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# Variability and controls of stable carbon isotopic fractionation during aerobic methane oxidation in temperate lakes

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The aerobic oxidation of methane (CH<sub>4</sub>) by methanotrophic bacteria (MOB) is the major sink of this highly potent greenhouse gas in freshwater environments. Yet, CH<sub>4</sub> oxidation is one of the largest uncertain components in predicting the current and future CH<sub>4</sub> emissions from these systems. While stable carbon isotopic mass balance is a powerful approach to estimate the extent of CH<sub>4</sub> oxidation *in situ*, its applicability is constrained by the need of a reliable isotopic fractionation factor ( $\alpha_{ox}$ ), which depicts the slower reaction of the heavier stable isotope (<sup>13</sup>C) during CH<sub>4</sub> oxidation. Here we explored the natural variability and the controls of  $\alpha_{ox}$  across the water column of six temperate lakes using experimental incubation of unamended water samples at different temperatures. We found a large variability of  $\alpha_{ox}$  (1.004–1.038) with a systematic increase from the surface to the deep layers of lake water columns. Moreover,  $\alpha_{ox}$  was strongly positively coupled to the abundance of MOB in the  $\gamma$ -proteobacteria class ( $\gamma$ -MOB), which in turn correlated to the concentrations of oxygen and CH<sub>4</sub>, and to the rates of CH<sub>4</sub> oxidation. To enable the applicability in future isotopic mass balance studies, we further developed a general model to predict  $\alpha_{ox}$  using routinely measured limnological variables. By applying this model to  $\delta^{13}C$ -CH<sub>4</sub> profiles obtained from the study lakes, we show that using a constant  $\alpha_{ox}$  value in isotopic mass balances can largely misrepresent and undermine patterns of the extent of CH<sub>4</sub> oxidation in lakes. Our  $\alpha_{ox}$  model thus contributes towards more reliable estimations of stable carbon isotope-based quantification of CH<sub>4</sub> oxidation and may help to elucidate large scale patterns and drivers of the oxidation-driven mitigation of CH<sub>4</sub> emission from lakes.

## KEYWORDS

methane, aerobic oxidation, isotopic mass balance, fractionation factor, lakes

## Introduction

Freshwater aquatic systems are important sources of methane (CH<sub>4</sub>)—a highly potent greenhouse gas—to the atmosphere (Bastviken et al., 2011; Saunois et al., 2016). However, large uncertainties exist in the estimates of global inland water CH<sub>4</sub> emissions, partly due to the lack of a clear understanding on the various processes driving CH<sub>4</sub> saturation and emission in aquatic environments. Aerobic methane oxidation (MOX), for example, plays a central role in regulating the global CH<sub>4</sub> budget by biologically mitigating CH<sub>4</sub> emissions from various natural (e.g., wetlands, rivers, lakes, and ocean) and anthropogenic (e.g., rice paddy fields, landfills) sources (King, 1992; Guérin and Abril, 2007; Reeburgh, 2007; Bastviken et al., 2008; Chanton et al., 2009; Chanton et al., 2011; Serrano-Silva et al., 2014; Cai et al., 2016; Sawakuchi et al., 2016; Li et al., 2022). Despite its pivotal role in controlling CH<sub>4</sub> emissions, accurate quantification of MOX is constrained methodologically and often represents one of the largest uncertain components in the budgets of various CH<sub>4</sub> sources. While experimental incubations of isolated samples (soil, sediment, or water) and tracking the decrease in CH<sub>4</sub> concentration over time is the most often used method, *in situ* estimations based on the stable isotopic composition of CH<sub>4</sub> offers a non-invasive whole-ecosystem technique to estimate the extent of CH<sub>4</sub> oxidation (Happell et al., 1994; Tyler et al., 1997; Liptay et al., 1998; Bastviken et al., 2002; Chanton et al., 2008; Chanton et al., 2011; Zhang et al., 2012; Preuss et al., 2013; Sawakuchi et al., 2016; Gebert and Streese-Kleeberg, 2017; Sparrow et al., 2019; Komiya et al., 2020; Bakkaloglu et al., 2021).

The stable carbon isotopic signature of CH<sub>4</sub> ( $\delta^{13}\text{C-CH}_4$ ) is extensively used for isotopic mass balance-based oxidation estimations. This approach relies on the behaviour of the two stable carbon isotopes (<sup>12</sup>C and <sup>13</sup>C) in terms of mass, steric properties, and diffusion rates leading to unequal behaviour in the rates of transport and at the enzyme bonding sites of CH<sub>4</sub>-oxidizing bacteria (MOB or methanotrophic bacteria). As a result of the slightly faster diffusion and oxidation of <sup>12</sup>C, the residual CH<sub>4</sub> pool gets enriched in <sup>13</sup>C (Barker and Fritz, 1981; Jahnke et al., 1999; Whiticar, 1999; De Visscher et al., 2004; Templeton et al., 2006; Gebert and Streese-Kleeberg, 2017). From the shift in the  $\delta^{13}\text{C}$  signature of CH<sub>4</sub> at the point of sampling ( $\delta^{13}\text{C-CH}_4\text{-}_{\text{sample}}$ ) relative to the anoxic source ( $\delta^{13}\text{C-CH}_4\text{-}_{\text{source}}$ ), the fraction of CH<sub>4</sub> oxidized ( $f_{\text{ox}}$ ) can be estimated using either open system models at steady state (Happell et al., 1994; Tyler et al., 1997) or non-steady state Rayleigh model for closed systems (Liptay et al., 1998).

Due to the slightly faster uptake of <sup>12</sup>C relative to <sup>13</sup>C by MOB, the first order rate constant ( $k$ ) of the oxidation of <sup>12</sup>CH<sub>4</sub> is greater than that of <sup>13</sup>CH<sub>4</sub>; therefore, the ratio of <sup>12</sup> $k$ /<sup>13</sup> $k$  represents the isotopic fractionation factor ( $\alpha_{\text{ox}}$ ). In practice, the  $\alpha_{\text{ox}}$  is determined from the incubation of samples at a desired

temperature, with or without CH<sub>4</sub> amendments (Chanton and Liptay, 2000; Börjesson et al., 2001; Bastviken et al., 2002; Börjesson et al., 2007; Chanton et al., 2008; Aghdam et al., 2018; Obersky et al., 2018; Sparrow et al., 2019; Fjølsted et al., 2020; but see Gebert and Streese-Kleeberg, 2017). From the concomitant change in the concentration ( $[\text{CH}_4]$ ) and stable isotopic signature ( $\delta^{13}\text{C-CH}_4$ ) of CH<sub>4</sub> during the incubation, the fractionation factor  $\alpha_{\text{ox}}$  can be derived from the slope ( $\beta$ ) of the regression between  $\ln [\text{CH}_4]$  and  $\ln (\delta^{13}\text{C-CH}_4 + 1000)$  (approximate Rayleigh approach, Mahieu et al., 2006) as:

$$\alpha_{\text{ox}} = \frac{\beta}{1 + \beta} \quad (1)$$

While the isotopic method is a simple and powerful tool to estimate CH<sub>4</sub> oxidation *in situ*, an accurate  $\alpha_{\text{ox}}$  value associated with oxidation in the given ecosystem is important in ensuring reliable estimations of the amount of CH<sub>4</sub> consumed by methanotrophy. The use of inaccurate  $\alpha_{\text{ox}}$  can induce large differences in the  $f_{\text{ox}}$  estimate (see, Cabral et al., 2010; Capanema and Cabral, 2012; Gebert and Streese-Kleeberg, 2017). It is thus critical to use suitable fractionation factors that are appropriate for each environment or for each place and time within a system.

Even though  $\alpha_{\text{ox}}$  is often used as a constant, experimentally determined stable carbon isotopic fractionation factors for methanotrophic bacterial cultures and various terrestrial and aquatic environments together vary widely (1.003–1.049; Coleman et al., 1981; Bergamaschi et al., 1998; Bastviken et al., 2002; Venkiteswaran and Schiff, 2005; Chanton et al., 2008; Templeton et al., 2006; Gebert and Streese-Kleeberg, 2017; Aghdam et al., 2018; Obersky et al., 2018; Sparrow et al., 2019; Fjølsted et al., 2020; Bakkaloglu et al., 2021). This large variability of  $\alpha_{\text{ox}}$  across environments can be attributed to diverse mechanisms; from the supply of CH<sub>4</sub> to the oxidizing zone to the rate of enzyme-catalysed oxidation of CH<sub>4</sub> by MOB. In an environment with abundant CH<sub>4</sub> supply, the irreversible conversion of dissolved CH<sub>4</sub> to the cellular carbon pool of MOB controls the isotopic fractionation—a process dependent on the diffusion and dissolution of CH<sub>4</sub> in the system, enzyme activity of the MOB, and the rate of CH<sub>4</sub> oxidation (Templeton et al., 2006). Since the above factors can be regulated by a variety of environmental conditions such as temperature, production and diffusion of CH<sub>4</sub> to oxidation zone, and MOB cell abundance, strong links between these variables and  $\alpha_{\text{ox}}$  are expected. For example, an inverse relationship between temperature and  $\alpha_{\text{ox}}$  is often observed for the forests and landfill soil environments (Tyler et al., 1994; Jahnke et al., 1999; Chanton and Liptay, 2000; Chanton et al., 2008; Nihous et al., 2010; Gebert and Streese-Kleeberg, 2017). The underlying mechanism behind such a pattern is suggested to be the increased oxidation rate at elevated temperature resulting in little fractionation, while low MOX rate is associated with greater discrimination of <sup>13</sup>C. However, it should be also noted that an

increase of  $\alpha_{\text{ox}}$  with temperature was observed in enriched methanotrophic bacterial cultures (Coleman et al., 1981), which was supported by field estimates in the Alaskan soils (King et al., 1989) and European landfills (Bergamaschi et al., 1998). Furthermore, temperature response of  $\alpha_{\text{ox}}$  is highly inconsistent in aquatic environments; from a complete absence of temperature effect in large lakes and reservoirs (Bastviken et al., 2002; Venkiteswaran and Schiff, 2005) to an inverse relationship between temperature for a small temperate lake (Venkiteswaran and Schiff, 2005). These inconsistent temperature responses in various environments suggest that there may be factors other than MOX rates at play to regulate  $\alpha_{\text{ox}}$  variability. Experimental studies in terrestrial systems and/or methanotrophic bacterial cultures have shown that in addition to MOX rates,  $\alpha_{\text{ox}}$  is dependent on the availability of  $\text{CH}_4$  and oxygen ( $\text{O}_2$ ), and the abundance of MOB (Börjesson et al., 2001; Templeton et al., 2006; Chanton et al., 2008; Gebert and Streese-Kleeberg, 2017).

