Methane oxidation pathways and associated methanotrophic communities in the water column of a tropical lake

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Abstract

We examined methane (CH4) oxidation pathways and associated methanotrophic communities in the water column of Lake Kivu using abundance and isotopic compositions of CH4 and phospholipid fatty acids (PLFA), distributions of glycerol dialkyl glycerol tetraethers, and catalyzed reporter deposition fluorescence in situ hybridization (CARD–FISH) analysis. The carbon isotopic data of CH4 indicate that aerobic CH4 oxidation is the predominant pathway of microbial CH4 consumption with an isotopic fractionation factor (α) of 1.022–1.038. A small amount of CH4 is oxidized anaerobically, with an α of 1.002–1.006. Aerobic CH4 oxidation is mediated by type II methane–oxidizing bacteria (type II MOB) based on the 13C depletion (δ13C of −40.5‰ to −43.7‰) of diagnostic C18:1ω7 fatty acids in the surface waters. CARD–FISH images and PLFA components C16:1ω7 and C16:1ω5 indicate the presence of type I MOB in the methane and nutrient–rich deep–water region. 13C depletion of C16:1ω7 and C16:1ω5 (δ13C, 238.2‰ to 245.0‰) of anteiso–methyl–C15:0 fatty acid in the lake water below 52 m suggests the involvement of type I MOB in methane oxidation in the anoxic deep–water regions of the lake. A novel cluster of anaerobic methane–oxidizing archaea (ANME) rather than the known ANME–1 and ANME–2 appear to be involved in anaerobic oxidation of methane (AOM). Sulfate reducing bacteria are associated with AOM in the lake based on the 13C depletion (δ13C, −38.2‰ to −45.0‰) of anteiso–methyl–C15:0 fatty acid. Methane constitutes an important carbon and energy source (up to 38%) for the heterotrophic and autotrophic communities in the lake.

The emission of CH4 from freshwater lakes (estimated at 8–48 Tg CH4 per annum) constitutes an important component of the global CH4 budget (about 6–16% of annual global CH4 emissions; Bastviken et al. 2004) and is higher than the annual CH4 emissions from the oceans (2% of annual global CH4 emissions; Reeburgh 2007). The amount of CH4 emitted from freshwater and marine systems to the atmosphere is just a small fraction of the CH4 produced in these systems as microbially mediated CH4 oxidation in the sediments and water columns of these systems mitigates CH4 flux to the atmosphere. Studies show that ~80–90% of CH4 produced in freshwater and marine systems is degraded or removed through anaerobic and aerobic CH4 oxidation (Reeburgh 2007).

Anaerobic oxidation of methane (AOM) can be driven by sulfate as the electron acceptor and is mediated by anaerobic methane–oxidizing archaea (ANME) alone (Milucka et al. 2012) or ANME in a consortium with sulfate reducing bacteria (SRB) (Boetius et al. 2000); this process has been observed in sediments and water columns of marine aquatic systems (Knittel and Boetius 2009). Alternative electron acceptors such as nitrate (Ettwig et al. 2008; Haroon et al. 2013) and nitrite (Ettwig et al. 2010) have been observed to oxidize CH4 in sediments but not in the water columns of aquatic systems, whereas iron and manganese have been observed in the sediments (Beal et al. 2009) and water columns (Crowe et al. 2011).

In contrast to AOM, aerobic methane–oxidizing bacteria (MOB) mediate CH4 oxidation using molecular oxygen (O2) as the terminal electron acceptor (Hanson and Hanson 1996), and the process is thought to be influenced by the concentrations of dissolved CH4, oxygen, copper, and inorganic nitrogen. Previous studies in the water columns of freshwater lakes have reported a half–saturation constant (the concentration needed for oxidation rate of one–half of the maximum rate) of 17–25 μmol O2 L−1 and optimal
Lake Kivu is a meromictic lake in the East African Rift Valley, with a maximum depth and volume of 485 m and 580 km$^3$, respectively. Lake Kivu has a surface area of 2370 km$^2$ and is one of 60 km$^3$ of CH$_4$ at standard temperature and pressure; Schmid et al. 2005). The very high CH$_4$ concentrations in the lake (up to 20 mmol L$^{-1}$; Schmid et al. 2005) make Lake Kivu an ideal system for investigating the microbial communities involved in CH$_4$ oxidation in aquatic environments. CH$_4$ oxidation rates in Lake Kivu have previously been examined by Jan-nasch (1975) and Pasche et al. (2011) and reported to be 31–33 g C m$^{-2}$ yr$^{-1}$. Although Lilrós et al. (2012) provided important insights into the general microbial ecology of the lake, the microbial communities involved in CH$_4$ oxidation in the lake have only been examined by Pasche et al. (2011). Using particulate CH$_4$ monooxygenase (pmoA) gene analysis, Pasche et al. (2011) reported that a type X MOB (Methylococcus capsulatus) was involved in aerobic methane oxidation in the lake, although they also pointed out that their primer set has a potential bias toward type X MOB and that diverse communities could be involved in aerobic methane oxidation in the lake. Also, Pasche et al. (2011) used denaturing gradient gel electrophoresis (DGGE) and methyl coenzyme M reductase (mcrA) gene analysis and noted that the SRB Desulfocapsa and an ANME Methanomicrobiales may be involved in anaerobic methane oxidation coupled to sulfate reduction in the anoxic region of the water column.

In this study, we sought to enhance our understanding of the methane oxidation pathways and methanotrophic communities in Lake Kivu using compound-specific isotope analysis and microscopy. We addressed the following research questions in Lake Kivu: (1) What is the predominant pathway of CH$_4$ oxidation in the water column? (2) How much CH$_4$ is entering the food web of the lake? (3) Which aerobic methanotrophs are mainly responsible for most of the CH$_4$ oxidation in the oxic zone? and (4) Which microbial communities are involved in AOM in the lake? We used concentration and carbon isotopic composition of dissolved CH$_4$ (and dissolved inorganic carbon [DIC]) to examine the predominant pathway (aerobic vs. anaerobic) of CH$_4$ oxidation in the lake. The carbon isotopic composition of biomarkers of heterotrophic (and autotrophic) organisms including phy-tol (3,7,11,15-tetramethyl-2-hexadecen-1-ol), brassicasterol (24-methyl cholesterol-5,22-dien-3$\beta$-ol), dinosterol (4a,23,24-tri-methyl-5z-cholest-22E-en-3$\beta$-ol), and cholesterol (cholest-5-en-3$\beta$-ol) (Wakeham et al. 1997; Schubert et al. 1998) was used to determine whether CH$_4$ is a significant carbon and energy source for the lake food web. Finally, the microbial communities associated with CH$_4$ oxidation in the lake were characterized from a detailed vertical profile of suspended particulate matter (SPM) from the lake water column by using CARD-FISH and the distribution and carbon isotopic composition of PLFA, archaeol (2,3-Di-O-phytanyl-sn-glycerol) and glycerol diakyl glycerol tetraethers (GDGTs).

Methods

**Study site**

Lake Kivu has a surface area of 2370 km$^2$ and is one of the deep lakes in the East African Rift Valley, with a maximum depth and volume of 485 m and 580 km$^3$, respectively.
The lake is surrounded by active volcanoes (e.g., Nyiragongo in DR Congo) and is fed by subaquatic springs entering the lake at different depths below 120 m water depth (Schmid et al. 2005). Approximately 130 rivers within the lake catchment feed the lake with ~ 2.4 km$^3$ yr$^{-1}$ of water (Muvundja 2010). The lake is meromictic with the oxic mixolimnion at ~ 30 m in the rainy season (September–May) and 60 m in the dry windy season (June–August). The anoxic monimolimnion has a hydrologic residence time of 800–1000 years and is rich in nutrients and dissolved CH$_4$ (Schmid et al. 2005). Most of the CH$_4$ in the lake (~ 65%) is thought to be of geogenic origin (Pasche et al. 2009). Primary production in the lake is dominated by diatoms during the dry season and by cyanobacteria during the rainy season (Sarmiento et al. 2012). Based on phylogenetic analysis of planktonic archaea, most of the Euryarchaeota in the deep anoxic waters are reported to belong to methanogenic lineages (Methanosarcinales and Methanocellales) (Llirós et al. 2010).

