Methane paradox in tropical lakes? Sedimentary fluxes rather than water column production in oxic waters sustain methanotrophy and emissions to the atmosphere

Cédric Morana1,2*, Steven Bouillon1, Vimac Nolla-Ardèvol1, Fleur A.E. Roland2, William Okello3, Jean-Pierre Descy2, Angela Nankabirwa3, Erina Nabafu3, Dirk Springael1, Alberto V. Borges2.

1Department of Earth & Environmental Sciences, KU Leuven, Belgium
2Chemical Oceanography Unit, Université de Liège, Belgium
3Limnology Unit, National Fisheries Resources Research Institute, Uganda

Correspondence to: Cédric Morana (Cedric.Morana@kuleuven.be)

Abstract. Despite growing evidence that methane (CH4) formation could also occur in well-oxygenated surface freshwaters, its significance at the ecosystem scale is uncertain. Empirical models based on data gathered at high latitude predict that the contribution of oxic CH4 increases with lake size and should represent the majority of CH4 emissions in large lakes. However, such predictive models could not directly apply to tropical lakes which differ from their temperate counterparts in some fundamental characteristics, such as year-round elevated water temperature. We conducted stable isotope tracer experiments which revealed that oxic CH4 production is closely related to phytoplankton metabolism, and is a common feature in five contrasting African lakes. Nevertheless, methanotrophic activity in surface waters and CH4 emissions to the atmosphere were predominantly fuelled by CH4 generated in sediments and physically transported to the surface. Indeed, measured CH4 bubble dissolution flux and diffusive benthic CH4 flux were several orders of magnitude higher than CH4 production in surface waters. Microbial CH4 consumption dramatically decreased with increasing sunlight intensity, suggesting that the freshwater “CH4 paradox” might be also partly explained by photo-inhibition of CH4 oxidizers in the illuminated zone. Sunlight appeared as an overlooked but important factor determining the CH4 dynamics in surface waters, directly affecting its production by photoautotrophs and consumption by methanotrophs.

1. Introduction

Emissions from inland waters are an important component of the global CH4 budget (Bastviken et al. 2011), in particular from tropical latitudes (Borges et al. 2015). While progress has been made in evaluating the CH4 emission rates, much less attention has been given to the underlying microbial production (methanogenesis) and loss (methane oxidation) processes. It is generally assumed that CH4 in lakes originates from the degradation of organic matter in anoxic sediments. Because most methanogens are considered to be strict anaerobes and net vertical diffusion of CH4 from anoxic bottom waters is often negligible (Bastviken et al. 2003), physical processes of CH4 transport from shallow sediments are usually invoked to explain patterns of local CH4 concentration maximum in surface waters (Encinas-Fernandez et al. 2016, Peeters et al. 2019, Martinez-Cruz et al. 2020). Indeed, CH4-rich pore water is regularly released from littoral sediment into the water column during resuspension events associated with surface waves (Hofmann et al. 2010).

The view that CH4 is formed under strictly anaerobic conditions has been challenged by several recent studies which proposed that acetoclastic methanogens directly attached to phytoplankton cells are involved in epilimnetic CH4 production (Grossart et al. 2011, Bogard et al. 2014), and are responsible of distinct near-surface peaks of CH4 concentration in certain thermally stratified, well-oxygenated waterbodies (Tang et al. 2016). It has also been showed that Cyanobacteria (Bizic et al. 2020) and widespread marine phytoplankton (Klintzsch et al. 2019) are able to release substantial amount of CH4 during a culture study, and this CH4 production mechanism might be linked to photosynthesis. From a model-based approach, epilimnetic CH4 production was shown to sustain most of the CH4 oxidation in 14 Canadian lakes (DeSontro et al. 2018), and would even
represent up to 90% of the CH4 emitted from a temperate lake (Donis et al. 2017). Further, empirical models based on data gathered in boreal and temperate lakes predict that the contribution of oxic CH4 increases with lake size (Gunthel et al. 2019) and should represent the majority of CH4 emissions in lakes larger than 1 km². Still, aerobic CH4 production has so far only been documented in temperate and boreal lakes so that such predictive models could not directly apply to tropical lakes which differ from their temperate counterparts in some fundamental characteristics, such as year-round elevated water temperature.

Among others, primary production, methanogenic and methanotrophic activities, and cyanobacterial dominance are potentially much higher in tropical lakes due to favorable temperature (Lewis 1987, Kosten et al. 2012). It has also been shown that CH4 emissions are positively related to temperature at the ecosystem scale (Yvon-Durocher et al. 2014).

Here, we tested the hypothesis that phytoplankton metabolism could fuel CH4 production in well-oxygenated waters in five contrasting tropical lakes in East Africa covering a wide range of size, depth, and productivity (L. Edward, L. George, L. Katinda, L. Nyamusingere and L. Kyambura). Phytoplankton activity could provide diverse substrates required for CH4 production mediated by methanogenic Archaea, or alternatively CH4 could be directly released by phytoplankton cells. Additionally, the significance of epilimnetic CH4 production at the scale of the aquatic ecosystem was assessed by quantifying CH4 release from sediments, CH4 production and oxidation rates in the water column, and CH4 diffusive and ebullitive emissions to the atmosphere.