Despite the long-recognised potential of isotopic mass balance in estimating  $\text{CH}_4$  oxidation in lakes (Bastviken et al., 2002), this approach is not extensively used in freshwater systems compared to terrestrial environments such as forest soils, landfills, and rice paddy fields. The number of  $\delta^{13}\text{C}\text{-CH}_4$  measurements has been increasing recently to derive greater insights on aquatic  $\text{CH}_4$  dynamics (Schubert et al., 2010; Brees et al., 2014; Rinta et al., 2015; Morana et al., 2015; Cadieux et al., 2016; Begum et al., 2021). Yet, only very few studies have derived the extent of  $\text{CH}_4$  oxidation based on isotopic data (Kankaala et al., 2007; Itoh et al., 2015; Thottathil et al., 2018; Grasset et al., 2020; Soued and Prairie, 2022) and studies that experimentally determined  $\alpha_{\text{ox}}$  are even lesser (Bastviken et al., 2002; Venkiteswaran and Schiff, 2005; Itoh et al., 2015). Nevertheless, these studies showed variable  $\alpha_{\text{ox}}$  values for lakes and reservoirs; between 1.018 and 1.021 for three Swedish boreal lakes (Bastviken et al., 2002), between 1.019 and 1.021 in a small temperate pond, 1.013–1.014 in temperate reservoirs (Venkiteswaran and Schiff, 2005), and 1.010 in a subtropical reservoir (Itoh et al., 2017). Since the number of  $\alpha_{\text{ox}}$  estimates are limited in lakes, knowledge of the natural variability and environmental control of  $\alpha_{\text{ox}}$  in these ecosystems is critically lacking. Methanotrophy in lakes is controlled by a suite of environmental factors including temperature, pH, total phosphorus (TP), total nitrogen (TN), and dissolved organic carbon (DOC) through regulating MOB population, supply of substrates ( $\text{CH}_4$  and  $\text{O}_2$ ), and by affecting water column light attenuation (Crevecoeur et al., 2019; Thottathil et al., 2019; Guggenheim et al., 2020; Reis et al., 2020; Sawakuchi et al., 2021; Kashi et al., 2022; Nijman et al., 2022). As response to these multiple environmental factors, MOX rates in freshwater lakes vary widely (Bastviken et al., 2002; Bastviken et al., 2008; Thottathil et al., 2019; Denfeld et al., 2016; Sawakuchi et al., 2021; D'Ambrosio and Harrison, 2021). However, whether  $\alpha_{\text{ox}}$  values equally vary along with the MOX

rates and/or how multiple environmental factors simultaneously shape the isotopic fractionation during  $\text{CH}_4$  oxidation in highly dynamic environments such as freshwater lakes remain elusive.

Here, we explicitly examine the variability and controls of stable carbon isotopic fractionation during aerobic  $\text{CH}_4$  oxidation in temperate lakes. Given the wide variability of MOX rate, we hypothesize that, 1) the inter- and intra-lake variability of  $\alpha_{\text{ox}}$  can be large in the stratified water column of lakes, and 2) the environmental variables relevant in shaping the rate of  $\text{CH}_4$  oxidation and MOB abundance (e.g., temperature, pH, and concentrations of  $\text{CH}_4$ ,  $\text{O}_2$ , nutrients, and DOC), are also relevant in regulating isotopic fractionation factor. To test these hypotheses, we conducted extensive incubation experiments with unamended lake water samples obtained from the stratified water column of six Canadian Shield lakes in southeastern Quebec during the summer stratification period. Using this unique dataset, we show that  $\alpha_{\text{ox}}$  variability in freshwater lakes is large and comparable to the variability observed in terrestrial environments and is modulated by the abundance of a specific MOB group as well as by the rates of  $\text{CH}_4$  oxidation, which are in turn regulated by a suite of environmental factors. Furthermore, we developed an empirical model using routinely measured limnological variables that can be used to estimate  $\alpha_{\text{ox}}$  in a wide range of freshwater environments. Finally, we show that  $\text{CH}_4$  oxidation extent in lakes can be significantly misrepresented at both seasonal and spatial scales if the variability of  $\alpha_{\text{ox}}$  is poorly constrained.

## Materials and methods

### Study area and sampling strategy

We sampled six northern temperate lakes of variable morphometry (surface area: 0.008–0.44  $\text{km}^2$ ; maximum depth: 4.3–20.3 m) during the summer stratification period (June to October) of 2016. Details on the limnological features of study lakes, sampling strategy, and experimental setups were given elsewhere (Thottathil et al., 2019; Reis et al., 2020). In each study lake, a single station was sampled onboard a small aluminium boat anchored at the deepest point located based on the known bathymetry (<https://crelaurentides.org/dossiers/eau-lacs/atlasdeslacs>). We performed the water column profiling at 1 m interval for temperature, pH, and  $\text{O}_2$  using a multiparameter probe (Yellow Spring Instruments, YSI; OH, United States). Along with the YSI-profiling, the water samples were collected for dissolved  $\text{CH}_4$  measurements from discrete depths at 1 m intervals using a continuous flow submersible pump (Proactive Environmental Products, FL, United States) attached to the YSI. At each discrete depth, water was allowed to pump for more than 5 minutes before initiating the sampling for dissolved  $\text{CH}_4$  using headspace equilibration method and the YSI was monitored continuously to ensure that pump was placed at the desired depth throughout the

sampling. For the headspace equilibration, a 60 ml syringe was connected to the pump outlet through a two-way luer-lock valve in such a way that water is directly transferred to the syringe without getting exposed to the air and subsequent degassing. After filling two 60 ml syringes, 30 ml water was removed and the equivalent volume of ultrapure zero air (Praxair Canada Inc., Canada) was transferred to the syringe from a small pressurized zero air tank through a needle (23 G1, Becton–Dickinson) injected through a rubber septum of the zero-air tank outlet. The headspace was equilibrated manually for 2 min by vigorous shaking and the equilibrated headspace gas was then transferred into 12-ml pre-evacuated exetainer vials (Labco Ltd., United Kingdom) using a needle. The water sample for the incubation experiment (~40 L) was collected in flexible cubitainers (VWR International, Canada) from two to three depths of the oxygenated layer of the water column of each lake using a submersible pump. The pump outlet was placed at the bottom of the cubitainers and filled slowly without bubbling and allowed to overflow for several minutes to replace the entire volume of the cubitainers and closed without trapping air bubbles. Except for the surface samples, the sampling depths for the incubation experiments were chosen differently in each lake due to large difference in total depth and the extent of stratification. Our sampling depths represented the surface mixed layer (surface), metalimnion (intermediate), and the deepest oxic waters in the hypolimnion or above the sediment (deep). This approach allowed us to cover a range of environmental conditions in terms of O<sub>2</sub> and CH<sub>4</sub> concentrations, as well as in terms of general water column chemistry (pH, TP, TN, and DOC) which are important drivers of the MOB community and CH<sub>4</sub> oxidation (Sawakuchi et al., 2016; Crevecoeur et al., 2019; Thottathil et al., 2019; Guggenheim et al., 2020; Reis et al., 2020; Sawakuchi et al., 2021; Kashi et al., 2022; Nijman et al., 2022).

From the depths chosen for incubation experiments, samples for TN and TP were collected in duplicates directly into acid washed (10% Hydrochloric acid) and oven-dried (at 200°C) 50 ml screwcap tubes. For DOC, water samples were first collected into 60 ml plastic syringes directly from the submersible pump outlet and subsequently filtered through a 0.45 µm syringe filters (Sarstedt AG & Co., Germany) into acid-washed 40 ml glass vials. The vials were sealed with plastic caps lined with Teflon and rubber septa. All samples were kept cold in the dark until analyses. Except for L. Triton and L. en Coeur, where the water column was weakly stratified and therefore only two depths (surface and deep) were sampled, in all other lakes three depths (surface, intermediate, and deep) were sampled for the experiment. Thus, a total of 16 samples were collected from six lakes for the experimental incubations.

## Experimental setup

In the laboratory, water from the cubitainers were dispersed into thirty 500 ml glass flasks, allowed to overflow and plugged air-tight using silicon stoppers without trapping air bubbles. A

set of ten flasks were placed in temperature-controlled incubators set at three different temperatures: surface samples were at 10, 20, and 25°C; intermediate and deep water samples were incubated at 5, 10, and 20°C. All experiments were performed using unamended lake water samples since the addition of CH<sub>4</sub> and/or O<sub>2</sub> may yield higher than actual fractionation effect (Templeton et al., 2006). The first time point sampling for concentration and the stable carbon isotopic signature of CH<sub>4</sub> (δ<sup>13</sup>C-CH<sub>4</sub>) was collected after about 3 h of acclimatization period in the incubators and a total of three to five time point samplings were conducted for each set of incubation. In order to examine presence of O<sub>2</sub> in the incubation flasks for the aerobic oxidation, the concentration of O<sub>2</sub> was monitored in two out of ten incubation flasks using optical O<sub>2</sub> sensors spots and a fiber optic meter (PreSens GmbH, Regensburg, Germany). At each time point, two flasks were removed from the incubation system and two 30 ml sub-samples were withdrawn from each flask using 60 ml plastic syringes attached with a long needle to ensure that water was withdrawn from the bottom of the flask to avoid the contact of water with the air. The headspace (30 ml) was created by adding zero air into the syringes from a pressurized tank using a needle attached the luer-lock valve of the syringe and piercing through the rubber septa at the outlet of the regulated pressure valve. The headspace was equilibrated for 2 minutes by vigorous shaking and the equilibrated headspace was transferred into 12 ml pre-evacuated exetainer vials.