Previous investigations across the various provinces of the lake show that the offshore regions of the main basin of Lake Kivu are homogeneous, exhibiting little horizontal variability in terms of trace metals (e.g., zinc, copper, iron, and manganese) and physicochemical properties (e.g., temperature, pH, dissolved organic carbon, dissolved oxygen) (Julius 2011). Other properties that are similar in different regions of the lake include the chlorophyll concentration and primary production (Sarmiento et al. 2012), nutrient concentrations and internal recycling (Pasche et al. 2009), as well as CH$_4$ concentrations and emissions (Borges et al. 2011), and distribution of crenarchaeota and methanogens (Llirós et al. 2010). The aerobic CH$_4$ oxidation rate has been estimated at 31–33 g C m$^{-2}$ yr$^{-1}$ (Jannasch 1975; Pasche et al. 2011), and CH$_4$ emission rates amount to as little as 0.01 g C m$^{-2}$ yr$^{-1}$ (Borges et al. 2011). Sulfate (SO$_4^{2-}$) concentration is reported to be ~ 0.15 mmol L$^{-1}$ in the oxic surface waters and decreases sharply from 0.14 mmol L$^{-1}$ to 0.01 mmol L$^{-1}$ between 60 m and 90 m, with values below detection at 100 m (Pasche et al. 2009). Previous studies have reported methane $^{13}$C values of ~$-60^\circ$ below 90 m, increasing to ~$-43^\circ$ in the oxic surface waters in the lake (Pasche et al. 2011).

**Sample collection**

The lake was sampled from the permanent sampling platform kept by the Lake Kivu Monitoring Program, Ministry of Infrastructure, Gisenyi, Rwanda (1.074°S, 22.226°N; Fig. 1) between 14 January 2013 and 30 January 2013. This site at the northern main basin of the lake (total water depth ~ 350 m) was chosen to take advantage of the data from adjacent sediment trap moorings. Depth profiles of temperature, conductivity, pH, and turbidity were obtained in situ with a RBR multiparameter probe (RBR XRX-620). Water samples for CH$_4$ concentration and CH$_4$ stable carbon isotopic composition ($^{13}$C–CH$_4$) were transferred directly from the Niskin bottle into precleaned 120 mL serum bottles by rinsing twice with sample and then overflowing with two volumes of the sample. Samples were immediately poisoned with cupric chloride (CuCl$_2$; Pasche et al. 2011), and sealed airtight with butyl-rubber stoppers and aluminum crimp caps. All samples were measured within six weeks of collection.

Water samples for DIC concentration were collected by overflowing two volumes of the lake water into 1 L precombusted (~ 4 h at 450°C) glass bottles; aliquots of these samples were immediately filtered via 20 mL syringe and syringe-filters (Millex-GP PES membrane, 0.22 μm pore size) into 12 mL air-tight exetainers and preserved with cupric chloride (Pasche et al. 2011). For measuring stable carbon isotopes of DIC ($^{13}$C-DIC), ~ 1-mL aliquots of syringe-filtered water were collected directly into air-tight capped 12 mL exetainers prefilled with ~ 0.5 mL of concentrated phosphoric acid and preflushed with helium gas to remove carbon dioxide. DIC and $^{13}$C-DIC samples were kept cool in the dark until analyses. DIC samples were measured within four weeks of collection.

SPM samples were obtained using an in situ filtration device (Water Transfer System; McLane Laboratories) to filter 41–135 L of lake water through precombusted double-packed Whatman GF/F filters (142 mm diameter; 0.7 μm nominal pore size). The filters with the retained particulate matter (SPM sample) were folded and placed in previously combusted aluminum foil and stored frozen at ~20°C until analysis. To ensure that the SPM samples remained frozen during transport (by air) to Switzerland, the frozen filters were stored between frozen cooling elements in a cooler.

**Measurement of dissolved O$_2$, S(II), NH$_4^+$, and NO$_2$**

Oxygen concentrations were measured in situ using needle-type optodes (Presens). Winkler concentrations were 237.5 ± 6.3 μmol L$^{-1}$ at 2 m depth and were used to calibrate the optodes. The optode logging system consisted of a EOM-O2-mini transducer (Presens), which was recorded by an additional data logger (Schmid Engineering). Details on the electrodes and logging system can be found in Kirf et al. (2014). The detection limit of the trace oxygen probe was 0.02 μmol L$^{-1}$. Concentrations of S(II), NH$_4^+$, and NO$_2$ were measured in lake water from selected depths using standard protocols as described by Pasche et al. (2009).

**Measurements of CH$_4$ and DIC concentrations**

CH$_4$ concentrations were measured by the headspace analysis. In brief, a headspace of 30 mL was created with N$_2$ gas; after equilibration in an ultrasonic bath with the samples stored upside down, the CH$_4$ concentration in the headspace was measured on a gas chromatograph (GC; Agilent 6990) with a flame ionization detector (FID). DIC concentration was measured on a Shimadzu total organic carbon (Shimadzu TOC-L$_{CSH}$/CPH) analyzer. The analyzer was calibrated with primary-standard grade sodium carbonate and sodium bicarbonate. The inorganic carbon in the sample was
volatilized with 25% (by weight) phosphoric acid in a CO$_2$-free closed reaction vessel and the evolved CO$_2$ was measured by a nondispersive infrared detector.

**Measurement of stable carbon isotopes of CH$_4$, DIC, and SPM**

Stable carbon isotope distributions in CH$_4$ samples were measured using an Isoprime isotope ratio mass spectrometer (IRMS) coupled to a trace gas preconcentrator (Micromass UK). An in-house CH$_4$ standard was analyzed at the beginning and after every four samples to assess instrumental performance. Isotopic ratios are reported in the standard delta notation relative to Vienna Pee Dee Belemnite. Instrumental precision based on multiple analyses of this CH$_4$ standard was $\sim 0.5\%_{\text{oo}}$. The stable carbon isotopic composition of DIC was measured using a GasBench II coupled to a Delta V IRMS with a ConFlo IV interface (Thermo Fisher Scientific). Typical instrumental precision based on multiple analyses of an external standard was 0.2$\%_{\text{oo}}$. SPM samples were freeze-dried, homogenized, and fumigated with 12 mol L$^{-1}$ HCl overnight to remove carbonate; they were then oven-dried at 60°C and cooled in a desiccator. Portions were loaded into tin capsules and analyzed for stable carbon ($\delta^{13}$C) and nitrogen ($\delta^{15}$N) isotopes on an elemental analyzer (EA) coupled to an Isoprime IRMS (Micromass). The EA-IRMS was calibrated with acetonilide (Merck), sulfanilic acid (Sigma Aldrich), and

![Map of Lake Kivu showing sampling site (star symbol) in the main basin of the lake.](image)
an in-house sediment standard. Typical instrumental precision based on multiple analyses of external standards was 0.2% for δ13C and 0.3% for δ15N.

Lipid biomarker analyses

SPM samples were extracted with methylene chloride (DCM) and methanol (MeOH) (DCM : MeOH 7 : 3, v : v) by microwave digestion (MLS 1200 Mega) to obtain total lipid extracts. The lipid extract was eluted over an activated carbon column to remove elemental sulfur (S) and through a sodium sulfate (Na2SO4) column to remove water. The neutral fraction was further separated into polar and apolar fractions using an NH2 column over a Na2SO4 column. The neutral fraction was further separated into polar and apolar fractions using an NH2 column with hexane : DCM (9 : 1, v : v) and DCM : isopropanol (99 : 1, v : v) for 5 min and then with a linear gradient to 18% hexane : isopropanol (9 : 1, v : v) over 34 min with a flow rate of 0.2 mL min−1.

