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 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). The use of an enzyme known as CH_4 monooxygenase 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), but 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 would be 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).

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 km^2) 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). 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 aerobic CH_4 oxiders using molecular tools (Pasche et al. 2011) and lipid analysis (Zigah et al. 2015), and a few incubations carried out almost 40 years ago (Jannasch 1975).

In this study, we used the 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 CH4 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. The GC-FID was calibrated with CH_4:CO_2:N_2O:N_2 mixtures (Air Liquide Belgium) of 1.05 ± 0.02, 10.2 ± 0.2, 30.3 ± 0.6 and 509 ± 10 ppm CH_4. Precision estimated from multiple injections of gas standards was better than ± 3% for the 1.05 ppm standard and better than ± 0.5% for the other 3 standards. The precision estimated from duplicated samples was ±3.9%.The concentrations were computed using the CH_4 solubility coefficient given by Yamamoto et al. (1976). Samples for the determination of the δ^{13}C signature of CH_4 (δ^{13}C-CH_4) were collected in 250 ml glass serum bottles similarly to CH_4 concentration samples. δ^{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 for $\delta^{13}C$ analysis (IAEA-CO1 and LSVEC) were used to calibrate $\delta^{13}C$-CH_4 data. Reproducibility of measurement estimated based on duplicate injection of a selection of samples was typically better than ± 0.5 ‰, or better than ± 0.2 ‰ when estimated based on multiple injection of standard gas.

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) and the reproducibility of the measurement was always better than ± 0.2 ‰.

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 of standard, was typically better than 0.2 ‰.

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}. δ^{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 δ^{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 δ^{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}), $\%^{13}C$-POC_f and $\%^{13}C$-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 $\%^{13}C$-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}), $\%^{13}C$-DIC_f and $\%^{13}C$-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).

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. Phospholipid 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.

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).

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.

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.

Figure 2. Vertical profiles of dissolved O_2 concentration (μmol L^{-1}), CH_4 concentration (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. Horizontal error bars represent standard deviation of replicates, when larger than the data point size. The grey zone corresponds to waters with dissolved O_2 concentration < 3 μmol L^{-1}.

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. Error bars representing standard deviation of replicates were smaller than the data point size. 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. Symbols are mean of duplicates, error bars represent standard deviation of duplicates when higher than data point size.

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).

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. Symbols represent mean MBP values, vertical error bars represent standard deviation of replicates. The CH_4:O_2 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 *in situ* CH_4:O_2 molar ratio. Symbols represent mean MBGE values, vertical error bars represent standard deviation of replicates. The CH_4:O_2 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 1.

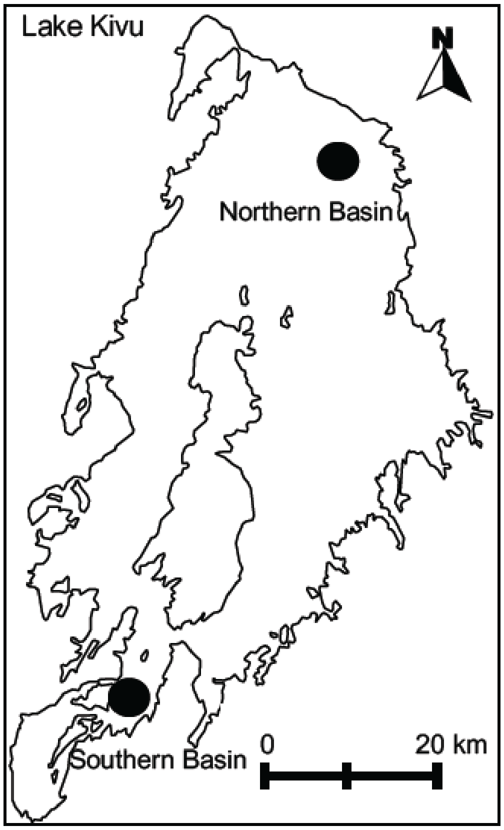


Figure 2.

