10th February 2015

Dear Editor,

Please find herewith a revised version of our manuscript (bg-2014-505), entitled *"Methanotrophy within the water column of a large meromictic tropical lake (Lake Kivu, East Africa)"*.

This updated manuscript provides a thorough revision addressing the minor issues raised by the reviewers. A point-by-point responses to all reviewer comments is enclosed below. Sections substantially modified in the revised manuscript are highlighted and annotated so that the corrections to the initial manuscript can be easily identified.

We sincerely hope that the present version of the manuscript can be considered for publication in *Biogeosciences* and thank the associate editor, and the two anonymous reviewers for their constructive comments.

Best regards,

Cédric Morana

Reply to the reviewer comments

First of all, we would like to thank the reviewers for their positive and very constructive comments. In the section below, we will provide a point-by-point reply to the suggestions and comments provided by the reviewers.

Anonymous Referee 1

Reviewer comment 1: *The paper is well written and the study well executed. My only major scientific criticism is that the authors focus solely on sulphate-AOM but do not present concentrations of alternative electron acceptors. It's possible that AOM in lake coupled to another electron acceptor such as, nitrate, nitrite, Fe(III), or Mn(IV): Why do the authors assume (e.g. on page 15680 line 6) that sulphate-AOM is occurring and not another form of AOM ? Did they measure concentrations of sulphate, nitrate, nitrite, Fe (III), Mn (IV) ?*

> Reply : This is an interesting comment. It has been reported frequently in the literature that 10Me16:0 and C17 MUFA are especially abundant in sulphate reducing bacteria, as mentioned in the text. However, it is true that the phylogenetic resolution of PLFA analysis is rather low, and hence it is difficult to unambiguously identify the organisms involved in this anaerobic oxidation process. Recent studies have revealed that marine anaerobic oxidation of CH_4 (AOM) is indeed coupled to a larger variety of electron acceptors than previously thought. For instance, it has been shown that the sulphate-reducing bacterial partners of methanotrophic archaea could also reduce iron (Coleman et al. 1993). Moreover, anaerobic oxidation of methane could also be carried out syntrophically by a consortium between methanotrophic archaea and denitrifying bacteria (Raghoebarsing et al. 2005), or between methanotrophic archaea and manganese reducing bacteria (Beal et al. 2009). The "discussion" section of our manuscript has been modified to take these studies into account. However, the major aims of our study were (i) to quantify the contribution of CH_4-derived carbon to the biomass, (ii) to quantify methanotrophic bacterial production, (iii) to quantify methanotrophic bacterial growth efficiency, and (iv) to identify which were the aerobic methanotrophs involved in CH_4 oxidation based on PLFA analyses. Our experiments were not designed to identify which electron acceptors were linked to anaerobic methane oxidation (although this is an interesting research topic), and hence we did not measure the concentrations of Fe(III), Mn (IV) and SO_4^{2-} (in September 2012) in the water column of Lake Kivu. Further investigations, with a special focus on the coupling between anaerobic CH_4 oxidation and other processes, would be needed to shed more light on this. The revised text now reads : "A significant MBP rate (1.3 µmol L^{-1} d^{-1}) was measured under low-oxygen conditions (< 3 µmol L^{-1}) at 60 m during the rainy season (February 2012). Moreover, the PLFA labelling pattern was drastically different, with a more important specific ^{13}C incorporation into 10Me16:0 and C17 MUFA instead of the C16 MUFA, relative to their concentrations. This different labelling pattern suggests that a different population of methanotrophs was active in CH_4 oxidation deeper in the water column. Archaea lack ester-linked fatty acids in their membrane and are therefore undetectable in PLFA analysis. However 10Me16:0 and C17 MUFA are known to be especially abundant in sulphate-reducing bacteria (Macalady et al. 2000, Boschker and Middelburg 2002), one of the syntrophic partner of anaerobic CH_4 oxidizing archaea (Knittel

and Boetius et 2009). Hence, the specific labelling of 10Me16:0 and C17 MUFA under low-oxygen conditions could indicates that a fraction of the upward flux of CH_4 was oxidized syntrophically by an archaea/bacteria consortium, and might support the hypothesis that the bacterial partner grow on CH_4-derived carbon source supplied by anaerobic methane oxidizers within the consortium, as already suggested by the results of an in vitro labelling ($^{13}CH_4$) study (Blumenberg et al. 2005). However, our data does not necessary imply that anaerobic methane oxidation would be coupled with SO_4^{2-} reduction, as some sulphate-reducing bacteria have been also found to be able to reduce iron (Coleman et al. 1993). Furthermore, the phylogenetic resolution of SIP-PLFA analyses in rather low (Uhlík et al. 2009), and recent studies showed that anaerobic methane oxidation could be carried out syntrophically by consortium between methanotrophic archaea and denitrifying bacteria (Raghoebarsing et al. 2005), or between methanotrophic archaea and manganese reducing bacteria (Beal et al. 2009). Further investigations would be needed to address more accurately which is the electron acceptors coupled to anaerobic CH_4 oxidation".

Reviewer specific comments :

> Reply : The text was corrected for grammatical errors following the suggestions provided by the reviewer.

Anonymous Referee 2

Reviewer comment 1 : *The introduction is too pedestrian. I find no motivation for this particular study in the introduction, neither are new, more recent general and lake-specific knowledge introduced and/or discussed. For instance, the specific types of methanotrophs and their identifying characteristics are not explored at all and the AOM coupled to sulfate reduction is presented as though it was the ONLY process of AOM. Recent studies show ANME are able to oxidize methane without the SRB partners. This should be noted in the introduction. Also, the authors should thoroughly discuss previous methane-based studies in Lake Kivu. They should then discuss gaps in research that their study sought to address.*

> Reply : The introduction was modified to present more recent literature and provide a better motivation of our work.

Reviewer comment 2 : p. 15666 Line 14 – *'bur' should be 'but'*

> Reply **:** This has been corrected.

Reviewer comment 3 : p. 15667 Line 3 – *Which inherent characteristics ? Please list/discuss them.*

> Reply **:** The permanent stratification and the high methane concentration in deep waters of Lake Kivu throughout the year. The revised text now reads : "Because of the permanently stratified nature of its water column and the large amount of CH_4 dissolved in its deep water, the meromictic Lake Kivu offers an ideal natural laboratory to investigate the role of methanotrophy in large tropical lakes."

Reviewer comment 4 : p. 15667 Line 23 – *Is "wanve" supposed to be wave ?*

> Reply **:** Yes, the text has been corrected.

Reviewer comment 5 : p. 15668 Line 19 – *What exact volume of headspace was created ?*

> Reply **:** 20 ml. This is now specified in the text.

Reviewer comment 6 : p. 15670 Line 1 – *Estimation of reproducibility was based on what ? Please be specific.*

> Reply **:** Based on triplicate measurements on a selection of samples. This is now specified in the text

Reviewer comment 7 : p. 15670 Line 17 – *Here and elsewhere, change "tube" to "tubing". Specify if PE, PP, PC, PTFE, etc*

> Reply **:** Silicone tubing. Changes have been made in the text.

Reviewer comment 8 : p. 15672 Line 15 – *Oxycline seems to start at 40 m rather than 50 m. Please check.*

> Reply **:** To avoid confusion, the text now reads : "CH_4 was abundant in deep waters, with a maximum concentration of 899 μmol L^{-1} at 80 m, however CH_4 decreased abruptly at the bottom of the oxycline, being 4 orders of magnitude lower in surface waters (Figure 1a)."

Reviewer comment 9 : p. 15672 Line 17 – *Here and elsewhere, how could you tell where the 'oxic-ANOXIC transition' was when the detection limit of your oxygen sensor was as high as 3 umol L-1? This means the depths you consider anoxic could actually contain as much as 3 uM oxygen... meaning a much deeper depths could be actually oxic. Are you sure the detection limit was not lower than this? If it was as high as reported, it would be advisable to avoid using the term 'oxic-anoxic' or at the least, you should use literature references to support your data in establishing this transitional layer.*

> Reply **:** We agree with the reviewer that using the word "anoxic" might be confusing, and not appropriate given the limit of detection of our oxygen sensor. In the revised version of the manuscript, "oxic-anoxic transition" has been replaced by "transition between oxic and O_2-depleted waters". Also, the term "O_2-depleted waters" is clearly defined in the material and methods as waters where the O_2 concentration was below 3 μmol L^{-1}

Reviewer comment 10 : p. 15674 Line 13-15 – *The use of 'oxic-anoxic condition' and 'low-oxygen conditions' is confusing. How can you have transition from oxic to anoxic condition, and then below the anoxic condition, you somehow also have a low-oxygen condition? This is an issue because of the detection limit of the oxygen sensor used. However, you can work around this by avoiding using the term "oxic-anoxic transition"*

> Reply **:** We agree with the reviewer that using the word "anoxic" might be confusing. See our reply to the previous comment : changes have been made in the text to replace "oxic-anoxic transition" by "transition between oxic and O_2-depleted waters".

Reviewer comment 11 : p. 15675 Line 19-22 – *105% and 142% are confusing. Please recheck your computations. The microbes cannot oxidize any more methane than what is available. I suggest to either report the average values or use fractionation factors that give reasonable estimates of the fraction of methane that is oxidized.*

> Reply **:** The reviewer raised an interesting issue. Actually, Bastviken et al. (2002) reported also values higher than 100 % for the flux of CH_4 oxidized. It is maybe due to the use of linear approximation of the Rayleigh equation, that might not be valid in Lake Kivu because of the high fraction of CH_4 that is oxidized. We revised our calculation using the original equations, and refer now to Coleman et al. (1981). The text now reads : "The fraction of the upward CH_4 flux oxidized within a depth interval can be estimated from a closed-system Rayleigh model of isotope fractionation (Blees et al. 2014) described by the following equation (rearranged from Eq. 11; Coleman et al. 1981):

$$\ln(1-f) = \ln((\delta^{13}CH_{4t}+1000)/(\delta^{13}CH_{4b}+1000))/((1/\alpha)-1) \quad (8)$$

where f is the fraction of CH_4 oxidized within the depth interval, $\delta^{13}CH_{4b}$ and $\delta^{13}CH_{4t}$ are the $\delta^{13}C$ values of CH_4 at the bottom and the top of the depth interval, respectively, and α is the isotope fractionation factor for CH_4 oxidation estimated in Lake Kivu in September 2012 (α = 1.016 ± 0.007). Based on this equation and using a range of isotope fractionation factors (from 1.009 to 1.023), we can estimate that 51-84% of the upward flux of CH_4 was microbially oxidized within a 10 m depth interval in the oxycline (60-70 m) in the Southern Basin during the dry season (September 2012). Similarly, 51-84% of the CH_4 flux was oxidized between 50 m and 55 m in the Northern Basin during the dry season, and 58-89% of the CH_4 flux was oxidized within a wider depth interval (45-70 m) during the rainy season (February 2012). The relatively wide range of the estimated percentage of CH_4 flux oxidized is due to the uncertainty on the isotope fractionation factor. Nevertheless, these calculations illustrate clearly the importance of microbial CH_4 oxidation processes in preventing CH_4 to reach the surface waters of the lake".