Compound-specific isotope analyses (CSIA)

Compound-specific carbon isotopic compositions (δ13C) of FAMES and silylated alcohols were analyzed on an Isoprime IRMS coupled to a GC (Agilent 6890N) with a combustion interface. The extracted compounds were dissolved in ethyl acetate prior to separation by GC using the same GC column and temperature programs used for the quantification of the compounds by GC-FID. Samples were analyzed in duplicate. In-house standards (methyl stearate and heptadecane) with known δ13C were used to assess and correct for isotopic fractionation during GC separation. Measured δ13C values of compounds were also corrected for methyl and trimethyl carbon introduced during derivatization during isotopic-mass balance calculation. Instrumental precision of δ13C based on duplicate analyses was ≤1%.

Calculations of biomarker source and isotope fractionation

The contribution of CH4-derived carbon or methanotrophic bacterial biomass to biomarkers including phytoplankton (non-specific phytoplankton including cyanobacteria), dinosterol (diatoms and dinoflagellates), brassicaterol (diatoms), and cholesterol (e.g., zooplankton) was estimated using a binary mixing model as follows:

\[
\delta^{13}C_{\text{Biomarker}} = f \times \delta^{13}C_{\text{CH}_4-C} + (1 - f) \times \delta^{13}C_{\text{NonCH}_4-C}
\]

where \(f\) denotes the fraction of CH4-derived carbon in the biomarker, \(1 - f\) stands for the fraction of non-CH4 carbon in the biomarker, and the subscripts “CH4−C” and “Non CH4−C” refer to methane-derived carbon and non-methane carbon, respectively. We used a δ13C value of −78‰, as the methane-derived carbon endmember based on our measured deep-water (200 m) methane δ13C of −62‰ and assuming a fractionation of −16‰ (Summons et al. 1994; Bastviken et al. 2003). For the non-methane carbon endmember, we used a δ13C value of −23‰ computed from the δ13C value of CO2(air) (−8‰) based on our measured average surface water [\(<40\) m] DIC-δ13C of ~4‰ as CO2(air) is typically 13C-depleted by ~10‰ relative to the bulk DIC; McCa1ister and del Giorgio 2008) and assuming an algal photosynthetic fractionation of 17‰. We did not use the typical C3 algal
photosynthetic fractionation of 20\(^\%\) because several studies have observed lower fractionation (~17\%) in lakes (Kluijver et al. 2014; McCallister and del Giorgio 2008).

The fractionation factors (\(\alpha\)) for aerobic CH\(_4\) oxidation and anaerobic CH\(_4\) oxidation were calculated after Hayes (1993) as:

\[
\alpha = \frac{\delta^{13}C_{\text{Sub-CH}_4} + 1000}{\delta^{13}C_{\text{Prod-CH}_4} + 1000}
\]  

(2)

where the subscript “Sub-CH\(_4\)” refers to substrate CH\(_4\) before microbial oxidation and “Prod-CH\(_4\)” indicates the product CH\(_4\) left behind after microbial oxidation.

The proportional contribution of methanotrophic lipids to the PLFA was estimated using a binary isotopic mixing model below:

\[
\delta^{13}C_{\text{PLFA}} = f \cdot \delta^{13}C_{\text{methanotrophic}} + (1 - f) \cdot \delta^{13}C_{\text{non-methanotrophic}}
\]  

(3)

where “PLFA” is phospholipid fatty acids, \(f\) is the proportion of methanotrophic lipids, and \((1 - f)\) is the proportion of non-methanotrophic lipids. We used a \(\delta^{13}C\) value of −23\(\%\) (as explained in Eq. 1) as the non-methanotrophic lipid end-member and a \(\delta^{13}C\) value of −78\(\%\) (as explained in Eq. 1) as the methanotrophic lipid end-member for the PLFA of type I and type II MOBs. As a much larger isotope fractionation (≥35\%) is associated with biomass synthesis by methanotrophic archaea during AOM (Blumenberg et al. 2004; Niemann and Elvert 2008), we used a \(\delta^{13}C\) value of −97\(\%\) (based on our measured 200 m methane \(\delta^{13}C\) of −62\(\%\)) and assuming a fractionation of −35\(\%\)) as the methanotrophic lipid end-member for the PLFA of SRB.

**Statistical analyses**

We used STATISTICA (StatSoft) for the analyses. Statistical significance was tested at the 95% confidence level.

**Quantification of bacterial and archaeal cells using CARD-FISH and 4',6-diamidino-2-phenylindole**

Five milliliter of lake water from 35 m, 50 m, 60 m, and 70 m (water-column depths) were fixed with formaldehyde (2\%, \(v : v\)) overnight at 4°C in the dark. Samples were then filtered onto 0.2 \(\mu\)m polycarbonate GTTP filters (Millipore) and stored at −20°C until further processing. CARD-FISH

**Table 1.** CARD-FISH probes used in this study along with their specificity, applied permeabilization, \%(v : v) formamide in the hybridization buffer, and reference.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Specificity</th>
<th>Permeabilization</th>
<th>% formamide</th>
<th>Probe sequence (5'—3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARCH915</td>
<td>Most archaea</td>
<td>Proteinase K</td>
<td>35</td>
<td>GTG CTC CCC CGC CAA TAC CT</td>
<td>Stahl and Amann 1991</td>
</tr>
<tr>
<td>AAA-FW-641</td>
<td>AOM-associated archaea, freshwater cluster</td>
<td>Proteinase K</td>
<td>60</td>
<td>GGT CCC AAG CCT ACC AGT</td>
<td>Schubert et al. 2011</td>
</tr>
<tr>
<td>AAA-FW-834</td>
<td></td>
<td>Proteinase K</td>
<td>60</td>
<td>TGC GGT CGC ACC GCA CCT</td>
<td>Schubert et al. 2011</td>
</tr>
<tr>
<td>ANME-1-350</td>
<td>ANME-1</td>
<td>Proteinase K</td>
<td>40</td>
<td>AGT TTT CGC GCC TGATGC</td>
<td>Boetius et al. 2000</td>
</tr>
<tr>
<td>ANME-2-538</td>
<td>ANME-2</td>
<td>SDS</td>
<td>40</td>
<td>GCC TAC CAC TCG GGC</td>
<td>Treude et al. 2005</td>
</tr>
<tr>
<td>EUJ338 I-III</td>
<td>Most bacteria</td>
<td>Lysozyme</td>
<td>35</td>
<td>GCT GCC TCC CGT AGG AGT GCA GCC ACC GTT GCT GCT ACC CGT AGG TGG</td>
<td>Daims et al. 1999</td>
</tr>
<tr>
<td>Alf 968</td>
<td>alphaproteobacteria</td>
<td>Lysozyme</td>
<td>35</td>
<td>GGT AAG GTT CTG CCG GIT</td>
<td>Neef 1997</td>
</tr>
<tr>
<td>Ma450</td>
<td>Type II methanotrophs</td>
<td>SDS</td>
<td>20</td>
<td>ATC CAG GTA CCG TCA TTA TC</td>
<td>Eller and Frenzel 2001</td>
</tr>
<tr>
<td>Mgamma705</td>
<td>Type I methanotrophs</td>
<td>Lysozyme</td>
<td>20</td>
<td>CTG GTG TTT CAG ATC</td>
<td>Eller and Frenzel 2001</td>
</tr>
<tr>
<td>Mgamma84</td>
<td></td>
<td>SDS</td>
<td>20</td>
<td>CCA GTT GTC AGC GCC</td>
<td>Eller and Frenzel 2001</td>
</tr>
<tr>
<td>NON338</td>
<td>Negative control</td>
<td>Lysozyme</td>
<td>35</td>
<td>ACT CCT ACG GGA GCC</td>
<td>Wallner et al. 1993</td>
</tr>
</tbody>
</table>