## Measurement of CH<sub>4</sub> concentration, δ<sup>13</sup>C-CH<sub>4</sub> values, methanotrophic bacteria abundance, and environmental variables

The partial pressure and δ<sup>13</sup>C signature of CH<sub>4</sub> in the headspace-equilibrated samples were measured using a Cavity Ring Down Spectrometer (CRDS) equipped with Small Sample Isotopic Module (SSIM, Picarro G2201-*i*, Picarro Inc., CA, United States). For high concentration samples (i.e., headspace concentration > 200 ppm), CH<sub>4</sub> concentration was first measured using gas chromatograph (GC) with a flame ionization detector (GC-2014, Shimadzu, Kyoto, Japan) then the isotopic measurements were performed after dilution using zero air *via* SSIM. The original *p*CH<sub>4</sub> in the water was calculated using the headspace ratio, incubation temperature, and temperature of the water in the syringe during equilibration using appropriate solubility constants (Yamamoto et al., 1976). The standard curve for δ<sup>13</sup>C-CH<sub>4</sub> signature was prepared using certified isotopic standards obtained from Isometric Instruments, Canada (Liso1: -66.5 ± 0.2‰, Tiso1: -38.3 ± 0.2‰, and Hiso1: -23.9 ± 0.2‰) and values are reported in delta (δ) notation expressed in ‰ relative to the standard Vienna Pee Dee Belemnite.

To estimate the abundance of MOB cells, 40 ml water samples collected at the start of the incubation were fixed

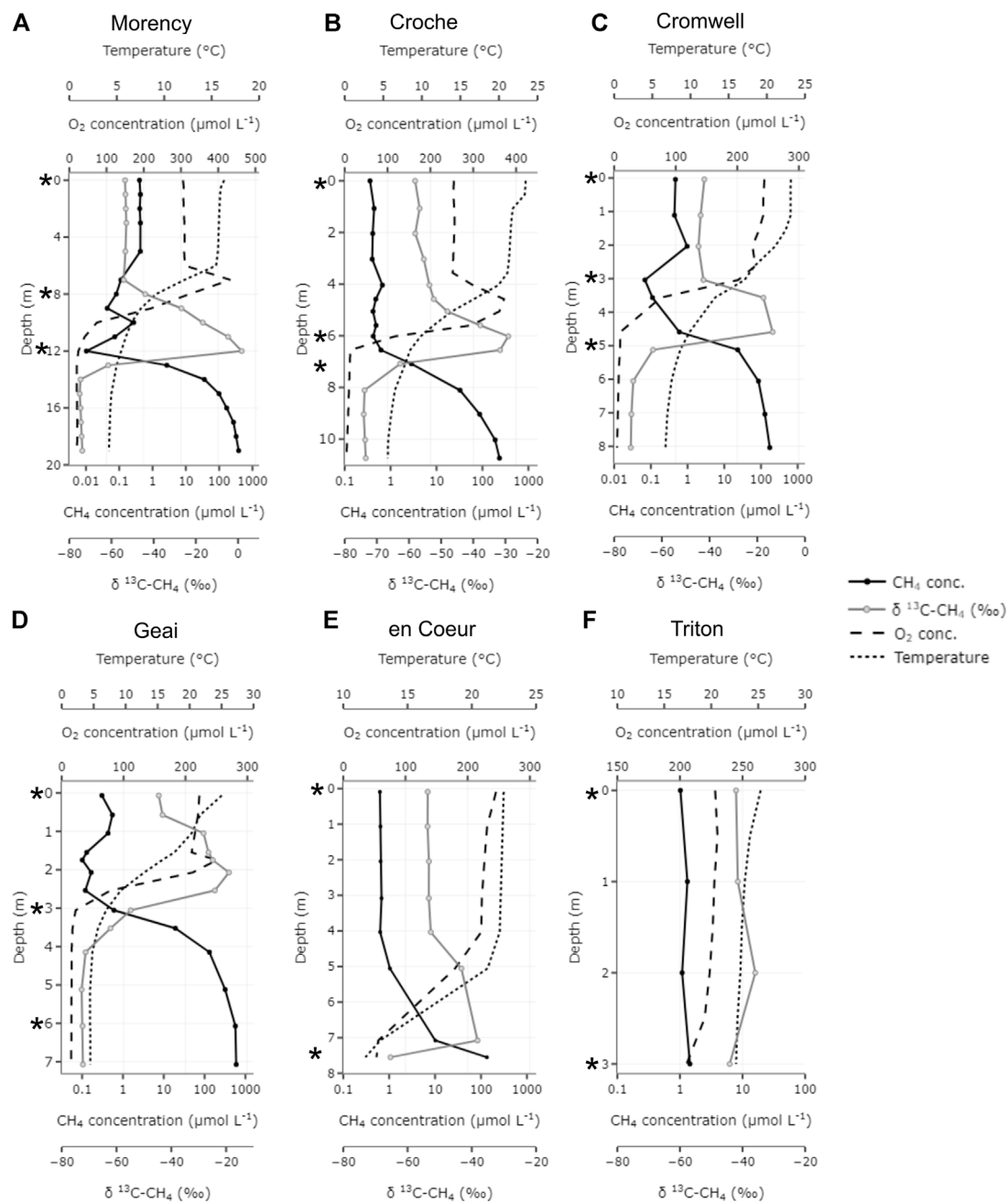


FIGURE 1

Vertical distribution of temperature, dissolved oxygen, methane concentration and stable carbon isotopic signature (<sup>13</sup>C-CH<sub>4</sub>) in each study lake (A–F). Symbol (\*) in the Y-axis panel indicate the sampling depths for experimental incubations.

with buffered paraformaldehyde and filtered through 0.2 μm polycarbonate filters (Millipore GTTP, 25 mm). The filters were kept frozen until microscopic analysis through Catalysed Reporter Deposition—Fluorescence *In Situ* Hybridization (CARD-FISH) with probes for aerobic MOB belonging to α- and γ-proteobacteria and epifluorescence microscopy (see Reis et al., 2020 for details). We used extensively applied CARD-FISH

probes (Malpha450, Mgamma84, and Mgamma705) to detect MOB cells and the effectiveness of these probes for detecting α-MOB and γ-MOB cells were described previously (see Figure 1; and Supplementary Material in Reis et al., 2022).

The concentration of TP was measured after potassium persulfate digestion of unfiltered samples following ammonium molybdate colorimetric method (Wetzel and

Likens, 2000). For the measurement of TN, unfiltered samples were first digested using alkaline persulfate and analysed using a continuous flow analyser (ALPKEM Flow Solution 3100) equipped with a cadmium reactor (Patton and Kryskalla, 2003). The concentration of DOC was measured after sodium persulfate digestion of filtered water samples using a Total Organic Carbon analyzer (OI 1010, OI Analytical, TX, United States).

## Calculations and statistical analyses

To estimate the kinetic isotopic fractionation during CH<sub>4</sub> oxidation, first we calculated the slope of the regression between  $\ln[\text{CH}_4]$  and  $\ln(\delta^{13}\text{C-CH}_4 + 1000)$  for each incubation and then the fractionation factor ( $\alpha_{\text{ox}}$ ) was derived from the slope following Eq. 1 (see Introduction). This estimation is based on the “Simplified Rayleigh approach” recommended for incubation experiments using unamended and unlabelled CH<sub>4</sub> (Mahieu et al., 2006). In this approach, the range of CH<sub>4</sub> concentration has been shown to have a limited influence on the error of  $\alpha_{\text{ox}}$ . Since our experiments are conducted over a large range of CH<sub>4</sub> concentrations, error associated with CH<sub>4</sub> concentrations will be minimal with the Simplified Rayleigh approach and therefore can be compared across the experiments. Moreover, this approximation of  $\alpha_{\text{ox}}$  was shown to be superior to the Coleman method (Coleman et al., 1981) since its difference to the original Rayleigh method is relatively smaller (<0.05%) than that of the Coleman method (up to 5%).

Further, we explored the environmental control of  $\alpha_{\text{ox}}$  using multiple regression analysis with Least Square Regression method. We used the incubation temperature, pH, concentrations of DOC, O<sub>2</sub>, CH<sub>4</sub>, TN, TP, cell abundance of  $\alpha$ -MOB and  $\gamma$ -MOB, and MOX rates as candidate independent variables and only statistically significant variables were selected in the final model. The final environmental model was applied to the data collected in 2014 and 2015 in the same study lakes to explore the implications of our model on the seasonal and vertical variability of  $\alpha_{\text{ox}}$  and the fraction of CH<sub>4</sub> oxidized ( $f_{\text{ox}}$ ). First, we estimated the seasonality in  $f_{\text{ox}}$  in the surface layers of the study lakes using closed system model (Eq. 2).

$$\ln(1 - f_{\text{ox}}) = \frac{\ln(\delta^{13}\text{C-CH}_{4\text{source}} + 1000) - \ln(\delta^{13}\text{C-CH}_{4\text{sample}} + 1000)}{\alpha_{\text{ox}} - 1} \quad (2)$$

where  $\delta^{13}\text{C-CH}_{4\text{sample}}$  is the seasonal  $\delta^{13}\text{C-CH}_4$  data obtained from our study lakes during May–November 2015 and  $\delta^{13}\text{C-CH}_{4\text{source}}$  is a fixed value for each lake. For the comparison, we used modelled  $\alpha_{\text{ox}}$  along with a fixed value of 1.020 which is the average  $\alpha_{\text{ox}}$  estimated in the previous studies for the northern lakes (Bastviken et al., 2002; Venkiteswaran and Schiff, 2005). Secondly, we estimated the vertical variability of  $\alpha_{\text{ox}}$

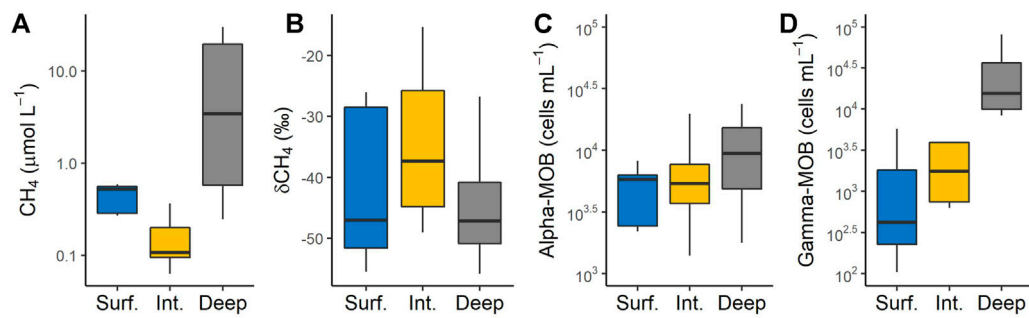
using data obtained from three of our study lakes during the summer 2014.