2. Material and methods

2.1. Site description

The sampled lakes cover a wide range of size (<1 to 2300 km²), maximum depth (3-117 m), mixing regimes, phytoplankton biomass and primary productivity (Table S1). Oligotrophic L. Kyamwinga (-0.18054°N, 30.14625°E) and eutrophic L. Katinda (-0.21803°N, 30.10702°E) are stratified, small but deep tropical lakes located in Western Uganda. Neighboring L. Nyamusingere (-0.28436°N, 30.03763°E) is a small but shallow and polymictic eutrophic lake. L. George is a larger (250 km²), hypereutrophic, shallow lake located at the equator (-0.02273°N, 30.19724°E). A single outlet (Kazinga Channel) flows from L. George to the neighboring Lake Edward (-0.28971°N, 29.73327°E), a holomictic, mesotrophic large lake (2325 km²). Water samples from pelagic stations of L. Katinda, L. George (2 km offshore) and L. Edward (15 km offshore) were collected in April 2017 (rainy season) and January 2018 (dry season). Pelagic sites of L. Kyamwinga and L. Nyamusingere were sampled only once, in April 2017 and January 2018, respectively.

2.2. Environmental setting of the study sites

Conductivity, temperature and dissolved oxygen concentration measurements were performed with a Yellow Spring Instrument EXO II multiparametric probe. Samples for particulate organic carbon (POC) concentration were collected on glass fiber filters (0.7 µm nominal pore size) and analyzed with an elemental analyzer coupled to an isotope ratio mass spectrometer (EA-IRMS) (Morana et al. 2015). Pigment concentrations were determined by high performance liquid chromatography (Descy et al. 2016) after filtration of water samples through glass fiber filters (0.7 µm nominal pore size).

Water samples for determination of dissolved CH4 concentration were transferred with tubing from the Niskin bottle to 60 ml borosilicate serum bottles that were poisoned with 200µL of a saturated solution of HgCl2, closed with a butyl stopper and sealed with an aluminum cap. The concentrations of dissolved CH4 was measured with the headspace equilibration technique (20 ml headspace) using a gas chromatograph with flame ionization detection (GC-FID, SRI8610C).

Samples for δ13C-CH4 determination were collected in 60 ml serum bottles following the same procedure than samples for CH4 concentration determination. In the laboratory, δ13C-CH4 was measured as described in Morana et al. (2015). Briefly, a 20ml helium headspace was created in the serum bottles, then samples were vigorously shaken and left to equilibrate overnight. The sample gas was flushed out through a double-hole needle and purified of non-CH4 volatile organic compounds.
in a liquid N\textsubscript{2} trap, CO\textsubscript{2} and H\textsubscript{2}O were removed with a soda lime and a magnesium perchlorate traps, and the CH\textsubscript{4} was converted to CO\textsubscript{2} in an online combustion column similar to that in an elemental analyzer (EA). The resulting CO\textsubscript{2} was subsequently preconcentrated in a custom-built cryo-focussing device by immersion of a stainless-steel loop in liquid N\textsubscript{2}, passed through a micro-packed GC column (HayeSep Q 2 m, 0.75mm ID; Restek), and finally measured on a Thermo Scientific Delta V Advantage isotope ratio mass spectrometer (IRMS). CO\textsubscript{2} produced from certified reference standards for δ\textsubscript{13}C analysis (IAEA-CO\textsubscript{1} and LSVEC) were used to calibrate δ\textsubscript{13}C-CH\textsubscript{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.

2.3. Diffusive CH\textsubscript{4} flux calculation

Surface CH\textsubscript{4} concentrations were used to compute the diffusive air-water CH\textsubscript{4} fluxes (FCH\textsubscript{4}) according to eq. (1):

\[ F_{CH_4} = k \times \Delta CH_4 \]  

Where k is the gas transfer velocity of CH\textsubscript{4} computed from wind speed (Cole & Caraco 1998) and the Schmidt number of CH\textsubscript{4} in freshwater (Wanninkhof 1992), and \( \Delta CH_4 \) is the air-water gradient. Wind speed data were acquired with a Davis Instruments meteorological station located in Mweya peninsula (0.11°S 29.53°E).

2.4. CH\textsubscript{4} ebullition flux

CH\textsubscript{4} ebullition flux was investigated in L. Edward, George, and Nyamusingere only. Bubble traps made with an inverted funnel (24 cm diameter) connected to a 60 ml syringe were deployed for a period between 24 h and 48 h at 0.5 m below the water surface (4 replicates). Measurements were performed at sites with water depth of 20 m, 2.5 m and 3 m for L. Edward, George and Nyamusingere, respectively. After measuring the gas volume collected within the trap during the sampling period, the gas bubbles were transferred in a tightly closed 12 ml Exetainer vial (Labco) for subsequent analysis of their CH\textsubscript{4} concentration. Variability of the gas volume in the 4 replicates was less than 10%. We used the SiBu-GUI software (McGinnis et al. 2006, Greinert et al. 2009) to correct for gas exchange within the water column during the rise of bubbles and thus obtained the CH\textsubscript{4} ebullition and CH\textsubscript{4} bubble dissolution fluxes. Calculations were made following several scenarios: two extreme bubble-size scenarios considering a release of many small (3 mm diameter) bubbles or fewer large (10 mm) bubbles, and an intermediate scenario of release of 6 mm diameter bubbles.

2.5. CH\textsubscript{4} flux across the sediment-water interface

CH\textsubscript{4} flux across the sediment-water interface was determined from short-term intact core incubations in L. Edward, L. George and L. Nyamusingere only. CH\textsubscript{4} flux was quantified from the change of CH\textsubscript{4} concentration in overlying waters at 5 different time steps, every 2 hours. Briefly, in every lake, 2 sediment cores (6 cm wide; ~ 30cm sediment and 30cm of water) were collected taking care to avoid disturbance at the sediment-water interface. Cores were kept in the dark until back in the laboratory, typically 6h later. Overlying water was carefully removed and replaced by bottom lake water filtered through 0.2µm polycarbonate filters (GSWP, Millipore) in order to remove water column methanotrophs. It was then degassed with helium during 20 minutes in order to remove background O\textsubscript{2} and CH\textsubscript{4}, and gently returned in the core tubes, on top of the sediments. Core tubes were tightly closed with a thick rubber stopper equipped with two sampling valves. A magnetic stirrer placed ~ 10 cm above the sediments was allowed to rotate gently in order to homogenize the overlying water layer during the incubation. At each time step, 60 ml of overlying water was sampled by connecting a syringe to the first sampling valve while an equivalent volume of degassed water was allowed to flow through the second valve in order to avoid any pressure disequilibrium. Subsamples of overlying water were transferred into a two 20 ml serum bottles filled without headspace and
poisoned with HgCl$_2$. Determination of the dissolved CH$_4$ concentration was performed with a GC-FID following the same procedure as described above.