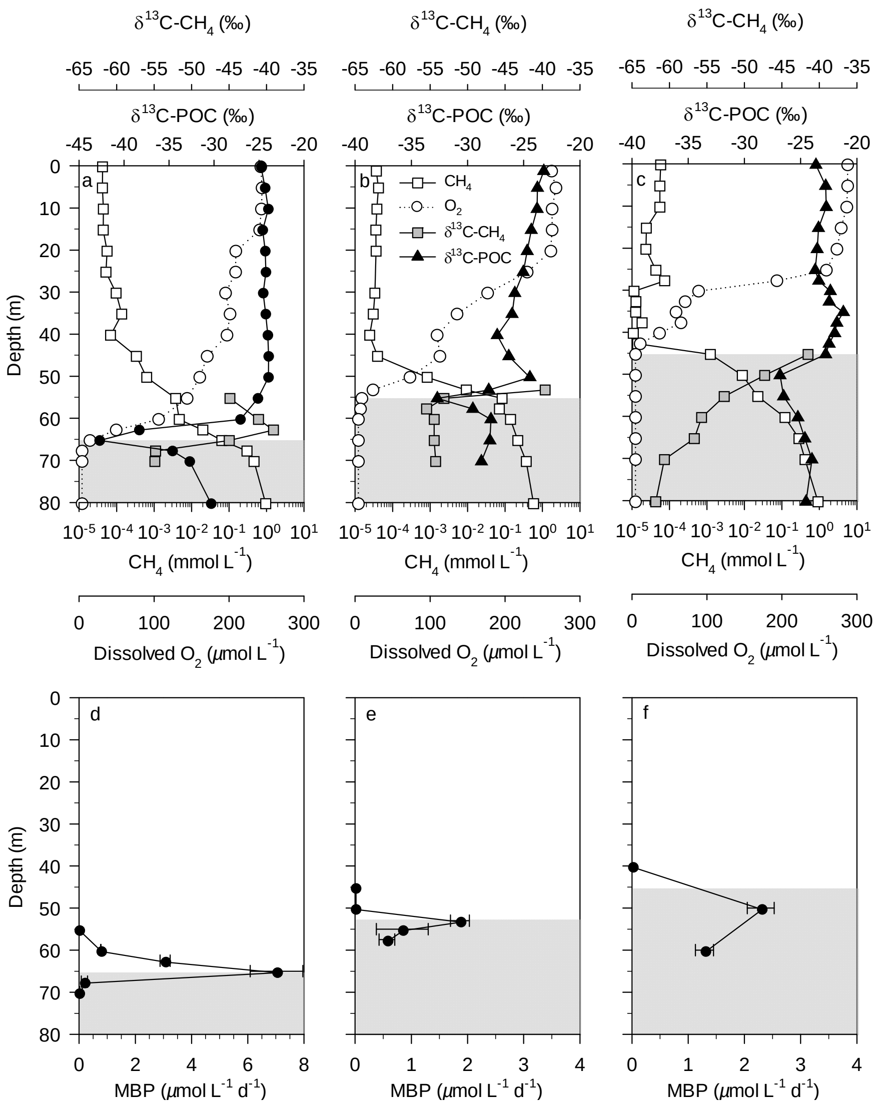
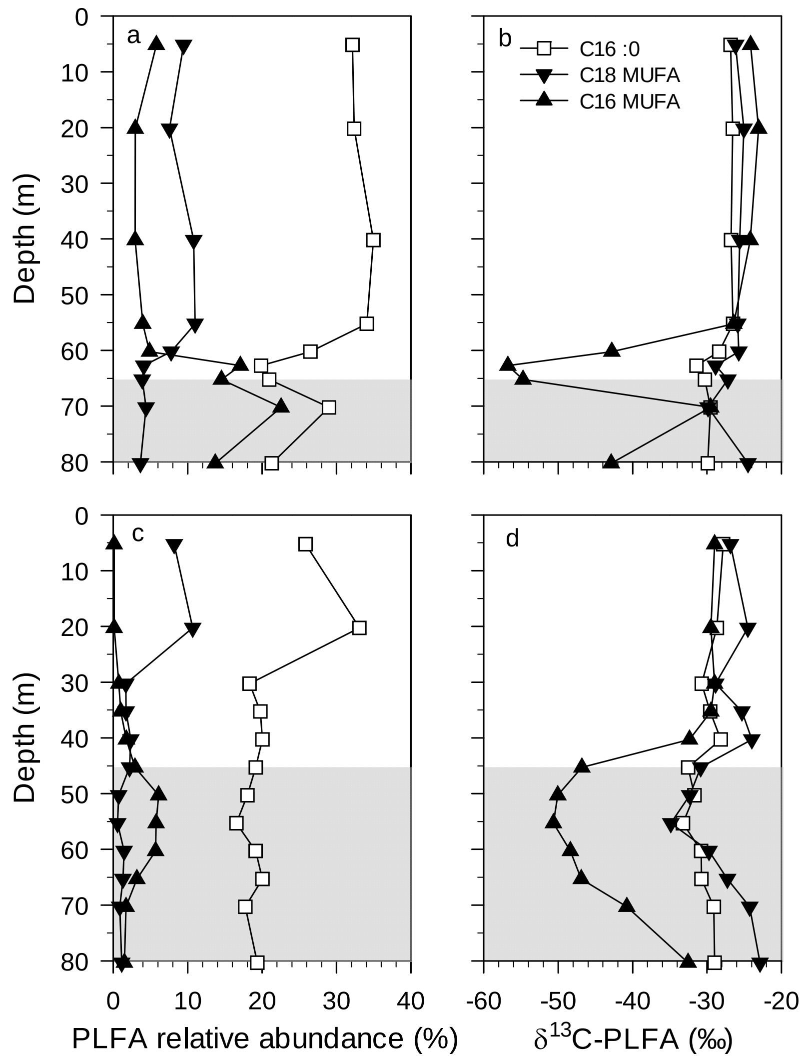
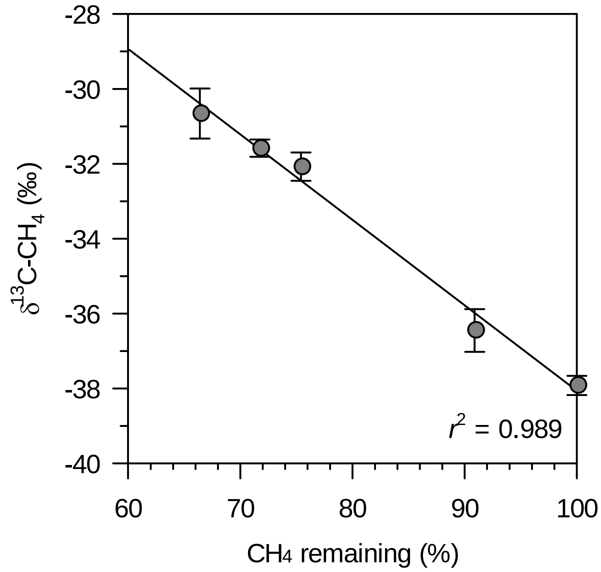