Reviewer comment 12 : p. 15676 Line 4-18 – *I have issues with the end-members used in the mixing model. The bulk POC should already contain some methanotrophic biomass so it should not be used as the end member of the sedimenting organic matter. Either completely remove these estimates from the paper or you should use C13 of diagnostic biomarkers or better still, use C13 of CO2/DIC (and correct for photosynthetic fractionation) as the sedimenting OM end-member.*

> Reply **:** We used the δ^{13}C-POC value in surface waters (5 m) as a sedimenting-OM end-member. This value was -22.9‰ in the Northern Basin during the rainy season, -24.4‰ in the Southern Basin during the dry season, and -23.9‰ in the Northern Basin during the dry season. In a manuscript recently published in Biogeosciences Discussions (Morana et al. 2014), we report that the δ^{13}C-POC values stayed almost constant throughout the year in surface waters (- 23.8 ± 0.8‰, n = 19). Similarly, the δ^{13}C-DIC was also rather constant, with values oscillating from +2.4‰ to +3.4‰. Phytoplankton is typically ~ 20‰ more depleted in ^{13}C than its source (see Fogel and Cifuentes 1993). Almost 95% of the DIC in surface waters of Lake Kivu is HCO_3^- (water temperature ~ 24°C ; pH ~9), therefore, using the isotope

fractionation between HCO_3^- and CO_2 reported by Vogel et al. (1970) or Zhang et al. (1995), the $\delta^{13}C$ signature of the dissolved CO_2 would approximate ~ -5‰. Our $\delta^{13}C$-POC values measured in the surface waters of Lake Kivu are then in a good agreement with an almost exclusive phytoplankton origin of the POC. These data are extensively discussed in Morana et al. (2014). Even if minimal, a contribution of methanotrophic biomass to the POC pool in surface waters would have led to drastically lower $\delta^{13}C$-POC, because of the much lower $\delta^{13}C$ values of CH_4 ($\delta^{13}CH_4$ was never higher than -39 ‰).

Reviewer comment 13 : p. 15679 Line 4 – *^{13}C-depletion of C16 MUFA was not within the oxycline (Fig 2d). The ^{13}C depletion rather appears to start right below (or at) the transition between the 'oxycline' and the 'low-oxygen waters', extending way deeper into the 'the low-oxygen' depths which could potentially be anoxic depths. This observation should be discussed. Why are the type I methanotrophs active within these depths?*

> Reply **:** This study is not the first to report a strong ^{13}C-depletion of bacterial lipid markers for aerobic methanotrophic bacteria at the bottom of the oxycline, or in O_2-depleted waters (see Schubert et al. 2006 for the Black Sea, Blees et al. 2014 for Lake Lugano). This is now discussed in the revised version of the manuscript. See the comment below for a more detailed reply.

Reviewer comment 14 : p. 15679 Line 4 – *Also, in the 13C-labelled methane tracer studies that the authors performed, all the samples that were used for the incubations were taken from depths below the oxycline (except 40 m in Feb 2012) (Fig. 4a &b). In September 2012, the 'oxycline' was above 55 m and the 'low-oxygen waters' below this depth. The incubated samples were from 62.5 -70 m (Fig 4a). Similarly, in February 2012, the 'oxycline' was above 45 m with low oxygen waters below this depth. The samples used for the incubations were from 40m, 50m and 60m (Fig 4b). The incubations that showed labelled 13C-CH4 incorporation by C16 MUFA (type I methanotrophs) were all from depths below the oxycline, that is, 65 m in September and 50 m in February. So both the in situ PLFA and tracer PLFA 13C data show that the type I methanotrophs are active way below the oxycline, in the 'low-oxygen' and potentially anoxic depths. The authors should discuss these observations. Based on the data, I am not convinced that the type I methanotrophs are active in methane oxidation in the oxycline or oxic zone as suggested by the authors.*

> Reply **:** This needs to be clarified : *all the samples that were used for the incubations were taken from depths below the oxycline (except 40 m in Feb 2012)*. As mentioned in the caption of the Figure 4., our incubations were performed in the Southern Basin during the dry season (Fig 4a), where oxygen concentrations were higher than 3 µmol L^{-1} down to 65 m (Fig1a). Therefore, our incubations at 62.5 m and 65 m (fig 4a) were indeed carried out under oxic conditions (at O_2 concentration of 61 µmol L^{-1} and 20 µmol L^{-1}, respectively). The reviewer's comment that "*The incubations that showed labelled 13C-CH4 incorporation by C16 MUFA (type I methanotrophs) were all from depths below the oxycline, that is, 65 m in September and 50 m in February"*: might arise from a misreading and inversion of the profile obtained from the Southern Basin (Fig1a) and the Northern Basin (Fig1b, where the water column was oxic down to 55 m). The ^{13}C-CH_4 incorporated in C16 MUFA at 65 m in the Southern Basin

(September 2012) was fixed in presence of oxygen, and a substantial amount of tracer was also incorporated in C16 MUFA under oxic conditions in the Northern Basin (February 2012), at 40 m (Fig 4b). To us, the fact that C16 MUFA, not C18 MUFA, were labelled during our incubation under oxic conditions unambiguously indicates that type I methanotrophs were active in methane oxidation at the bottom of the oxycline, and at the transition between oxic and O_2-depleted waters. It is however correct that some $^{13}CH_4$ was incorporated inC16 MUFA at 50 m (5 m below the bottom of the oxycline) in February 2012, in the Northern Basin. Due to the limit of detection of our oxygen sensor, it is difficult to know if the waters at 50 m was truly anoxic, or micro-oxic, with O_2 concentration lower than 3 µmol L^{-1} but still sufficiently high to support some aerobic CH_4 oxidation. In a recently published paper, Blees et al. (2014) provided multiple lines of evidence for micro-aerobic methane oxidation, 40 m below the chemocline of Lake Lugano. They measured aerobic methane oxidation at nanomolar O_2 concentration, well below the limit of detection of our oxygen sensor. We revised our text and extended our discussion on this aspect. The revised text now reads : "Nevertheless, in February 2012 the C16 MUFA appeared to be strongly depleted in ^{13}C below the transition between oxic and O_2-depleted waters (Figure 2d). This study is not the first to report strong ^{13}C-depletion of bacterial lipid markers for aerobic methanotrophic bacteria in O_2-depleted waters (see Schubert et al. 2006 for the Black Sea, Blees et al. 2014 for Lake Lugano). The presence of methanotrophic bacterial biomass below the oxycline could simply result from gravity-driven physical particle transport from oxic waters, but it has been also demonstrated that some aerobic methanotrophs are able to persist under low oxygen conditions in a reversible state of reduced metabobic activity (Roslev & King 1995). By constrast, the recovery of these aerobic methanotrophs after CH_4 deprivation under oxic conditions is less successful because they have been found to degrade a significant amount of cell proteins (Roslev & King 1995). Blees et al. (2014) suggested that this physiological preference for O_2 starvation than CH_4 starvation under oxic conditions would drive aerobic methanotrophs toward the O_2-depleted part of the oxygen continuum. This concept seems particularly important in tropical lakes because the thermal stratification of the water column is usually very dynamic in these systems due to the small temperature gradient, allowing episodic, yet frequent, O_2 intrusion into deeper waters. Aerobic methanotrophs in dormancy would recover quickly after the episodical O_2 injection, and resume rapidly micro aerobic CH_4 oxidation (Blees et al. 2014)".

Reviewer comment 15 : p. 15680 Line 2 – *While previous studies have also noted the involvement of sulfate reducing bacteria in the deep waters of the lake, as was observed in this study, 10 Me 16:0 and C17 MUFA are not typically used as biomarkers for sulfate-reducing bacteria. The use of these biomarkers should be thoroughly and convincingly discussed in the context of wider literature.*

> Reply **:** Due to the low phylogenetic resolution of PLFA analyses, we acknowledge that the labelling of 10me16:0 and C17 MUFA does not necessarily reflect a coupling between SO_4^{2-} reduction and anaerobic methane oxidation. This issue was also raised by reviewer 1, see our reply to his/her comment. The discussion now reads : "A significant MBP rate (1.3 µmol L^{-1} d^{-1}) was measured under low-oxygen conditions (< 3 µmol L^{-1}) at 60 m during the rainy

season (February 2012). Moreover, the PLFA labelling pattern was drastically different, with a more important specific ^{13}C incorporation into 10Me16:0 and C17 MUFA instead of the C16 MUFA, relative to their concentrations. This different labelling pattern suggests that a different population of methanotrophs was active in CH_4 oxidation deeper in the water column. Archaea lack ester-linked fatty acids in their membrane and are therefore undetectable in PLFA analysis. However 10Me16:0 and C17 MUFA are known to be especially abundant in sulphate-reducing bacteria (Macalady et al. 2000, Boschker and Middelburg 2002), one of the syntrophic partner of anaerobic CH_4 oxidizing archaea (Knittel and Boetius 2009). Hence, the specific labelling of 10Me16:0 and C17 MUFA under low-oxygen conditions could indicates that a fraction of the upward flux of CH_4 was oxidized syntrophically by an archaea/bacteria consortium, and might support the hypothesis that the bacterial partner grow on CH_4-derived carbon source supplied by anaerobic methane oxidizers within the consortium, as already suggested by the results of an in vitro labelling ($^{13}CH_4$) study (Blumenberg et al. 2005). However, our data does not necessary imply that anaerobic methane oxidation would be coupled with SO_4^{2-} reduction, as some sulphate-reducing bacteria have been also found to be able to reduce iron (Coleman et al. 1993). Furthermore, the phylogenetic resolution of SIP-PLFA analyses in rather low (Uhlík et al. 2009), and recent studies showed that anaerobic methane oxidation could be carried out syntrophically by consortium between methanotrophic archaea and denitrifying bacteria (Raghoebarsing et al. 2005), or between methanotrophic archaea and manganese reducing bacteria (Beal et al. 2009). Further investigations would be needed to address more accurately which is the electron acceptors coupled to anaerobic CH_4 oxidation".

