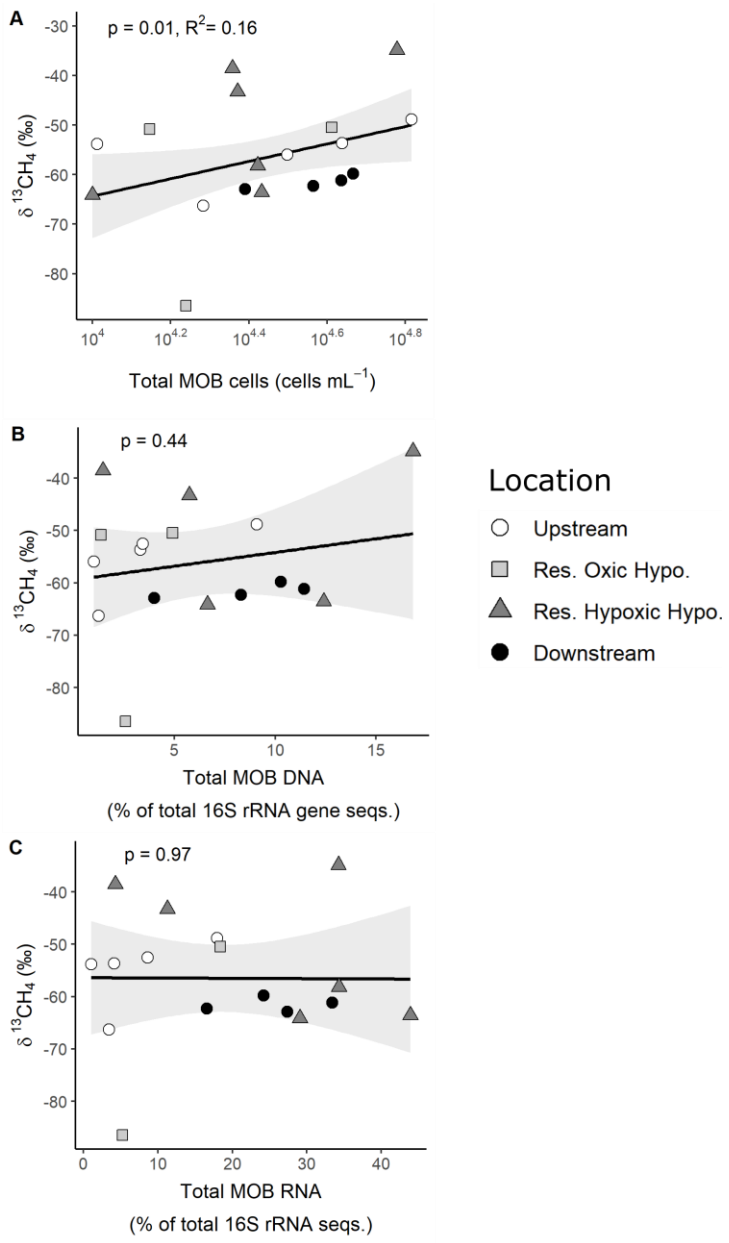

Figure S 2.1. Correlation between methane-oxidizing bacteria (MOB) and $\delta^{13}CH_4$ in Batang Ai river-reservoir system. A) Total MOB cells (determined by CARD-FISH). B) Total MOB DNA (% of total 16S rRNA gene sequences). C) Total MOB RNA (% of total 16S rRNA sequences). Plotted data does not include the reservoir mixed layer, since MOB cells count was not determined in this layer due to the very low MOB sequences abundance (<0.1%). Note log scale in the x axis of A.

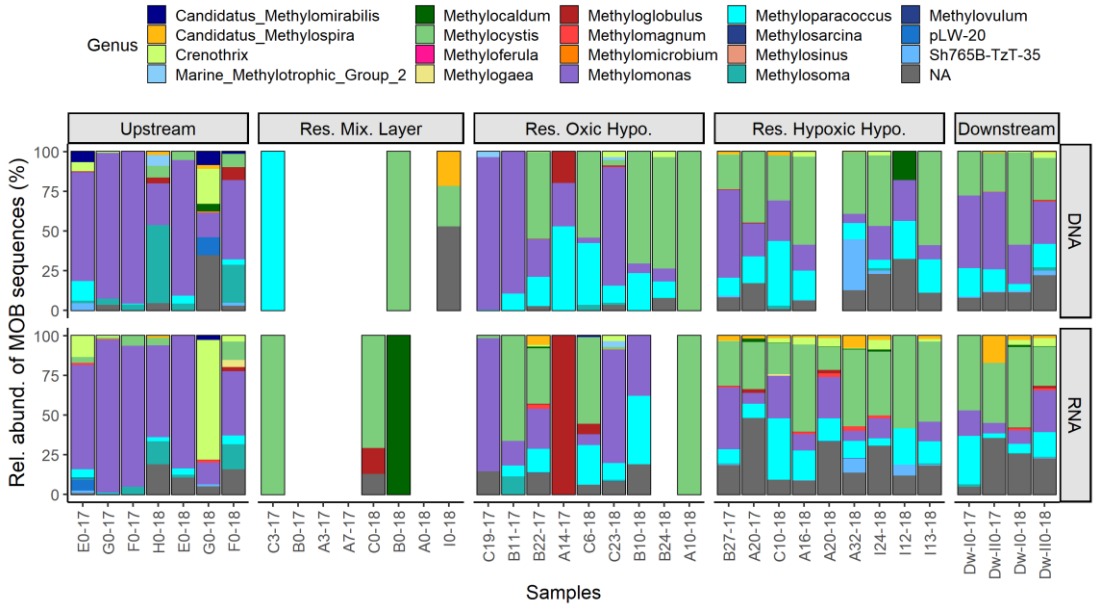

Figure S 2.2. Relative abundance (%) of sequences belonging to all methane-oxidizing bacteria (MOB) genera detected across the dataset (sum of all MOB sequences in a sample = 1). Each bar represents a sample taken at a different campaign, site, and depth. In the x axis, the letters indicate the site code as in Fig. 1, followed by two numbers indicating the sampling depth and the sampling campaign, respectively (2017 or 2018). Empty slots mean that no MOB was detected in the sample (Res. Mixed Layer) or that sample was lost during rarefication (Res. Oxic Hypo. and Res. Anoxic Hypo.). NA genus includes unclassified Methylomonaceae and Methylococcaceae (Gamma-MOB) ASVs.

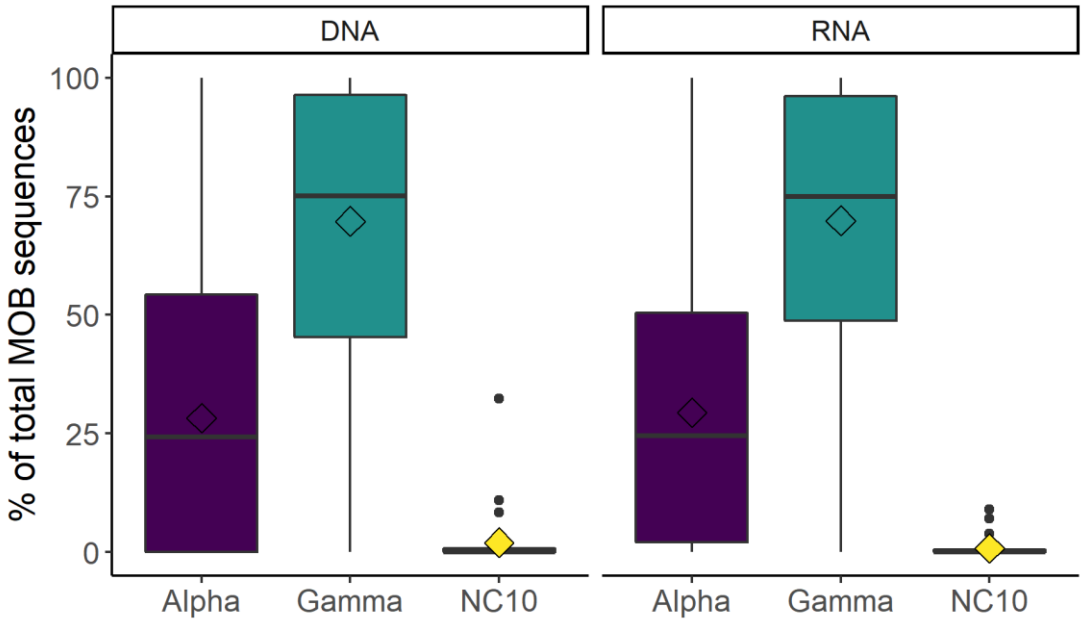

Figure S 2.3. Contribution of each methane-oxidizing bacteria (MOB) group detected in this study to the total MOB 16S rRNA sequences across samples. Alpha: Alphaproteobacteria MOB; Gamma: Gammaproteobacteria MOB; NC10: candidate phyla NC10 MOB. Boxplots represent median, first and third quartiles (hinges), and 1.5 *x* interquartile range (whiskers). Diamonds denote means.