2.6. Primary production and N$_2$ fixation

Primary production and N$_2$ fixation rates were determined from dual stable isotope photosynthesis-irradiance experiments using NaH$^{13}$CO$_3$ (Eurisotop) and dissolved $^{15}$N$_2$ (Eurisotop) as tracers for incorporation of dissolved inorganic carbon (DIC) and N$_2$ into the biomass. The $^{15}$N$_2$ tracer was added dissolved in water (Mohr et al. 2010). Incident light intensity was measured by a LI-190SB quantum sensor during day time during the entire duration of the sampling campaign. At each station a sample of surface waters (500 ml) was spiked with the tracers (final $^{15}$N atom excess ~5%). Three subsamples were preserved with HgCl$_2$ in 12-ML Exetainers vials (Labco) for the determination of the exact initial $^{13}$C-DIC and $^{15}$N-N$_2$ enrichment. The rest of the sample was divided into nine 50-ml polycarbonate flasks, filled without headspace. Eight flasks were placed into a floating incubation device providing a range of light intensity (from 0 to 80% of natural light) using neutral density filter screen (Lee Filters). The last one was immediately amended with neutral formaldehyde (0.5% final concentration) and served as killed control sample. Samples were incubated in situ during 2 hours around mid-day just below the surface at lake surface temperature. After incubation, biological activity was stopped by adding neutral formaldehyde into the flasks, and the nine samples were filtered on pre-combusted GF/F filters when back in the lab. Glass fiber filters were decarbondated with HCl fumes overnight, dried, and their delta V Advantage). For the measurement of the initial $^{15}$N$_2$ enrichment, a 2-ml helium headspace was created, and after 12h equilibration, a fraction of the headspace was injected into the above-mentioned EA-IRMS equipped with a Cu column warmed at 640°C and a CO$_2$ trap. Initial enrichment of $^{13}$C-DIC was also measured.

Photosynthetic (P) (Hama et al. 1983) and N$_2$ fixation (N$_2$fix) (Montoya et al. 1996) rates in individual bottles were calculated, and corrected for any abiotic tracer incorporation by subtraction of the killed control value. For each experiment, the maximum photosynthetic and N$_2$ fixation rates (P$_{max}$, N$_2$fix$_{max}$) and the irradiance at the onset of light saturation ($I_{I_P}$, $I_{N2fix}$) were determined by fitting P, and N2fix, to the light intensity gradient provided by the incubator (I) using the equation (eq. 2) for photosynthesis activity (Vollenweider 1965) and (eq. 3) for N$_2$ fixation (Mugidde et al. 2003).

\[
P_l = 2P_{max}\left(\frac{l_I/2I_{I_P}}{1 + (l_I/2I_{I_P})^2}\right)
\]

\[
N_2fix_l = 2N_2fix_{max}\left(\frac{l_I/2I_{N2fix}}{1 + (l_I/2I_{N2fix})^2}\right)
\]

2.7. Determination of CH$_4$ oxidation rates.

CH$_4$ oxidation rates in surface waters (1 m depth) were determined from the decrease of CH$_4$ concentrations measured during short (typically < 24h) time course experiments. Samples for CH$_4$ oxidation rate measurement were collected in 60 mL glass serum bottles filled directly from the Niskin bottle with tubing, left to overflow, and immediately closed with butyl stoppers previously boiled in milli-Q water, and sealed with aluminum caps. The first bottle was then poisoned with a saturated solution of HgCl$_2$ (100 µl) injected through the butyl stopper with a polypropylene syringe and a steel needle and corresponded to the initial CH$_4$ concentration at the beginning of the incubation (T0).

The remaining bottles were incubated in the dark, at in situ (~26°C) temperature during ~12h or ~24h except in L. George and Nyamusingere where the incubation was shorter (~6h). At 4 different times step one bottle was poisoned with 100 µL of HgCl$_2$ and stored in the dark until measurement of the CH$_4$ concentrations with the above-mentioned GC-FID. CH$_4$ oxidation rates were calculated as a linear regression of CH$_4$ concentrations over time ($r^2$ generally better than 0.80) during the course of the incubation.
2.8. Sunlight inhibitory effect on CH₄ oxidation

The influence of light intensity on methanotrophy was investigated in Lake Edward and Lake George by means of a stable isotope (¹³C) labelling experiment. For each experiment, 12 serum bottles (60 mL) were filled with lake surface waters (1 m) as described above. All bottles were spiked with 100 µL of a solution of dissolved ¹³CH₄ (50 µmol L⁻¹ final concentration, 99% enrichment) added in excess. Half of the bottles were amended with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU, 0.5 mg L⁻¹) in order to inhibit photosynthesis (Bishop 1958) and investigate the hypothetical inhibitory effect of dissolved O₂ production by phytoplankton. Two bottles were poisoned immediately with pH-neutral formaldehyde (0.5% final concentration) and served as killed controls. The ten others were incubated during 24h at 26°C in a floating device providing 5 different light intensities (from 0 to 80% of natural light using neutral density filter screens (Lee Filters). For every bottle at the end of the incubation, one 12-mL vial (Labco Exetainer) was filled with the water sample and preserved with 50 µL HgCl₂.