Reviewer comment 16 : *Please include a map of the Lake showing the study sites.*

> Reply **:** Figure was added as suggested.

Reviewer comment 17 : *Fig 1 (a,b,c) : Please use different scales for the 13C of CH4 and 13C of POC. Also, the CH4 concentrations should be reported in mmol L-1 . I reckon that using the same unit as oxygen allows for easy comparison, but in this particular instance, it is better to keep the methane concentration in mmol L-1. With this figure and all others, it would be informative to the reader to indicate the precision of the measurements by way of error bars.*

> Reply: Units were changed as suggested

Reviewer comment 18: Fig 3. It will help the reader if you include the time (hours) on each data point.

> Reply : Figure was changed as suggested.

Reviewer comment 19 : Fig 4. You should note in the figure title that all the samples in Fig 4a that were incubated were from depths below the oxycline and within the low-oxygen region. Similarly, include in the title that 50 m and 60 m are below the oxycline, and are within the low oxygen depths.

> Reply : Only the samples “67.5 m” and “70 m” of the Fig 4a and the samples “50 m” and “60 m” from the Fig 4b were incubated under low oxygen conditions (> 3 µmol L^{-1}), as explained above. The caption of the Figure 4 now reads : “Figure 4. Specific CH_4-derived C incorporation pattern into phospholipid fatty acids (PLFA) (incorporation rates of C into PLFA normalized on PLFA concentration, d^{-1}) in (a) September 2012 (dry season) in the Southern Basin and (b) in February 2012 (rainy season) in the Northern Basin. Dissolved oxygen concentration was lower than 3 µmol L^{-1} at 67.5 m and 70 m (a), and 50 m and 60 m (b).

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Methanotrophy within the water column of a large meromictic tropical lake (Lake Kivu, East Africa)

C. Morana[1], A. V. Borges[2], F. A. E. Roland[2], F. Darchambeau[2], J-P. Descy[3], and S. Bouillon[1]

[1] Department of Earth and Environmental Sciences, KU Leuven, Leuven, Belgium

[2] Chemical Oceanography Unit, Université de Liège, Liege, Belgium

[3] Research Unit in Environmental and Evolutionary Biology, UNamur, Namur, Belgium

Correspondence to: C. Morana (cedric.morana@ees.kuleuven.be)

Abstract

The permanently stratified Lake Kivu is one of the largest freshwater reservoirs of dissolved methane (CH_4) on Earth. Yet CH_4 emissions from its surface to the atmosphere have been estimated to be 2 orders of magnitude lower than the CH_4 upward flux to the mixed layer, suggesting that microbial CH_4 oxidation is an important process within the water column. A combination of natural abundance stable carbon isotope analysis ($\delta^{13}C$) of several carbon pools and $^{13}CH_4$-labelling experiments was carried out during the rainy and dry season to quantify (i) the contribution of CH_4-derived carbon to the biomass, (ii) methanotrophic bacterial production (MBP), and (iii) methanotrophic bacterial growth efficiency (MBGE), defined as the ratio between MBP and gross CH_4 oxidation. We also investigated the distribution and the $\delta^{13}C$ of specific phospholipid fatty acids (PLFA), used as biomarkers for aerobic methanotrophs. Maximal MBP rates were measured in the oxycline, suggesting that CH_4 oxidation was mainly driven by oxic processes. Moreover, our data revealed that methanotrophic organisms in the water column oxidized most of the upward flux of CH_4, and that a significant amount of CH_4-derived carbon was incorporated into the microbial biomass in the oxycline. The MBGE was variable (2-50%) and negatively related to CH_4:O_2 molar ratios. Thus, a comparatively smaller fraction of CH_4-derived carbon was incorporated into the cellular biomass in deeper waters, at the bottom of the oxycline where oxygen was scarce. The aerobic methanotrophic community was clearly dominated by type I methanotrophs and no evidence was found for an active involvement of type II methanotrophs in CH_4 oxidation

in Lake Kivu, based on fatty acids analyses. Vertically integrated over the water column, the MBP was equivalent to 16-60% of the average phytoplankton particulate primary production. This relatively high magnitude of MBP, and the substantial contribution of CH_4-derived carbon to the overall biomass in the oxycline, suggest that methanotrophic bacteria could potentially sustain a significant fraction of the pelagic food-web in the deep, meromictic Lake Kivu.

1 Introduction

Although the atmospheric methane (CH_4) concentration is low compared to carbon dioxide (CO_2), CH_4 contributes significantly to the anthropogenic radiative forcing (18%) because of its 25 times higher global warming potential than CO_2 (Forster et al. 2007). CH_4 has several natural and anthropogenic sources and sinks, whereby natural and artificial wetlands are recognized as major CH_4 sources to the atmosphere (e.g. Kirschke et al. 2012). Bastviken et al. (2011) estimated that CH_4 emissions to the atmosphere from freshwater ecosystems (0.65 Pg C yr^{-1} as CO_2 equivalent) would correspond to 25% of the global land carbon (C) sink (2.6 ± 1.7 Pg C yr^{-1}, Denman et al. 2007). Tropical regions are responsible for approximately half of the estimated CH_4 emissions from freshwater ecosystems to the atmosphere, although they have been consistently undersampled (Bastviken et al. 2011). Thus, more information on both the magnitude and controlling factors of CH_4 emissions from tropical inland waters are warranted. CH_4 is produced mainly in anoxic sediments by methanogenic archaea following two different pathways: acetoclastic methanogenesis, using acetate produced from organic matter degradation, or CO_2 reduction. Although both methanogenic pathways may co-occur, CO_2 reduction is dominant in marine sediments, while acetate fermentation is the major pathway in freshwater sediments (Whiticar et al. 1986).

CH_4 production rates are typically higher than CH_4 emission fluxes to the atmosphere, since aerobic and anaerobic microbial CH_4 oxidation within lacustrine sediments or in water columns are effective processes that limit the amount of CH_4 reaching the atmosphere, in particular when vertical CH_4 transport occurs mainly through diffusive transport, rather than through ebullition. A wide variety of electron acceptors can be used during microbial CH_4 oxidation, including but not limited to oxygen (O_2, Rudd et al. 1974). Micro-organisms using O_2 as electron acceptors belong to the Proteobacteria phylum. The use of an enzyme known as CH_4 monooxygenase (either under a soluble or membrane-bound form) to catalyze the oxidation of CH_4 to methanol is a defining characteristic of aerobic methanotrophs (Hanson & Hanson 1996). Methanol is then oxidized to formaldehyde, which is assimilated to form intermediates of central metabolic routes that are subsequently used for biosynthesis of cell material (Hanson & Hanson 1996 and references therein). Hence, aerobic methanotrophs use CH_4 not only as an energy source, but also as a C source. Aerobic methanotrophs are typically classified into two phylogenetically distinct groups that use different pathways for the formaldehyde assimilation : the type I methanotrophs belong to the *Gammaproteobacteria* and use the ribulose monophosphate pathway while the type II methanotrophs belong to the *Alphaproteobacteria* and use the serine pathway.

Besides aerobic processes, anaerobic CH_4 oxidation coupled with SO_4^{2-} reduction has been found to be carried out by a syntrophic consortium of CH_4-oxidizing archaea and sulphate-reducing bacteria. The association between the archaea and bacteria is commonly interpreted as an obligate syntrophic interaction in which the archaeal member metabolizes CH_4 leading to the production of an intermediate, which in turn is scavenged as an electron donor by its SO_4^{2-} reducing partner (Knitell and Boetius 2009). The identity of the intermediates transferred between the CH_4 oxidizers and the SO_4^{2-} reducers is still uncertain. In contrast to aerobic CH_4 oxidation, the contribution of CH_4 as a C source is minimal as only ~ 1% of the oxidized CH_4 is channelled to biosynthesis pathway and the growth of the partners of the consortium is slow, with generation times of months to years (Knitell and Boetius 2009). Both partners of the consortium are strictly intolerant to O_2 (Knitell and Boetius 2009). Initially reported in marine sediments (Boetius et al. 2000), this consortium was later identified in the water column of marine euxinic basins, such as the Black Sea (Schubert et al. 2006), but rarely in lacustrine systems probably because fresh waters are usually depleted in SO_4^{2-} in comparison with other electron acceptors (NO_3^-, Fe^{3+}, Mn^{4+}) in contrast with the oceans. Nevertheless, it appeared during the last decade that anaerobic CH_4 oxidation could be coupled to a wider variety of electron acceptors that previously thought, including nitrite (NO_2^-), nitrate (NO_3^-), manganese (Mn), and iron (Fe) (Raghoebarsing et al. 2006 ; Beal et al. 2009).

Comment [CMorana1]: Following the comment 1 of reviewer 2, this section was considerably modified to provide more details on microbial CH_4 oxidation and present more recent information on anaerobic CH_4 oxidation processes.

Aerobic methanotrophic organisms not only use CH_4 as electron donors but they are also able to incorporate a substantial fraction of the CH_4-derived C into their biomass, and could therefore contribute to fuel the pelagic food web (Bastviken et al. 2003; Jones and Grey 2011; Sanseverino et al. 2012). A recent study carried out in small boreal lakes (surface area < 0.01

km²) demonstrated that methanotrophic bacterial production (MBP, i.e. incorporation rates of CH_4-derived carbon into the biomass) contributed to 13-52% of the autochtonous primary production in the water column (Kankaala et al. 2013). However, in spite of the potential importance of this alternative C source in aquatic ecosystems, most of the studies carried out in aquatic environments reported gross CH_4 oxidation, while direct measurements of MBP in lakes are still scarce. Also, the methanotrophic bacterial growth efficiency (MBGE), defined as the amount of biomass synthesized from CH_4 per unit of CH_4 oxidized, was found to vary widely in aquatic environments (15-80% according to King 1992 ; 6-77% according to Bastviken et al. 2003), but little is known about the factors driving its variability so that it is currently not possible to derive accurate estimations of the MBP based solely on gross CH_4 oxidation rates. A better understanding of the environmental control of MBGE would help to assess more accurately the importance of methanotrophic organisms as carbon sources for higher trophic level of the food-web.