**Zigah et al.**

Lake Kivu methane oxidation pathways

**Table 1.** CARD-FISH probes used in this study along with their specificity, applied permeabilization, \%(v : v) formamide in the hybridization buffer, and reference.
was performed using horseradish peroxidase (HRP)-labeled oligonucleotide probes to target specific microbial groups. Details of the probes (purchased from Biomers, Ulm, Germany) used for this study along with the respective formamide concentrations of hybridization buffer are provided in Table 1. Permeabilization with lysozyme (10 mg mL⁻¹) was done for 1 h at 37°C. With proteinase K (15 µg mL⁻¹) and sodium dodecyl sulfate (SDS; 0.5%, v:v) cell walls were permeabilized for 3 min and 10 min at room temperature, respectively. Filter pieces were then bleached with 0.1 mol L⁻¹ HCl for 10 min at room temperature for inactivation of endogenic peroxidase activity. Hybridization with the respective probes and corresponding hybridization buffers was completed for 2.5 h at 46°C. Probes were used in a 1 : 1 mix where the same group was targeted, that is, AOM-associated archaea (AAA-FW) (Schubert et al. 2011) and type I methanotrophs (Mγamma) (Eller and Frenzel 2001). Probes were amplified with the tyramide Oregon Green 488 (1 μl mL⁻¹) for 30 min at 37°C. Finally, filters were counterstained with 4’,6-diamidino-2-phenylindole (DAPI) (1 μl mL⁻¹) and dried before they were embedded (4 : 1, Citifluor : Vectashield) and mounted onto glass slides. Cells were enumerated with an epifluorescence microscope (Zeiss Axioskop 2) by counting 20 independent fields of view. On average, 200 cells per field of view were counted with DAPI and probes EUB338 I–III (Daims et al. 1999). If signals were detected, averages of 10 cells per field of view were counted for all other probes used (Table 1). The percentages of the probe signals were calculated based on total cell numbers, which were counted with DAPI. Background signal (negative control) based on the probe NON338 (Wallner et al. 1993) was negligible in all the samples.

**Results**

**Temperature, dissolved oxygen, and reduced species**

Dissolved O₂ concentration in the surface waters (≤ 13 m) was ~237 µmol L⁻¹ (Fig. 2a). The depth profile of dissolved O₂ shows two oxyclines; one at 13–26 m where the concentration decreased slightly and steadily from 237 µmol L⁻¹ to 210 µmol L⁻¹, and the other at 26–31 m showing a sharp decrease in oxygen concentration from 237 µmol L⁻¹ to 30 µmol L⁻¹ (Fig. 2a). The dissolved O₂ level remained fairly stable at 30 µmol L⁻¹ until 48 m where the value dropped to 1 µmol L⁻¹ (Fig. 2a). Concentrations below the sensor detection limit of 0.02 µmol L⁻¹ (referred to as “anoxic” throughout the rest of the article) were observed below 52 m water depth (Fig. 2a). The temperature profile exhibited two thermoclines, decreasing gradually from 25.5°C to 23.9°C at 1–25 m depth layer and a slight but sharp decline from 23.9°C to 23.4°C at ~26–32 m (Fig. 2a). The temperature was around 23°C in the deep waters (below 50 m) and showed an increasing trend with depth after 80 m (Fig. 2a). Concentrations of the reduced species showed a pronounced chemo-cline in the deep anoxic waters (Fig. 2b). The deep source of the reduced species was reflected in the increase with depth of the concentration of NH₄ from 18 µmol L⁻¹ to 434 µmol L⁻¹, of S(II) from 9 µmol L⁻¹ to 196 µmol L⁻¹, and of NO₂⁻ from 0.1 µmol L⁻¹ to 38.0 µmol L⁻¹ within the anoxic depths 60–90 m (Fig. 2b). The values of ammonium and sulfide were low within the oxic-anoxic transition zone (31–52 m) and negligible in the oxic region of the lake (Fig. 2b).

**Stable carbon and nitrogen isotopic composition of SPM**

The SPM from the lake at 60–90 m showed relatively more ³¹C-depletion (δ³¹C, -29.8%o to -28.1%o) than the SPM in the region of 5–55 m (δ³¹C, -25.4%o to -21.9%o) and in the deeper layers at 140–200 m (δ³¹C, -24.4%o to -24.1%o) (Fig. 3). The SPM in the surface 5–10 m was more ¹⁵N-depleted (δ¹⁵N, 0.1–0.9%o) than the other depth regions in the lake (Fig. 3). The most ¹⁵N-enriched SPM (δ¹⁵N of 7.4–9.6%o) was observed at 50–70 m, and interestingly, coincided with the minimum in the δ¹³C-profile of SPM (Fig. 3).

**Concentration and isotopic composition of CH₄ and DIC**

Between 50 m and 200 m, CH₄ and DIC concentrations increased from 0.02 mmol L⁻¹ to 2.9 mmol L⁻¹ and from 11.5 mmol L⁻¹ to 51 mmol L⁻¹, respectively (Fig. 4a,b). Above 50 m water depth, CH₄ and DIC concentrations in the lake varied slightly and were ~0.01 mmol L⁻¹ and ~10 mmol L⁻¹, respectively (Fig. 4a,b). The δ¹³C values of CH₄ in the deep water at 140–200 m were ~ -62%o and were slightly but significantly (t-test, p < 0.05) more ¹³C-depleted than the values at 50–90 m (~60%o to ~56%o) (Fig. 4a). In the surface waters, CH₄ was more ¹³C-enriched with a δ¹³C-Ch₄ of ~57%o at 50 m and ~30%o at 5 m (Fig. 4a). The δ¹³C of DIC was ¹³C-depleted in the anoxic waters (e.g., 1.6%o at 200 m) relative to the surface waters (e.g., 4.5%o at 5 m; Fig. 4b).

**Fatty acids**

The fatty acid C16:1ω5 from the SPM had a concentration of ≤0.03 µg L⁻¹ and exhibited more ¹³C-depletion (δ¹³C of -50.6%o to -44.8%o) in the deep anoxic waters (≥ 52 m) than in the surface waters (δ¹³C of -32.8%o to -29.5%o; Table 2, Fig. 5a). Similarly, the fatty acid C16:1ω7 also showed more ¹³C-depletion in the anoxic waters (δ¹³C of -42.6%o to -38.9%o), one exception being the δ¹³C of -33.0%o, seen at 140 m (Fig. 5b). However, C18:1ω7 fatty acid from the SPM, with a concentration of ≤24.1 µg L⁻¹, was more ¹³C-depleted (~43.7%o to ~40.5%o) in the oxic surface waters than in the deep anoxic waters (~35.3%o to ~28.9%o; Fig. 5c). The fatty acid anteiso-methyl-C15:0 (iα-C15:0) from the SPM had concentration of ≤0.9 µg L⁻¹ and was more ¹³C-depleted (δ¹³C of ~45.0%o to ~38.2%o) in the deep anoxic waters than in the surface waters (δ¹³C of ~32.8%o to ~26.6%o) of the lake (Table 2, Fig. 5d).

**GDGTs and archaeal**

Archaeal in the SPM from the anoxic waters had a concentration of 0.9–7.8 ng L⁻¹, with the highest concentration (7.2–7.8 ng L⁻¹) observed at 70–80 m in the lake (Fig. 6a).
Table 2. Concentrations and stable carbon isotope distributions of individual fatty acids within SPM from the water column of Lake Kivu. Fatty acids were analyzed as FAMES. Precision of δ^{13}C values, based on duplicate analyses, was ≤ 1‰ and precision of the concentration, based on multiple analyses of external standard, was ≤ 4%.