We used Analysis of Variance (ANOVA) and Tukey’s HSD (Honest Significant Difference) to explore the variations in the water chemistry, CH<sub>4</sub> concentrations, stable isotopic signature, and  $\alpha_{\text{ox}}$  across depth zones. All the statistical analyses were performed with JMP® Pro software version 14.0 (SAS Institute, United States) and graphs were plotted using ggplot2 (v3.3.3), cowplot (v1.1.1) and plotly (v4.10.0) packages (Wickham, 2016; Wilke et al., 2019; Sievert, 2020) in R software (v4.0.4) or using Grapher™ (Golden Software LLC, United States).

## Results

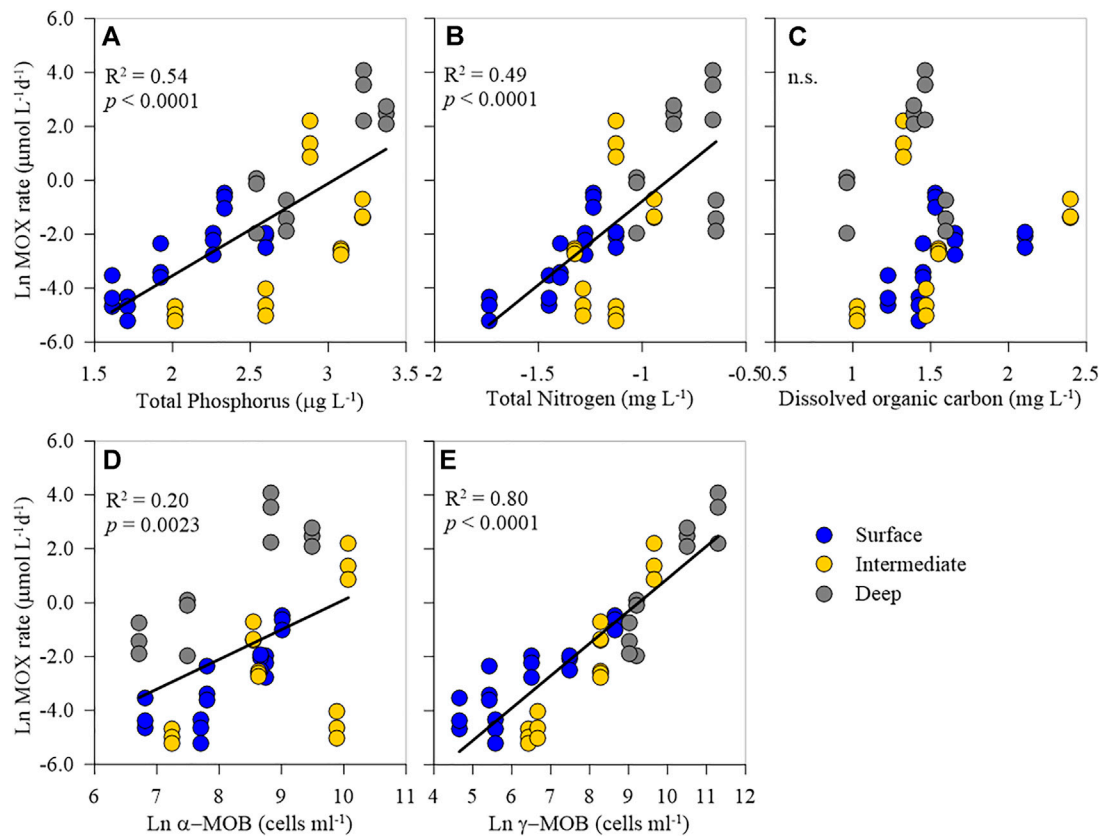
### Environmental conditions, concentration and $\delta^{13}\text{C}$ signature of CH<sub>4</sub>, and methanotrophic bacteria abundance

All sampling lakes, but L. Triton, were thermally stratified and the vertical distribution of CH<sub>4</sub> concentration and  $\delta^{13}\text{C-CH}_4$  values were mirror images of each other with lowest CH<sub>4</sub> concentration and most enriched  $\delta^{13}\text{C-CH}_4$  values coinciding in the metalimnion (Figure 1). Since we sampled the thermally stratified water column for the experiment, various chemical variables (pH, TN, TP, and DOC) at the beginning of the incubations varied largely across layers (Supplementary Figure S1). Similarly, the initial CH<sub>4</sub> concentration in the incubations varied widely with significant difference across depth zones; surface and intermediate layers showed a narrower range (0.27–0.59 and 0.06–0.36  $\mu\text{mol L}^{-1}$ , respectively) than the deep layer (0.25–30.02  $\mu\text{mol L}^{-1}$ ; Figure 2A). While initial  $\delta^{13}\text{C-CH}_4$  values showed a narrower range in the surface (–55.48‰ to –26.07‰) and deep layer (–55.79‰ to –26.76‰), the intermediate layer exhibited a much larger variability (–49.04‰ to –15.32‰) (Figure 2B). The abundance of  $\alpha$ -MOB and  $\gamma$ -MOB at the start of each incubation also varied widely; the abundance of  $\gamma$ -MOB ranged from  $1.05 \times 10^2$  to  $8.11 \times 10^4$  cells  $\text{ml}^{-1}$  (average:  $1.13 \times 10^4$  cells  $\text{ml}^{-1}$ ) and  $\alpha$ -MOB abundance ranged from  $8.27 \times 10^2$  to  $2.37 \times 10^4$  cells  $\text{ml}^{-1}$  (average:  $6.93 \times 10^3$  cells  $\text{ml}^{-1}$ ; Figures 2C,D). While the abundance of  $\alpha$ -MOB was unrelated to the concentration of O<sub>2</sub> and only slightly related to the CH<sub>4</sub> concentrations, the abundance of  $\gamma$ -MOB increased with decreasing O<sub>2</sub> concentrations and positively correlated with CH<sub>4</sub> concentrations (Supplementary Figure S2). Furthermore,  $\gamma$ -MOB abundance was strongly positively and linearly correlated with TP ( $R^2 = 0.74$ ,  $p < 0.0001$ ) and TN ( $R^2 = 0.64$ ,  $p < 0.0001$ ), but unrelated to DOC (Supplementary Figure S3). On the other hand, abundance of  $\alpha$ -MOB was not significantly linearly related to the above variables, except a weak positive



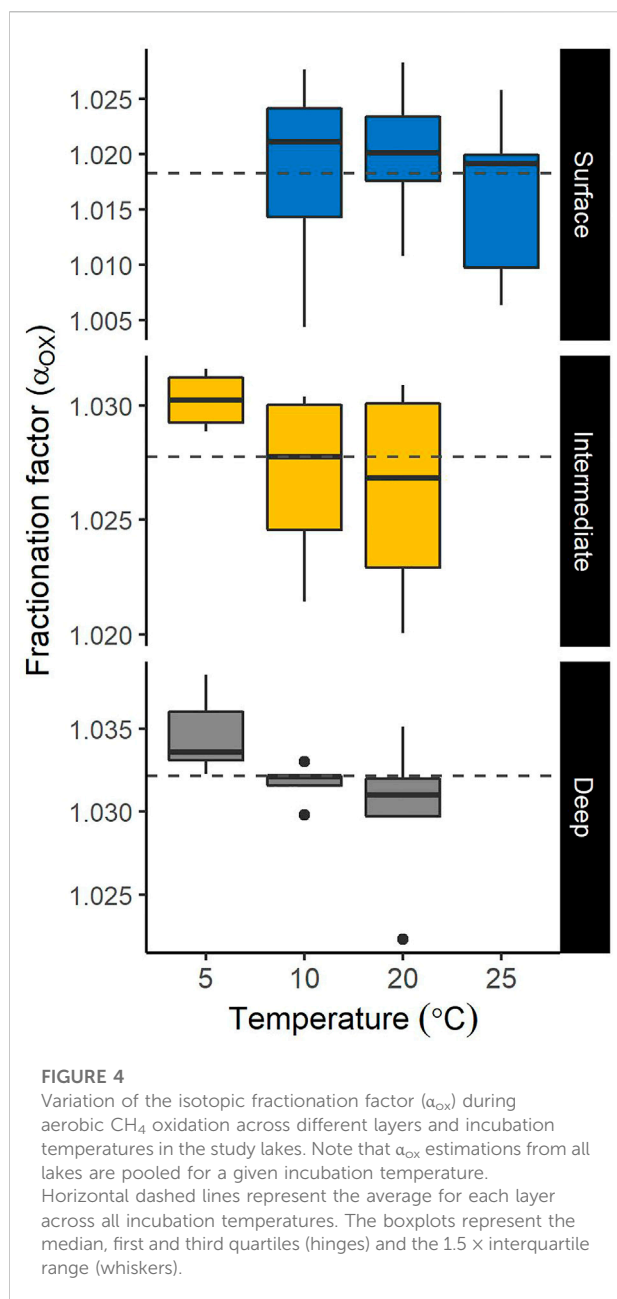
**FIGURE 2**

Variability of methane (CH<sub>4</sub>) concentration (A), stable carbon isotopic signature of CH<sub>4</sub> (δ<sup>13</sup>C-CH<sub>4</sub>) (B), abundance of α (C) and γ (D) groups of methanotrophic bacteria (α-MOB and γ-MOB, respectively) at the start of the incubation experiments across all study lakes. Surf., Surface layer; Inter., Intermediate layer; Deep, Deeper layer. The boxplots represent the median, first and third quartiles (hinges) and the 1.5 × interquartile range (whiskers).



**FIGURE 3**

Relationships between methane oxidation rate (MOX) and total phosphorus (A), total nitrogen (B), dissolved organic carbon (C), and abundance of α-MOB (D) and γ-MOB (E).



correlation with TP ( $R^2 = 0.31$ ,  $p < 0.0001$ ; [Supplementary Figure S3](#)).