Lake Kivu, located in a volcanic area, is one of the largest freshwater CH_4 reservoirs, with approximately 60 km³ (at standard temperature and pressure) dissolved in its permanently stratified water (Schmid et al. 2005). One third of the CH_4 accumulated in its deep waters is estimated to be produced via the acetoclastic pathway and two thirds by reduction of geogenic CO_2 (Schoell et al. 1988). Based on a modelling approach, Schmid et al. (2005) estimated that CH_4 production recently increased by threefold since the 1970s for a still unknown reason. Although the deep layers of the lake contain a huge amount of dissolved CH_4, Lake Kivu ranks globally among the lakes with the lowest CH_4 emissions to the atmosphere (Borges et al. 2011). Moreover, the emission of CH_4 from surface waters to the atmosphere (0.038 mmol m^{-2} d^{-1}, Borges et al. 2011) is several orders of magnitude lower than the upward flux of CH_4 to the mixed layer (9.38 mmol m^{-2} d^{-1}, Pasche et al. 2009), suggesting that CH_4 oxidation prevents most of CH_4 to reach the surface of the lake. Our knowledge on bacterial CH_4 oxidation in Lake Kivu has so far been based on circumstantial evidence such as mass balance considerations (Borges et al. 2011; Pasche et al. 2011), identification of *Gammaproteobacteria* aerobic CH_4 oxiders in the oxycline using molecular tools (Pasche et al. 2011), and a few incubations carried out almost 40 years ago (Jannasch 1975).

Because of the permanently stratified nature of its water column and the large amount of CH_4 dissolved in its deep water, the meromictic Lake Kivu offers an ideal natural laboratory to investigate the role of methanotrophy in large tropical lakes. In this study, we used the

Comment [CMorana2]: Following the comment 1 of reviewer 2, this section was considerably modified to provide better modification to our work.

Comment [CMorana3]: Following the comment 3 of reviewer 2, this section was modified to clarify which of the "inherent characteristic of Lake Kivu" make it an ideal natural laboratory to study methanotrophy in large tropical lakes difference in C stable isotope abundance ($\delta^{13}C$) of different C sources to estimate the fraction of CH_4 inputs to the mixed layer from deep waters that is microbially oxidized within the water column, and to quantify the relative contribution of CH_4-derived C to the particulate biomass. Additionally, phospholipid fatty acids (PLFA) and their $\delta^{13}C$ signatures were analyzed to characterize the populations of methanotrophic bacteria present in the water column. We also carried out $^{13}CH_4$-labelling experiments to trace the incorporation of CH_4-derived C into the biomass, to quantify methanotrophic bacterial production, and its conversion to CO_2, to quantify methanotrophic bacterial growth efficiency). Finally, stable isotope probing (SIP) of specific PLFA (SIP-PLFA) after ^{13}C-CH_4 labelling allowed to characterize the bacterial populations active in methanotrophy.

2 Material and methods

2.1. Study site description and sampling

Lake Kivu (East Africa) is a large (2370 km²) and deep (maximum depth of 485 m) meromictic lake. Its vertical structure consists of an oxic and nutrient-poor mixed layer (seasonally variable depth, up to 70 m), and a permanently anoxic monimolimnion rich in dissolved gases (CH_4, CO_2) and inorganic nutrients (Damas, 1937; Degens et al., 1973; Schmid et al., 2005). Seasonal variations of the vertical position of the oxycline are driven by contrasting hygrometry and long wave radiation between rainy (October-May) and dry (June-September) seasons (Thiery et al. 2014), the latter being characterized by a deepening of the oxic zone, and an increased input of dissolved gases and inorganic nutrients into the mixed layer (Sarmento et al. 2006, Borges et al. 2011). Sampling was carried out in the Northern Basin (1.72°S, 29.23°E) in February 2012 (rainy season), and in the Northern Basin and Southern Basin (2.34°S, 28.98°E) in September 2012 (dry season) (Figure 1).

O_2 concentration was measured with a YSI-proODO probe with a optical O_2 sensor (detection limit is 3 μmol L^{-1}), calibrated using air saturated water. Hereafter, "O_2-depleted waters" stands for waters with concentration < 3 µmol L^{-1}. Lake water was collected with a 7 L Niskin bottle (Hydro-Bios) at a depth interval of 5 m from the lake surface to the top of the monimolimnion, at 80 m.

2.2. Chemical analyses

Samples for CH_4 concentrations were collected in 50 ml glass serum bottles from the Niskin bottle with a silicone tubing, left to overflow, poisoned with 100 µl of saturated $HgCl_2$ and sealed with butyl stoppers and aluminium caps. Concentrations of CH_4 were measured by headspace technique (Weiss 1981) using gas chromatography with flame ionization detection (GC-FID, SRI 8610C), after creating a 20 ml headspace with N_2 in the glass serum bottles, and then analyzed following the method described by Borges et al. (2011). Samples for the determination of the $\delta^{13}C$ signature of CH_4 ($\delta^{13}C$-CH_4) were collected in 250 ml glass serum bottles similarly to CH_4 concentration samples. $\delta^{13}C$-CH_4 was determined by a custom developed technique, whereby a 20 ml helium headspace was first created, and CH_4 was flushed out through a double-hole needle, CO_2 was removed with a CO_2 trap (soda lime), and the CH_4 was converted to CO_2 in an online combustion column similar to that in an Elemental Analyzer (EA). The resulting CO_2 was subsequently preconcentrated by immersion of a stainless steel loop in liquid nitrogen in a custom-built cryofocussing device, passed through a micropacked GC column (HayeSep Q 2m, 0.75mm ID ; Restek), and finally measured on a Thermo DeltaV Advantage isotope ratio mass spectrometer (IRMS). Certified reference standards (IAEA-CO1 and LSVEC) were used to calibrate $\delta^{13}C$-CH_4 data.

Comment [CMorana4]: Following the comment 7 of reviewer 2, the material of the tubing used for collecting the waters is now specify in the text

Comment [CMorana5]: Following the comment 5 of reviewer 2, the exact volume of headspace is now specify in the text

Samples for the determination of $\delta^{13}C$ signatures of dissolved inorganic carbon (DIC) were collected by gently overfilling 12 ml glass vial (Labco Exetainer), preserved with 20 μl of saturated $HgCl_2$. For the analysis of $\delta^{13}C$-DIC, a 2 ml helium headspace was created and 100 μl of H_3PO_4 (99 %) was added into each vial to convert all DIC species into CO_2. After overnight equilibration, a variable volume of the headspace was injected into an EA coupled to an isotope ratio mass spectrometer (EA-IRMS; Thermo FlashHT with Thermo DeltaV Advantage). Calibration of $\delta^{13}C$-DIC measurements was performed with certified reference materials (LSVEC and either NBS-19 or IAEA-CO-1).

Samples for particulate organic carbon (POC) concentrations and its stable C isotope signature ($\delta^{13}C$-POC) were filtered on pre-combusted (overnight at 450°C) 25 mm glass fiber filters (Advantec GF-75; 0.3 μm), and dried. These filters were later decarbonated with HCl fumes for 4 h, dried and packed in silver cups. POC and $\delta^{13}C$-POC were determined on an EA-IRMS (Thermo FlashHT with Thermo DeltaV Advantage). Calibration of POC and $\delta^{13}C$-POC was performed with IAEA-C6 and acetanilide, and reproducibility of $\delta^{13}C$-POC measurements, estimated based on triplicate measurements, was typically better than 0.2 ‰.

Comment [CMorana6]: Following the comment 6 of reviewer 2, the text has been modified to specify how the reproducibility of the measurement was estimated

Samples (~ 2 L) for measurements of phospholipid fatty acid concentrations (PLFA) and their $\delta^{13}C$ signature were filtered on pre-combusted 47 mm glass fiber filters (Advantec GF-75; 0.3 µm), and kept frozen until further processing. Extraction and derivatisation of PLFA was performed following a modified Bligh and Dyer extraction, silica column partitioning, and mild alkaline transmethylation as described by Boschker et al. (2004). Analyses were made on a Isolink GC-c-IRMS coupled to a Thermo DeltaV Advantage. All samples were analyzed in splitless mode, using an apolar GC column (Agilent DB-5) with a flow rate of 2 ml min^{-1} of helium as carrier gas. Initial oven temperature was set at 60°C for 1 min, then increased to 130°C at 40°C min^{-1}, and subsequently reached 250°C at a rate of 3°C min^{-1}. $\delta^{13}C$-PLFA were corrected for the addition of the methyl group by a simple mass balance calculation, and were calibrated using internal (C19:0) and external (mixture of C14:0, C16:0, C18:0, C20:0, C22:0) fatty acid methyl ester (FAME) standards. Reproducibility estimated based on replicates measurement was ± 0.6 ‰ or better for natural abundance samples.

2.3. Determination of the isotope fractionation factor

In September 2012 (Southern Basin), the isotope fractionation factor (ε) was estimated by monitoring the changes in CH_4 concentration and $\delta^{13}C$-CH_4 over time in microcosms at several depths (60 m, 62.5 m , 65 m, 67.5 m) across the oxycline. Six glass serum bottles (60 ml) were gently overfilled at each depth and tightly capped with a butyl rubber stopper and an aluminium cap. They were then incubated in the dark at the lake temperature during 0, 24, 48, 72, 96 or 120 h. The incubation was stopped by poisoning the bottles with 100 µl of saturated $HgCl_2$. The measurement of the concentration of CH_4 and the $\delta^{13}C$-CH_4 in every bottle was performed as described before. The isotope fractionation factor was calculated according to Coleman et al. (1981).