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Nd = not determined or lost during processing of samples in the field or lab.
The $\delta^{13}C$ of archaeol was $-39.8_{\text{so}}$ to $-26.2_{\text{so}}$ with the exception of a more $^{13}C$-depleted archaeol ($-52.1_{\text{so}}$) value at 80 m (Fig. 6a). The concentration of GDGT-0 in the SPM was 0.1–4.3 ng L$^{-1}$ and GDGT-IV (crenarchaeol) concentration was 0.2–38.3 ng L$^{-1}$ (Fig. 6b). The ratios GDGT-0 : GDGT-IV and GDGT-0 : IsoGDGTs were invariant in the oxic surface waters, being $\sim$ 0.10 and 0.06 units, respectively (Fig. 6b) but increased with depth in the anoxic waters (55–200 m) from 0.10 to 6.9 and 0.06 to 0.64, respectively (Fig. 6b).

Phytol, brassicasterol, dinosterol, and cholesterol

Concentrations of phytol, brassicasterol, dinosterol, and cholesterol were higher in the oxic surface waters than in the anoxic region of the lake (Fig. 7b–e), and ranged from 6-916 ng L$^{-1}$, 3–83 ng L$^{-1}$, 7–147 ng L$^{-1}$ and 8–257 ng L$^{-1}$, respectively. $\delta^{13}C$ values of phytol, brassicasterol, dinosterol, and cholesterol within the SPM from the lake ranged from $-42.3_{\text{so}}$ to $-31.8_{\text{so}}$, $-43.8_{\text{so}}$ to $-29.9_{\text{so}}$, $-29.1_{\text{so}}$ to $-19.1_{\text{so}}$, and $-41.5_{\text{so}}$ to $-20.3_{\text{so}}$, respectively (Fig. 7b–e).

Depth distribution of bacteria and archaea in the water column of Lake Kivu

Bacteria dominated the prokaryotic community in the lake with cell numbers ranging from $4.6 \times 10^5$ cells mL$^{-1}$ at 70 m to $8.0 \times 10^5$ cells mL$^{-1}$ at 50 m, representing 57–81% of the total DAPI stained cells ($6.8 \times 10^5$–$8.2 \times 10^6$ cells mL$^{-1}$; Fig. 8a,b). Archaeal cell numbers were consistently lower than those of bacteria in the lake and ranged from $0.1 \times 10^5$ cells
mL\(^{-1}\) at 60 m to \(1.5 \times 10^5\) cells mL\(^{-1}\) at 35 m, representing 1–12\% of the total DAPI stained cells (Fig 8a,b). Alphaproteobacteria were less abundant in the lake, with cell numbers <3\% of the total DAPI stained cells. The cell numbers of type I MOB were \(0.13 \times 10^5\) cells mL\(^{-1}\) (1.3\% of DAPI stained cells), \(0.05 \times 10^5\) cells mL\(^{-1}\) (0.6\% of DAPI stained cells), and \(0.04 \times 10^5\) cells mL\(^{-1}\) (0.6\% of DAPI stained cells) at 50 m, 60 m, and 70 m, respectively (Fig. 8a,b). Type II MOB were less abundant than type I MOB with cell numbers of \(0.03 \times 10^5\) cells mL\(^{-1}\) (0.3\% of DAPI stained cells) and \(0.01 \times 10^5\) cells mL\(^{-1}\) (0.2\% of DAPI stained cells) at 50 m and 70 m, respectively (Fig. 8a,b). Type I MOB cells were not observed at 35 m, and type II MOB cells were not observed at either 35 m or 60 m in the lake (Fig. 8a). ANME-1 and ANME-2 (probes ANME-1-350 (Boetius et al. 2000) and ANME-2-538 (Treude et al. 2005)) and AOM-associated archaea (probes AAA-FW-641 and AAA-FW-834; Schubert et al. 2011) were not detected in the lake (Fig. 8a).

Discussion

Bulk CH\(_4\) isotopic insights into microbial CH\(_4\) oxidation

The observed \(^{13}\)C-enrichment of CH\(_4\) between 52 m and 90 m (relative to CH\(_4\) from deeper depths, 140–200 m; Fig. 4a) is due to preferential microbial oxidation of lighter CH\(_4\) (\(^{12}\)CH\(_4\)) leading to \(^{13}\)C-enrichment of the residual CH\(_4\) pool. This implies that anaerobic CH\(_4\) oxidation occurs within this anoxic region of the lake. However, the slight offset in \(^{13}\)C of CH\(_4\) between 140–200 m and 52–90 m (\(\Delta^{13}\)C of 2–6\%; Fig. 4a), corresponding to an anaerobic methane oxidation isotope fractionation factor (\(\alpha\)) of 1.002–1.006 (Eq. 2), indicates that a small amount of CH\(_4\) is consumed through anaerobic CH\(_4\) oxidation in the lake. In contrast, the large offset in \(^{13}\)C of CH\(_4\) between the anoxic region (52–90 m) and the oxic region (5–35 m; \(\Delta^{13}\)C of 21–36\%), corresponding to an aerobic methane oxidation \(\alpha\) of 1.022–1.038, indicates intense aerobic microbial CH\(_4\) consumption in the lake, which is corroborated by the drastic decrease in CH\(_4\) concentration in the oxic region (Fig. 4a).

Our observation is consistent with other studies that observed that aerobic methane oxidation was the main sink of methane in Lake Kivu (Pasche et al. 2009). Pasche et al. (2009) noted a relatively higher total oxygen flux (57 g C m\(^{-2}\) yr\(^{-1}\)) compared to the methane upward flux (35 g C m\(^{-2}\) yr\(^{-1}\)) in the oxic surface waters (0–60 m), hence, aerobic methane oxidation may be primarily responsible for methane removal in Lake Kivu.

The observed fractionation factor for aerobic CH\(_4\) oxidation in Lake Kivu is consistent with the reported value (\(\alpha = 1.037\)) in the water column of Lake Mekkojärvi, Southern Finland (Kankaala et al. 2007). The aerobic CH\(_4\) oxidation \(\alpha\) in Lake Kivu is, however, larger than the \(\alpha\) of 1.005 observed in Lake Matano (Indonesia) (Crowe et al. 2011). The fractionation factor (\(\alpha = 1.002 – 1.006\)) for anaerobic CH\(_4\) oxidation in Lake Kivu is consistent with reported values (\(\alpha = 1.005 – 1.008\)) in other anoxic systems (Whiticar 1999).

Preferential oxidation of \(^{12}\)CH\(_4\) should lead to the production of \(^{12}\)C-enriched CO\(_2\) (Templeton et al. 2006). However, this was not observed in Lake Kivu (Fig. 4b) most likely because of the enormous volume of CO\(_2\) in the lake (300 km\(^3\); Schmid et al. 2005) masking the isotopic effect of the CO\(_2\) produced from CH\(_4\) oxidation (estimated at 31–33 g C m\(^{-2}\) yr\(^{-1}\); Jannasch 1975; Pasche et al. 2011). Also, other biochemical processes such as photosynthesis in the surface waters (which result in \(^{12}\)C-depletion of residual CO\(_2\) or DIC) and respiration in the deep waters (which result in \(^{12}\)C-enrichment of residual CO\(_2\) or DIC) could be more important than CH\(_4\) oxidation in modulating the isotopic signature of the CO\(_2\) or DIC in the lake.