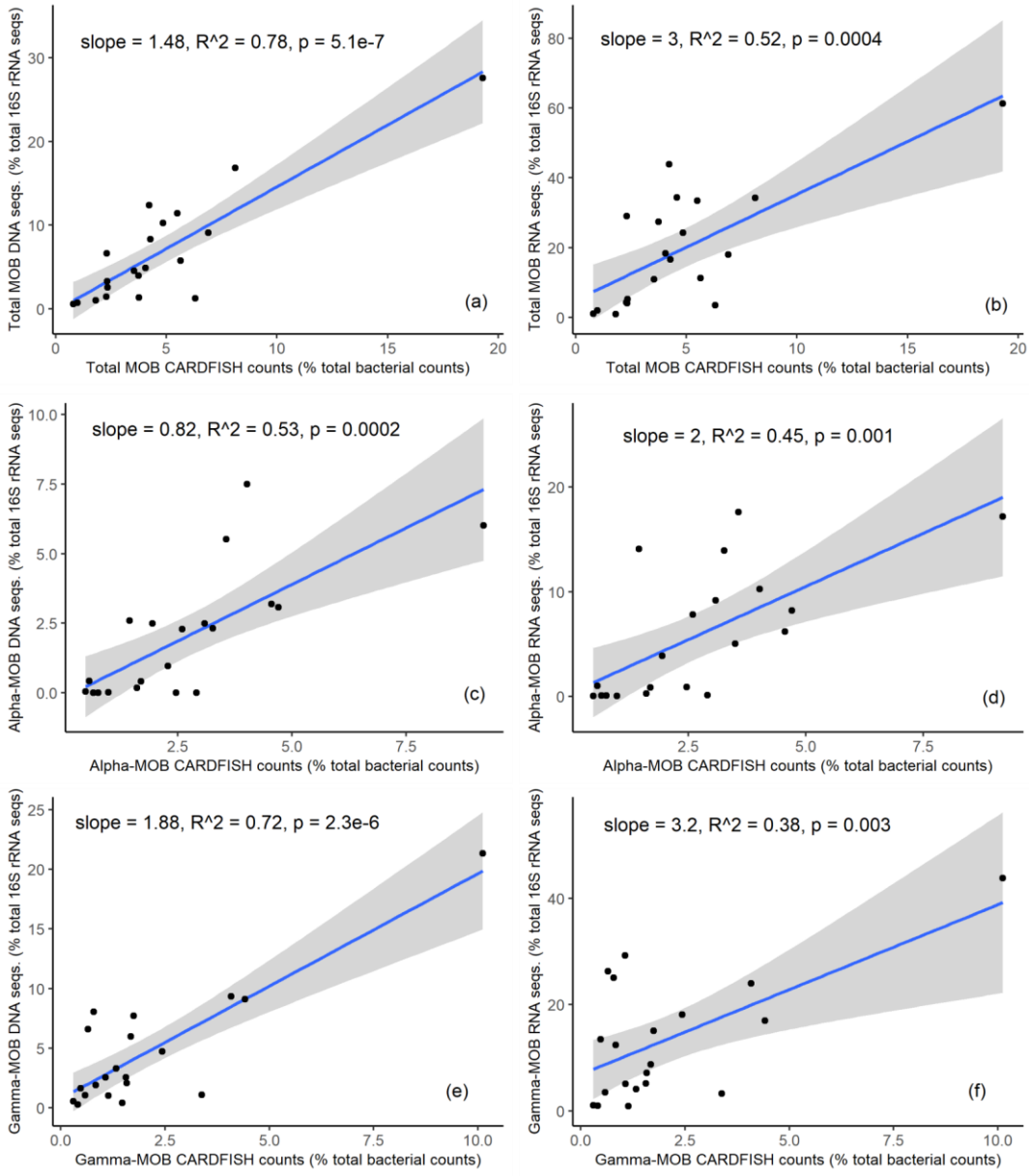

Figure S 2.4. Correlation between CARD-FISH MOB cell counts (% of total DAPI counts) and MOB 16S rRNA sequences (DNA and RNA) (% of total bacterial 16S rRNA sequences) across samples in Batang Ai reservoir. a-b) total Proteobacteria MOB (Alpha- and Gamma-MOB). c-d) Alphaproteobacteria MOB. e-f) Gammaproteobacteria MOB.

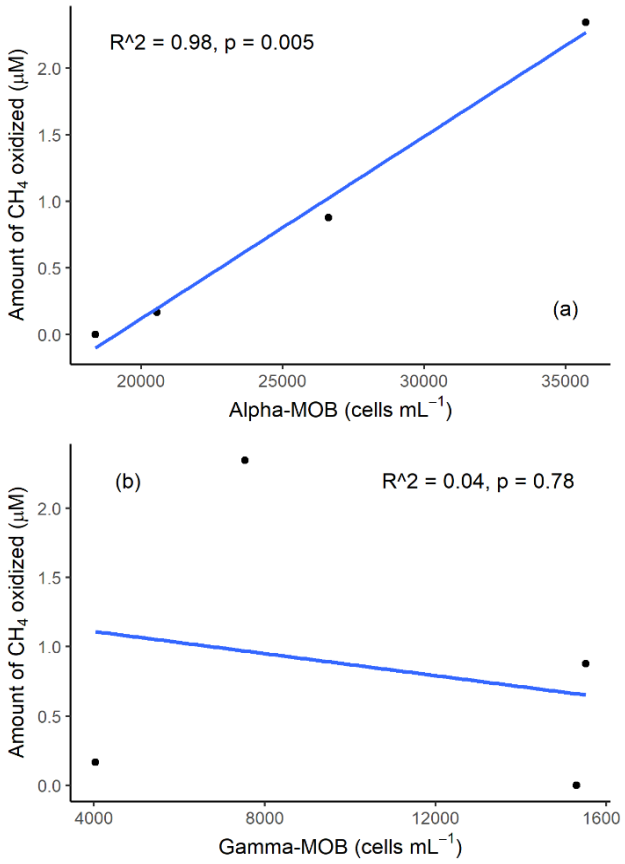

Figure S 2.5. Correlation between MOB cells and amount of CH_4 oxidized from the intake of the turbines in the hypolimnion of the reservoir to the power station (0 km) and from 0 km to 2.7 km further downstream at both sampling campaigns. a) Alphaproteobacteria MOB cells. b) Gammaproteobacteria MOB cells.