2.4. Methanotrophic bacterial production and growth efficiency measurement

At several depths throughout the water column, the methanotrophic bacterial production and methanotrophic bacterial growth efficiency were estimated by quantifying the incorporation of ^{13}C-labelled CH_4 (^{13}C-CH_4, 99.9%, Eurisotop) into the POC and DIC pool. Water from each sampling depth was transferred with a silicone tubing into 12 serum bottles (60 ml), capped with butyl stoppers and sealed with aluminium caps. Thereafter, 4 different volumes (50 μl, 100 μl, 150 μl, or 200 μl) of a ^{13}C-CH_4 gas mixture (1:10 in He) were injected in triplicate and 100 µl of saturated $HgCl_2$ was immediately added to one bottle per gas concentration treatment, serving as control bottle without biological activity. After vigorous shaking, the bottles were incubated in the dark during 24 h at the lake temperature. The incubation was stopped by filtration of a 40 ml subsample on 25 mm glass fiber filters (Advantec GF-75; 0.3 µm) to measure the ^{13}C-POC enrichment, and a 12 ml Exetainer was filled and poisoned with the addition of $HgCl_2$ in order to measure the ^{13}C-DIC enrichment. The exact amount of $^{13}C-CH_4$ added in the bottles was determined from the bottles poisoned at the beginning of the experiment. The measurements of the concentration of POC, the $\delta^{13}C$-POC, the $\delta^{13}C$-DIC and the $\delta^{13}C-CH_4$ were performed as described above. Methanotrophic bacterial production (MBP, µmol L^{-1} d^{-1}) rates were calculated according to Hama et al. (1983) :

$$MBP = POC_f * (\%^{13}C\text{-}POC_f - \%^{13}C\text{-}POC_i)/(t * (\%^{13}C\text{-}CH_4 - \%^{13}C\text{-}POC_i)) \quad (1)$$

where POC_f is the concentration of POC at the end of incubation (µmol L^{-1}), $\%^{13}C\text{-}POC_f$ and $\%^{13}C\text{-}POC_i$ are the percentage of ^{13}C in the POC and the end and the beginning of incubation, t is the incubation time (d^{-1}) and $\%^{13}C\text{-}CH_4$ is the percentage of ^{13}C in CH_4 directly after the inoculation of the bottles with the ^{13}C tracer. The methanotrophic bacterial respiration rates (MBR, µmol L^{-1} d^{-1}) were calculated according to :

$$MBR = DIC_f * (\%^{13}C\text{-}DIC_f - \%^{13}C\text{-}DIC_i)/(t * (\%^{13}C\text{-}CH_4 - \%^{13}C\text{-}DIC_i)) \quad (2)$$

where DIC_f is the concentration of DIC after the incubation (µmol L^{-1}), $\%^{13}C\text{-}DIC_f$ and $\%^{13}C\text{-}DIC_i$ are the final and initial percentage of ^{13}C in DIC. Finally, the methanotrophic bacterial growth efficiency (MBGE, %) was calculated according to :

$$MBGE = MBP/(MBP+MBR) * 100 \quad (3)$$

The CH_4 concentration in the bottles sometimes increased drastically because of the $^{13}C-CH_4$ addition, which could have induced a bias in the estimation of MBP and MBR in case of CH_4-limitation of the methanotrophic bacteria community. However, performing incubation along a gradient of CH_4 concentrations allowed us to assess if the measured MBP and MBR were positively related to the amount of tracer inoculated in the bottles. In case of such an effect (only at 50 m in the Northern Basin in February 2012 and at 60 m in the Southern Basin in September 2012) we applied a linear regression model (r^2 always better than 0.90) to estimate the intercept with the y-axis, which was assumed to correspond to the MBP or MBR rates at in-situ CH_4 concentration.

2.5. Stable isotope probing of PLFA (SIP-PLFA) with $^{13}C-CH_4$

At each sampling depth and in parallel with the MBP measurement, 4 serum bottles (250 ml) were filled with water, overflowed and sealed with butyl stopper and aluminium caps. Bottles were spiked with 500 µl of ^{13}C-CH_4 (99.9%). After 24 h of incubation in the dark at lake temperature, the water from the 4 bottles was combined and filtered on a single pre-combusted 47 mm glass fiber filter (Advantec GF-75; 0.3µm) to quantify the incorporation of the tracer in bacterial PLFA. The filters were kept frozen until further processing. The extraction, derivatisation and analysis by GC-c-IRMS were carried out as described above.

3 Results

3.1. Physico-chemical parameters

In September 2012, the water column in the Southern Basin was oxic (> 3 µmol L^{-1}) from the surface to 65 m (Figure 2a). CH_4 was abundant in deep waters, with a maximum concentration of 899 μmol L^{-1} at 80 m, however CH_4 decreased abruptly at the bottom of the oxycline, being 4 orders of magnitude lower in surface waters (Figure 2a). Consistent with its biogenic origin, CH_4 was depleted in ^{13}C in deep waters ($\delta^{13}C$-CH_4 : -55.0 ‰) but became abruptly enriched in ^{13}C at the transition between oxic and O_2-depleted waters, where CH_4 concentrations sharply decreased, to reach a maximal value of -39.0 ‰ at 62.5 m depth (Figure 2a). The $\delta^{13}C$-POC values mirrored the pattern of $\delta^{13}C$-CH_4: they were almost constant from the surface to 55 m (-24.4 ± 0.3 ‰), then showed an abrupt excursion towards more negative values at the bottom of the oxycline, with a minimum value (-42.8 ‰) at 65 m depth (Figure 2a). Similar results were found in September 2012 in the Northern Basin, where the water was oxic (> 3 μmol L^{-1}) down to 55 m (Figure 2b). At the transition between oxic and O_2-depleted waters, an abrupt isotopic enrichment of the CH_4 was also observed and the $\delta^{13}C$-POC was relatively depleted in ^{13}C, similarly as in the Southern Basin (Figure 2b).

Comment [CMorana7]: Following the comment 8 of reviewer 2, the text has been modified to avoid confusion

Comment [CMorana8]: Following the comment 9 of reviewer 2, the term "oxic-anoxic transition" has been replaced here and elsewhere by "transition between oxic and O_2-depleted waters" as the term anoxic might not be appropriate because of the limit of detection of our oxygen sensor. A clear definition of O_2-depleted waters is given in the material and methods section

In February 2012 in the Northern Basin, the water was oxic (> 3 μmol L^{-1}) until 45 m depth but the O_2 concentrations were below the limit of detection deeper in the water column (Figure 2c). The gradual decrease in the CH_4 concentration between 60 m and 45 m (from 110 μmol L^{-1} to 3 μmol L^{-1}) was accompanied by a parallel increase of the $\delta^{13}C$-CH_4 signature in the same depth interval (from -55.9‰ to -41.7‰), the residual CH_4 becoming isotopically enriched as CH_4 concentration decreased (Figure 2c). $\delta^{13}C$-POC values were also slightly lower below the oxic zone, with a minimum at 50 m (-26.9‰) (Figure 2c).

3.2. Phosphlipid fatty acid concentration and stable isotopic composition

Figure 3 show profiles of the relative concentration and the $\delta^{13}C$ signature of specific PLFA in September 2012 (Figure 3a, 3b ; Southern basin) and February 2012 (Figure 3c, 3d ; Northern Basin). Irrespective of station, season and depth, the C16:0 saturated PLFA was always the most abundant PLFA (18-35% of all PLFA). The relative abundance of the C16 monounsaturated fatty acids (C16 MUFA) significantly increased at the bottom of the oxycline in February and September 2012. The $\delta^{13}C$ signature of the C16 MUFA was comparable to the $\delta^{13}C$ signature of the C16:0 in oxic waters, oscillating around -27‰ or -29‰ in February and September 2012, respectively. However, C16 MUFA were largely depleted in ^{13}C in the oxycline, with minimal $\delta^{13}C$ values as low as -55.3‰ at the transition between oxic and O_2-depleted waters in September 2012, and -49.5‰ in February 2012. This very strong depletion in $\delta^{13}C$ was only observed for this particular type of PLFA (C16 MUFA). The C18 MUFA were slightly more abundant in oxic waters (on average 9%) than in deeper waters (1-4%). Their isotopic composition varied with depth following the same vertical pattern than C16 MUFA, but with a lower amplitude. C18 MUFA minima in $\delta^{13}C$ were observed in O_2-depleted waters in February 2012 (55 m, -35.1‰) and September 2012 (70m, -30.5‰). The relative abundance of iso- and anteiso-branched C15:0 PLFA was systematically low (1-5%) and did not follow any depth pattern. Their isotopic signature was however slightly lower in O_2-depleted waters than in oxic waters.

3.3 Isotope fractionation factor determination

During the isotope fractionation factor experiment, a significant decrease of the CH_4 concentration over time and a parallel enrichment of the residual CH_4 (Figure 4) were monitored in every bottle incubated under oxic conditions. However, no consumption of CH_4 was measured in O_2-depleted waters. The isotope fractionation factor measured at several depths across the oxycline ranged between 1.008 and 1.024, and averaged 1.016 ± 0.007 (n = 5).

3.4. Methanotrophic bacterial production

MBP rates within the oxycline were variable (from 0 to 7.0 μmol C L^{-1} d^{-1}). Maximum values were always observed at the bottom of the oxycline, near the transition between oxic and O_2-depleted waters (Figure 2d, 2e, 2f), however substantial MBP (up to 2.2 μmol L^{-1} d^{-1}) were also recorded in O_2-depleted waters in February 2012 (Figure 2f). Vertically integrated over the water column, MBP rates were estimated at 28.6 mmol m^{-2} d^{-1} and 8.2 mmol m^{-2} d^{-1} in

September 2012 in the Southern and Northern Basin, respectively, and 29.5 mmol m^{-2} d^{-1} in February 2012 in the Northern Basin. MBGE was found to be highly variable in the water column ranging between 50% at 52.5 m in the Northern Basin (September 2012) and 2% at 67.5 m in the Southern Basin (September 2012). Computed from depth-integrated MBP and MBR rates, the water column mean MBGE were 23% in September 2012 in the Southern and Northern Basins, and 42% in February 2012 in the Northern Basin.

Specific CH_4-derived C incorporation rates in PLFA (d^{-1} ; incorporation rates normalized on PLFA concentration) show that bacteria containing C16 MUFA and C14:0 were particularly active in CH_4-derived C fixation in the oxycline in February and September 2012 (Figure 5a, 4b). In contrast, the specific incorporation pattern was dominated by C17 MUFA, and to a lesser extent 10Me16:0 and C16 MUFA in O_2-depleted waters in February 2012 (Figure 5b).

4. Discussion

The sharp decrease of CH_4 concentration and the isotopic enrichment of the residual CH_4 in the oxycline, mirrored by the isotopic depletion of the POC pool at these depths indicated that microbial CH_4 oxidation is a strong CH_4 sink within the water column of Lake Kivu. Similar patterns characterized by a strong isotopic depletion of the POC pool in the oxycline were reported in other systems, such as the meromictic Northern Basin of Lake Lugano (Lehmann et al. 2004, Blees et al. 2014). The fraction of the upward CH_4 flux oxidized within a depth interval can be estimated from a closed-system Rayleigh model of isotope fractionation (Blees et al. 2014) described by the following equation (rearranged from Eq. 11 in Coleman et al. 1981):

$$\ln(1-f) = \ln((\delta^{13}CH_{4t}+1000)/(\delta^{13}CH_{4b}+1000))/((1/\alpha)-1) \quad (4)$$

where f is the fraction of CH_4 oxidized within the depth interval, $\delta^{13}CH_{4b}$ and $\delta^{13}CH_{4t}$ are the $\delta^{13}C$ values of CH_4 at the bottom and the top of the depth interval, respectively, and α is the isotope fractionation factor for CH_4 oxidation estimated in Lake Kivu in September 2012 (α = 1.016 ± 0.007). Based on this equation and using a range of isotope fractionation factors (from 1.009 to 1.023), we can estimate that 51-84% of the upward flux of CH_4 was microbially oxidized within a 10 m depth interval in the oxycline (60-70 m) in the Southern Basin during the dry season (September 2012). Similarly, 51-84% of the CH_4 flux was oxidized between 50 m and 55 m in the Northern Basin during the dry season, and 58-89% of the CH_4 flux was oxidized within a wider depth interval (45-70 m) during the rainy season (February 2012). The relatively wide range of the estimated percentage of CH_4 flux oxidized is due to the uncertainty on the isotope fractionation factor. Nevertheless, these calculations illustrate clearly the importance of microbial CH_4 oxidation processes in preventing CH_4 to reach the surface waters of the lake.