The rest of the sample (~50 mL) was filtered on a precombusted GF/F filter for subsequent δ¹³C-DIC measurement. δ¹³C-DIC and δ¹³C-POC were determined with an EA-IRMS as described above. The methanotrophic bacterial production, defined at the CH₄-derived ¹³C incorporation rates into the POC pool was calculated as in eq. (4) (Morana et al. 2015):

\[
MBP = \frac{POC_f \times (\%^{13}C_{POC}/\%^{13}C_{CCH})}{t \times (\%^{13}C_{DIC}/\%^{13}C_{CCH})} \times 100
\]  

(4)

Where POCᵢ is the concentration of POC after incubation, %¹³C-POCᵢ and %¹³C-POC are the final and initial percentage of ¹³C in the POC, t is the incubation time and %¹³C-CH₄ is the percentage of ¹³C in CH₄ after the inoculation of the bottles with the tracer. Similarly, the methanotrophic bacterial respiration rates, defined as the CH₄-derived ¹³C incorporation rates into the DIC pool, were calculated as in eq. (5):

\[
MBR = \frac{DIC_f \times (\%^{13}C_{DIC}/\%^{13}C_{CCH})}{t \times (\%^{13}C_{DIC}/\%^{13}C_{CCH})} \times 100
\]  

(5)

Where DICᵢ is the concentration of DIC after the incubation, %¹³C-DICᵢ and %¹³C-DIC are the final and initial percentage of ¹³C in DIC and %¹³C-CH₄ is the percentage of ¹³C in CH₄ after the inoculation of the bottles with the tracer.

Potential CH₄ oxidation rates (MOX) were calculated as the sum of MBP and MBR rates. The fraction (%) of MOX inhibited by light was calculated at every light intensity as eq.(6):

\[
MOX_{inhibition}(\%) = (1 - MOX_{i}/MOX_{dark}) \times 100
\]

(6)

Where MOXᵢ is the potential CH₄ oxidation for a given light treatment and MOX_dark is the potential CH₄ oxidation in the dark.

2.9. Determination of pelagic CH₄ production rates.

Time course ¹³C tracer experiments were carried out in well oxygenated surface waters at every sampling site. Measurement of the isotopic enrichment of the CH₄ during this experiment allowed to estimate production rates of CH₄ issued from 3 different precursors: ¹³C-DIC (NaH¹³CO₃), ¹³C₁(₂) acetate and ¹³C₅-hydroxy-methionine. Serum bottles (60 ml) were spiked with 1 ml of ¹³C tracer solution, or with an equivalent volume of distilled water for the control treatment. NaH¹³CO₃ was added in the bottles at a tracer level (less than 5% of ambient HCO₃⁻ concentration) while ¹³C₁(₂)-acetate and ¹³C₅-hydroxy-methionine were added largely in excess (>99% of ambient concentration). Therefore, we assume the CH₄ production rates measured from ¹³C-DIC could be representative of in-situ rates, but the production rates measured from ¹³C-acetate and ¹³C-methionine should...
instead be viewed as potential rates. The exact amount of $^{13}$C-DIC added in the bottles was determined filling a borosilicate 12 ml exetainer vials preserved and analysed for $\delta^{13}$C-DIC as described above.

The control bottles and the bottles amended with the different $^{13}$C tracer were incubated under constant temperature conditions (26°C) following three different treatments: (1) one third were incubated under constant light (PAR of ~ 200 µmol photon m$^{-2}$ s$^{-1}$), (2) another third were incubated under the same light intensities conditions but were first amended with DCMU (0.5 mg L$^{-1}$; final concentration), an inhibitor of photosynthesis, (3) and the last third were incubated in the dark.

At each time step (typically every 6-12h, 5-time steps), the biological activity was stopped by adding 100 µL of a saturation solution of HgCl$_2$. Bottles were kept in the dark until CH$_4$ concentration measurement and $\delta^{13}$C-CH$_4$ determination as described above.

The term CH$_4$$_{prod}$ (nmol L$^{-1}$ h$^{-1}$) defined as the amount of CH$_4$ produced from a specific tracer during a time interval t (h), was calculated following this equation (eq. 7) derived from Hama et al. (1983):

$$CH_4_{prod} = \frac{CH_4_t \times (\%^{13}CCH_4_t/\%^{13}CCH_4_i)}{t \times (\%C tracer/\%^{13}CCH_4_i)}$$  \hspace{1cm} (7)

Where CH$_4_i$ and $\%^{13}CCH_4_i$ represent the CH$_4$ concentration (nmol L$^{-1}$) and the $\%^{13}$C atom of the CH$_4$ pool at a given time step, respectively. $\%^{13}CCH_4_t$ represent the $\%^{13}$C atom of the pool of CH$_4$ at the beginning of the experiment. $\%C$ tracer represent the $\%^{13}$C atom of the isotopically enriched pool of the precursor molecule tested (NaHCO$_3$, methionine or acetate, depending of the treatment). $\%^{13}$C-tracer was assumed constant during the full course of the incubation given the high concentration of ambient DIC in the sampled lakes (~ 2 mmol L$^{-1}$ in L. George, > 6 mmol L$^{-1}$ in the other lakes) and that acetate and methionine were spiked in large excess (>99%).