Contribution of methane carbon to the Lake Kivu food web

CH\(_4\) is a significant carbon reservoir in Lake Kivu, therefore, we used biomarkers to evaluate whether CH\(_4\) was an
important source of carbon and energy for the lake food web. Consumption of CH$_4$ by methanotrophic bacteria during CH$_4$ oxidation converts CH$_4$ carbon into microbial biomass. Subsequently, this methanotrophic biomass can serve as a carbon and energy source for heterotrophic organisms via zooplankton grazing on methanotrophic bacteria, or the use of inorganic carbon from mineralized methanotrophic biomass by autotrophs. In Lake Kivu, the $\delta^{13}$C values of biomarkers for phytoplankton including cyanobacteria (phytol; $\delta^{13}$C of $-42.3‰$ to $-31.8‰$), diatoms (brassicaterol; $\delta^{13}$C of $-43.8‰$ to $-29.9‰$), dinoflagellates (dinosterol; $\delta^{13}$C $-29.1‰$ to $-19.1‰$), and a biomarker for zooplankton and other heterotrophs (cholesterol; $\delta^{13}$C of $-41.5‰$ to $-20.3‰$; Fig. 7) indicate that eukaryotic phototrophs and heterotrophic grazers in the lake derive some of their carbon and energy from CH$_4$. Using a binary isotopic mixing model (Eq. 1), the contribution of CH$_4$ carbon to phytol, brassicasterol, and cholesterol within SPM in the lake was estimated at 16–35%, 13–38%, and $\leq$34%, respectively. The autotrophic plankton community in Lake Kivu is dominated by cyanobacteria, mainly *Synechococcus* sp. and *Planktolyngbya limnetica*, and diatoms, mainly *Nitzschia bacata* and *Fragilaria Danica* (Sarmento et al. 2012). Other phytoplankton groups such as dinoflagellates and cryptophytes are also present in the lake. In their detailed study of the vertical distribution of phytoplankton groups in the main basin of Lake Kivu off Kibuye, Sarmento et al. (2012) reported that diatoms were the dominant phytoplankton in the lake during deep mixing conditions (where the upper mixed layer is deeper than 50 m) in the dry season (June–September), whereas diazotrophic cyanobacteria and picocyanobacteria are dominant during the rainy season (October–May). The use of methane-derived carbon by photoautotrophs in Lake Kivu is interesting. However, both the mineralization of methanotroph-derived organic matter (e.g., from dead aerobic methanotrophic bacteria and zooplankton supported by methanotrophic bacteria and archaea) and the oxidation of methane to CO$_2$ could generate methane-derived dissolved carbon dioxide that could be utilized by the autotrophic plankton during photosynthesis. This agrees with the assertion by Pasche et al.

**Fig. 4.** Vertical profiles of the concentration and stable carbon isotopic compositions of (a) methane and (b) DIC in the water column of Lake Kivu.
that uptake of methane by picoplankton could be a sink of methane in the surface oxic layers of the lake and is consistent with the observation by Llirós et al. (2012) that mineralization of organic matter coupled to oxidation of reduced species including methane can result in a rapid decrease in oxygen concentrations in the lower mixolimnion (25–30 m to ~65 m) after thermal stratification begins in October–November. Also, Morana et al. (2014) observed that methane contributes significantly to carbon fixation in the oxic-anoxic interface in Lake Kivu. The heterotrophic community in Lake Kivu could derive some of their carbon from methane through grazing on methanotrophic archaea and bacteria, as well as methane-supported phototrophs. For instance, Descy et al. (2012) noted that methanotrophic archaea and bacteria likely contribute to planktonic crustacean production in Lake Kivu. Support of the food web by CH$_4$ carbon in Lake Kivu is consistent with previous studies in other aquatic systems. Werne et al. (2002) used the isotopic composition of biomarkers to trace methane-derived carbon through microbes into heterotrophic ciliates. Also, in their study of zooplankton carbon isotope signatures in three lakes in Sweden (Illersjön, Marn, and Lillsjön), Bastviken et al. (2003) reported that 5–15% of zooplankton production was supported by methanotrophic biomass. In Lake Kivu, it was interesting to observe that below the chemocline in the anoxic waters (70–80 m depth), the $\delta^{13}C$ values of the biomarkers of phototrophs and heterotrophs were $^{13}C$-depleted relative to shallower oxic waters (Fig. 7). The $^{13}C$-depletion of heterotrophic organisms at this depth range could be from anaerobic methane-oxidizing archaea, SRB involved in AOM and/or their heterotrophic grazers. Also, chemoautotrophic and chemoheterotrophic bacteria or archaea (Llirós et al. 2012) supported by methane or methane-derived CO$_2$ and their heterotrophic grazers could contribute to the observed $^{13}C$-depletion of heterotrophic organisms observed at this depth region. The $^{13}C$-depletion of phototrophs at this depth suggests deeper anoxygenic photosynthetic activity. The anoxygenic photoautotrophs, green sulfur bacteria (GSB), are commonly found in the upper anoxic layers of the lake (Llirós et al. 2012). Although a small amount of light (≤1% of the light intensity at the surface) potentially reaches this depth region below the euphotic zone, the
obligate anaerobic bacteria GSB are known to be able to photosynthesize under extreme low-light conditions using hydrogen sulfide, hydrogen, or Fe (II) as an electron donor (Lliròs et al. 2012). Carbon fixation by GSB using methane-derived CO₂ could explain the ¹³C-depleted biomarkers of phototrophs observed at this depth. Also, some minimal carbon fixation by phototrophs such as cyanobacteria could contribute to the observed ¹³C-depletion of the biomarkers of phototrophs at this depth region.

Methanotrophic communities mediating methane oxidation in the oxic ozone

Molecular isotopic evidence of aerobic CH₄ consumption by MOB comes from the ¹³C-depletion of the fatty acid C₁₈:₁ω₇ seen in the oxic surface waters but not in the deep anoxic waters of the lake (Fig. 5c). The fatty acids C₁₈:₁ω₈ and C₁₈:₁ω₇ are diagnostic of type II MOB (Hanson and Hanson 1996; Bodelier et al. 2009). Therefore, the higher concentrations coupled to ¹³C-depletion of C₁₈:₁ω₇ fatty acids in the surface waters (Fig. 5c) indicate that type II MOB are consuming CH₄ in the oxic surface waters. In contrast, C₁₆:₁ω₅ (and C₁₆:₁ω₇) fatty acids, which are diagnostic of type I MOB (Kankaala et al. 2007; Bodelier et al. 2009), were not ¹³C-depleted in the surface waters (Fig. 5a,b), implying that type I MOB were not involved in CH₄ oxidation in the surface waters. In a related study in Lake Kivu, Pasche et al. (2011) detected the methane monooxygenase (pmoA) gene (a molecular marker for aerobic methanotrophs) in particles at 40 m and 60 m (both oxic depths in that study) and observed that the pmoA sequences were closely related to Methylococcus capsulatus (type X MOB). It should be noted that while this is not consistent with our

**Fig. 6.** Vertical distributions of (a) the concentration and stable carbon isotopic composition of archaeol and (b) the concentration of GDGT-IV, and the values of the ratios of GDGT-0 to isoGDGTs and GDGT-0 to GDGT-IV in the water column of Lake Kivu. Values < 1 of the ratio of GDGT-0 to GDGT-IV and GDGT-0 to isoGDGT (Fig. 6b) indicate relatively lesser contributions from methanogenic archaean lipids compared to lipids from crenarchaeota and other archaea groups throughout the water column. The increasing values of GDGT-0:isoGDGTs and GDGT-0:GDGT-IV with depth in the anoxic region shows the increasing contribution from methanogens to the archaeal biomass.
observation that type II MOB is mainly responsible for aerobic methane oxidation in Lake Kivu, Pasche et al. (2011) also indicated that the primer set used in that study had a potential bias toward type X MOB and that different and/or diverse communities could have been involved in aerobic methane oxidation.

Fig. 7. Vertical distribution of the concentration and stable carbon isotopic composition of (b) phytol, (c) brassicasterol, (d) dinosterol, and (e) cholesterol in the water column of Lake Kivu. The isotopic composition of (a) SPM has been included here for comparison.