Comment [CMorana9]: Following the comment 11 of reviewer 2, we susbtantilly rewrite this paragraph and revised our calculation. See the point-by-point reply to reviewer comments for further details.

The theoretical $\delta^{13}C$ signature of methanotrophs can be estimated at each depth from $\delta^{13}C$-CH_4 values and the experimental isotope fractionation factor (α, ranged between 1.009-1023). Applying a simple isotope mixing model with the $\delta^{13}C$ signature of methanotrophs as an end-member and the $\delta^{13}C$-POC in the surface (5 m) as a sedimenting organic matter end-member, it is possible to estimate the contribution of CH_4-derived C to the POC pool. Indeed, the contribution of CH_4-derived C appeared to be substantial at the bottom of the mixolimnion. In September 2012 in the Southern Basin, 32-44% of the depth-integrated POC pool in the oxycline (between 60 m and 70 m) originated from CH_4 incorporation, with a local maximum at the transition between oxic and O_2-depleted waters (65 m, 44-54%). In the Northern Basin, 13-16 % of the POC in the oxycline (between 50 m and 60 m) derived from CH_4. However, the contribution of CH_4 to the POC pool was relatively lower during the rainy season, as only 4-6% of the POC in the 50-70 m depth interval, below the oxycline, had been fixed by methanotrophic organisms in the Northern Basin in February 2012 (local maximum slightly below the oxycline at 50 m, 8-10%).

$^{13}CH_4$ tracer experiments allowed estimation of the net MBP and the MBGE. Whatever the season, the highest MBP (0.8-7.2 µmol C L^{-1} d^{-1}) rates were found near the transition between oxic and O_2-depleted waters. Hence, CH_4 oxidation in Lake Kivu seems to be mainly driven by oxic processes. Furthermore, maximal MBP rates were observed where the *in situ* CH_4:O_2 ratio ranged between 0.1 and 10 (molar units, Figure 6), encompassing the stoichiometric CH_4:O_2 ratio for aerobic microbial CH_4 oxidation (0.5) and the optimal ratio estimated in culture experiment (0.9, Amaral & Knowles 1995). This relationship highlights the importance of the regulation of aerobic methanotrophic production by both CH_4 and O_2 availability. Vertically integrated over the water column, the MBP was estimated at 29.5 mmol m^{-2} d^{-1} during the rainy season in the Northern Basin, and 28.6 mmol m^{-2} d^{-1} and 8.2 mmol m^{-2} d^{-1} during the dry season in the Southern Basin and the Northern Basin, respectively. These rates are comparable to the gross CH_4 oxidation rate reported earlier by

Jannasch (1975) in Lake Kivu (7.2 mmol m^{-2} d^{-1}) and the upward CH_4 flux recently estimated (9.38 mmol m^{-2} d^{-1}) by Pasche et al (2009). Areal MBP in Lake Kivu are equivalent to 16-60% of the mean annual phytoplankton primary production (49 mmol m^{-2} d^{-1}, Darchambeau et al. 2014), suggesting that biomass production by methanotrophs has the potential to sustain a significant fraction of the pelagic food-web. For example, it has been shown that cyclopoid copepods (mesozooplankton) of Lake Kivu escape visual predators by migrating below the euphotic zone, sometimes down to O_2-depleted waters (Isumbisho et al. 2006), where they might feed on CH_4-derived C sources.

The relative contribution of MBP to the autochtonous production in Lake Kivu was distinctly higher than those reported in 3 Swedish lakes during summer, where MBP was equivalent to 0.3 and 7.0% of the phytoplankton production (Bastviken et al. 2003). This was unrelated to the phytoplankton production rates in the Swedish lakes that ranged between 7 and 83 mmol m^{-2} d^{-1} and encompassed the average phytoplankton production value in Lake Kivu (49 mmol m^{-2} d^{-1}). The MBP rates in the Swedish lakes (based on ^{14}C incubations) were, however, distinctly lower than in Lake Kivu, ranging between 0.3 and 1.8 mmol m^{-2} d^{-1}. This difference is probably related to the high CH_4 concentrations at the transition between oxic and O_2-depleted waters in Lake Kivu, as MBP peaked in the Swedish lakes at CH_4 concentrations < 100 µmol L^{-1}, while MBP peaked in Lake Kivu at CH_4 concentrations one to two orders of magnitude higher. Kankaala et al. (2013) reported seasonally resolved (for the ice-free period) MBP in five small (0.004 to 13.4 km^2) boreal humic lakes (with dissolved organic C concentrations ranging between 7 and 24 mgC L^{-1}) in southern Finland. In these lakes phytoplankton production and MBP were highly variable, ranging between 5 and 50 mmol m^{-2} d^{-1} and <0.2 mmol C m^{-2} d^{-1} and 41 mmol m^{-2} d^{-1}, respectively. MBP was significantly higher in the two smallest lakes (0.004-0.008 km^2), characterized by high CH_4 concentrations (< 750 µmol L^{-1}) and permanent anoxia throughout the year in bottom waters. Considering a MBGE of 25%, their MBP estimates corresponded to a highly variable percentage of phytoplankton production, between 35% and 100% in the two smallest lakes, and between 0.4% and 5.0% in the three larger lakes (0.04 to 13.4 km^2), and therefore they proposed that the relative contribution of methanotrophic bacteria to the total autotrophic production in a lake is related to its size (Kankaala et al. 2013). However, the results reported for the large (2370 km^2) Lake Kivu do not fit with this general pattern, probably because of the permanent and strong stratification of its water column that on one hand promotes a long residence time of deep waters and the accumulation of CH_4, and on the other hand leads to very slow upward diffusion of solutes, promoting the removal of CH_4 by bacterial oxidation as it diffuses to the surface.

The MBGE found during this study was variable (2-50%), but within the range of reported values in fresh waters (15-80%, King 1992; 6-72 %, Bastviken et al. 2003). MBGE was negatively related to the CH_4:O_2 ratio (Figure 7), i.e., a smaller fraction of the oxidized CH_4 was incorporated into the biomass at the bottom of the oxycline, where O_2 availability was relatively limited compared to CH_4. It has been recently suggested that under O_2-limiting conditions, methanotrophic bacteria are able to generate energy (adenosine triphosphate) by fermentation of formaldehyde (Kalyuzhnaya et al. 2013), the key intermediate in the oxidation of CH_4. This CH_4-based fermentation pathway would lead to the production of excreted organic acids (lactate, formate, ...) from CH_4-derived C instead of converting CH_4 into cellular biomass. If the metabolic abilities for this process are ubiquitous in methanotrophic organisms, it may potentially occur within the water column of Lake Kivu, at the bottom of the oxycline or in micro-oxic zone, as suggested by the low MBGE values found at high CH_4:O_2 molar ratio.

Almost all known aerobic methanotrophic bacteria are phylogenetically affiliated to Proteobacteria, belonging either to the *Gammaproteobacteria* (also referred to type I methanotrophs) or *Alphaproteobacteria* (type II methanotrophs) classes (Hanson & Hanson 1996). The two distinct groups differ in some important physiological characteristics. Notably, they use different C fixation pathway (ribulose monophosphate for type I; the serine pathway for type II) and possess different patterns of PLFA. C16 MUFA are especially abundant in the type I methanotrophs while the type II methanotrophs contain mainly C18 MUFA (Le Bodelier et al. 2009). Therefore, the much larger ^{13}C depletion of C16 MUFA than C18 MUFA and the strong labelling of C16 MUFA during the incubation with ^{13}C-CH_4 indicate that the aerobic methanotrophic community was dominated by type I methanotrophs in the water column during this study. In contrast, Type II methanotrophs did not appear to contribute much to the overall CH_4 oxidation in Lake Kivu, in good agreement with the results of Pasche et al. (2011). Nevertheless, in February 2012 the C16 MUFA appeared to be strongly depleted in ^{13}C below the transition between oxic and O_2-depleted waters (Figure 3). Strong ^{13}C-depletion of bacterial lipid markers for aerobic methanotrophic bacteria in O_2-depleted waters has also been reported in the Black Sea (Schubert et al. 2006) and in Lake

Lugano (Blees et al. 2014). The presence of methanotrophic bacterial biomass below the oxycline could simply result from gravity-driven physical particle transport from oxic waters, but it has been also demonstrated that some aerobic methanotrophs are able to persist under low oxygen conditions in a reversible state of reduced metabobic activity (Roslev and King 1995). In constrast, the recovery of these aerobic methanotrophs after CH_4 deprivation under oxic conditions is less successful because of a significant degradation of cell proteins (Roslev and King 1995). Blees et al. (2014) suggested that this physiological preference for O_2 starvation than CH_4 starvation under oxic conditions would drive aerobic methanotrophs towards the O_2-depleted part of the oxygen continuum. This concept seems particularly important in tropical lakes because the thermal stratification of the water column is usually very dynamic in these systems due to the small temperature gradient, allowing episodic, yet frequent, O_2 intrusion events into deeper waters. Aerobic methanotrophs in dormancy would recover quickly after the episodical O_2 injection, and resume rapidly micro aerobic CH_4 oxidation (Blees et al. 2014).

Comment [CMorana10]: Following the comment13 and 14 of reviewer 2, the text has been amended with this section in order to discuss the observation of C16 MUFA depleted fatty acids in O2-depleted waters. See the point-by-point reply to reviewer comments for further details.