δ13C-CH4 (‰)
-65 -60 -55 -50 -45 -40 -35
δ13C-POC (‰)
-45 -40 -35 -30 -25 -20
-40 -35 -30 -25 -20
a
b
c
CH4
O2
δ13C-CH4
δ13C-POC
Depth (m)
CH4 (mmol L-1)
Dissolved O2 (µmol L-1)
0 100 200 300
d
e
f
MBP (µmol L-1 d-1)

Figure 3.



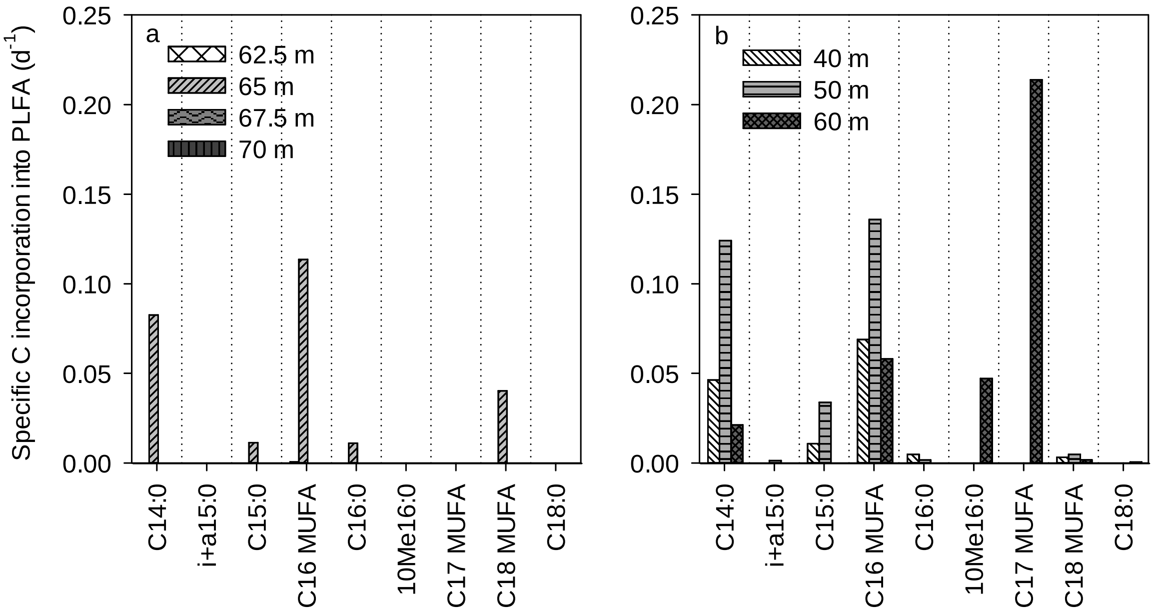
a
b
c
d
C16 :0
C18 MUFA
C16 MUFA
Depth (m)
PLFA relative abundance (%)
δ13C-PLFA (‰)

