Niche separation within aerobic methanotrophic bacteria across lakes and its link to methane oxidation rates

Running head: Linking MOB to methane oxidation in lakes

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Originality-Significance Statement

Our study shows for the first time that inter- and intra-lake variations in oxygen and colored dissolved organic matter concentrations affect the distribution and abundance of methane-oxidizing bacteria (MOB) groups, which, in turn, is related to methane oxidation rates. Combining an absolute quantification method (CARD-FISH) to determine Alpha- and Gamma-MOB abundances and incubations to measure ambient methane oxidation rates from different lakes, we show that Gamma-MOB dominate the overall methanotrophic activity, but that Alpha-MOB may be an important player particularly in well-mixed lakes. Including Gamma-MOB cell abundance improved environmental models of methane oxidation rates, explaining part of the variation that could not be explained by environmental factors alone. This implies that changes in microbial abundance can impact methane oxidation rates and thus may affect lake methane emissions. In a broader sense, our findings shed light on the link between the microbiome and ecosystem processes and on how environmental microbial information can improve our understanding and predictions of biogeochemical processes.

Summary

Lake methane (CH₄) 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 CH₄ 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 CH₄ 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 appear 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 CH₄ 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 CH₄ oxidation rates in lakes and that information on the MOB community can help predict CH₄ oxidation rates and thus emissions from lakes.

Introduction

Freshwater lakes are disproportionally large sources of methane (CH₄) – a highly potent greenhouse gas – to the atmosphere, emitting roughly 71.6 Tg CH₄ per year despite their small global area (Downing *et al.*, 2006; Bastviken *et al.*, 2011; Kirschke *et al.*, 2013). To a large extent, lake CH₄ emissions are controlled by the activity of aerobic methane-oxidizing bacteria (MOB), which oxidize a substantial, but variable portion of the total CH₄ produced before it is emitted (Utsumi *et al.*, 1998; Bastviken *et al.*, 2008; Morana *et al.*, 2015; Thottathil *et al.*, 2018). CH₄ oxidation rates in lakes have been shown to vary widely depending on water column stratification (Kankaala *et al.*, 2007; 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 (CH₄ and oxygen, O₂) 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 CH₄ 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) (Hanson and Hanson, 1996; Bowman, 2006). Alpha- and Gamma-MOB are known to differ in many metabolic and ecological traits (Hanson and Hanson, 1996b; Semrau *et al.*, 2010; Knief, 2015), such as different carbon conversion efficiencies of the C1 assimilation pathways (Trotsenko and Murrell, 2008; Kalyuzhnaya *et al.*, 2015), the possession of two particulate methane monooxygenases (pMMO) isozymes within Alpha-MOB (Baani and Liesack, 2008), the ability of Gamma-MOB to oxidize CH₄ in virtually anoxic environments (Blees *et al.*, 2014; Oswald *et al.*, 2016a; Rissanen *et al.*, 2018), and different life strategies (Ho *et al.*, 2013). Yet, the environmental factors which 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 CH₄ under natural conditions. For example, some studies have detected the presence or activity of MOB groups under highly variable CH₄ and O₂ levels (Amaral and Knowles, 1995; Dunfield *et al.*, 1999; Biderre-Petit *et al.*, 2011; Graef *et al.*, 2011; Crevecoeur *et al.*, 2015; Zigah *et al.*, 2015) while others have found a strong temperature effect on MOB composition (Börjesson *et al.*, 2004; Knoblauch *et al.*, 2008; He *et al.*, 2012) or no discernable pattern (Urmann *et al.*, 2009).

Highest methanotrophic activity in lakes is normally encountered at oxic-anoxic interfaces (Lidstrom and Somers, 1984; Kankaala *et al.*, 2006; Schubert *et al.*, 2010; Reim *et al.*,