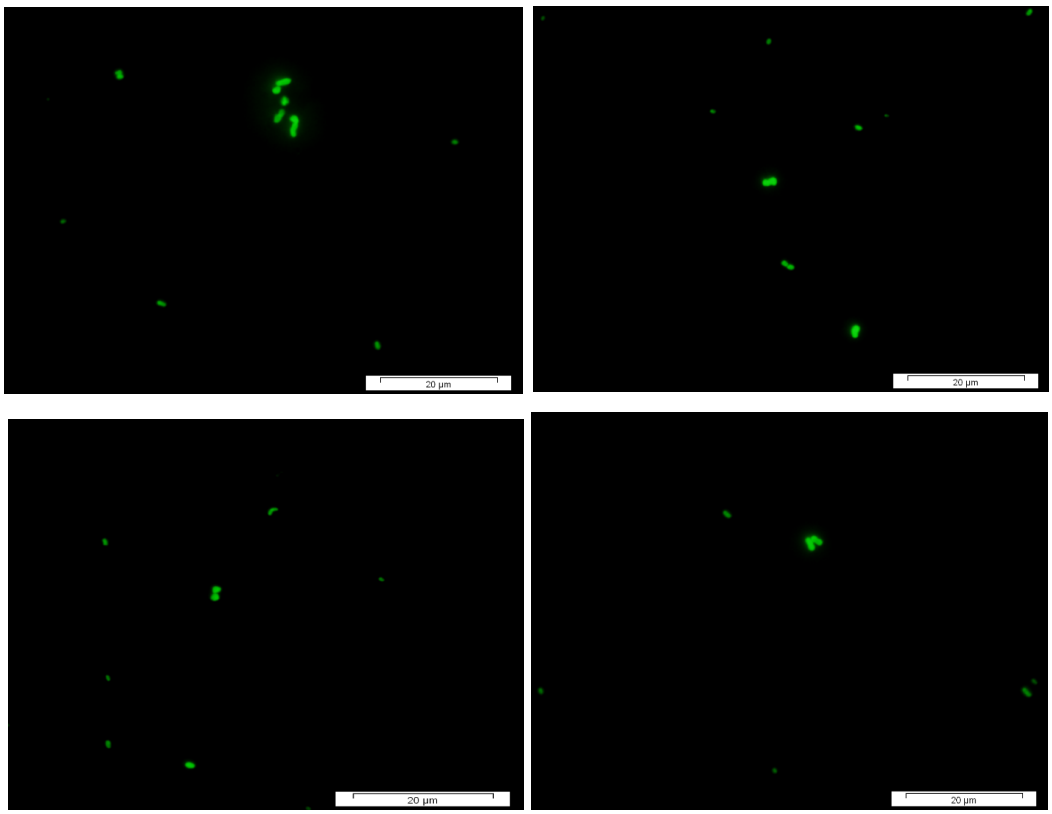

Figure S 2.6. Potentially dividing Alphaproteobacteria MOB cells in the downstream river section between 0 and 2.7 Km downstream of the dam. Cells under the microscope visualized by CARD-FISH (catalyzed reporter fluorescent in situ hybridization) using specific probes. Whole scale bars represent 20 µm.

Calculating MOB cell growth in the downstream segment

We estimated the potential increase in MOB cell abundance over the downstream segment (from 0 to 2.7 Km downstream of the dam) using the equation:

$$\Delta CellsMOB = \left(\frac{Coxid \times MBGE}{BactC \times MOBVol}\right) \div 1000$$

where $\Delta CellsMOB$ and $Coxid$ are the MOB cells produced (cells mL^{-1}) and the measured amount of CH_4 oxidized (µgC L^{-1}; see model developed in Soued and Prairie 2020 for details) within the river segment, respectively. *MBGE* is the assumed methanotrophic bacterial growth efficiency, *BactC* is bacterial carbon content in µgC μm^{-3} (63×10^{-9} µgC μm^{-3}; Fagerbakke et al. 1996), and *MOBVol* is the average MOB cell volume in μm^3 calculated from the measured area of the DAPI subset of FISH positive cells in this dataset assuming a round shape of cells.

CHAPTER III

MICROBIAL METABOLISM AND LAKE CHARACTERISTICS DETERMINE THE RELATIVE IMPORTANCE OF METHANOTROPHY TO WHOLE-LAKE BACTERIAL CARBON CYCLING

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N.B. References cited in this chapter are presented at the end of the thesis.

3.1 Abstract

Previous evidence indicates that methanotrophy is an important pathway of carbon (C) transformation and basal C source to food webs in lakes. Yet, the mechanisms enabling such a pivotal role despite the relatively low cell abundance of methane-oxidizing bacteria (MOB) and methane (CH_4) concentrations – relative to dissolved organic C (DOC) – are unclear. To address this question, we combined incubations, microscopy, and water column profiles in six temperate lakes to determine metabolic features of MOB and heterotrophic bacteria and to estimate the relative importance of methanotrophy at the whole-lake level. We show that MOB has a larger average cell size and higher activity per biomass than heterotrophic bacteria, partially compensating for lower MOB abundance. MOB activity per biomass and total C consumption are regulated by CH_4 and O_2 concentrations with total C consumption by MOB surpassing that of heterotrophic bacteria at CH_4:O_2 ratios > 0.2 (± 0.1). Thus, the role of methanotrophy at the ecosystem level is greater in lakes where a larger volume of the water column meets this condition, which is the case of well-stratified, DOC-rich lakes. Further, our results show that methanotrophy could be the main C processing pathway in lakes with DOC concentrations > 6 mg L^{-1}. Given that a large suite of the world's lakes has DOC concentrations above this value, MOB may be the main player in the microbial C cycling of many lakes globally, challenging our current concept of the microbial loop in lakes.

3.2 Introduction

Methane-oxidizing bacteria (MOB or methanotrophs) are well-known for playing a central role in lake methane (CH_4) budgets and mitigating aquatic CH_4 emissions to the atmosphere (Bastviken *et al.*, 2008). MOB use CH_4 as source of metabolic energy and structural carbon (C), producing bacterial biomass and CO_2 from the oxidation of

CH_4 (Hanson et Hanson, 1996). Given that a substantial portion of the total CH_4 produced in lakes is oxidized by MOB (e.g. Bastviken et al., 2008; Kankaala et al., 2007b; Thottathil et al., 2018), methanotrophy is a potentially important pathway in the total C budget of lakes. Indeed, methanotrophic production is comparable in some cases to the total C fixation by heterotrophic bacteria and a considerable proportion of primary production (Bastviken *et al.*, 2003 ; Kankaala *et al.*, 2013 ; Morana *et al.*, 2015). Evidence for preferential grazing on MOB (Kankaala *et al.*, 2007a) and widespread isotopic and biomarkers evidence for CH_4-derived C support of aquatic food webs (Grey, 2016; Jones and Grey, 2011 and references therein) also point to an important role of methanotrophy in the C flow to higher trophic levels in lakes. Similarly, CO_2 produced from CH_4 during methanotrophy may contribute to the general CO_2 oversaturation found in freshwaters, influencing the balance between CH_4 and CO_2 emissions by these ecosystems (Guérin et Abril, 2007).

The relative contribution of individual microbial groups to total C processing in lakes can change depending on environmental conditions, intrinsic metabolic features of taxa, and the interplay between these two (Del Giorgio et Gasol, 2008). For instance, microbial cell abundance, size, total biomass, and activity may vary due to the metabolic potential of each taxa and environmental conditions, affecting the relative importance of each microbial process at the ecosystem level. For MOB, the availability of substrates, namely CH_4 and O_2, are the most important factors regulating methanotrophic abundance and activity (Bastviken et al., 2003; Reis et al., 2020b; Thottathil et al., 2019). Although MOB cell abundance in freshwaters are generally low (<1% of total bacterial cells), MOB can peak under favorable conditions such as in the hypoxic layer of stratified water columns (Kankaala et al., 2007a; Milucka et al., 2015; Reis et al., 2020a; Reis et al., 2020b; Zigah et al., 2015). Culture-based and few environmental studies suggest that MOB cells can be large (Brown *et al.*, 1964 ; Bussmann *et al.*, 2006 ; Kankaala *et al.*, 2007a ; Khmelenina *et*

al., 2010), which is indicative of high metabolic activity and susceptibility for preferential grazing as demonstrated previously (Kankaala *et al.*, 2007a).

Although evidence suggests an important role of methanotrophy as a process of C transformation and C source to lake food webs, quantitative estimations under ambient CH_4 concentrations across lakes are lacking. Moreover, the mechanisms enabling such potential importance of methanotrophy are unclear due to the low MOB cell abundance (often <1% of total bacterial cells) and relatively lower CH_4 concentration than dissolved organic carbon (DOC) in lakes. To address this issue, we performed incubations at ambient CH_4 and O_2 concentrations to determine MOB and HB abundance and metabolic features at different depths of six temperate lakes spanning across large DOC gradient. Further, we used profiles of CH_4, O_2, and temperature taken across the summer in these systems to estimate the relative importance of methanotrophy at the whole-lake level. We hypothesized that the despite their relatively low total abundances, high metabolic efficiency of MOB enables an important role of methanotrophy in the pelagic C cycling of lakes. We also expected that CH_4 and O_2 availability exert a strong control on the relative importance of methanotrophy in lakes with a greater role of methanotrophy in pelagic C cycling where CH_4-rich hypolimnia contribute to a larger fraction of the total lake volume.