2.10. DNA extraction

Surface water sample for DNA analysis (between 1 L and 0.15 L, depending on the biomass) were first filtered through 5.0 µm pore size polycarbonate filters (Millipore). The eluent was then subsequently filtered through 0.2 µm pore size polycarbonate filters (Millipore) to retain free living prokaryotes. Filters were stored frozen (-20°C) immerged in a lysis buffer until processing in the laboratory. Total DNA was extracted from the 0.2 µm and 5.0 µm 47 mm filters using DNeasy PowerWater kit (Qiagen) following the manufacturer’s instructions. Quality and quantity of the extracted DNA were estimated using the NanoDrop ND-1000 spectrophotometer (ThermoFisher) and the Qubit 3.0 fluorometer (Life technology). Extracted DNA was stored at -20 °C until further use.

2.11. Quantification of mcrA via qPCR

Quantification of mcrA gene copies was performed by quantitative PCR (qPCR) on the total extracted DNA. The used primer pair consisted of forward primer qmcrA-F 5'-TTCGGTGGATCDCARAGRGC-3' and qmcrA-R 5'-GBARGTGAWACCTGATAATCC-3' (Denman et al. 2007). The reaction mixture contained 3 µL of total community DNA extract, 7.5 µL ABsolute qPCR SYBR Green Mix (ThermoFisher, Cat. AB1158B), 0.3 µL of 10 µM forward primer mcrF, 0.3 µL of 10 µM reverse primer mcrR, 1.5 µL of a 1% w/v Bovine Serum Albumin solution (Amersham Bioscience) and 2.4 µL of Nuclease/DNA-free water. The qPCR was performed in a Rotorgene 3000 (Corbett Research) using the following conditions: 95 °C (15 min) followed by 40 cycles of 20 s at 95 °C, 20 s at 58 °C, 20 s at 72 °C and a final extension step of 5 s at 80 °C. Standard curves were prepared from serial dilutions of a prequantified mcrA PCR fragment amplified using primers mcrF and mcrR from a plasmid extract carrying the complete mcrA gene using concentrations ranging from 1x10$^2$ to 1x10$^8$ copies µL$^{-1}$. Samples were analyzed in triplicates.

2.12. 16S rRNA gene amplicon sequencing
Sequencing of the 16S rRNA gene was done on the total extracted DNA to assess community composition. 16S rRNA gene sequencing was done with the Illumina MiSeq v3 Chemistry following the “16S Metagenomic Sequencing Library Preparation” protocol with the following universal 16S rRNA gene primers targeting the V4 region, forward UniF/A519F-(S-D-Arch-0519-a-S-15) 5’- CACGMGCCGGTAA-3’ and reverse UniR/802R-(S-D-Bact-0785-b-A-18) 5’- TACNVGGGTATCTAATCC-3’ (Klindworth et al. 2013). Sequenced read quality was checked using FastQC v0.11 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Short reads were trimmed to 250 bp with FastX Toolkit v0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit) in order to remove trailing Ns and low quality bases. Operational Taxonomical Units (OTU) for each analyzed sample were obtained from the quality trimmed reads using mothur v1.39.5 (Kozich et al. 2013) and following the online MiSeq SOP (https://mothur.org/wiki/MiSeq_SOP - accessed April 2018) using the Silva v128 16S rRNA database with the following parameters: maxambig = 0 bp; maxlength = 300 bp; maxhomop = 8; and classify OTUs to 97% identity. Generated OTU table was used to calculate relative abundances of each OTU per sample.

3. Results and discussion

3.1. Patterns of phytoplankton biomass and dissolved CH₄

The sampled lakes cover a wide range of size (<1 to 2300 km²), maximum depth (3-117 m), mixing regimes, phytoplankton biomass and primary productivity (Table S1, Fig. S1). Phytoplankton biomass (Chlorophyll-a from 3.6 µg L⁻¹ to 190.2 µg L⁻¹) was dominated by Cyanobacteria (>95%) in the most productive lakes, while Diatoms (<20%) and Chrysophytes (<40%) also contributed in the less productive ones (Fig. S2). Maximum potential photosynthetic activity (Pₘₐₓ) varied from 1.5 µmol C L⁻¹ h⁻¹ in L. Edward to 199.0 µmol C L⁻¹ h⁻¹ in L. George and was linearly related to chlorophyll a concentration. Light-dependent N₂ fixation was detected in every lake with the exception of L. Kyanwming. No significant N₂ fixation rates were measured in the dark. Maximum potential N₂ fixation rates (N₂fixₘₐₓ) ranged between 1 nmol L⁻¹ h⁻¹ and 128 nmol L⁻¹ h⁻¹ and were positively related to Pₘₐₓ (Fig. S1).

We detected and quantified the abundance of the archaeal alpha subunit of methyl-coenzyme M reductase gene (mcrA), a proxy for methanogens, in the surface waters of each lake. mcrA gene copy abundance (mcrA copy ng DNA⁻¹) ranged between 319 ± 41 (L. Edward) and 7537 ± 476 (L. Katinda) in the fraction of seston < 5 µm, and between 541 ± 19 (L. Edward) and 7968 ± 167 (L. Katinda) in the fraction of seston > 5 µm (Fig. S3). Illumina 16S rRNA gene amplicon sequencing indicated that methanogens accounted for a small fraction of the prokaryotic community in the surface waters of L. Edward (0.01 %), L. Kyanwninga (0.03 %) and L. Nyamunsingere (0.08 %). They represented a substantially higher fraction of the community in L. Katinda (0.38%) and L. George (0.57 %) (Fig. S4). In all lakes, hydrogenotrophic (Methanomicrobiales and Methanobacteriales) were always more abundant than acetoclastic (Methanosarcinales) microorganisms, representing at least 65% of the methanogens (up to 95% in L. Katinda, Fig. S4).