The dominance of type I over type II methanotrophs has been frequently reported in various stratified freshwater (Sundh et al. 1995, Blees et al. 2014) or marine environments (Schubert et al. 2006, Schmale et al. 2012), but this recurrent observation is still difficult to explain. In a recent review, Ho et al. (2013) attempted to classify several genera of methanotrophs according to their life strategies, using the competitor/stress tolerator/ruderal functional classification framework (Grime 1977). Since type I methanotrophs dominate the active community in many environments and are known to respond rapidly to substrate availability, they classified them as competitors, or competitors-ruderals. In contrast, they proposed that type II members would be more tolerant to environmental stress, and thus classified them as stress tolerator, or stress tolerator-ruderal. Relatively large availability of CH_4 and O_2 (O_2:CH_4 ratio close to 1, Figures 2 and 6) at the bottom of the oxycline of Lake Kivu is a favourable environment for the competitor-ruderal bacterial communities that could explain the dominance of type I methanotrophs over type II methanotrophs in this lake.

A significant MBP rate (1.3 µmol L^{-1} d^{-1}) was measured in O_2-depleted waters (< 3 µmol L^{-1}) at 60 m during the rainy season (February 2012). Moreover, the PLFA labelling pattern was drastically different, with a more important specific ^{13}C incorporation into 10Me16:0 and C17 MUFA instead of the C16 MUFA, relative to their concentrations. This different labelling pattern suggests that a different population of methanotrophs was active in CH_4 oxidation deeper in the water column. Archaea lack ester-linked fatty acids in their membrane and are therefore undetectable in PLFA analysis. However 10Me16:0 and C17 MUFA are known to be especially abundant in sulphate-reducing bacteria (Macalady et al. 2000, Boschker and Middelburg 2002), one of the syntrophic partner of anaerobic CH_4 oxidizing archaea (Knittel and Boetius 2009). Hence, the specific labelling of 10Me16:0 and C17 MUFA in O_2-depleted waters could indicates that a fraction of the upward flux of CH_4 was oxidized syntrophically by an archaea/bacteria consortium, and might support the hypothesis that the bacterial partner grow on CH_4-derived carbon source supplied by anaerobic methane oxidizers within the consortium, as already suggested by the results of an in vitro labelling ($^{13}CH_4$) study (Blumenberg et al. 2005). However, our data does not necessary imply that anaerobic methane oxidation would be coupled with SO_4^{2-} reduction, as some sulphate-reducing bacteria have been also found to be able to reduce iron (Coleman et al. 1993). Furthermore, the phylogenetic resolution of SIP-PLFA analyses in rather low (Uhlík et al. 2009), and recent studies showed that anaerobic methane oxidation could be carried out syntrophically by consortium between methanotrophic archaea and denitrifying bacteria (Raghoebarsing et al. 2006), or between methanotrophic archaea and manganese reducing bacteria (Beal et al. 2009). Further investigations would be needed to address more accurately which is the electron acceptors coupled to anaerobic CH_4 oxidation.

Comment [CMorana11]: Following the comment1 of reviewer 1 and the comment 15 of reviewer 2, the text has been amended with this section in order to discuss the limitation of fatty acids analysis (low phylogenetic resolution, non detection of archaeal lipids) and the putative implication of other electron acceptors in anaerobic CH4 oxidation

5. Conclusions

We provide conclusive evidences on the occurrence of CH_4 oxidation in the oxycline of Lake Kivu using stable isotopic characterisation of a suite of carbon pools (CH_4, POC, PLFA) as well as rate measurements (MBP). Vertically integrated MBP ranged between 8 and 29 mmol m^{-2} d^{-1}, and was higher than previously reported in other lakes (Bastvinken et al. 2003, Kankaala et al. 2013). MBP was equivalent to 16-60% of the average annual phytoplankton primary production, a fraction distinctly higher than previously reported in other lakes, usually < 10% (Bastvinken et al. 2003, Kankaala et al. 2006). Hence, methanotrophic bacteria could potentially sustain a significant fraction of the pelagic food-web in this oligotrophic CH_4-rich lake. Lake Kivu ranks globally among the lakes with the lowest CH_4 emissions to the atmosphere (Borges et al. 2011), despite the huge amount of CH_4 dissolved in its deep waters and a relatively high upward flux of CH_4 to the mixed layer (9.38 mmol m^{-2} d^{-1}, Pasche et al. 2009). This apparent paradox is linked to its strong meromictic nature that on one hand promotes a long residence time of deep waters and the accumulation of CH_4, and on the other hand leads to very slow upward diffusion of solutes, promoting the removal of CH_4 by microbial oxidation as it diffuses to the surface.

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8. Figure captions

Figure 1. Map of Lake Kivu.

Comment [CMorana12]: As requested by reviewer 2, a map has been added

Figure 2. Vertical profiles of dissolved O_2 concentration (μmol L^{-1}), CH_4 concentration (~~μmol~~ mmol L^{-1}), $\delta^{13}C$-CH_4 (‰) and $\delta^{13}C$-POC (‰) in Lake Kivu, in September 2012 (dry season) in the Southern Basin (a) and Northern Basin (b), and in February 2012 (rainy season) in the Northern Basin (c). Information about the precision of measurement can be found in the material and methods section. Vertical profiles of methanotrophic bacterial production rates (MBP, μmol L^{-1} d^{-1}) in September 2012 in the Southern Basin (d) and Northern Basin (e) and in February 2012 in the Northern Basin (f). Symbols in (d), (e), and (f) represent mean values, vertical bars represent standard deviation of replicates. The grey zone corresponds to waters with dissolved O_2 concentration < 3 μmol L^{-1}.

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Comment [CMorana13]: Figure has been modified following the recommandation of reviewer 2

Figure 3. Vertical profiles of the relative abundance of phospholipid fatty acids (PLFA, %) and their respective carbon isotopic signature ($\delta^{13}C$-PLFA, ‰) in (a, b) the Southern Basin in September 2012 (dry season) and (c, d) in the Northern Basin in February 2012. The grey zone corresponds to waters with dissolved O_2 concentration < 3 μmol L^{-1}.

Figure 4. Example (62.5 m) of relationship between the $\delta^{13}C$-CH_4 and the fraction of CH_4 remaining in the bottles during the incubation (%) to determine the isotope fractionation factor carried out in September 2012 in the Southern Basin. Data points were gathered at a 24 h interval.

Comment [CMorana14]: Sampling time is now given in the caption, following the recommendation of reviewer 2

Figure 5. Specific CH_4-derived C incorporation pattern into phospholipid fatty acids (PLFA) (incorporation rates of C into PLFA normalized on PLFA concentration, d^{-1}) in (a) September 2012 (dry season) in the Southern Basin and (b) in February 2012 (rainy season) in the Northern Basin. Dissolved O_2 concentration was lower than 3 µmol L^{-1} at 67.5 m and 70 m (a), and 50 m and 60 m (b).

Comment [CMorana15]: Samples incubated under O2-depleted conditions are now clearly identified in the figure caption, as resquested by reviewer 2

Figure 6. In Lake Kivu, relationship between the methanotrophic bacterial production rates (MBP, μmol C L^{-1} d^{-1}) and the *in situ* CH_4: O_2 molar ratio. The ratio was calculated with an O_2 concentration value of 3 μmol L^{-1} when observed in situ values were below the detection limit of the sensor (3 μmol L^{-1}).

Figure 7. In Lake Kivu, relationship between the methanotrophic bacterial growth efficiency and the the *in situ* CH_4: O_2 molar ratio. The ratio was calculated with an O_2 concentration

value of 3 μmol L^{-1} when observed in situ values were below the detection limit of the sensor (3 μmol L^{-1}).

Comment [CMorana16]: An error in the drawing of these two figures has been corrected, they are therefore different

Figure 1.

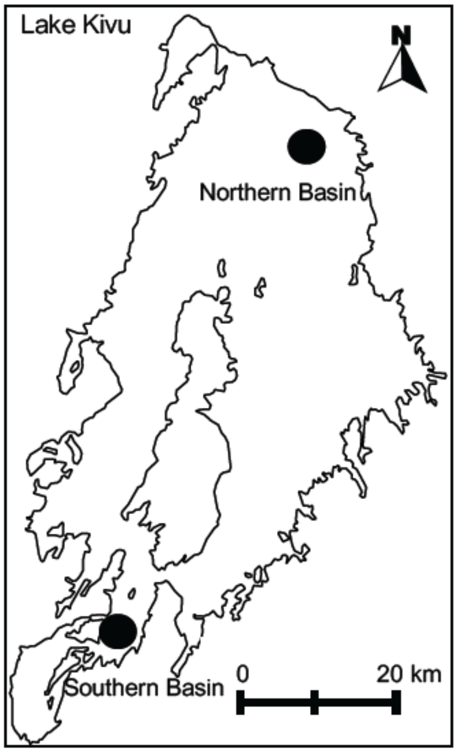
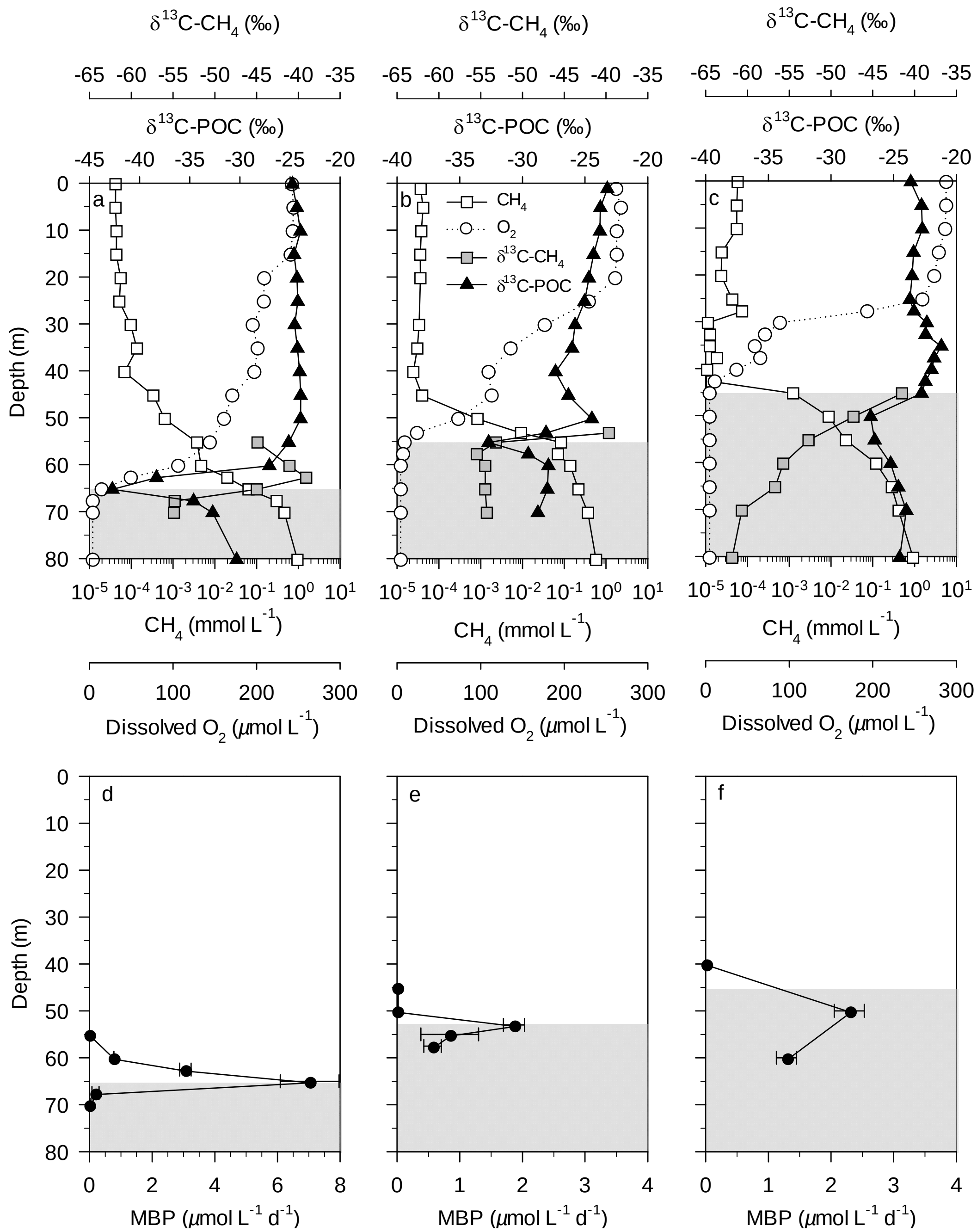