3.3 Methods

3.3.1 Study sites and sampling

We sampled six temperate lakes located in the Laurentians region of Québec during the summer in 2015 and 2016. These lakes largely differ in size, depth, nutrients, chlorophyll-*a*, and dissolved organic carbon (DOC) (details can be found in Reis et al., 2020b). In 2015, profiles of temperature, O_2, and CH_4 concentrations were

performed monthly in the six studied lakes from May to November. Few additional profiles were performed in 2016 between June and September. Temperature and O_2 measurements were taken at each meter using a YSI probe (Yellow Springs Instruments, USA) and the concentration of CH_4 was determined using the headspace technique followed by gas chromatography (GC-8A/GC-2014, Shimadzu, Japan). Vertical profiles of photosynthetically active radiation (PAR) were performed once in each lake in 2015 using an underwater light sensor (LI-192, LI-COR Biosciences, USA). PAR attenuation coefficients (K_d) were calculated as the absolute linear slope between depth and the natural logarithm of the measured radiation. Incubations for the determination of methanotrophy and heterotrophy C consumptions were performed during the summer in 2016. Water for the incubations was collected with a peristaltic pump at the surface and another one or two depths within the oxic water column in each lake, covering a large gradient in CH_4 and O_2 concentrations (0.02–455 μM and 3–265 μM, respectively). The water was pumped into acid-rinsed collapsible bags until overflow and taken to the laboratory where they were gently transferred to incubation flaks with the aid of a hose.

3.3.2 Methanotrophic C consumption rate

Since CH_4 is the sole source of C and energy for obligate methanotrophs, we determined total methanotrophy C consumption (MCC) by measuring CH_4 oxidation rates in incubations with untreated lake water. In the laboratory, ten 500 mL-flasks per depth were filled with lake water allowing to overflow and sealed with silicone stoppers without headspace. Flasks were incubated at in situ temperature (± 2°C) in dark circulation water baths for three to eight days. CH_4 concentration was measured by headspace technique with ultra-high purity zero air (Praxair Canada Inc., Canada) and cavity ring down spectrometry (Picarro G2201-i, Picarro Inc, USA) or GC by sampling duplicate flasks three to five times during each incubation. CH4 oxidation rates were determined by differentiation of the first order CH_4 concentration decay

curve at each time point of the experiment. The measured CH_4 oxidation rates represent actual rather than potential rates since the incubations were performed at in situ gases concentrations (CH_4 and O_2) and in the presence of potential grazers. At the start and end of each incubation, water was collected for the identification and enumeration of MOB and HB cells as described below.

3.3.3 Heterotrophic C consumption rate

Total heterotrophic carbon consumption (HCC) was determined as the sum of heterotrophic bacterial production (HBP) and respiration. The HBP was measured by incorporation of ^{3}H-leucine performed with the same water of the start of incubations for CH_4 oxidation rates. For that, three replicates of 1.5 mL of water from each depth and one trichloroacetic acid (TCA)-killed control was inoculated with ^{3}H-leucine at a final concentration of 20 nM and incubated for 1 h in the dark at room temperature. Incubations were stopped by the addition of TCA and vials were kept at 4°C until processing through the centrifugation method (Smith et Azam, 1992). Heterotrophic bacterial respiration (HBR) was measured in incubations held simultaneously with the methanotrophic C consumption (MCC) incubations. HBR was determined by the change in dissolved oxygen (O_2) concentration in 500 mL-flasks equipped with O_2 optodes (Fibox 3, PreSens, Germany) filled with the same water used for MCC but gently filtered through 1.2 µm filters. Incubations were held for at least 48 h at in situ temperature (± 2°C) using dark circulation water baths and O_2 was measured three to five times. Respiration rate was calculated as the absolute slope of linear regression of O_2 (mg L^{-1}) vs. time (h). Rates of O_2 consumption were converted to C consumption using a respiratory quotient (RQ) of 1.

3.3.4 Methanotrophic and total bacterial cells counts, size, and biomass

Methanotrophic and total bacteria cells were microscopically quantified at the start and at end of each incubation. Methanotrophic bacterial cells were identified by

catalyzed-reporter deposition-fluorescence in situ hybridization (CARD-FISH) with specific probes for the detection of Alpha- and Gammaproteobacteria methanotrophs (Eller *et al.*, 2001). Total bacterial cells were identified using 4',6-diamidino-2-phenylindole (DAPI) staining. Cells were visualized at 630x magnification under an automated epifluorescence microscope and CARD-FISH and DAPI cells counts were determined using the ACME tool3 software (Zeder, 2014). Details on the CARD-FISH and DAPI staining protocols can be found in Reis et al. (2020b).

Cell size was measured for every bacterial cell in pixels using the ACME tool software (Zeder, 2014) and then converted to μm^2. Measurements were done in the DAPI subset for MOB (cells with positive signals for CARD-FISH and DAPI, visualized in the DAPI channel) and in the DAPI set for other bacteria to avoid bias from different staining methods on the cell size determination. Bacterial volume (μm^3) of both MOB and other bacteria was calculated from the measured cell size assuming a spherical shape of bacterial cells (measured mean circularity of total cells: 0.71 ± 0.17, n = 23 738; circularity of 1 indicates perfect circle). Bacterial biomass was then calculated from cell volume assuming a bacterial C content factor of 63 fgC μm^{-3} (Fagerbakke *et al.*, 1996).

3.3.5 C consumption per bacterial biomass

C consumption per unit biomass for methanotrophy and heterotrophy was calculated using the measured total amount of C consumed by methanotrophy and heterotrophy in incubations and the measured total bacterial biomass of MOB and heterotrophic bacteria as described above. For that, we assumed that all non-methanotrophic bacterial cells (cells with positive signal in the DAPI channel but negative signal in the CARD-FISH channel) were heterotrophic bacterial cells.

3.3.6 Whole-lake methanotrophy and heterotrophy during summer

Whole-lake methanotrophic C consumption in the six lakes was calculated at every meter of the water column using the stratified profiles performed in 2015 and 2016 and a model that predicts CH_4 oxidation rates based on CH_4, O_2, and temperature developed in the same lakes of this study (Thottathil *et al.*, 2019). Oxygen profiles were calibrated so that dissolved oxygen concentrations < 0.2 mg L^{-1} were considered anoxic. Total heterotrophic C consumption and heterotrophic production were measured in incubations as described above and assumed to be constant in each layer of the water column during the summer stratification. Whole-lake volume-weighted rates of both total methanotrophic and heterotrophic C consumptions were determined for each profile using the volume of 1 m or 2 m layer derived from bathymetric maps.

3.4 Results

3.4.1 Cells abundance, size, and biomass

MOB accounted for a small fraction of the total bacterial cells across incubations, with average Alpha- and Gamma-MOB cell abundances being orders of magnitude lower than that of other bacterial cells (Fig. 3.1A). Together, MOB cells comprised between 0.1 and 5% of the total DAPI counts across lakes and incubations. Gamma-MOB cell volume was on average three times greater than the bulk bacteria cell volume, but Alpha-MOB was not significantly larger than bulk bacteria (ANOVA and Tukey HSD; Fig. 3.1B). Average cell diameter and volume were 1.2 μm and 0.9 μm^3 for Gamma-MOB, 0.9 μm and 0.5 μm^3 for Alpha-MOB, and 0.8 μm and 0.3 μm^3 for other bacteria, respectively. Due to the larger cell size, the low abundance of MOB cells was partially compensated in terms of biomass, with Alpha- and Gamma-MOB biomass being on average 1.5 and 3 times higher than their cell numbers, respectively.