Surface waters were super-saturated in CH₄ in all lakes, with surface concentrations (at 1 m) ranging between 78 and 652 nmol L⁻¹ (atmospheric equilibrium ~ 2 nmol L⁻¹). Vertical patterns of CH₄ and stable carbon isotope composition of CH₄ (δ¹³C-CH₄) were variable among the different lakes. In L. Kyanwninga and Katinda, higher CH₄ concentrations and lower δ¹³C-CH₄ values were observed in the well-oxygenated epilimnion compared to the metalimnion showing a source of relatively ¹³C-depleted CH₄ to the epilimnetic CH₄ pool (Fig. 1). The CH₄ concentrations and δ¹³C-CH₄ were homogeneous in the water column of L. Edward that is much larger than the other studied lakes (2300 km², Table S1) and characterized by a higher wind exposure and a substantially weaker thermal stratification (Fig. 1). However, a clear horizontal gradient in CH₄ concentration and δ¹³C-CH₄ occurred between the littoral and pelagic zones (Fig. S5). Vertical gradients were also observed at much smaller scale in the near sub-surface (top 0.3 m) in the shallow and entirely well oxygenated L. George and L. Nyamunsingere (Fig. 2). In both lakes CH₄ concentrations were relatively modest in the hypolimnion (< 50 nmol L⁻¹) but increased abruptly in the thermal gradient (0.3 m interval) to reach a surface maximum > 240 nmol L⁻¹ (Fig. 2). δ¹³C-CH₄ mirrored this pattern with
significantly lower values in surface than at the bottom of the water column indicating that a source of relatively $^{13}$C-depleted CH$_4$ contributed to the higher epilimnetic CH$_4$.

3.2. Occurrence of microbial CH$_4$ production in surface waters

Despite the prevalence of oxic conditions, $^{13}$C-labelling experiments revealed that CH$_4$ was produced in surface waters of each lake with the exception of L. Kyamwinga (Fig. 3). The kinetic of incorporation of NaH$^{13}$CO$_3$ into the CH$_4$ pool revealed that a substantially higher amount of CH$_4$ was produced from dissolved inorganic carbon (DIC) in illuminated waters, and this mechanism of CH$_4$ formation appears to be related to photosynthesis, as none or only modest quantities of CH$_4$ were produced from $^{13}$C-labelled DIC under darkness or when photosynthesis was inhibited by DCMU (Figs. 3a and S6). Furthermore, CH$_4$ production from DIC appeared strongly correlated ($r^2 = 0.91$) to the photosynthetic activity (Fig. 4a) and N$_2$ fixation rates (Fig 4b), supporting the view that CH$_4$ formation in oxic waters was directly linked to phytoplankton metabolism (Bizic et al. 2020).

Aside from DIC, an appreciable amount of CH$_4$ was generated in all lakes from the sulfur bonded methyl group of methionine when bottles were incubated under light, irrespective of the addition of DCMU (Fig. 3b and S6), that were approximately 4 times higher than in the dark. In addition, a positive relationship between CH$_4$ production from methionine in the light and the photosynthetic activity was found (Fig. 4c).

$^{13}$C-labelled acetate, the substrate of acetoclastic methanogenesis, supported the production of CH$_4$ in all lakes with the exception of L. Kyamwinga, but at much lower rates compared to light-dependent CH$_4$ production from DIC (50 times lower, n=7) or methionine (10 times lower, n=4) (Fig. 3c and S6). $^{13}$C analysis of the DIC in the bottles spiked with $^{13}$C-labelled acetate showed that the acetate was mineralized at rates of 5-6 orders of magnitude higher than acetoclastic methanogenesis so that added acetate appeared to be used almost exclusively by heterotrophic micro-organisms other than methanogens. Pattern of acetate-derived production of CH$_4$ were similar in light and dark treatments (Figs. 3c and S6) and this mode of CH$_4$ production appeared unrelated to phytoplankton activity (Fig. 4d).

3.3. Mechanisms of epilimnitic CH$_4$ production

Only a minimal fraction of the CH$_4$ produced under aerobic conditions originated from acetate in contrast with several earlier studies (Bogard et al. 2014, Donis et al. 2017) which proposed, based on the apparent fractionation factor of $\delta^{13}$C-CH$_4$, that acetoclastic methanogenesis linked to phytoplankton production of organic matter would be the dominant biochemical pathway of pelagic CH$_4$ production in oxic freshwaters. Instead, our results suggest that epilimnetic CH$_4$ production in well-oxygenated conditions was related to DIC fixation by photosynthesis (Fig. 3), and correlated to primary production (Fig. 4a) and N$_2$ fixation (Fig 4b). When normalized to POC concentrations, the average DIC-derived CH$_4$ production rates (0.08 ± 0.05 nmol mmol$^{-1}$POC h$^{-1}$, n = 7) was remarkably similar to the CH$_4$ production rates recently reported in Cyanobacteria cultures (0.04 ± 0.02 nmol mmol$^{-1}$POC h$^{-1}$) grown at 30°C, among which the freshwater Microcystis aeruginosa (Bizic et al. 2020), the dominant Cyanobacterium species in the tropical lakes investigated in our study (see Fig S2). These CH$_4$ production rates are 2 orders of magnitude higher than rates reported in an axenic culture of the eukaryote Emiliania huxleyi (0.19 ± 0.07 pmol mmol$^{-1}$POC-d$^{-1}$) (Lenhart et al. 2016), but they are 4 orders of magnitude lower than typical anoxic CH$_4$ production rates by methanogenic Archaea (Mountford & Asher 1979). Although it seems improbable that $^{13}$C-DIC acted as a direct precursor molecule for the CH$_4$ released by phytoplankton (Lenhart et al. 2016, Klintzsch et al. 2019) $^{13}$C-DIC could have been taken up by phytoplankton cells and then used as a C source for the synthesis of many different organic molecules that may serve as the actual CH$_4$ precursors. Indeed, healthy phytoplankton cells actively release a variety of low molecular weight molecules which are generally highly labile and rapidly consumed (Baines & Pace 1991, Morana et al. 2014). Phytoplankton metabolism could have fuelled CH$_4$ production pathways, at least partially, excreting substrates involved in CH$_4$ production via biochemical
processes such as demethylation of a variety of organic molecules like methionine, one of the S-bonded methylated amino acids (Lenhart et al. 2016), trimethylamine (Bizic et al. 2018), or methylphosphonate (Yao et al. 2016).