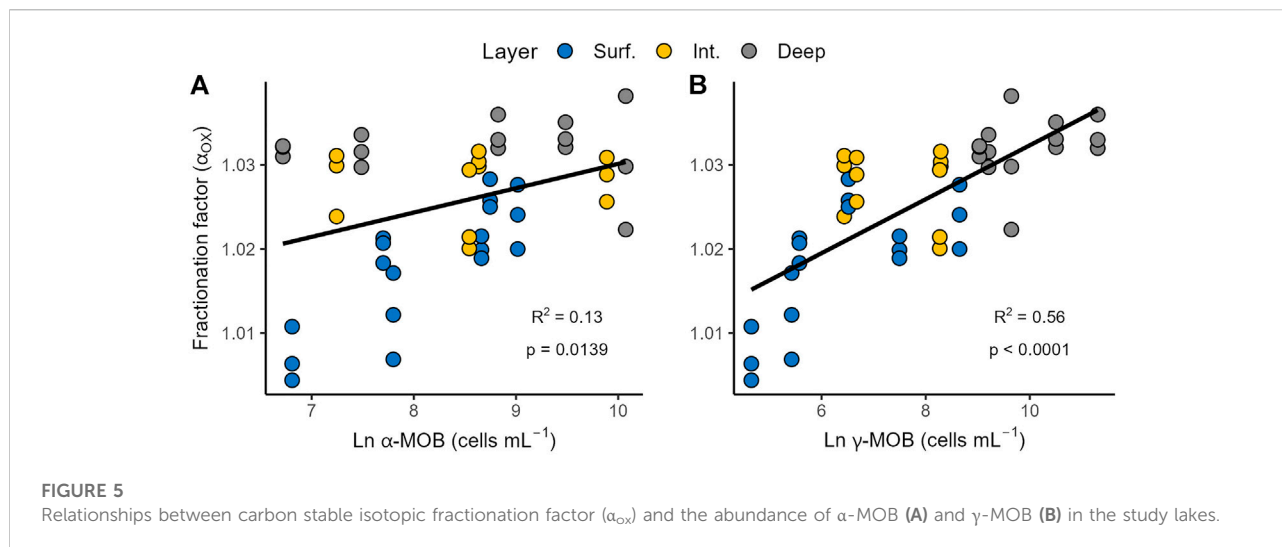
## Methane oxidation rate and patterns of isotopic fractionation factor

A total of 48 incubation experiments encompassing samples from six different lakes, two-three depths from each system, and three different incubation temperatures were conducted. The MOX rates across our study lakes, depth layers, and incubation

temperature ranged widely from 0.01 to  $59.71 \mu\text{mol L}^{-1} \text{d}^{-1}$  with consistently high rates in the deep layer ( $16.27 \pm 20.33 \mu\text{mol L}^{-1} \text{d}^{-1}$ ) than in the surface ( $0.13 \pm 0.18 \mu\text{mol L}^{-1} \text{d}^{-1}$ ) and intermediate layer ( $0.11 \pm 0.15 \mu\text{mol L}^{-1} \text{d}^{-1}$ ). The MOX rate was highly positively correlated with the concentrations of TP ( $R^2 = 0.64$ ;  $p < 0.0001$ ) and TN ( $R^2 = 0.55$ ;  $p < 0.0001$ ) while unrelated to DOC ([Figures 3A–C](#)). Furthermore, MOX rate was strongly positively correlated with  $\gamma$ -MOB abundance ( $R^2 = 0.84$ ;  $p < 0.0001$ ; [Figure 3E](#)), while weakly correlated with the abundance of  $\alpha$ -MOB ( $R^2 = 0.33$ ;  $p < 0.0001$ ; [Figure 3D](#)).

For the water samples from the deeper layers of L. Geai, the initial  $O_2$  concentrations were quite low ( $<26 \mu\text{mol O}_2 \text{L}^{-1}$ ). Given the measured MOX rate ( $55.8$ ,  $47.7$ , and  $31.3 \mu\text{mol L}^{-1} \text{d}^{-1}$  at  $20$ ,  $10$ , and  $5^\circ\text{C}$  respectively) and assuming a  $CH_4:O_2$  stoichiometry of 1:1.8 for aerobic  $CH_4$  oxidation ([Naguib 1976](#)), incubations would have run out of  $O_2$  before the second time point sampling within 12 h. Therefore, those results were excluded to limit our analysis to the controls of isotopic fractionation factor during strict aerobic oxidation. Hence, a total of 45  $\alpha_{ox}$  estimations representing the water column of six temperate lakes were used for further statistical analysis to understand the controls of  $\alpha_{ox}$ . During the incubation, declines in  $CH_4$  concentration were generally mirrored by the  $\delta^{13}\text{C}-CH_4$  value and the isotopic fractionation factor ( $\alpha_{ox}$ ) was estimated for each depth and incubation temperature ([Supplementary Figure S4](#)). Across the water column of the six study lakes, two—three depths, and different incubation temperatures ( $5$ – $25^\circ\text{C}$ ),  $\alpha_{ox}$  varied from 1.004 to 1.038 ([Figure 4](#); [Supplementary Table S1](#)). Notably, this large variability of  $\alpha_{ox}$  observed in our experiments is not only introduced by the difference in incubation temperature, but the sampling depth also mattered substantially. For instance,  $\alpha_{ox}$  varied from 1.011 to 1.028 for the surface samples of all lakes incubated at  $20^\circ\text{C}$ , while for the intermediate and deep layer samples at the same temperature,  $\alpha_{ox}$  values varied from 1.020 to 1.031 and 1.030 to 1.032, respectively ([Supplementary Table S1](#)). Moreover, combining our estimates at different temperatures by layers showed a remarkably low  $\alpha_{ox}$  in the surface ( $1.018 \pm 0.007$ ) relative to the intermediate ( $1.028 \pm 0.004$ ) and the deep ( $1.032 \pm 0.004$ ) layers ([Figure 4](#)). However, a strong decrease in  $\alpha_{ox}$  with increasing temperature was generally observed for the intermediate and deep layers ([Figure 4](#)). Except for the surface layer of shallow L. Triton, where  $\alpha_{ox}$  strongly negatively correlated with temperature, a hump-shaped relationship between  $\alpha_{ox}$  and temperature was observed for the surface of other lakes ([Supplementary Figure S5](#)). Together, temperature was negatively correlated with  $\alpha_{ox}$  ( $R^2 = 0.12$ ;  $p = 0.0190$ ; [Supplementary Figure S5](#)). For a given depth where MOX rate is high and an increase of MOX rate was observed with increasing temperature, a general decline of  $\alpha_{ox}$  with increasing temperature and therefore MOX rate was evident ([Supplementary Figure S6](#)). This pattern suggests that in several cases, particularly in the





intermediate and deep layers, MOX rate was strongly negatively correlated to the isotopic fractionation (Supplementary Figure S6).

We explored the regulation of  $\alpha_{ox}$  in the water column of our study lakes;  $\alpha_{ox}$  was most significantly correlated with  $\gamma$ -MOB abundance ( $R^2 = 0.56$ ,  $p < 0.0001$ ), while only weakly correlated with  $\alpha$ -MOB ( $R^2 = 0.13$ ,  $p = 0.0139$ ; Figure 5). We developed a simple predictive model for  $\alpha_{ox}$  using multiple regression. Among all the tested variables,  $\gamma$ -MOB abundance ( $\text{cells mL}^{-1}$ ) and MOX rate ( $\mu\text{mol L}^{-1} \text{d}^{-1}$ ) emerged as the predictive variables in the multiple regression model (both MOB abundance and MOX rate were highly statistically significant;  $p < 0.0001$ ) and the resulting model took the form of:

$$\alpha_{ox} = 0.965 + (-0.003 \times \text{Ln MOX rate}) + (0.007 \times \text{Ln } \gamma\text{-MOB}) \quad (3)$$

$$p < 0.0001, n = 45$$

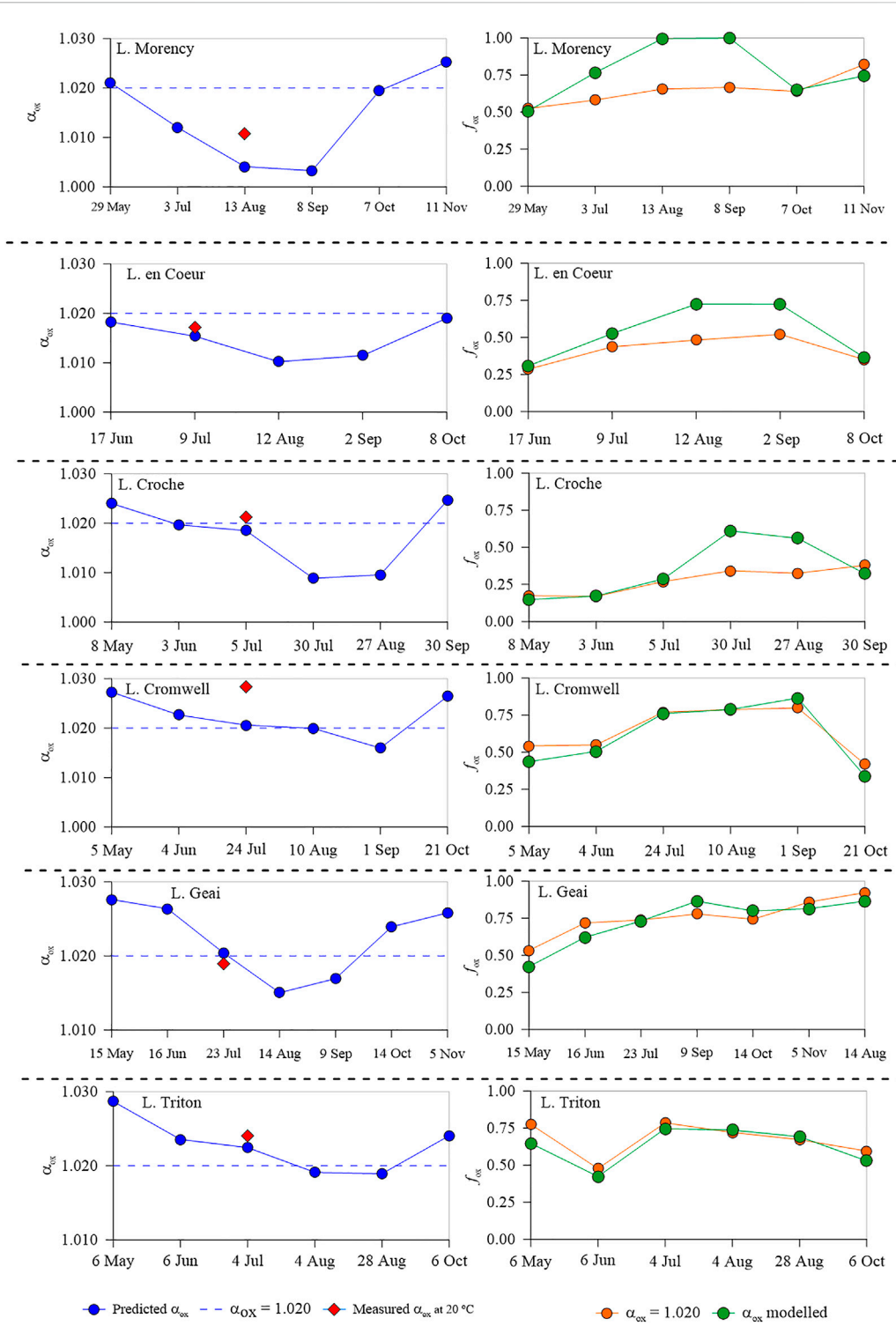
While this simple model explains MOB abundance and MOX rates as the key drivers of  $\alpha_{ox}$ , MOB abundance and MOX rates are not frequently measured variables and therefore may not be suitable as a predictive model for  $\alpha_{ox}$  to be applied in the isotopic mass balance of other systems. Therefore, we further explored the data to develop a predictive model for  $\alpha_{ox}$  using routinely measured limnological variables. To do so, we have included all measured variables such as temperature, pH, concentrations of  $\text{O}_2$ ,  $\text{CH}_4$ , DOC, TP, TN, and Chlorophyll *a* in the statistical analysis. However, the final multiple regression model with statistically significant predictive variables took the form of:

$$\alpha_{ox} = 1.0698 + (0.0067 \times \text{Ln TP}) + (-0.0059 \times \text{pH}) + (-0.0144 \times \text{Ln DOC}) + (-0.0002 \times \text{Temperature}) \quad (4)$$

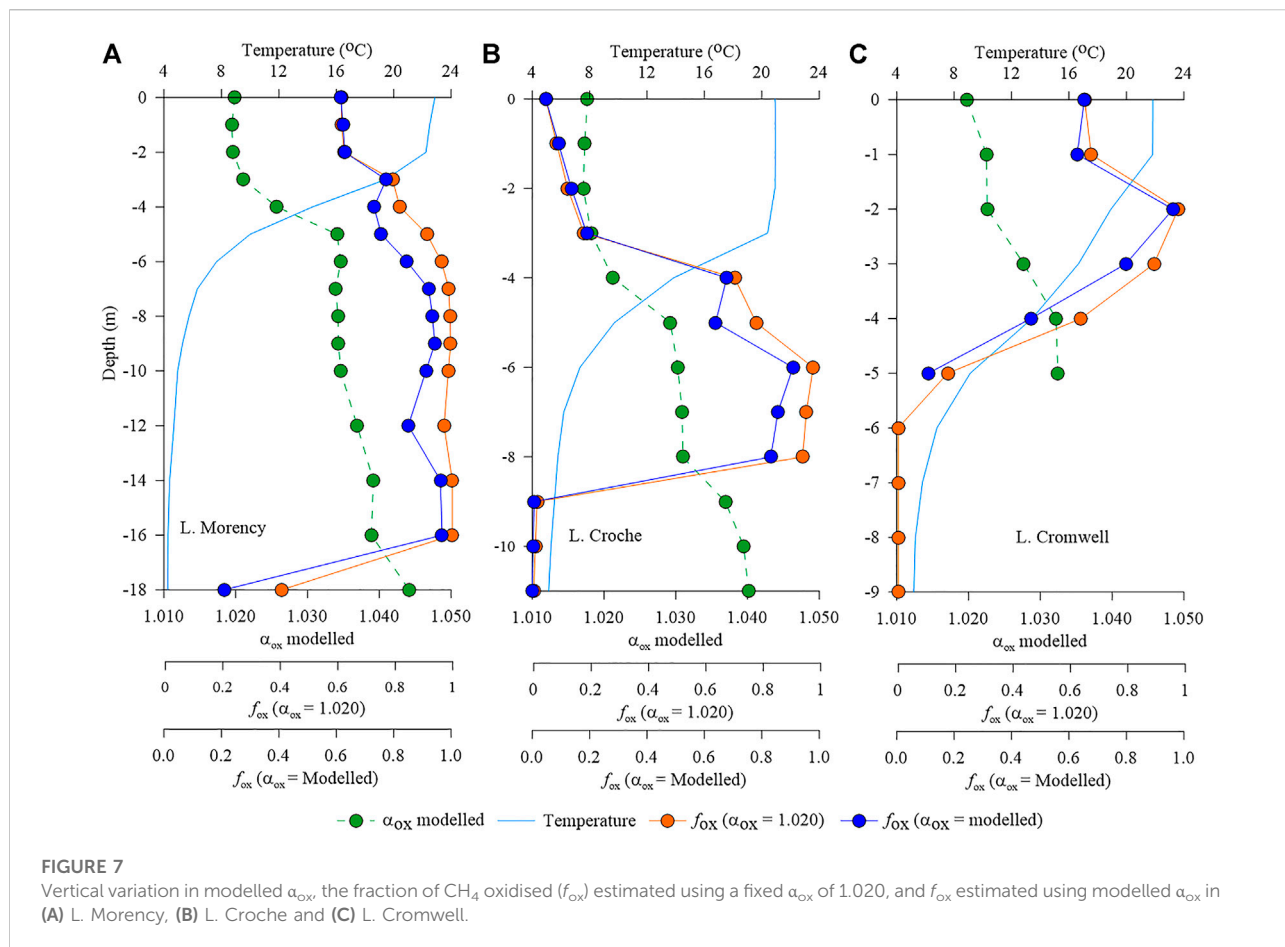
$R^2 = 0.78$ ;  $p < 0.0001$ ;  $n = 45$ , where TP and DOC are in  $\mu\text{g L}^{-1}$  and  $\text{mg L}^{-1}$ , respectively and temperature is in  $^{\circ}\text{C}$  (Supplementary Figure S7). All the parameter estimates of the model were statistically significant;  $p < 0.0001$  for DOC and pH,  $p = 0.00025$  for TP, and  $p = 0.02045$  for temperature.

## Variability of modelled $\alpha_{ox}$ and implications to $f_{ox}$ estimates

Further, we used the temperature, pH, TP, and DOC data of the surface layer of the study lakes in 2015 to predict the fractionation factor by applying the  $\alpha_{ox}$  model (Eq. 4). We observed a clear seasonality in the estimated  $\alpha_{ox}$  values with lower values consistently observed during the peak summer stratification period (Figure 6). Across our study lakes, modelled  $\alpha_{ox}$  varied widely with a larger seasonal variability in L. Morency (1.003–1.025) and L. Croche (1.009–1.025) compared to L. Geai (1.015–1.028) and L. Cromwell (1.016–1.027), while the seasonal  $\alpha_{ox}$  variability was minimal in L. en Coeur and L. Triton (1.010–1.019 and 1.019–1.029, respectively). Subsequently, the variability of the extent of oxidation (i.e.,  $f_{ox}$ ) was different across lakes; much pronounced in some lakes (Morency, Croche, and Geai) while moderate in the others (Figure 6). Further, we compared  $f_{ox}$  estimates derived from the modelled  $\alpha_{ox}$  with a fixed value of 1.020 previously used for temperate lakes (Bastviken et al., 2002). Our comparison indicates that using a fixed  $\alpha_{ox}$  clearly masks seasonality and can substantially misrepresent oxidation estimates at ecosystem scale. Similarly, when we applied the  $\alpha_{ox}$  model to water column profiles performed in 2014, we observed a large variability of  $\alpha_{ox}$  with depth (Figure 7), but the extent of the variability was different across lakes.  $\alpha_{ox}$  ranged from 1.019 to 1.044 in L. Morency, while the variability was only



**FIGURE 6**  
 Seasonal variation in predicted  $\alpha_{ox}$  (left panel) and fraction of  $CH_4$  oxidised ( $f_{ox}$ ) estimated using a fixed  $\alpha_{ox}$  of 1.020 or the modelled  $\alpha_{ox}$  in the surface layers of each study lake.



marginal (1.020–1.032) in L. Cromwell. Overall, our estimation showed a large spatial and temporal variability of  $\alpha_{\text{ox}}$  across lakes and therefore shows that inaccurate  $\alpha_{\text{ox}}$  values can cause large errors in the estimation of  $\text{CH}_4$  oxidation.

## Discussion

The estimation of  $\text{CH}_4$  oxidation using stable isotopic approach fundamentally relies on the isotopic fractionation factor. Despite the growing number of stable isotopic measurements of  $\text{CH}_4$  in inland waters (Bastviken et al., 2002; Kankaala et al., 2007; Schubert et al., 2010; Rinta et al., 2015; Sawakuchi et al., 2016; Thottathil et al., 2018; Grasset et al., 2020; Begum et al., 2021), the use of isotopic mass balance to estimate the extent of  $\text{CH}_4$  oxidation is restricted to very few studies, and wherever attempted, a single  $\alpha_{\text{ox}}$  was applied across systems (Bastviken et al., 2002; Sawakuchi et al., 2016; Thottathil et al., 2018; Grasset et al., 2020). Our extensive experimental incubations of unamended water samples from six temperate lakes have shown wide variability of  $\alpha_{\text{ox}}$  in natural conditions. Furthermore, we show that  $\alpha_{\text{ox}}$  variability in lake water column is

predominantly shaped by the dominant group in the methanotrophic bacterial assemblage ( $\gamma$ -MOB) and MOX rates. However, in a practical point of view, we further developed a model for predicting  $\alpha_{\text{ox}}$  in natural waters using routinely measured limnological variables that are in turn known to regulate  $\gamma$ -MOB assemblage and MOX rates.