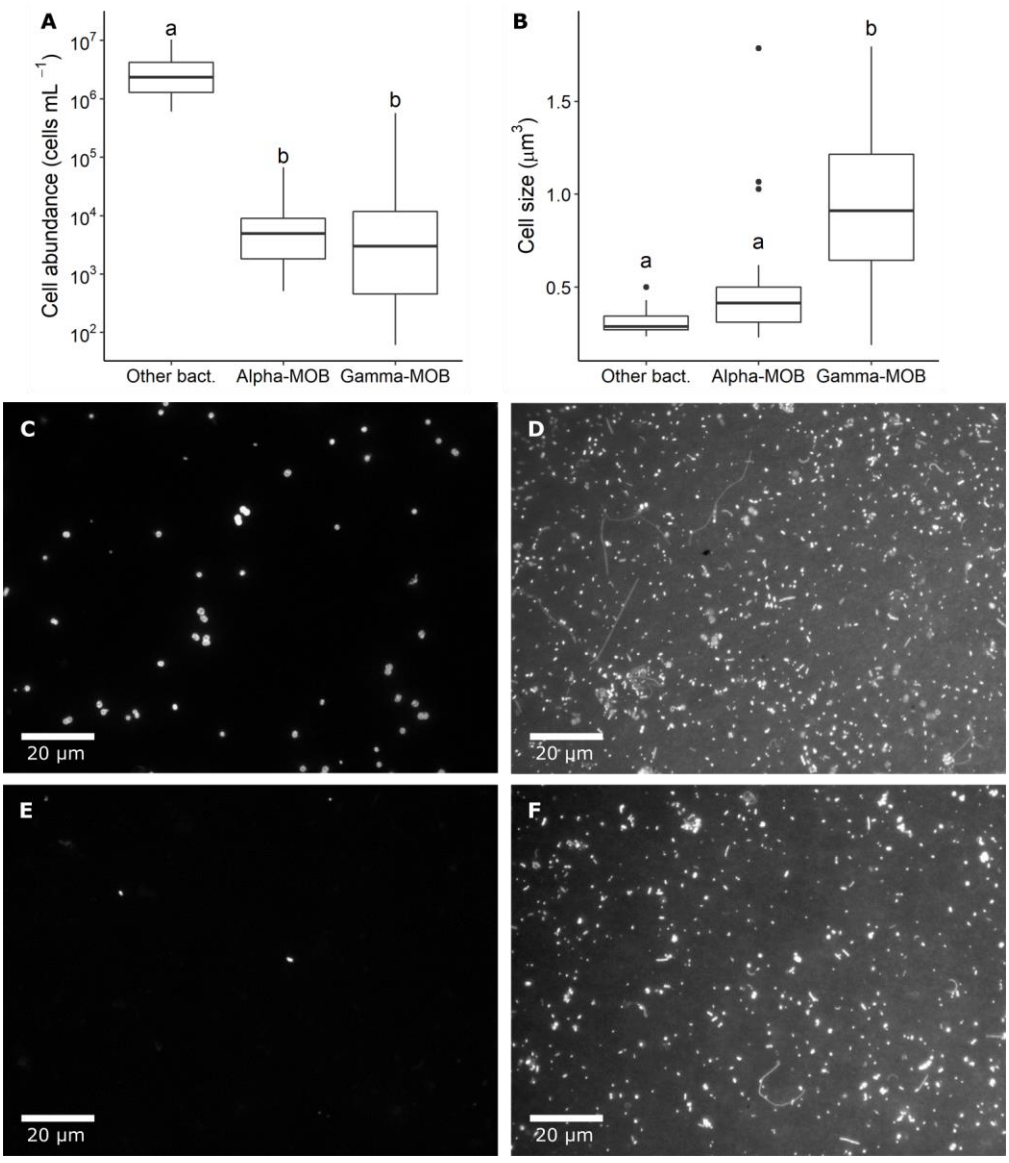

Figure 3.1. Difference in the cell abundance and size of methanotrophic (Alpha-MOB and Gamma-MOB) and bulk bacteria (Other bact.) across incubations with water from different lakes and depths. A) Cell abundance. B) Cell size. C-D) Microscopic picture of Gamma-MOB cells visualized by CARD-FISH and corresponding picture of the same field of view showing all cells visualized by DAPI staining, respectively. E-F) Microscopic picture of Alpha-MOB cells visualized by CARD-FISH and corresponding picture of the same field of view showing all cells visualized by DAPI staining, respectively. White scale bars in C to F represent 20 µm. Different letters in

A and B indicate significant difference between means ($p < 0.05$, ANOVA and Tukey HSD). Note log scale in y axis of A.

3.4.2 C consumption per biomass

The consumption of C per unit biomass of MOB was significantly higher than that of HB across incubations ($p = 0.001$, t-test; Fig. 3.2A). However, C consumption per MOB biomass was more variable than per HB biomass. C consumption per MOB biomass varied across the gradient in CH_4 concentration, increasing with increase in CH_4 until a maximum around 20 µM and declining after that probably because O_2 becomes limiting (Fig. 3.2B).

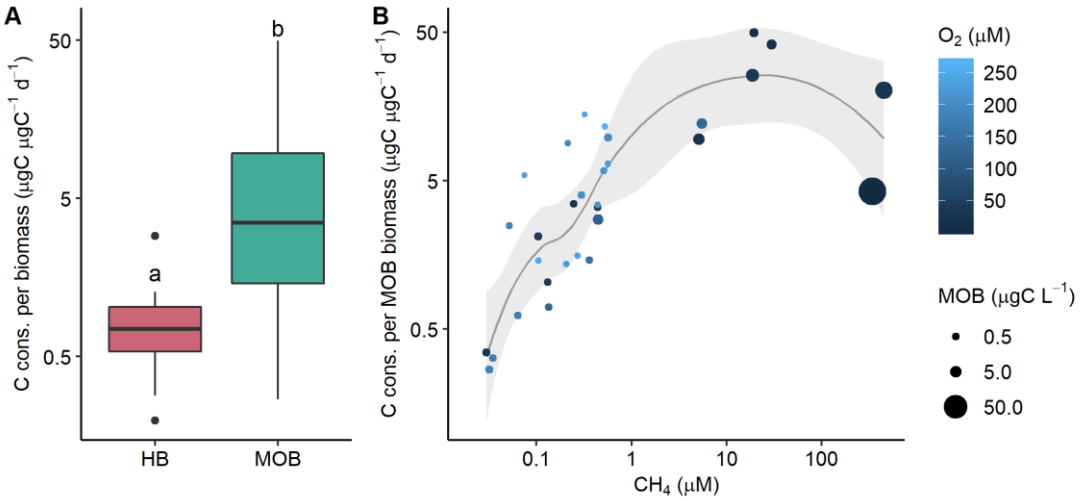

Figure 3.2. Carbon (C) consumption per bacterial biomass across incubations. A) C consumption per unit biomass in methane-oxidizing bacteria (MOB) and in heterotrophic bacteria (HB). Different letters indicate statistical difference ($p < 0.05$; t-test). B) Change in C consumption per MOB biomass along the gradient in CH_4 concentration. Plotted trend line is a loess curve and shaded area around line indicates 95% confidence interval. Color of points show the O_2 concentration. Note log scale of y axis in A and of y and x axes in B.

3.4.3 C consumption by methanotrophy and heterotrophy in incubations

Methanotrophic C consumption (MCC) across incubations ranged between 0.06 and 433 µgC L^{-1} d^{-1} and changed monotonically along the gradient of CH_4:O_2 molar ratios. Heterotrophic C consumption (HCC) was much less variable, ranging between 17 and 87 µgC L^{-1} d^{-1}, and did not change along the gradient of CH_4:O_2 molar ratios (Fig. 3.3A). MCC was up to three orders of magnitude lower than HCC at CH_4:O_2 ratios below 0.2 (±0.1) but surpassed HCC at CH_4:O_2 molar ratios above this value (Fig. 3.3B). At higher CH_4:O_2 molar ratios, MCC was up to six times higher than HCC, but stabilized at the maximum CH_4:O_2 molar ratio measured in our incubations (~100).