While the source of methylphosphonate in freshwaters is obscure and its actual natural abundance remains to be determined, dissolved free amino acids would represent up to 4% of the DOC produced by phytoplankton and are rapidly consumed by heterotrophic bacteria (Sarmento et al. 2013). Our incubations indeed demonstrated that the methyl group of methionine was a potential precursor of CH₄ in all lakes investigated, in line with recent findings showing that Emiliania huxleyi could act as a direct source of CH₄ in oxic conditions using methionine as precursor, without involvement of any other micro-organisms (Lenhart et al. 2016). We found that CH₄ production from methionine was clearly stimulated under light, even when photosynthetic activity was inhibited by DCMU, while little CH₄ from methionine was produced in darkness (Fig. 3b). DCMU notably prevents reduction of plastoquinone at photosystem II and generates singlet oxygen (Petrillo et al. 2014). The mechanism of CH₄ production from methionine is still unclear, but its residue in proteins is particularly sensitive to oxidation to methionine sulfoxide by radical oxygen species (ROS) (Levine et al. 1996) so that methionine would act as an effective ROS scavenger and play important protective roles under photooxidative stress conditions, as shown in vascular plants (Bruhn et al. 2012). The side chain of methionine sulfoxide is identical to dimethyl sulfoxide which is known to react with hydroxyl radicals (OH) to form CH₄ (Repine et al. 1979). Besides its photoprotective role for phytoplankton, methionine could also be catabolized by a wide variety of microorganisms to methanethiol, which could in turn be transformed to CH₄ as shown in Arctic Ocean surface waters (Damm et al. 2010). Nevertheless, occurrence of this latter mechanism in the tropical lakes investigated seems unlikely as this mode of CH₄ production would be expected to be insensitive to light irradiance and no CH₄ was produced from methionine in the dark during the incubations.

3.4. Relevance of epilimnetic CH₄ production compared to CH₄ loss terms at ecosystem scale

Net CH₄ oxidation was detected in all 5 investigated lakes ranging from 11 to 5212 nmol L⁻¹ d⁻¹ (Fig. 5), and was by far the largest loss term of dissolved CH₄ at ecosystem scale (8 to 46 times higher than the diffusive emission to the atmosphere). Surface water CH₄ turnover times were particularly short in the shallow and eutrophic L. George (2h) and L. Nyamusingere (3h) and slightly longer in the deeper and less productive L. Katinda (11h), L. Kyamwinga (77h) and L. Edward (100h). In all studied lakes, pelagic CH₄ production rates measured during the stable isotope tracer experiments represented between 0.1% and 8.5% of net CH₄ oxidation rates, regardless of the CH₄ precursors tested.

All of the major sources and sinks of CH₄ at ecosystem scale were experimentally determined offshore in three lakes (L. Edward at 20 m depth, George and Nyamusingere) (Fig. 6). In these three lakes, surface CH₄ production rates from the diverse precursors molecules investigated were modest relative to the diffusive CH₄ efflux to the atmosphere (0.4 – 20.0 %) and microbial CH₄ oxidation (0.1 – 13.2 %). In opposition, the combined CH₄ bubble dissolution flux and diffusive benthic CH₄ flux were several orders of magnitude higher than CH₄ production in surface waters, and met the CH₄ loss terms (emission and oxidation) (Fig. 6). These results gathered in tropical lakes of various size (from 0.44 to 2300 km²) and depth are in sharp contrast with the estimation of an empirical model (Gunthel et al. 2019) which proposed that mechanisms of oxic CH₄ production represents the majority of CH₄ emissions in lakes larger than 1 km². This discrepancy highlights the need to consider the unique limnological characteristics of a vast region of the world that harbours 16% of the total surface of lakes (Lehner & Doll 2004). One of the most distinctive features of tropical aquatic environment is the persistent elevated water temperature in the hypolimnion and at the water-sediment interface which favours methanogenic activity in sediment and decreases CH₄ solubility, enhancing bubbles formation.

3.5. Origin of ¹³C depleted CH₄ in surface waters

Epilimnetic CH₄ production was a marginal flux at ecosystem scale and could not explain alone the accumulation of ¹³C-depleted CH₄ in the epilimnion of most of the lakes of our dataset (Figs. 1, 2), for which we propose two other alternative
mechanisms: dissolution of arising CH\textsubscript{4} bubbles in the epilimnion combined with inhibition by light of CH\textsubscript{4} oxidation. The partial dissolution of the CH\textsubscript{4} bubbles as they rise in the epilimnion should allow a rapid transport of 
\textsuperscript{13}C-depleted CH\textsubscript{4} from the sediment, bypassing the hotspot of CH\textsubscript{4} oxidation at the sediment-water interface and representing an alternative source of 
\textsuperscript{13}C-depleted CH\textsubscript{4} in water column. The shallower L. George and L. Nyamusingere were notably characterized by sharp thermal density gradients (Fig. 2) and extreme phytoplankton biomass largely dominated by \textit{Microcystis aeruginosa} (Chlorophyll a up to 190 µg L\textsuperscript{-1}). \textit{Microcystis aeruginosa} cells form large aggregates (>1 mm) embedded in a matrix of extracellular polymeric substance that might act as a barrier to trap small CH\textsubscript{4} bubbles arising from the sediment (Fig S7). Dissolution of CH\textsubscript{4} bubbles could be enhanced at the very near surface due to the entrapments of bubbles at the air-water interface by abundant surface organic films that delay the bubble “burst”. The presence of a sharp sub-surface temperature gradient would further enhance CH\textsubscript{4} accumulation during day-time near the air-water interface (by blocking vertical redistribution of CH\textsubscript{4} by mixing). We hypothesize that this process could be widespread in shallow tropical lakes which are characterized by high productivity and are susceptible to be simultaneous large benthic CH\textsubscript{4} sources.