2012; Morana *et al.*, 2015), but CH₄ oxidation rates are often detected throughout the water column, even in surface waters with low CH₄ and high O₂ concentrations (Kankaala *et al.*, 2006; Zigah *et al.*, 2015; Thottathil *et al.*, 2018). 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 CH₄ oxidation rates throughout the water column. Although studies have reported a link between the taxonomic composition of methanotrophs and CH₄ oxidation in soils (Levine *et al.*, 2011; Bodelier *et al.*, 2013; Nazaries *et al.*, 2013; Judd *et al.*, 2016), no study has evaluated the extent to which vertical changes in the abundances of MOB groups are related to CH₄ 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 CH₄ 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 CH₄ 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.* CH₄ oxidation rates) along the water column of six freshwater temperate lakes which differed in their concentrations of CH₄, O₂, and other environmental variables. More specifically, we investigated the drivers of the abundance of each MOB group and the relationship between MOB groups and CH₄ 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 CH₄ oxidation rates and abundance changes of the two MOB groups. Incubations were performed with unamended lake water (*i.e. in situ* CH₄ and O₂ concentrations) and at three different temperatures to explore the effect of CH₄ and O₂ levels and temperature on MOB composition and activity. Alpha- and Gamma-MOB cells were microscopically identified and quantified using Catalyzed 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 CH₄ oxidation rates along the water column. We further expected that including MOB abundance data would improve CH₄ oxidation models and help increase our understanding of how environmental and biotic factors influence CH₄ dynamics in lakes.

Results

Vertical variations in temperature, O₂, CH₄, and MOB community across lakes

All lakes, except L. Triton, were thermally stratified during sampling with dissolved O₂ concentrations decreasing sharply below the oxycline (Figure 1a-e). Conversely, CH₄ concentrations increased steeply below the oxycline and varied up to 4 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 O₂ and increase in CH₄ concentrations from surface to bottom waters (Figure 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% to 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 (Figure 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, $p=0.004$) (Figure 2a). However, in L. Triton Alpha-MOB cells were more abundant than Gamma-MOB cells in both surface and bottom waters although differences were not statistically significant ($p>0.05$; Figures 1f, 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 (Figure 2a).

[Preferred position for Figures 1 and 2.]

CH₄ oxidation rates and MOB dynamics during incubations

CH₄ oxidation was detected in every sampled lake and depth (Figure 3a). Decreases in CH₄ concentration through time were highly log-linear, a clear indication of first-order kinetics (minimum $R^2=0.92$). CH₄ oxidation rates varied widely from 0.004 to 65.7 $\mu\text{mol CH}_4 \text{ L}^{-1} \text{ d}^{-1}$ across lakes, depths, and incubation temperatures, with the highest CH₄ oxidation rates in each lake happening in deep waters (Figure 3a). In L. 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 (Figure 3b). In addition, the highest CH₄ oxidation rates across lakes were observed in the deep waters of L. Geai and Cromwell where the largest increases in the Gamma:Alpha-MOB ratio were also observed (Figure 3b). In these incubations, Gamma-MOB cell abundance increased by up to 66% in L. Cromwell and up to 300% in L. Geai (Figures S3c, S3d). In contrast, in L. en Coeur and Triton, there was a decrease in the Gamma:Alpha-MOB ratio during incubations, even though CH₄ oxidation rates were comparatively high in these lakes (Figure 3b). In summary, in the well-stratified lakes, highest CH₄ 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 CH₄ 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 (Figure S1-S3). However, in the deep waters of L. Geai, where the highest Gamma-MOB abundance was observed (7×10^5 cells mL⁻¹), temperature and Gamma-MOB abundance were inversely related (Figure S3d). In general, the most abundant MOB group *in situ* maintained its higher abundance relative to the other during incubations independent of the temperature treatment (Figures S1-S3).

[Preferred position for Figure 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 CH₄, O₂, 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 and del Giorgio, 2011). CH₄ (log₁₀ CH₄), temperature, DOC (log₁₀ DOC), and cDOM (log₁₀ cDOM) were used as linear variables, but dissolved O₂ 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 O₂ residuals (details not shown). We found that Alpha-MOB abundance was predicted well by CH₄ (p<0.0001), O₂ (p<0.0001), and cDOM (p=0.006) (R^2_{adj} =0.69, n=82, GAM; Table S2; Figure 4a-c), while temperature (p=0.82) and DOC (p=0.48) were not significant predictors. O₂ showed a *quasi* bell-shaped relationship with Alpha-MOB, with highest cell abundance around 150 µM O₂ and declining on either side of this 'optimum' O₂ but increasing again close to 0 (Figure 4a). CH₄ and cDOM were strongly and positively related to Alpha-MOB abundance (Figure 4b, c). Differently, Gamma-MOB cell abundance was predicted well by O₂ (p<0.0001; Figure 4d) and CH₄ (p<0.0001; Figure 4e) only

($R^2_{\text{adj}}=0.81$, $n=95$, GAM; Table S2), 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 (Figure 4d), while CH_4 had a positive effect on it (Figure 4e).