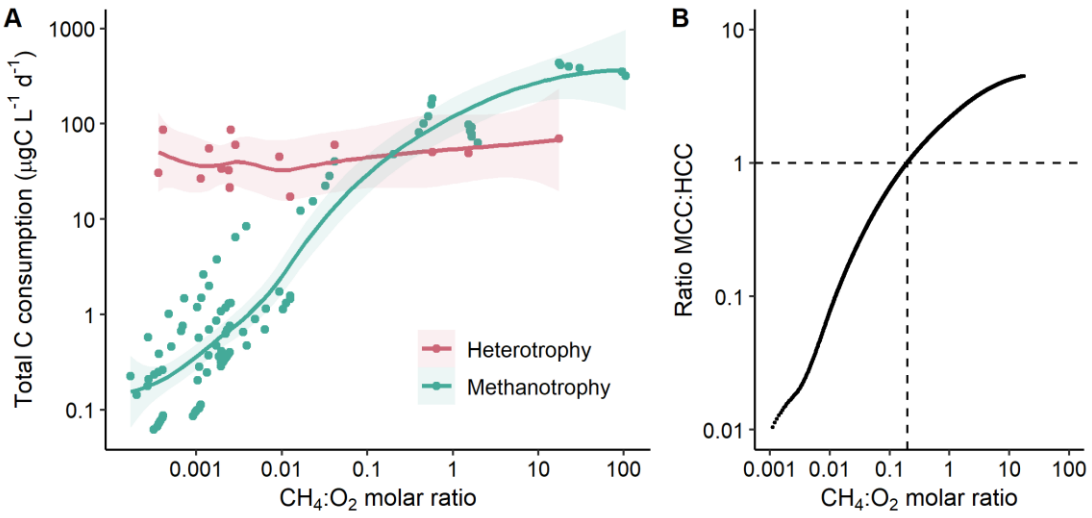

Figure 3.3. Methanotrophic and heterotrophic carbon (C) consumption rates measured in incubations. A) Change in methanotrophy and heterotrophy total C consumption along the gradient in CH_4:O_2 molar ratio. Plotted trend lines are loess curves and shaded area around line indicates 95% confidence interval. B) Ratio between methanotrophy and heterotrophy C consumption (MCC and HCC, respectively) – calculated on the predicted values of the loess curves in A – along the gradient in CH_4:O_2 molar ratio. The dashed horizontal line indicates MCC = HCC and the dashed vertical line crosses the x axis at 0.2. Note log scale of both axes in A and B.

3.4.4 Relative importance of methanotrophy to whole-lake bacterial C consumption

The contribution of methanotrophy to whole-lake bacterial C consumption varied largely among lakes and months during summer stratification (0.4-83%; Fig. 3.4). In lakes Triton and en Coeur, methanotrophy was responsible for only a small portion of the total bacterial C consumption across the season (up to 2.5% in Triton and 7.9% in en Coeur; Fig. 3.4). In these same lakes, the layer of the water column with CH_4:O_2 molar ratios > 0.2 was absent or negligible throughout the summer (Fig. 3.5A-B). In contrast, in the well-stratified lakes with clear hypolimnia and thicker layers of CH_4:O_2 molar ratios > 0.2 (Fig. 3.5C-F), methanotrophy represented a significant fraction of the whole-lake bacterial C processing (average: 42%). The highest contributions of methanotrophy were detected in Lake Geai, where methanotrophy accounted for up tp 83% of the total bacterial C consumption (Fig. 3.4) and where a larger proportion of the water column showed CH_4:O_2 molar ratios > 0.2 (Fig. 3.5F). Between 0 and 68% of the total water column volume of the studied lakes presented CH_4:O_2 molar ratios > 0.2, with such proportion depending on the water column stratification and lake morphometry (Fig. 3.5). Within the well-stratified lakes with clear hypolimnia, the average relative importance of methanotrophy during the summer stratification increased with surface dissolved organic carbon (DOC) concentration and was strongly correlated to the photosynthetically active radiation attenuation coefficient (K_d PAR) (Fig. 3.6).

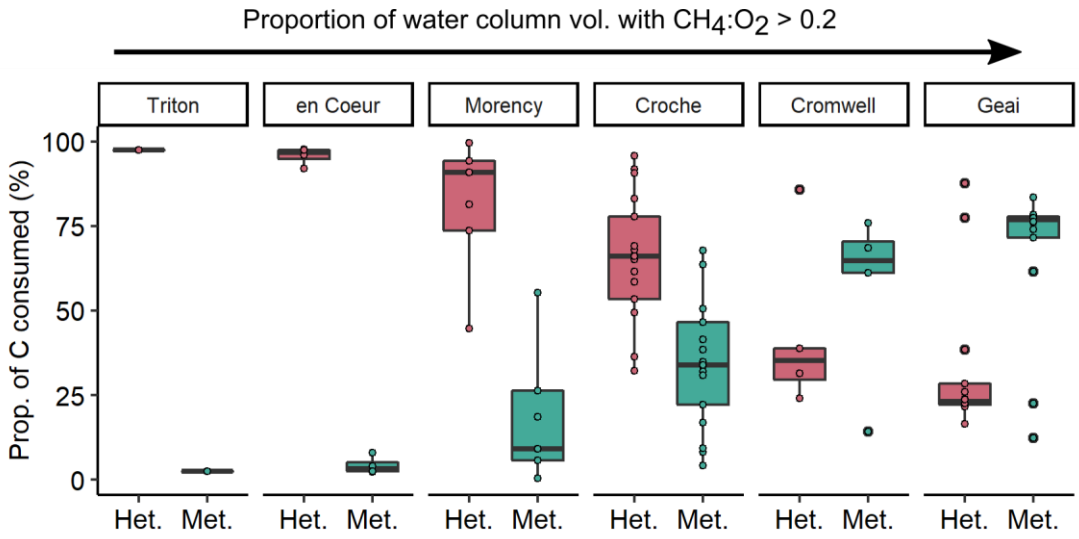

Figure 3.4. Contribution of heterotrophy (in rose) and methanotrophy (in green) to whole-lake bacterial carbon consumption (volume weighted rates) during summer in each studied lake. Lakes are plotted in order of increasing proportion of water column volume with CH_4:O_2 molar ratio > 0.2.

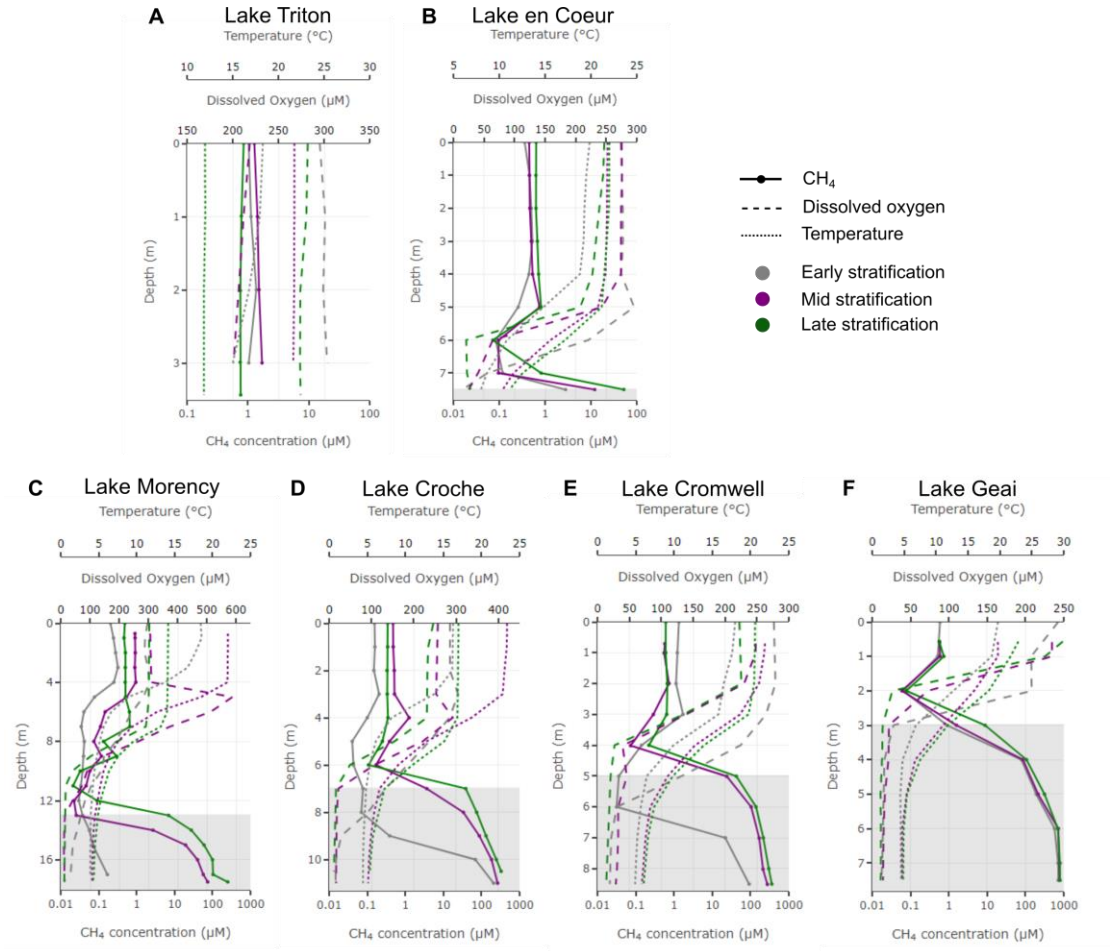

Figure 3.5. Vertical profiles of CH_4, O_2, and temperature taken at the early, mid, and late summer in 2015 in each studied lake. Shaded areas indicate the thickest layer of CH_4:O_2 molar ratio > 0.2 measured in that year.