Fig. 8. Distributions of the (a) cell numbers, and (b) relative percentages of bacteria, archaea, alphaproteobacteria, type I MOB, and type II MOB in the water column of Lake Kivu. Positive hybridization with ANME specific probes was not observed at these water depths in the lake.
The apparent habitat specialization of MOB in Lake Kivu as observed in this study, where type II MOB are active in the nutrient-limited oxic surface waters and type I MOB are active within the methane and nutrient-rich deep-water region with ≤20 nmol L⁻¹ oxygen (“anoxic” region) is interesting and could be linked to the chemical species regime in the lake. Tavormina et al. (2013) have also reported increased abundance of type I MOB at the upper and lower horizons of oxygen minimum zones off of Costa Rica. Previous studies have reported the predominance of type II MOB under nutrient-limiting (nitrogen or copper-depleted) conditions and the predominance of type I MOB in favorable growth (nitrogen and copper-rich) conditions (Amaral and Knowles 1995). In small, eutrophic Lake Rotsee in Switzerland, type I MOB predominated in the aerobic oxidation of CH₄ (Schubert et al. 2010) most likely due to nutrient (e.g., nitrogen; Schubert et al. 2010) rich conditions in the lake. Similarly, type I MOB is the predominant MOB in nutrient-rich Lake Constance (Rahalkar et al. 2009). In contrast, the predominance of type II MOB in the aerobic oxidation of CH₄ in Lake Kivu could be due to the oligotrophic, nutrient-poor (low levels of nitrogen and copper) conditions of the oxic surface waters (Lliros et al. 2010; Julius 2011).

**CH₄ oxidation by type I MOB in the deep waters**

Fatty acids C16:1ω8, C16:1ω7, and C16:1ω5 are diagnostic of type I MOB as noted earlier and are characteristic of the genera *Methylomonas* (C16:1ω8), *Methylobacter* (C16:1ω7 and C16:1ω5), *Methyllococcus* (C16:1ω7), and *Methylomicrobium* (C16:1ω5 and C16:1ω7) (Hanson and Hanson 1996; Kaneva et al. 2007; Bodelier et al. 2009). The presence of C16:1ω5 and C16:1ω7, and their considerable 13C-depletion in the deep-water region below 52 m (Figs. 5a,b), suggest the involvement of type I MOB in CH₄ oxidation in the anoxic deep-water region (with ≤20 nmol L⁻¹ oxygen concentration) of the lake. This is corroborated by the presence of type I MOB throughout the sampled deep-water depths as determined from CARD-FISH analysis (Fig. 8a).

Alternatively, the 13C-depleted fatty acids from SPM observed in the deep waters of the lake could be from sinking inactive or dead bacterial biomass of type I MOB from the oxic overlying surface waters. However, the same fatty acid species in surface-water SPM were not as 13C-depleted as in the deep waters, which should have been the case if the deep-water fatty acids had originated from the surface waters. Also, because of the consistent 13C depletion of these fatty acids at multiple depths in the deep-water region (which would not be the case for just an episodic sinking of SPM from the surface region), it is unlikely that the observed 13C-depleted C16:1ω5 and C16:1ω7 originated from the surface waters.

The exact physical or biochemical pathway or mechanism that is driving the aerobic methane oxidation in the deep waters is not known. It is possible that the type I MOB are utilizing very low O₂ concentrations (≤20 nmol L⁻¹) for methane oxidation, or that there is a temporal input of dissolved oxygen from the subaquatic springs feeding the anoxic regions of the lake. Coupling of the aerobic methane oxidation with deep-water photosynthetic activity or the reduction of oxidized nutrients or metals are also possible.

**Presence of SRB and potential coupling with ANME in anaerobic methane oxidation**

The pathway of anaerobic methane oxidation can be examined in the context of the vertical distribution of potential electron acceptors in the anoxic region. In Lake Kivu, the reduction of sulfate to sulfide is revealed by the opposite depth gradients of SO₄²⁻ and S(II) within 60–90 m in the anoxic region (Fig. 2b; Pasche et al. 2009). This observation, coupled to the inverse depth profiles of methane and sulfate within 60–90 m suggest that anaerobic methane oxidation via sulfate reduction occurs within this anoxic region in the lake (Pasche et al. 2009, 2011). By comparing the total sulfate flux (1 ± 0.5 g C m⁻² yr⁻¹) and methane upward flux (35 ± 0.5 g C m⁻² yr⁻¹), Pasche et al. (2009) noted that a maximum of only 3% of the methane could be anaerobically oxidized via sulfate reduction. The fluxes of nitrate, nitrite, and oxidized iron or manganese are too low, and likely play a negligible role in anaerobic methane oxidation in Lake Kivu (Pasche et al. 2011; Lliros et al. 2012).

A syntrophic cooperation between SRB and ANME oxidize CH₄ under anoxic conditions through electron transfer from the CH₄ to sulfate (Boetius et al. 2000). However, in a recent study, Milucka et al. (2012) observed that ANME alone (without SRB partnership) may be able to carry out AOM through dissimilatory reduction of sulfate to zero-valent sulfur.

The fatty acids ai-and i-C15:0 and -C17:0 are diagnostic of SRB (Wakeham et al. 1997; Pancost et al. 2000). Kohring et al. (1994) reported that ai-C15:0 was a major constituent of the membrane lipids of the SRBs *Desulfosarcina variabilis* and *Desulfococcus multivorans*. Although the fatty acids ai-and i-C15:0 and -C17:0 have also been found in some iron and manganese oxidizers, the observed 13C-depletion of the fatty acid ai-C15:0 (δ¹³C of −45.0‰ to −38.2‰; Fig. 5d) in anoxic waters overlapping the sulfate reduction zone suggests that this fatty acid most likely originated from SRB involved in AOM (rather than Mn or Fe-reducers with a negligible role in AOM) in the lake. This observation is consistent with Pasche et al. (2011) who used DGGE of PCR-amplified 16S rRNA gene fragments and found a deltaproteobacteria sequence with high similarity to the SRB *Desulfocapsa* within the sulfate reducing zone (60–90 m). Also, Schmitz (2011), using DGGE analysis, found deltaproteobacteria in the anoxic region (50–100 m) of the lake. In addition to the very low fluxes of iron and manganese, the Mn²⁺ depth profile in the lake (cf. Fig. 2a; Pasche et al. 2011) does not show coupling with AOM in the lake. Consequently, ai-C15:0 fatty acids from Mn reducing bacteria would not be
13C-depleted in contrast to ai-C15:0 fatty acids from SRB involved in AOM.

Our observations in Lake Kivu are consistent with studies in other systems. For instance, 13C-depletion of ai-C15:0 was linked to the involvement of SRB in AOM in the surface sediments of the Mediterranean ridge (δ13C of −68‰; Pancost et al. 2000), Black Sea sediments (δ13C of −83.5‰ to −78.9‰; Blumenberg et al. 2004), and sediments in freshwater Lake Cadagno, Switzerland (δ13C, down to −44.8‰; Schubert et al. 2011).

To identify the potential ANME partners of SRB involved in AOM in Lake Kivu, Pasche et al. (2011) used methyl coenzyme M reductase (mcrA) genes as markers for ANME (as well as methanogens) and reported that the sequenced clones within the sulfate reducing zone (90 m) were most closely related to the Methanomicrobiales (potentially a new ANME group). Also, in the deeper anoxic regions (below 90 m) of the lake, Pasche et al. (2011) observed mcrA gene sequences that were most closely related to the ANME-2ab clade. In our study, we used probes for known ANME-1 and ANME-2 (Blumenberg et al. 2004) but did not detect these in the lake (Fig. 8a), consistent with Pasche et al.’s (2011) observation that a novel cluster of ANME may be involved in AOM in the lake.