[Preferred position for Figure 4.]

Relationship between MOB groups and CH_4 oxidation rates

Combining all 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 ($R^2=0.65$, $p<0.0001$; Figure 5b) than that with Alpha-MOB ($R^2=0.33$, $p<0.0001$; Figure 5a). As the MOB community was vertically structured along the water column (Figures 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 (Figures 5c-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 (Figure 5c, d).

[Preferred position for Figure 5.]

Contribution of MOB abundances to the prediction of CH₄ oxidation rate

CH₄ oxidation rates could be strongly predicted by environmental variables, namely CH₄ concentration, temperature, and O₂ concentration ($R^2=0.90$, $p<0.0001$, $n=277$, Generalized Regression, Adaptive Elastic Net with AICc validation; Figure S4). 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 ($R^2=0.92$, $p<0.0001$; Figure 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 CH₄ 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 if Gamma-MOB abundance explained an additional amount of the variance in CH₄ oxidation rates than environmental factors alone, we fitted a linear regression between the residuals of the environmental model (which has temperature, CH₄, and O₂ 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 CH₄ oxidation rates ($R^2=0.23$, $p<0.0001$, $n=94$; Figure 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 CH₄ 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 CH₄ oxidation (Thottathil *et al.*, 2019).

[Preferred position for Figure 6.]

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 O₂ concentration. Changes in the ratio of MOB groups during incubations with deep waters indicated that lower O₂ concentrations in well-stratified lakes favor the growth and activity of Gamma-MOB while higher O₂ concentrations in weakly or non-stratified lakes favor 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 dominate 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 CH₄ oxidation rates and that quantitative microbial data help predict variations in lake methanotrophy.

Niche separation of MOB groups in lakes is mostly driven by O₂

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). In the

stratified lakes, the surface and intermediate waters were dominated by Alpha-MOB, whereas Gamma-MOB were dominant in deep waters (Figure 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 L. Triton, Alpha-MOB were more abundant than Gamma-MOB including in bottom waters. Accordingly, our GAMs results suggest that O₂, rather than CH₄ or temperature, drives the distribution of MOB groups in the water columns, with O₂ showing a non-linear relationship to Alpha-MOB with highest cell abundance around 150 µM (Figure 4a) but a strong negative relationship to Gamma-MOB (Figure 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 O₂ (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; Kits *et al.* 2015b; Skennerton *et al.*, 2015; Oswald *et al.*, 2017), or the ability to perform fermentation (Kalyuzhnaya *et al.*, 2013).

Still, although the GAMs showed that both MOB groups are positively correlated to CH₄ concentrations, the slope of the linear relationship between CH₄ and Alpha-MOB abundance (0.21) is lower than that between CH₄ and Gamma-MOB abundance (0.28; Table S2), suggesting a higher affinity for CH₄ of Alpha-MOB. Also, in L. Triton, where Alpha-MOB were more

abundant than Gamma-MOB including in deeper waters, CH₄ concentrations in the deep were much lower than that in the deep waters of the stratified lakes (Figure 1). The possession of genes encoding a high affinity particulate methane monooxygenase enzyme (pMMO₂) has been shown for *Methylocystis* sp. which allows these members of the Alpha-MOB to oxidize CH₄ even at atmospheric CH₄ levels (Baani and Liesack, 2008). If this is the case for the Alpha-MOB present in our studied lakes, a higher affinity for CH₄ combined to the preference for or tolerance to higher O₂ levels could together explain the higher abundance of Alpha-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 O₂ 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 O₂ 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 O₂ 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 CH₄ oxidation rates with Alpha-MOB than with Gamma-MOB in surface waters incubated in the dark does not support this idea (Figure 5c, d). Alternatively, as cDOM is a proxy of humic substances and thus of terrestrial organic matter (Cuthbert and 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).