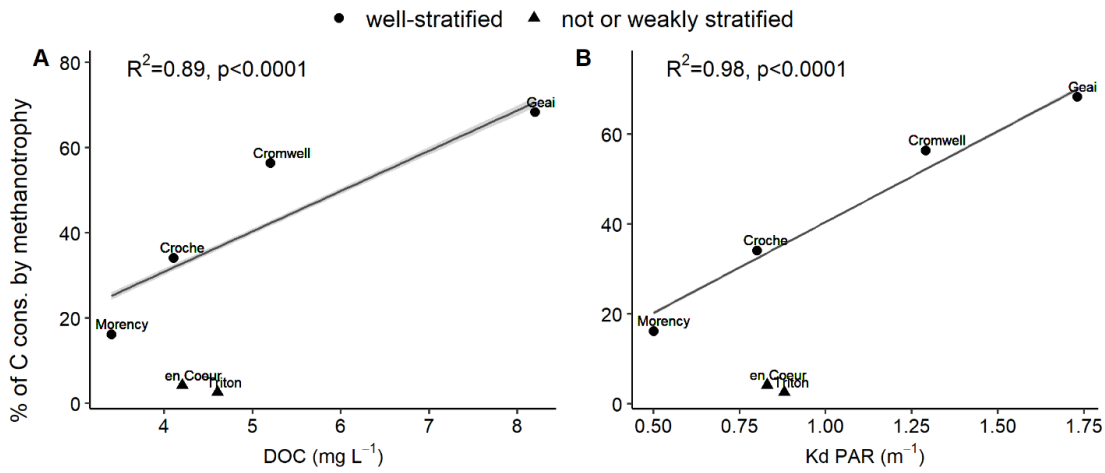

Figure 3.6. Relationships between the mean fraction of C consumed by methanotrophy over summer and lake characteristics in the studied lakes. A) Relationship with dissolved organic carbon concentration (DOC). B) Relationship with the attenuation coefficient of photosynthetically active radiation (K_d PAR). Lines indicate linear relationship between the percentage of C consumed by methanotrophy and DOC or K_d PAR in the well-stratified lakes only (i.e. excluding lakes Triton and en Coeur).

3.5 Discussion

Despite the well-recognized central role of methanotrophy in reducing lake CH_4 emissions, its relevance to the overall cycling of C at the whole-ecosystem scale remained poorly constrained. Likewise, while a handful of studies indicated a potentially large role of methanotrophy in lake food webs based on biomarkers and stable isotopes, quantitative estimations of its importance are rare. Here we show that despite their relatively low abundance, MOB cells can outpace heterotrophic metabolism to constitute the main microbial C processing pathway in lakes. The regulation of the relative importance of methanotrophy to microbial C processing in lakes is shaped by the interaction between CH_4 and O_2 concentrations. At high CH_4:O_2 molar ratios (> 0.2), MOB exhibit an ecological advantage over HB to process more C, in part because of their larger cell size and their high affinity with

their substrate (CH_4). At the whole-ecosystem scale, the importance of methanotrophy depends on the spatial extent of the hypoxic water column layer with high CH_4 concentrations, which is in turn regulated by lake morphometry, stratification, and DOC concentrations.

3.5.1 Larger MOB cells indicate selective grazing

Cell size and biomass are important characteristics that determine the ecological and biogeochemical roles of a microbial group. Microbial groups with large cells can be much more important in terms of C cycling than their abundances suggest (e.g. Garcia-Chaves et al., 2016). For instance, due to the size-selective bacterivory, larger bacterial cells are more likely to be grazed and to support food webs than smaller bacterial cells (Jürgens et Matz, 2002). Here we found that Gamma-MOB cells are on average larger than bulk bacterial cells across lakes, supporting previous notion on the selective grazing on MOB (Kankaala *et al.*, 2007a). Gamma-MOB cells are significantly larger than Alpha-MOB cells, indicating that Gamma-MOB may be responsible for most of the CH_4-derived C entering food webs in temperate lakes. In addition, a large cell size of Gamma-MOB agrees with a high metabolic activity observed by the tighter link between CH_4 oxidation rates and Gamma-MOB cells than Alpha-MOB cells across stratified temperate lakes (Reis et al., 2020b). However, the large variability in Gamma-MOB cell size detected here (Fig. 3.1B) suggests a wide range of single-cell activity and physiological status within Gamma-MOB across the sampled lakes and depths, demonstrating also a strong environmental control on methanotrophic metabolism.

3.5.2 High MOB activity per biomass is linked to CH_4 concentration and metabolic characteristics

The level of cell activity was on average one order of magnitude higher in MOB than in HB across all incubations. The highest methanotrophic C consumption by unit biomass measured at CH_4 concentrations around 20 μM (Fig. 3.2B) suggests a

surprisingly high apparent turnover rate (around 50 d^{-1}), meaning that a MOB cell can replace its biomass up to 50 times per day. Environmental factors, such as the availability of substrates, are important regulators of the physiological structure of the bacterioplankton (Del Giorgio et Gasol, 2008). Indeed, we found a strong response of MOB activity to CH_4 concentrations, with rates of C consumption per biomass increasing along the measured CH_4 concentration (0.02 – 455 µM) in our incubations. Methanotrophic activity per biomass was limited by CH_4 until reaching concentrations around 20 µM and started to decrease thereafter despite an increase in MOB biomass. This sharp decline in activity per biomass after 20 µM CH_4 indicates a metabolic saturation point after which CH_4 oxidation rates increase through an increase in MOB biomass rather than through increased single-cell activity. Accordingly, Steenbergh et al. (2010) found that the kinetics of CH_4 oxidation in soil bioassays were caused by increases in cell activity followed by cell growth. Similarly, Dumont et al. (2011) observed that *pmo*A transcripts and genes were labeled progressively after incubating lake sediments with $^{13}CH_4$, indicating that active MOB cells (transcribing the *pmo*A gene) started replicating.

Besides substrate availability, intrinsic phylogenetic characteristics between MOB taxa may also influence a large variation in metabolic activity of cells within MOB assemblages. For example, Gamma-MOB cells possibly show higher activity than Alpha-MOB cells in temperate lakes as indicated by their larger cell size as shown here. In fact, a distinctive feature between Gamma- and Alpha-MOB is their pathways for C assimilation, with the Alpha-MOB's serine pathway showing a lower C conversion efficiency (Hanson et Hanson, 1996 ; Trotsenko et Murrell, 2008). This metabolic difference suggests that Gamma- and Alpha-MOB may show different growth rates in the environment and therefore, microbial physiology may be important factors to be considered to understand lake CH_4 cycling.

3.5.3 Methanotrophy surpasses heterotrophy at high CH_4:O_2 molar ratios

Total C consumption by methanotrophy varied largely along a gradient of CH_4:O_2 molar ratios, reaching its maximum and stabilizing at ~100 (Fig. 3.3A). Interestingly, in boreal lakes, Kankaala et al. (2013) also reported that methanotrophy varied as a function of CH_4:O_2 molar ratios but highest CH_4 oxidation rate was observed between ratios of 0.5 and 12, with 12 being the highest ratio measured. By sampling at a wider range of CH_4 and O_2 concentrations, we found that methanotrophy keeps increasing until a CH_4:O_2 molar ratio of 100, where it stabilizes probably due to limiting O_2 concentrations.