Figure 4.



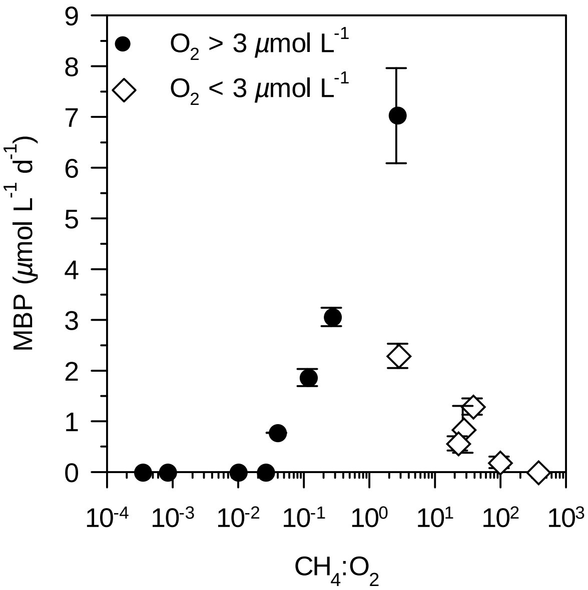
-28
-30
-32
-34
-36
-38
-40
δ13C-CH4 (‰)
r2 = 0.989
60
70
80
90
100
CH4 remaining (%)

Figure 5.



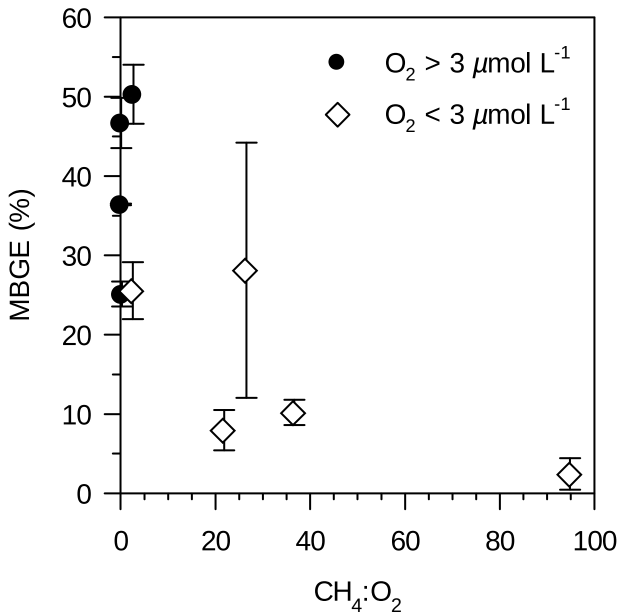
a
62.5 m
65 m
67.5 m
70 m
b
40 m
50 m
60 m
Specific C incorporation into PLFA (d^{-1})
0.25
0.20
0.15
0.10
0.05
0.00
C14:0
i+a15:0
C15:0
C16 MUFA
C16:0
10Me16:0
C17 MUFA
C18 MUFA
C18:0

Figure 6.



O_2 > 3 μmol L^{-1}
O_2 < 3 μmol L^{-1}
MBP (μmol L^{-1} d^{-1})
9
8
7
6
5
4
3
2
1
0
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
CH_4:O_2

Figure 7.



MBGE (%)
60
50
40
30
20
10
0
0
20
40
60
80
100
CH4:O2
O2 > 3 µmol L-1
O2 < 3 µmol L-1