Both MOB groups contribute to overall CH₄ oxidation, but Gamma-MOB dominate methanotrophy in lakes

The significant correlations between both MOB groups and CH₄ 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 (Figure 5c, 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 (Figure 3b). However, Gamma-MOB abundances showed a stronger relationship with CH₄ oxidation rates than Alpha-MOB in every layer (Figure 5c, 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 CH₄ even in the surface waters despite their lower cell abundance as they showed stronger relationship to CH₄ oxidation rates than Alpha-MOB including in this layer. Accordingly, the Gamma-MOB abundance *in situ* showed a stronger positive relationship with $\delta^{13}\text{CH}_4$ than Alpha-MOB across lakes surface waters (Figure S5), suggesting that the isotopic enrichment of CH₄ is mostly due to CH₄ oxidation by Gamma-MOB. A predominance of Gamma-MOB activity regardless of CH₄ and O₂ concentrations has been observed in other lakes (Schubert *et al.*, 2010; Biderre-Petit *et al.*, 2011; Oswald *et al.*, 2016b; Crevecoeur *et al.*, 2017; Rissanen *et al.*, 2018). The wide range of O₂ and CH₄ concentrations measured in our incubations (1.14 to 277 μM O₂ and 0.01 to 455 μM

CH₄) also supports a high tolerance to O₂ and CH₄ concentrations within Gamma-MOB, despite optimal conditions at low O₂ and high CH₄ concentrations (Figure 4d, e). Moreover, we found that in the well stratified lakes, highest CH₄ 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 L. en Coeur and Triton where thermal stratification was weak or absent, highest CH₄ 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 favoring 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 O₂.

An overall weaker relationship of Alpha-MOB to CH₄ 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 have 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 (Reim *et al.*, 2012; Ho *et al.*, 2013). 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 CH₄ oxidation rates. The capability of growing on other carbon compounds besides CH₄ 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 and Murrell, 2005).

The addition of Gamma-MOB cell abundance improves models of CH₄ 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 CH₄ oxidation rates, but also that the addition of specific taxon (Gamma-MOB) abundance to environmental predictors increased the explanatory power of CH₄ 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 CH₄ oxidation rate that could not be explained by environmental predictors alone. This suggests that the intrinsic metabolic characteristics of Gamma-MOB explain part of the variation on CH₄ 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 CH₄ oxidation rates and therefore potentially higher lake CH₄ emissions, consistent with the recent finding that trophic cascade effects can modulate CH₄ 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 CH₄ 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 CH₄ intracellularly under anoxic conditions (Ettwig *et al.*, 2010) while our incubations were performed under oxic conditions.

Conclusion

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 CH₄, O₂, 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 O₂. We showed that this separation was related, to a large extent, to the inter- and intra-lake variability in CH₄ oxidation rates, with low O₂ concentrations in deep waters of well-stratified lakes favoring Gamma-MOB activity and growth while Alpha-MOB are mostly favored under higher O₂ 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 CH₄ oxidation rates in lake water columns with a significant and independent effect of Gamma-MOB cell abundance. This direct link between Gamma-MOB and CH₄ oxidation rates indicates that a reduction in Gamma-MOB abundance – for example through grazing – leads to a reduction in CH₄ oxidation rates and potentially to an increase in lake CH₄ emissions. In summary, we showed that microbial composition is related to CH₄ oxidation rates in the water column of lakes and that microbial community information can improve our capacity to understand or predict CH₄ dynamics in lakes.

Experimental procedures

Study lakes and sampling

This study was conducted in six environmentally different lakes in the Laurentians region of Quebec, Canada (Table 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 O₂ 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 CH₄ 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 Inc., CA, USA). Water samples for incubation experiments were collected from either the surface and other two depths (range 3-12 m) in lakes showing three

distinct stratified layers (Morency, Croche, Cromwell, and Geai) or from the surface and bottom (range 3-7.5 m) in the other lakes (en Coeur and Triton). Samples were collected by pumping water into acid-rinsed collapsible bags which were allowed to overflow and closed bubble-free. Sampling depths for the incubation experiments were determined based on O₂ profiles in order to cover a large range of CH₄ and O₂ concentrations. The collapsible bags were kept cold in the dark for 2-4h until further processing in the laboratory.