We detected archaeal cells (Figs. 8, 9) and the biomarker archaeol (Fig. 6a) in the anoxic region of the lake. The observed predominance of bacteria (57–81% of total DAPI

Fig. 9. Cells of microbial communities from the water column of Lake Kivu showing: (a,b) DAPI staining from 50 m and the corresponding CARD-FISH image for bacterial cells (probes EUB338 I-II) at 50 m. (c,d) DAPI staining from 70 m and the corresponding CARD-FISH image of type I MOB (probes Mgamma705 + 84) from 70 m. (e, f) DAPI staining and corresponding CARD-FISH image for type II MOB from 70 m (probe Ma450). (g, h) DAPI staining and corresponding archaeal signals from 50 m water depth (probe ARCH915). Note all scale bars = 5 μm. All images show confocal scanning micrographs.
stained cells) over archaea (1–12% of total DAPI stained cells) throughout Lake Kivu agrees with Llirós et al. (2010), who reported bacterial (46–95% of total DAPI stained cells) vs. archaeal (0.3–4.6% of total DAPI stained cells) proportions in the surface and intermediate waters (0–100 m) of Lake Kivu, and is also consistent with observation in other freshwater lakes (Llirós et al. 2011). Archaeol is a diagnostic membrane lipid of archaea including methanogenic and methanotrophic archaea (Niemann and Elvert 2008). The isotopic composition of archaeol was moderately $^{13}$C-depleted and not conclusive as to the predominant source of the archaeol.

To gain more insights into the predominant source of archaeol in the SPM, we used the distribution of archaea-derived isoprenoid GDGTs (isoGDGTs). While the isoGDGTs can be derived from crenarcheota, methanogenic archaea, and methanotrophic archaea (Blumenberg et al. 2004; Schouten et al. 2013), methanogenic archaea produce predominantly and sometimes only GDGT-0 (Blaga et al. 2009; Schouten et al. 2013). Values < 1 of the ratio of GDGT-0 to GDGT-IV and GDGT-0 to isoGDGT (Fig. 6b) indicate relatively lesser contributions from methanogenic archaeal lipids compared to lipids from crenarcheota and other archaea groups (e.g., *Thaumarchaeota*) throughout the water column. The increased values of the ratios of GDGT-0 to GDGT-IV and GDGT-0 to isoGDGT with depth (Fig. 6b), and their higher values in the anoxic region imply higher contributions from methanogenic archaeal lipids in the anoxic region, in agreement with the reported biological origin of methane in the lake (Pasche et al. 2011). Llirós et al. (2010, 2012), in their study of the archaea community in Lake Kivu, noted that the phylotypes affiliated with the phyla Crenarchaeota, Thaumarchaeota, and Euryarchaeota comprised 32%, 43%, and 25% of the assigned operational taxonomic units. Consistent with our observation, they noted that most of the Crenarchaeota and Thaumarchaeota in the lake were from ammonia oxidizing archaea, whereas most of the Euryarchaeota from the anoxic water column were from methanogens (*Methanosarcinales* and *Methanocellales*).

**Isotope fractionation during methane oxidation and mixed-source contribution to methanotrophic biomarkers**

Isotopic fractionation during biomass synthesis by MOB is reported to range from 3–39%$\text{}_{\text{o}}$ and is influenced by the type of MOB (I, X, or II), dissolved CH$_4$ concentrations, cell densities, and the availability of copper (Summons et al. 1994; Jahnke et al. 1999; Templeton et al. 2006). While type I MOB synthesizes its biomass from CH$_4$-derived formaldehyde through the RuMP pathway, the type X MOB, in addition to using formaldehyde, is capable of using small amounts of CO$_2$ for biomass synthesis through the ribulose bisphosphate carboxylase pathway. In contrast, type II MOB, however, synthesizes 50–70% of its biomass from formaldehyde using the serine pathway, and produces 30–50% of its biomass from CO$_2$ using the phospho(enol)pyruvate carboxylase pathway (Hanson and Hanson 1996). These different pathways lead to varying isotopic fractionations in the different MOB. In their growth experiment studies of *Methyllococcus capsulatus*, a type X MOB, Summons et al. (1994) reported an isotopic fractionation of 16–31%$\text{}_{\text{o}}$ during growth, and Jahnke et al. (1999) observed a fractionation of $\sim 10\%$ for the same species. In their experiments with pure cultures without copper amendments, Templeton et al. (2006) observed $\sim 14–24\%$ and $\sim 1–13\%$ $^{13}$C-depletion of the biomass relative to substrate CH$_4$ by *Methylomonas methanica* (type I MOB) and *Methylosinus trichosporium* (type II MOB), respectively. They also observed that the biomass synthesized by *M. methanica* and *M. trichosporium* was 6%$\text{}_{\text{o}}$ and 15%$\text{}_{\text{o}}$ enriched in $^{13}$C compared to the dissolved CO$_2$. A much larger isotope fractionation (35–70%$\text{}_{\text{o}}$) was reported to be associated with biomass synthesis by methanotrophic archaea during AOM (Blumenberg et al. 2004; Niemann and Elvert 2008).

In Lake Kivu, as the $\delta^{13}$C of substrate CH$_4$ in the anoxic region of the lake was $-62.6\%$ to $-56.6\%$ (Fig. 4a), biomass from MOB directly consuming CH$_4$ would be on average $^{13}$C-depleted by 16%$\text{}_{\text{o}}$, relative to the source CH$_4$ ($\delta^{13}$C of $-78.6\%$ to $-72.6\%$). Similarly, biomass from SRB and ANME involved in AOM is expected to be $^{13}$C-depleted by at least 35%$\text{}_{\text{o}}$, relative to the source CH$_4$ ($\delta^{13}$C of $-97.6\%$ to $-91.6\%$). However, the biomarkers for type I MOB (fatty acids C16:1ω5, C16:1ω7), type II MOB (fatty acid C18:1ω7), and SRB partners of ANME (fatty acid ai-C15:0) were not as highly $^{13}$C-depleted as should be the case if CH$_4$ was the only source of carbon. This implies that: (1) the MOB (especially type II MOB) were facultative (rather than obligate) methanotrophs, using other C1-compounds or substrates (e.g., CO$_2$) in addition to CH$_4$ for their biomass synthesis, which could lead to the observed $^{13}$C-enrichment (or similarity) of the biomass relative to the source CH$_4$. (2) These PLFA biomarkers were derived from mixed sources. To further investigate the latter idea, we used a binary isotopic mixing model (Eq. 3) to tease apart the proportional contributions of methanotrophic lipids to the PLFA biomarkers. The model estimates indicate that 21–30% of the fatty acid ai-C15:0 in the SPM from the deep anoxic waters was derived from SRB involved in AOM. Also, lipids from type I MOB constituted 18–36% of the fatty acids C16:1ω7 and 40–50% of the fatty acid C16:1ω5 in the SPM from the anoxic waters of the lake. Finally, type II MOB contributed 32–38% of the fatty acid C18:1ω7 in the SPM from the oxic surface waters of the lake. It is worth noting that the fractionation factors previously published (and discussed above) for aerobic methanotrophs were from pure cultures and could be different in a mixed community under the prevailing environmental conditions in the lake.
This study showed that CH$_4$ in the water column of Lake Kivu was oxidized both anaerobically and aerobically, with anaerobic oxidation being a small sink for CH$_4$ compared to aerobic CH$_4$ oxidation in the lake. Molecular and isotopic data showed that aerobic CH$_4$ oxidation in the lake was mediated by type II MOB. Type I MOB were not involved in aerobic CH$_4$ oxidation in the surface waters but was involved in CH$_4$ oxidation in the anoxic deep waters of the lake. SRB were found in the deeper anoxic waters and were associated with AOM in the lake based on observed $^{13}$C depletion of al-C15:0 fatty acid. Methane constituted an important carbon and energy source (up to 38%) for the lake food web, including heterotrophic and autotrophic communities.

References


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