One of the key aspects in assessing the significance of methanotrophy in lake C cycling is to determine under which scenarios MOB may have an ecological advantage over HB. Here we showed that methanotrophic C consumption surpasses heterotrophic C consumption at CH_4:O_2 molar ratios higher than 0.2 (±0.1) (Fig. 3.3), which is close to the theoretical CH_4:O_2 stoichiometry for aerobic CH_4 oxidation (0.5). In this zone of high CH_4 concentrations but available O_2, methanotrophy can be up to six times higher than heterotrophy and this layer can constitute a variable portion of the water column of lakes during summer, from negligible to most of the water column volume. However, C consumption by methanotrophy may be limited to only a fraction of the hypolimion in lakes where the deeper layer is completely anoxic, unless the MOB assemblage has the metabolic capacity to oxidize CH_4 using other electron acceptors (Martinez-Cruz *et al.*, 2017 ; Oswald *et al.*, 2016b)

3.5.4 The relative importance of methanotrophy to whole-lake bacterial C processing is greater in well-stratified high-DOC lakes

The relative importance of methanotrophy at the lake level was higher in well-stratified lakes that form thick hypolimnia during summer, while it was much less important in the shallower lakes where stratification was weak or absent (Triton and en Coeur). In the well-stratified lakes, where a significant layer of CH_4:O_2 > 0.2 was

present, the relative importance of C consumption by methanotrophy increased linearly with DOC concentration and K_d PAR (Fig. 3.6). These strong positive relationships indicate that light penetration plays a key role in the importance of methanotrophy in lakes. First, less light penetration in higher DOC lakes limits O_2 production in deeper layers leading to thicker and shallower hypoxic hypolimnia with higher total CH_4 stocks and a greater portion of the water column dominated by methanotrophy (Fig. 3.5). In addition, while light may enable methanotrophy in deeper layers by providing photosynthetically produced O_2 (Oswald *et al.*, 2015 ; Savvichev *et al.*, 2019), algal biomass may also boost heterotrophic bacterial metabolism and thus not necessarily increase the relative importance of methanotrophy at the lake level. Finally, methanotrophic activity has been shown to be inhibited by light (Dumestre *et al.*, 1999b ; Murase et Sugimoto, 2005 ; Thottathil *et al.*, 2018), rendering an ecological advantage for methanotrophy in higher K_d PAR lakes.

The observed relationship between the relative importance of methanotrophy and DOC suggests that methanotrophy constitutes the main (> 50%) microbial C processing pathway in lakes with DOC concentrations > 5 mg L^{-1} (Fig. 3.6). Considering that the median DOC concentrations across lakes around the globe is 5.71 mg L^{-1} (Sobek *et al.*, 2007) and that many of these lakes stratify during summer, methanotrophy may be the main microbial C processing pathway in a large suite of the world's lakes. Even though we have not assessed here how much of the total C processed by methanotrophy is incorporated into food webs, our findings suggest that the dominance of MOB over HB in terms of whole-lake C processing is widespread and challenge the current view of DOC-centered microbial loop in lakes.

3.6 Conclusion

Our study shows that the role of methanotrophy at the lake level depends both on intrinsic microbial features and lake characteristics. A key finding of our study is that in terms of total C cycling, the larger MOB cell size and higher activity per biomass partially compensate for their low abundances and total biomass. MOB activity per biomass and total C consumption are controlled by the distribution of CH_4 and O_2 in the water column, and the role of methanotrophy in bacterial C cycling is greater in lakes where a larger volume of the water column shows CH_4:O_2 molar ratios > 0.2, which is the case of higher DOC lakes. We further show that the positive relationship between methanotrophy and DOC is likely through the DOC effect on light attenuation, which creates shallower and more volumetric hypoxic hypolimnia where methanotrophy has an ecological advantage over heterotrophy. Given that a large suite of the world's lakes has high DOC concentrations (Sobek *et al.*, 2007), our findings indicate that MOB may be a neglected but main player in the bacterial C processing of many lakes globally, challenging our current understanding of the microbial loop in lakes. Finally, our results suggest that changes in the DOC concentration and quality in lakes due to anthropogenic activity or climate change can affect the CH_4 and O_2 distributions in lake water columns, which impact MOB metabolism and the balance between methanotrophy and heterotrophy, ultimately altering the biogeochemical and ecological role of methanotrophy in lakes.

3.7 Acknowledgements

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strategic cluster of the Fonds de recherche du Québec Nature et technologies (FRQNT). PCJR was supported by a merit scholarship from FRQNT.

CONCLUSION

This thesis aimed at better understanding how the fate of methane (CH_4) in lakes and hydropower complexes is influenced by the interplay between the environment and methanotrophic composition, activity, and metabolism. The combination of different approaches, including incubations, field sampling, sequencing and microscopic techniques, enabled important insights into this issue.

Chapters I and II show that different methane-oxidizing bacteria (MOB) taxa may be responsible for local CH_4 oxidation rates depending on the environmental conditions. Specifically, chapter I shows a niche separation within Proteobacterial MOB along water columns, which is mostly driven by oxygen concentration. Interestingly, in chapter II, the faster growth of Alpha-MOB cells in the CH_4-rich but well-oxygenated downstream river below a tropical hydropower reservoir agrees with the niche separation observed in the temperate lakes studied in chapter I. The role of oxygen in structuring MOB communities may be related to the ability of using other electron acceptors for the oxidation of CH_4 by taxa within Gamma-MOB as indicated by their tolerance to low oxygen concentrations (chapter I) and suggested by other studies (Kits *et al.*, 2015b, 2015a ; Oswald *et al.*, 2016b ; Skennerton *et al.*, 2015). In addition, chapter I shows that Gamma-MOB is responsible for most CH_4 oxidized during summer in stratified temperate lakes and that their abundance adds explanation power to models of CH_4 oxidation rate. This finding suggests that intrinsinc microbial features, or other factors affecting microbial abundance such as grazing, directly affect CH_4 cycling in lakes. Chapter II highlights the importance of microbial CH_4 oxidation in reducing the carbon (C) footprint of hydropower impoundments,

including in the less studied rivers below dams where MOB cells grow rapidly. It also demonstrates that the damming of rivers for hydropower generation leads to changes in the inflowing MOB communities, with a steep increase in total MOB abundances in the hypoxic hypolimnion of the reservoir and community structure shifts caused by environmental filtering and a break in the hydrological connectivity between surface upstream and downstream waters. Finally, chapter III demontrates that microbial metabolic characteristics influence the contribution of methanotrophy to the C cycling and potential CH_4 support to food webs in lakes. The larger cell size and higher cell activity of MOB relative to the bulk heterotrophic bacteria partially compensate for MOB's relatively low abundance in lakes, with methanotrophy being responsible for up to 83% of the total bacterial C transformation in the studied lakes at the end of summer stratification. The relative importance of methanotrophy at the lake level depends on the concentrations of CH_4 and O_2 in water columns, which is in turn related to lake morphometry and dissolved organic C concentrations. Given the natural gradient in lake DOC concentrations around the globe (Sobek *et al.*, 2007), our findings indicate that MOB may be the main player in the bacterial C cycling of many lakes, challenging the current understanding of the microbial loop in lakes.

Given the important role of freshwaters in the global C and CH_4 cycles (Bastviken *et al.*, 2011 ; Deemer *et al.*, 2016 ; Tranvik *et al.*, 2018) and the ongoing anthropogenic and climate changes, a thorough understanding of the factors and mechanisms controlling CH_4 emissions from freshwaters is needed. In this context, CH_4 oxidation is a key process that ultimately regulates CH_4 emissions from freshwaters. Despite being a microbial process, most previous studies assessing the controls on CH_4 oxidation in aquatic ecosystems have focused on the geochemical and physical aspects of it. At the same time, other studies have focused solely on the microbial ecology of methane-oxidizing bacteria, not directly linking it to process rates. While microbial characteristics are not always linked to bulk rates in the environment in the case of biogeochemical processes widely distributed across taxa, these may be

relevant to processes more narrowly distributed across taxa such as methanotrophy (Bier *et al.*, 2015 ; Levine *et al.*, 2011 ; Schimel, 1995). Indeed, this thesis demonstrates that CH_4 oxidation in lakes and reservoirs is to a large extent related to MOB community structure and metabolic features. The environment dictates the basic conditions for CH_4 oxidation to occur, but it also shapes MOB composition, abundance, and activity. In turn, these microbial features can affect the fate of CH_4 due to intrinsic differences in niches, metabolism, and cell size both among MOB taxa and between MOB and other bacteria. Therefore, this thesis represents a novel contribution to the field and demonstrates how combining biogeochemistry and microbial ecology may render relevant insights into the regulation and ecological role of biogeochemical processes.

In summary, this thesis provides mechanistics explanations of how environmental and microbial characteristics affect the fate of CH_4 in lakes and hydropower complexes. Looking forward, these findings should allow more accurate evaluations and predictions of the impact of ongoing anthropogenic and climate changes on the functioning of freshwater ecosystems and their role in the global CH_4 and C cycles.

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