## Variability and patterns of $\alpha_{\text{ox}}$ in freshwater lakes

The existing data show a large variability of  $\alpha_{\text{ox}}$  (1.005–1.049) in terrestrial environments (landfill, rice paddy field, and soil) and in various methanotrophic bacterial cultures (Coleman et al., 1981; Bergamaschi, 1997; Templeton et al., 2006; Chanton et al., 2008; Gebert and Streese-keeberg, 2017; Aghdam et al., 2018; Obersky et al., 2018; Sparrow et al., 2019; Fjelsted et al., 2020; Bakkaloglu et al., 2021). Unlike these systems, only a few experimentally determined  $\alpha_{\text{ox}}$  values exist for aquatic systems: 1.009 in a subtropical reservoir (Itoh et al., 2015), 1.013–1.021 in experimentally flooded boreal reservoir (Venkiteswaran and Schiff, 2005), and 1.018 to 1.021 in three

boreal lakes (Bastviken et al., 2002). However, given that MOX rates vary several orders of magnitude across lakes (Bastviken et al., 2002; Thottathil et al., 2019; Denfeld et al., 2016; Sawakuchi et al., 2021; D'Ambrosio and Harrison, 2021) and considering that MOX rate is often coupled with isotopic fractionation (Chanton et al., 2008; Gebert and Streese-Kleeberg, 2017), existing estimates (1.009–1.021) likely underrepresent the extent of  $\alpha_{ox}$  variability in lakes. Unlike previous studies, we estimated the isotopic fractionation factor across and within several lakes covering large environmental gradients and range in methanotrophic activity. Therefore, our study revealed a large and previously unrealized variability of  $\alpha_{ox}$  (1.004–1.038; Figure 4) during aerobic CH<sub>4</sub> oxidation in aquatic environments.

Our experimentally determined  $\alpha_{ox}$  values for six temperate lakes encompass nearly the full spectrum of variability shown across methanotrophic bacterial cultures and diverse terrestrial environments (1.003–1.049; Bergamaschi, 1997; Chanton et al., 2008; Templeton et al., 2006; Bakkaloglu et al., 2021). Based on concurrently measured deep-water profiles of CH<sub>4</sub> and  $\delta^{13}CH_4$ ,  $\alpha_{ox}$  values were estimated to be 1.008 in the temperate L. Lugano (Blees et al., 2014), 1.037 in the boreal L. Mekkojarvi (Kankaala et al., 2007), and between 1.02 and 1.06 in three Arctic lakes (Cadieux et al., 2016). Unlike these data from different systems, our extensive estimates within the water column of temperate lakes showed a systematic vertical shift in  $\alpha_{ox}$  with significantly lower values in the surface relative to a few meters below (Figure 4). This systematic variability of  $\alpha_{ox}$  can be attributed to the observed increase of MOB abundance. While inhibition of CH<sub>4</sub> oxidation at high O<sub>2</sub> levels combined with low CH<sub>4</sub> supply reduce methanotrophic activity in the surface layers, “optimal O<sub>2</sub>” concentration (~15  $\mu\text{mol L}^{-1}$ ; Thottathil et al., 2019) and high CH<sub>4</sub> supply enhance MOB growth, particularly  $\gamma$ -MOB abundance in the deep layer (Zigah et al., 2015; Reis et al., 2020). Together, the vertical distribution of  $\alpha_{ox}$  in the water column of our study lakes shows greater discrimination of heavier isotopes (<sup>13</sup>C) at high MOB abundance while relatively less discrimination in the surface layer where MOB abundance is relatively low.

Studies in terrestrial environments shown that for a given site,  $\alpha_{ox}$  generally decreases with increasing MOX rate which is in turn linked to temporal changes in temperature (Chanton et al., 2008; Gebert and Streese-Kleeberg, 2017). Therefore, the low  $\alpha_{ox}$  in the surface layer where MOX rate is low and high  $\alpha_{ox}$  in the deeper layer where MOX rate is high in lakes seems contradictory to the pattern observed in the terrestrial environments. However, at a fixed MOB abundance for a given water layer, temperatures generally enhanced the MOX rates, which resulted in lower  $\alpha_{ox}$  (Figure 4; Supplementary Figures S5, S6). This is particularly pronounced in the intermediate and deep layers where MOX rate and MOB abundance are consistently high (Supplementary Figure S6). Thus, our data in general is in accordance with the pattern observed in the terrestrial environment where higher MOX rates lead to lower discrimination against heavier isotope and therefore lower values of  $\alpha_{ox}$  (Chanton et al., 2008;

Gebert and Streese-Kleeberg, 2017). However, for a given layer of the stratified water column,  $\alpha_{ox}$  can be highly sensitive to the factors affecting the temporal changes in CH<sub>4</sub> oxidation besides temperature (substrate supply, nutrients, etc.) and these need to be accounted for precise  $\alpha_{ox}$  estimates. Together, our study clearly shows that using literature-derived  $\alpha_{ox}$  values, or assuming a single isotopic fractionation factor over space and time within or across systems can lead to miscalculation of oxidation extent in lakes.

The emergence of  $\gamma$ -MOB as a key predictor of  $\alpha_{ox}$  in the lake water column is an important outcome of this study. Notably, the correlation between  $\gamma$ -MOB and  $\alpha_{ox}$  was evident within each layer of the water column—even in the surface waters where  $\gamma$ -MOB abundance was generally low (Figure 5). Although previous studies have linked isotopic fractionation to MOB abundance in growth medium with pure and/or mixed methanotrophic cultures (Templeton et al., 2006; Feisthauer et al., 2011), our study establish such connection in natural, unamended samples. It should be noted that the abundance of  $\gamma$ -MOB was higher than the abundance of  $\alpha$ -MOB (Figure 2) in our study lakes, particularly in the deeper layer of stratified lakes where multiple environmental factors (low pH, high TP concentration, and strong counter gradients of CH<sub>4</sub> and O<sub>2</sub>; Figure 1; Supplementary Figure S1) support the proliferation of  $\gamma$ -MOB cells (Reis et al., 2020; Nijman et al., 2021). Under these ideal environmental conditions, the greater  $\gamma$ -MOB abundance and higher CH<sub>4</sub> oxidation rates result in higher isotopic fractionation. This is further evident from the stronger correlation between  $\gamma$ -MOB abundance and MOX rates than that between  $\alpha$ -MOB abundance and MOX rates in our study lakes (Figure 3). However, the stronger relationship between  $\gamma$ -MOB abundance and  $\alpha_{ox}$  (Figure 5) does not necessarily indicate a greater discrimination against the heavier isotope (<sup>13</sup>C-CH<sub>4</sub>) in  $\gamma$ -MOB cells than in  $\alpha$ -MOB cells. In systems or environments where  $\alpha$ -MOB is dominant over  $\gamma$ -MOB and MOX rate is regulated by  $\alpha$ -MOB abundance, changes in  $\alpha$ -MOB abundance are expected to drive  $\alpha_{ox}$  variation. Our estimates suggest that under ideal conditions, enhanced uptake of lighter carbon (<sup>12</sup>C) can result in  $\alpha_{ox}$  values as high as 1.038, corroborating previous observation that where there is an ideal condition for methanotrophic growth (i.e., excess CH<sub>4</sub> availability and optimal O<sub>2</sub> oxygen concentration),  $\alpha_{ox}$  values should approach its maximum (Templeton et al., 2006). Our data shows that in the stratified lakes, such condition often exists in the deep layers of the water columns resulting in large increase of  $\alpha_{ox}$  from the surface to deeper layers. Further, multiple regression analysis suggested that  $\gamma$ -MOB abundance and MOX rates essentially control the variability of isotopic fractionation in the water column of our study lakes (Eq. 3), where  $\alpha_{ox}$  was shown to decrease with increasing oxidation rates while increases with  $\gamma$ -MOB abundance. While the depth-wise variation of  $\alpha_{ox}$  suggests an increase of isotopic fractionation with MOX rates (Figure 4), the multiple regression analysis shows an inverse

relationship between  $\alpha_{\text{ox}}$  and MOX rates consistent with studies from various terrestrial environments (Templeton et al., 2006; Chanton et al., 2008; Gebert and Streese-Kleeberg, 2017). This suggests that for a given  $\gamma$ -MOB cell abundance, an increase in MOX rate induced by increased temperature and  $\text{CH}_4$  concentration, and/or “optimal  $\text{O}_2$ ” supply would essentially result in less discrimination against heavier isotope.

## Modelling $\alpha_{\text{ox}}$ with routinely measured limnological variables

While the  $\alpha_{\text{ox}}$  model depicts that the abundance of  $\gamma$ -MOB and MOX rates are the most important drivers of isotopic fractionation (Eq. 3), we analysed the data further to develop a more “practical model” that enables the prediction of  $\alpha_{\text{ox}}$  using routinely measured limnological variables (Eq. 4; Supplementary Figure S7). Temperature emerged as a statistically significant parameter ( $p = 0.0245$ ) in explaining the variability of isotopic fractionation. Systematic decrease of  $\alpha_{\text{ox}}$  with increasing temperature was often observed in terrestrial environments for temperature range of 5–25°C (Tyler et al., 1994; Chanton et al., 2008; Gebert and Streese-Kleeberg, 2017). Despite a comparable temperature range for our incubations, inverse relationship between temperature and  $\alpha_{\text{ox}}$  was weak in our model where all measurements were combined. Yet, for samples where MOX rates were generally high and substantially enhanced by temperature, strong temperature response was observed (Supplementary Figure S6). For instance, small (0.017 km<sup>2</sup>) and shallow (maximum depth: 4.2 m) unstratified L. Triton where MOX rate was highest among all surface samples, a strikingly strong temperature dependence of  $\alpha_{\text{ox}}$  (Supplementary Figure S5) with a slope ( $-7.6 \times 10^{-4}$  and  $-1.6 \times 10^{-3}$ ) comparable with the values reported for soil environments ( $-4.0 \times 10^{-6}$  to  $-1.7 \times 10^{-4}$ ; Chanton et al., 2008) was observed. Although we lack estimates from similar systems, a previous study in a similarly sized boreal lake (0.024 km<sup>2</sup>) in the Experimental Lakes Area, Ontario (Canada) showed a comparable pattern with strong temperature dependence of  $\alpha_{\text{ox}}$  (slope:  $-2.0 \times 10^{-4}$ ; Venkiteswaran and Schiff, 2005). Thus, these data suggest a comparable temperature dependence of  $\alpha_{\text{ox}}$  in small lakes and soil environments, while the pattern is different for the surface layers of larger lakes (Supplementary Figure S5). However, further studies are needed to confirm whether there exists systematic shift in the temperature response of  $\alpha_{\text{ox}}$  in the continuum from soil to small and large lakes. In addition to small L. Triton, for most of the deep layer samples where MOX rates were relatively high, increasing incubation temperature resulted in a general decrease of  $\alpha_{\text{ox}}$  (Figure 4; Supplementary Figure S6). This temperature response of  $\alpha_{\text{ox}}$  that we observed for most our samples is likely linked to the increasing MOX rates with temperature; where a strong positive relationship with

temperature and MOX rate was observed, an inverse relationship between temperature and  $\alpha_{\text{ox}}$  was evident (Supplementary Figure S6) indicating that for a given sample, enhanced  $\text{CH}_4$  oxidation with temperature is associated with less isotopic discrimination. Our result is therefore consistent with the inverse relationship between site specific seasonal temperature changes and  $\alpha_{\text{ox}}$  observed for various terrestrial environments (Tyler et al., 1994; Chanton et al., 2008; Gebert and Streese-Kleeberg, 2017).