The influence of light on methanotrophy was investigated in the deep L. Edward, and shallow L. George and L. Nyamusingere, revealing that CH\textsubscript{4} oxidation rates decreased dramatically with increasing light intensity (Fig. 5). For instance, when exposed to full sunlight intensity, methanotrophs consumed only 42% (L. Edward), 54% (L. Nyamusingere) or 74% (L. George) of the CH\textsubscript{4} they were able to oxidize in the dark. The magnitude of this light-induced inhibition decreased substantially with decreasing sunlight intensities, as shown elsewhere (Murase & Sugimoto 2005). The physiological mechanism of photoinhibition of CH\textsubscript{4} oxidation could be related to the fact that the copper-containing methane monoxygenase enzyme and structurally close to the ammonia monoxygenase enzyme, and might be inactivated by ROS produced during photooxidative stress, as shown for ammonium oxidizers (French et al. 2012, Tolar et al. 2016). Altogether, our results emphasize the role of sunlight irradiance as an important, but frequently overlooked, environmental factor driving the CH\textsubscript{4} dynamics in lake surface waters, and possibly contributing to the occurrence of \textsuperscript{13}C depleted CH\textsubscript{4} in surface waters.

4. Supplement

Supplementary figures are available on-line on the \textit{Biogeosciences} website.

5. Data availability

All data included in this study are available upon request by contacting the corresponding author.

6. Author contributions

This study was designed by C. Morana, A.V. Borges & S. Bouillon. All authors participated to samples collection, data acquisition and analysis, and to the drafting of the manuscript. All authors approved the final version of the manuscript.

7. Competing interests

The authors declare that they have no conflict of interest.

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10. Figures

Figure 1. Depth profile. Depth profile of the temperature (°C; dashed line), CH$_4$ concentration (nmol L$^{-1}$; red symbols) and stable isotope carbon composition of CH$_4$ ($\delta^{13}$C-CH$_4$, ‰; blue symbols) in Lake Kyamwinga (left), Lake Katinda (middle), and Lake Edward (right).
Figure 2. Depth profile, focus on the surface. Depth profile of the temperature (°C; dashed line), CH$_4$ concentration (nmol L$^{-1}$; red symbols) and stable isotope carbon composition of CH$_4$ ($\delta^{13}$C-CH$_4$, ‰; blue symbols) in Lake George (left), Lake Nyamusingere (middle), and the surface waters (0-2 m) of Lake Edward (right).
Figure 3. Tracer experiments show CH$_4$ production in well-oxygenated surface waters. CH$_4$ production rates (nmol L$^{-1}$ d$^{-1}$) from dissolved inorganic carbon (a), the methyl group of methionine (b) and acetate (c) measured in the surface waters (0.3 m) of a variety of African tropical lakes. Green, grey and dark bars respectively represent rates measured under light, light in presence of a photosynthesis inhibitor (DCMU), or darkness. Values showed for L. Edward, L. George and L. Katinda are the average of 2017 and 2018 sampling campaign measurement. n.s = not significant, < 0.1 = below 0.1 nmol L$^{-1}$ d$^{-1}$. 
Figure 4. Direct link between CH₄ production and phytoplankton metabolism. Relationship between the maximum photosynthetic activity ($P_{\text{max}}$, µmol C L⁻¹ h⁻¹) or maximum nitrogen fixation rates ($N_{\text{max}}$, nmol L⁻¹ h⁻¹) and surface CH₄ production rates (nmol C L⁻¹ h⁻¹) from dissolved inorganic carbon (a, b), methyl group of methionine (c), and acetate (d).
Figure 5. Light inhibition patterns of CH₄ oxidation in surface waters. Left panel: CH₄ oxidation rates (nmol L⁻¹ d⁻¹) measured in the surface waters (0.3 m) in the dark of a variety of African tropical lakes. Right panel: relationship between illumination (fraction of incident sunlight irradiance, %) and CH₄ oxidation inhibition (fraction of CH₄ oxidation in the dark inhibited at a given irradiance, %) in Lake Edward, Lake George and Lake Nymusingere. Symbols represent the mean, and error bars represent the maximum and minimum of duplicate experiments.
Figure 6. Epilimninitic CH₄ production is a marginal source of CH₄ compared to sedimentary sources and CH₄ sinks in several contrasting African lakes. Summary of the different CH₄ flux experimentally measured in L. Edward, L. George and L. Nyamusingere. Values of CH₄ oxidation in brackets are values not considering CH₄ photoinhibition. Pelagic CH₄ production values determined from NaH¹³CO₃ (~5% final enrichment) and ¹³C-acetate (99% final enrichment), as described in the methods section. ¹³C-labelling experiment carried out under constant light irradiance. CH₄ flux at the water-air and sediment-water interface were determined experimentally as described in the Methods. CH₄ bubble dissolution and CH₄ ebullition flux.
were determined using the SiBu-GUI software (Greinert & McGinnis 2009); minimum and maximum represents the values obtained from two extreme bubble-size scenarios considering a release of many small (3 mm diameter) bubbles or fewer large (10 mm) bubbles.