Nutrients, chlorophyll-a, and organic carbon concentrations

Total phosphorus (TP), total nitrogen (TN), chlorophyll-a (chl-*a*), DOC, and colored 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 and Likens, 2000); TN was measured using a continuous flow analyzer (ALPKEM Flow Solution 3100) using an alkaline persulfate digestion method, coupled with a cadmium reactor (Patton and Kryskalla, 2003); and chl-*a* was analyzed 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 Analyzer using the persulfate oxidation method; and cDOM was determined at 440nm using a spectrophotometric absorbance scan (Ultrospec 3100pro spectrophotometer) (Cuthbert and del Giorgio, 1992).

[Preferred position for Table 1.]

CH₄ oxidation rates

Experimental incubations for the determination of CH₄ oxidation rates were conducted as explained in a parallel study (Thottathil *et al.*, 2019). Briefly, thirty 500 mL-flasks equipped with O₂ 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 CH₄ and stable isotopic signature ($\delta^{13}\text{CH}_4$) were measured 3 to 5 times during each incubation by measuring duplicate flasks and using the headspace technique, gas chromatography, and stable isotope analysis. Due to enzymatic fractionation against ¹³C-CH₄ during CH₄ oxidation, the increase in $\delta^{13}\text{CH}_4$ values in the remaining CH₄ pool during incubations was used to confirm that the reported decreases in CH₄ concentration reflected CH₄ oxidation. Because the incubation followed near perfect 1st order kinetics, instantaneous CH₄ oxidation rates were calculated by differentiation of the 1st order CH₄ depletion curve at each time point. Samples for MOB enumeration were also taken at the end of each incubation series.

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 Catalyzed Reporter Deposition-Fluorescence In Situ

Hybridization (CARD-FISH), using probes for aerobic methanotrophic bacteria belonging to Alpha- (Mα450) and Gammaproteobacteria (My84 and My705) (Table S1) (Eller *et al.*, 2001). 40 mL-samples were fixed with buffered paraformaldehyde (PFA, 1% final concentration) and kept refrigerated at 4 °C before filtration. 2 to 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 DAPI (4',6-diamidino-2-phenylindole) 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 630x 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 end of each incubation experiment to evaluate how they relate to environmental factors, to each other, and to the measured CH₄ oxidation rates.

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 CH₄ oxidation to O₂ [43]. To evaluate the contribution of MOB cell abundance in predicting CH₄ 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 (Sievert, 2018) and ggplot2 3.1.0 (Wickham, 2016).

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Table 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- <i>a</i> (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	0.0084	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.

Figure 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 CH₄ concentration and MOB cell abundance. Bars for MOB abundance indicate mean and whiskers indicate standard deviations.

Figure 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* (a) in stratified lakes (all, except Triton) (Alpha-MOB: N = 35; Gamma-MOB: N = 32) and (b) in L. 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 (as shown in Figure 1) and 'Deep' refers to the deepest layer sampled in each lake. Note log scale in the y axes of (a) and (b). Whole scale bars in (c) and (d) represent 20 µm.

Figure 3. CH₄ oxidation rates and MOB dynamics in incubations. (a) CH₄ oxidation rates and (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) in each studied lake (ordered from deepest to shallowest as shown in the legend). Plotted data include all CH₄ 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 Figure 1).

Figure 4. Different effects of environmental variables on the abundance of MOB groups. Partial effect plots (Generalized Additive Models-GAMs) of significant environmental predictors for 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.

Figure 5. Relationship between MOB groups and CH_4 oxidation rates in the water column of studied lakes. Linear regressions between measured CH_4 oxidation rates (y axes) and MOB cell abundances (x axes) across all lakes (a-b) and distinguishing between water column layers (c-d; circles: surface, triangles: intermediate, and squares: deep). Plotted data includes samples from the six studied lakes. Note log-scale in y axes.

Figure 6. Effect of the addition of Gamma-MOB cell abundance to environmental model predicting CH₄ oxidation rate. (a) Measured by predicted plot of the model of CH₄ 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, CH₄ concentration, and square of O₂ concentration as predictors and the residuals of the environmental model of CH₄ oxidation rates.