Despite the narrow pH range in our data, pH was a highly significant variable ( $p < 0.0001$ ) in shaping overall variability of isotopic fractionation. Given that in our study lakes  $\alpha_{\text{ox}}$  is shaped by  $\gamma$ -MOB abundance, the emergence of pH in the model is consistent with the effect that pH exerts on bacterial communities in general and on MOB community structure in lakes (Newton et al., 2011; Ren et al., 2015; Crevecoeur et al., 2019; Guggenheim et al., 2020). The pH effect on bacterial abundance can be direct since even small changes in pH can impose strong physiological changes, likely induce changes in competition for resources, and affect the net growth of the population (Madigan et al., 2011; Ren et al., 2015). Furthermore, at the ecosystem-scale, pH also exerts indirect effects on bacterial communities acting as an integrative factor of multiple environmental variables including nutrients and substrate availability (Ren et al., 2015; Crevecoeur et al., 2019; Guggenheim et al., 2020). Therefore, we believe that the effect of pH shown in these studies must be relevant for MOB communities in our study lakes, thereby emerging as a proxy of  $\gamma$ -MOB abundance, which directly controls  $\alpha_{\text{ox}}$ . However, we acknowledge that our understanding on the effect pH on MOB communities and isotopic fractionation is still limited and future studies are warranted to confirm the trend shown here.

The “practical model” for  $\alpha_{\text{ox}}$  showed a highly significant negative relationship between  $\alpha_{\text{ox}}$  and DOC concentrations ( $p < 0.0001$ ; Supplementary Figure S7). Although this pattern corroborates the role of DOC in shaping MOB community in northern lakes (Samad and Bertilsson, 2017; Crevecoeur et al., 2019), uncoupling of DOC and MOB abundance (Supplementary Figure S3) in our study lakes warrants other explanations for the DOC- $\alpha_{\text{ox}}$  relationship in the model. It should be noted that DOC concentration was positively correlated with MOX rates in the surface and intermediate layers, while a hump-shaped relationship was found in the deep layer (Supplementary Figure S8). This positive relationship between MOX rate and DOC in the upper layers can be attributed to the strong negative relationship between DOC and  $\text{O}_2$  concentrations which is in turn induced by higher heterotrophic bacterial respiration of organic carbon with increasing DOC concentration (Supplementary Figure S8). Since high  $\text{O}_2$  concentration inhibits  $\text{CH}_4$  oxidation (Rudd et al., 1976; Thottathil et al., 2019), observed decrease of  $\text{O}_2$  concentration with the increasing concentration of DOC is likely to relieve the methanotrophic community from  $\text{O}_2$  inhibition and

thereby enhances the oxidation rate. However, it should be noted that  $O_2$  is not selected as a predictive variable in the  $\alpha_{ox}$  model. Therefore, DOC not only integrates the effect of  $O_2$ , but also accounts for other potential factors such as the light attenuation-mediated enhancement of MOB activity (Dumestre et al., 1999; Murase and Sugimoto, 2005; Sawakuchi et al., 2016; Thottathil et al., 2018). Together, we suggest that increasing DOC concentration and thereby MOX rates resulted in less discrimination against the heavier isotope as shown in the model (Eq. 3); therefore, in our “practical model” DOC should be interpreted as proxy of  $CH_4$  oxidation.

Unlike temperature, pH, and DOC, the model showed a strong positive correlation between TP and  $\alpha_{ox}$  (Supplementary Figure S7;  $p = 0.0003$ ). Note that TP was correlated with the abundance of MOB, particularly with  $\gamma$ -MOB (Supplementary Figure S3) suggesting a greater role of P-availability in enhancing the methanotrophic abundance; a pattern previously shown for ice-covered boreal lake water column (Denfeld et al., 2016; Sawakuchi et al., 2021) and subtropical lake sediment (Nijman et al., 2022). Therefore, the positive correlation between TP and  $\alpha_{ox}$  indicates that TP in our “practical model” is a proxy of the abundance of dominant methanotrophic group (i.e.,  $\gamma$ -MOB). The identification of TP as a strong regulator of MOX rates and  $\alpha_{ox}$  has important implications. For example, a recent study reported strong correlation between the extent of oxidation (i.e.,  $f_{ox}$ ) and TP across lake mesocosms (Grasset et al., 2020). Since  $CH_4$  oxidation is known to be light inhibited (Dumestre et al., 1999; Murase and Sugimoto, 2005), the authors attributed this pattern to enhanced methanotrophic activity due to greater light attenuation in the P-amended mesocosms where phytoplankton biomass increased substantially (Grasset et al., 2020). However, our data suggests that the role of TP on  $CH_4$  oxidation can be rather direct by stimulating  $CH_4$  oxidation as shown in recent studies (Sawakuchi et al., 2021; Kashi et al., 2022; Nijman et al., 2022). Hence, our model suggests that overlooking the effect of TP and assuming a single  $\alpha_{ox}$  for the mass balance calculation across systems with different trophic status could underestimate the  $f_{ox}$ . It is interesting to note that despite the covariation of TP and TN in our study lakes, TN is not a significant predictive variable in the multiple regression analysis. This is in accordance with the recent study showing that MOB community is P-limited as evident from the increase of P content of the MOB biomass despite the elevated phosphorus and nitrogen levels in the experimental incubations (Nijman et al., 2022). Along with the above study, our data suggests that strong correlation between TP and MOB abundance is not just due to their concomitant increase with depth but caused due to the strong role of P in stimulating MOX rates and increasing MOB abundance in lakes. The  $\gamma$ -MOB abundance is positively correlated with  $\alpha_{ox}$  in our model (Eq. 3); therefore, the strong correlation between  $\alpha_{ox}$  and TP in our “practical model” suggests TP as a proxy of  $\gamma$ -MOB abundance and its increase leads to greater isotopic fractionation.

## The importance of using appropriate $\alpha_{ox}$ values

By applying our environmental model of  $\alpha_{ox}$  to water column profiles, we found that  $\alpha_{ox}$  and consequently the seasonal variability of  $f_{ox}$  was much more pronounced in some lakes (Morency, Croche, and Geai) than in others (Figures 6, 7). Therefore, assuming a single  $\alpha_{ox}$  value to all lakes would largely misrepresent the oxidation extent and thereby lead to errors in the estimation of ecosystem-scale  $CH_4$  oxidation rates. For instance, assuming a constant  $\alpha_{ox}$  of 1.020 throughout the year in the epilimnion of L. Morency would underestimate the  $f_{ox}$  by up to 34% during August–September period and underestimate by up to 8% in the spring and autumn period (Figure 6). Similarly, applying an  $\alpha_{ox}$  of 1.020 throughout the water column overestimates  $f_{ox}$  by up to 10% in the deeper layers of our study lakes (Figure 7). These potential errors in the  $f_{ox}$  estimation induced solely by the difference in  $\alpha_{ox}$  value is comparable with the error estimations reported previously (Cabral et al., 2010; Capanema and Cabral, 2012). Together, our “practical  $\alpha_{ox}$  model” with routinely measured limnological variables allows more accurate estimation of  $f_{ox}$  via  $\delta^{13}C$ - $CH_4$  mass balance and enable to derive large-scale patterns of  $CH_4$  oxidation across freshwater aquatic systems without the biases induced by the variability of  $\alpha_{ox}$ .

## Conclusion

In conclusion, we show a large variability in the stable carbon isotopic fractionation during aerobic  $CH_4$  oxidation in freshwater lakes with a systematic increase of  $\alpha_{ox}$  from the surface to deep layers of the water column. Our study showed that the rates of  $CH_4$  oxidation and the abundance of  $\gamma$ -MOB explain the variability of  $\alpha_{ox}$  in temperate lake water columns. However, we suggest that the observed relationship between  $\gamma$ -MOB abundance and  $\alpha_{ox}$  is due to the dominance of this MOB group in the temperate lake water column and does not necessarily suggest a group specific difference in isotopic fractionation. For a given  $\gamma$ -MOB abundance,  $\alpha_{ox}$  decreased with increasing MOX rates—a pattern consistent with the data from various terrestrial environments suggesting that with increasing  $CH_4$  oxidation rate, there is less discrimination against the heavier stable isotope ( $^{13}C$ ). For practical purposes, we developed a general model of  $\alpha_{ox}$  by integrating the effect of various factors on experimentally derived  $\alpha_{ox}$  values using routinely measured limnological variables. We showed that applying a single  $\alpha_{ox}$  value in  $CH_4$  stable isotopic mass balances can misrepresent its within lake variability, thereby hampering the detection of existing spatial and temporal patterns in the extent of  $CH_4$  oxidation across lakes. Our model of  $\alpha_{ox}$  in conjunction with the growing

number of  $\delta^{13}\text{C}-\text{CH}_4$  measurements should help to elucidate large scale patterns and drivers of the oxidation-driven mitigation of  $\text{CH}_4$  emission from freshwater aquatic systems.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

## Author contributions

ST and PR developed the research idea and conducted experiments. ST analyzed and plotted data and wrote the first version of the manuscript, and PR analyzed and plotted data, reviewed, and edited the manuscript. YP contributed to analyzing data, reviewed and edited the manuscript, supervised the research, and acquired funding.

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## Conflict of interest

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

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fenvs.2022.833688/full#supplementary-material>

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