Figure 2.



δ13C-CH4 (‰)
δ13C-POC (‰)
a
b
c
d
e
f
CH4
O2
δ13C-CH4
δ13C-POC
Depth (m)
CH4 (mmol L-1)
Dissolved O2 (μmol L-1)
MBP (μmol L-1 d-1)

Figure 3.

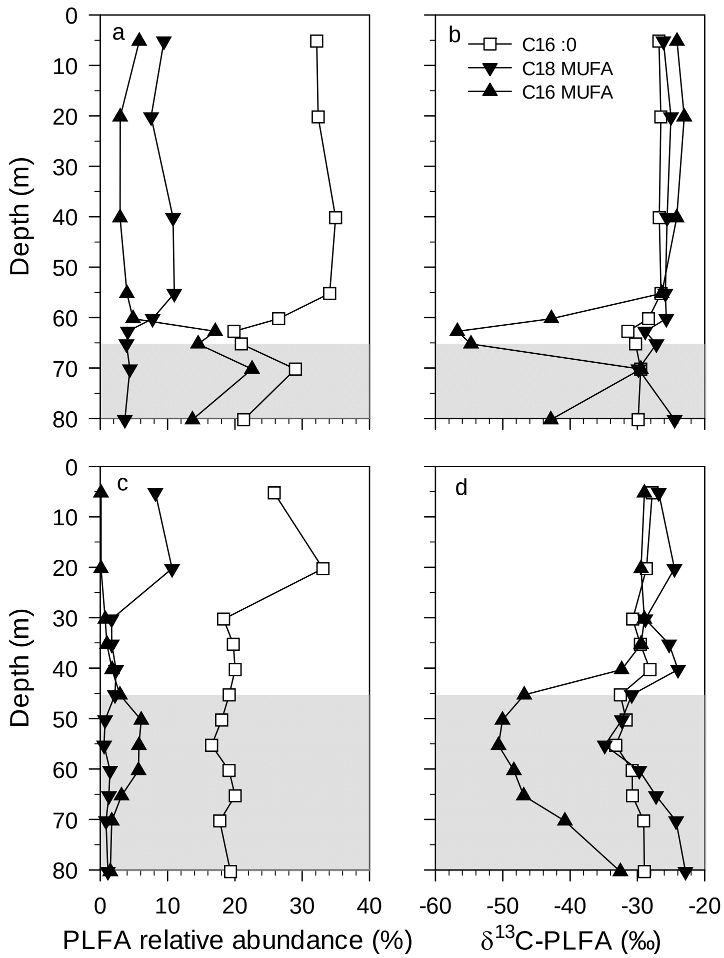
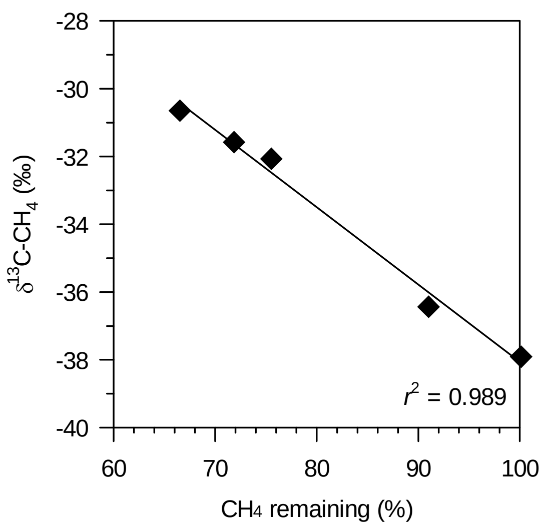
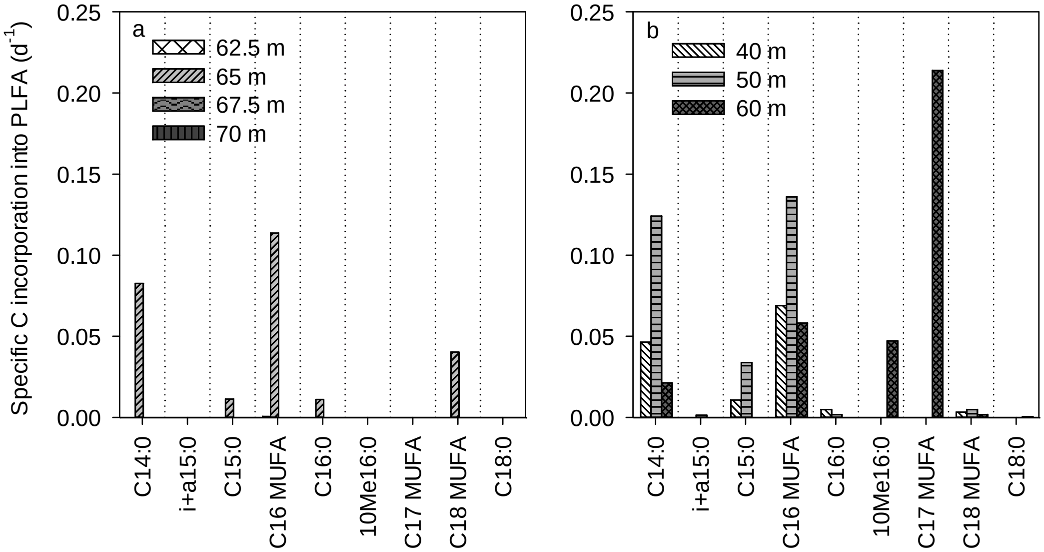

a
b
c
d
C16 :0
C18 MUFA
C16 MUFA
Depth (m)
Depth (m)
PLFA relative abundance (%)
δ13C-PLFA (‰)

Figure 4.



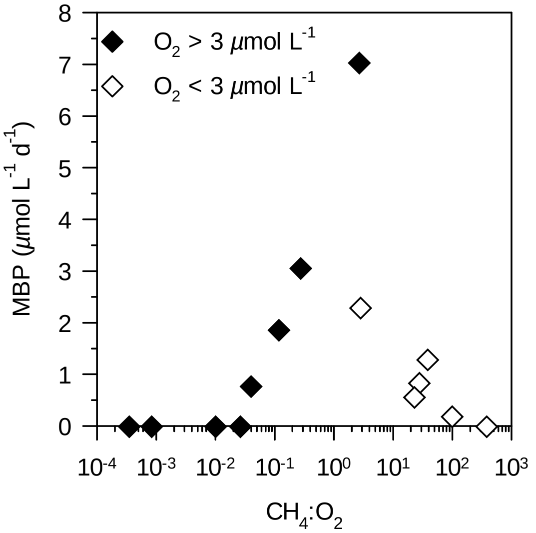
-28
-30
-32
-34
-36
-38
-40
60
70
80
90
100
δ^{13}C-CH_4 (‰)
r^2 = 0.989
CH_4 remaining (%)

Figure 5.



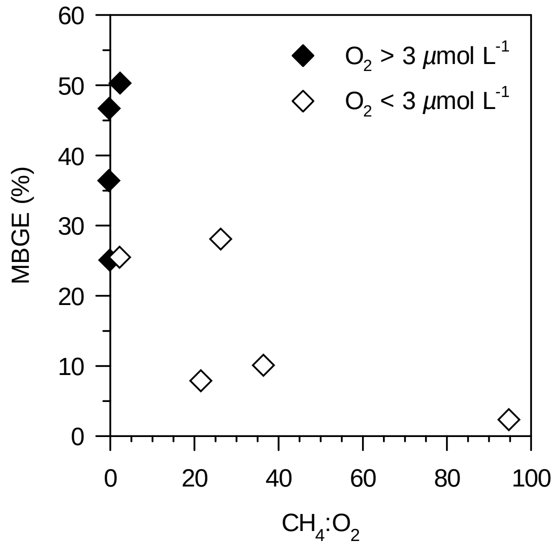
a
62.5 m
65 m
67.5 m
70 m
b
40 m
50 m
60 m
Specific C incorporation into PLFA (d^{-1})
0.25
0.20
0.15
0.10
0.05
0.00
C14:0
i+a15:0
C15:0
C16 MUFA
C16:0
10Me16:0
C17 MUFA
C18 MUFA
C18:0

Figure 6.



O_2 > 3 µmol L^{-1}
O_2 < 3 µmol L^{-1}
MBP (µmol L^{-1} d^{-1})
0
1
2
3
4
5
6
7
8
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
CH_4:O_2

Figure 7.



MBGE (%)
CH_4:O_2
O_2 > 3 μmol L^{-1}
O_2 < 3 μmol L^{-1}
0
10
20
30
40
50
60
0
20
40
60
80
100