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Imprint of eutrophication on methane-cycling microbes in freshwater sediment

Alice Bosco-Santos[1*], Eulalie Rose Beyala Bekono[1], Santona Khatun[1], Marie-Ève Monchamp[2], Joana Séneca[3,4], Petra Pjevac[3,4], Jasmine S. Berg[1]

[1]Institute of Earth Surface Dynamics (IDYST), University of Lausanne, Lausanne, Switzerland

[2]Biology Department, McGill University, Montreal, Canada

[3]Division of Microbial Ecology, Centre for Microbiology and Environmental Systems Science, University of Vienna, Vienna, Austria

[4]Joint Microbiome Facility of the Medical University of Vienna and the University of Vienna, Vienna, Austria

*(alice.boscosantos@unil.ch)

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Abstract. Eutrophication can alter methane (CH_4) cycling in lakes, yet its long-term effect on sediment microbial communities remains unclear. To elucidate these effects, we analyzed a 400-year-old sediment record from the historically eutrophied Lake Joux, Switzerland, combining porewater and solid-phase geochemistry with 16S rRNA gene amplicon analyses. Lithological and chemical stratification defined three intervals (deep eutrophic, middle carbonate, upper eutrophic) that were correlated with changes in organic matter sources. Methanogens were clearly depth-partitioned: methylotrophic Methanomassiliicoccales dominated deep eutrophic sediments, whereas hydrogenotrophic Methanomicrobiales and Methanobacteriales increased upward in shallower, more recent sediments with fresher organic matter. Paired isotopic data support this substrate-driven shift in CH_4 production. Although O_2 was not detected below ~0.4 cm, sequences of aerobic gammaproteobacterial methanotrophs (*Crenothrix* and *Methylobacter*) were abundant in surface sediments down to ~20 cm sediment depth, correlating with NO_3^- and PO_4^{3-} concentrations. The absence of anaerobic methanotrophs and C-isotopic evidence for ongoing methane oxidation suggest that these O_2-requiring, methane monooxygenase-utilizing Methylococcales constitute the dominant CH_4 sink in these surface sediments. These findings reveal that eutrophication can cause a stratification of methane-cycling microbial communities, highlighting the role of sedimentary legacies in regulating benthic CH_4 emissions from freshwater ecosystems.

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1. Introduction

Freshwater ecosystems are significant sources of the greenhouse gas methane (CH_4), with natural lakes estimated to contribute more than 70% to freshwater CH_4 emissions (Sanches et al., 2019). Despite their substantial contribution to atmospheric CH_4, the mechanisms regulating CH_4 emissions from lakes at regional and global scales remain poorly understood (Bastviken et al., 2011; Sanches et al., 2019). In freshwater sediments, CH_4 is abundantly produced via anaerobic methanogenesis by archaea (Bastviken et al., 2004; Bastviken et al., 2011; Conrad, 2020; Dean et al., 2018; Saunois et al., 2020; Tranvik et al., 2009). Methanogens can respire different substrates produced during organic matter remineralization and are classified according to three known pathways for CH_4 production: hydrogenotrophic (carbon dioxide reduction using hydrogen), acetoclastic (splitting acetate), and methylotrophic (using methylated compounds like methanol) (Garcia et al., 2000). Environmental factors such as substrate concentration, temperature, salinity, and pH influence the predominance of these pathways, with methylotrophic methanogenesis, for instance, being favored at higher salinity and acidity (Bueno De Mesquita et al., 2023; Yvon-Durocher et al., 2014).

Much of the CH_4 produced in lake sediments is oxidized through both aerobic and anaerobic microbial processes before it can reach the atmosphere (Bastviken et al., 2004; Bastviken et al., 2008; Martinez-Cruz et al., 2017; Martinez-Cruz et al., 2018; Oswald et al., 2016). Aerobic oxidation, predominantly performed by methane-oxidizing bacteria (MOB) from the Gammaproteobacteria and Alphaproteobacteria classes, occurs at the sediment-water interface and in the water column (Hanson and Hanson, 1996; Knief, 2015). MOB rely on the O_2-dependent methane monooxygenase enzymes to oxidize up to 90% of sediment-derived CH_4, thus helping to mitigate greenhouse gas emissions from freshwater ecosystems (Bastviken et al., 2004; Bastviken et al., 2008). Anaerobic oxidation of methane (AOM) is performed by methanotrophic archaea (ANMEs), often in partnership with bacteria that use electron acceptors other than oxygen (Knittel and Boetius, 2009; Milucka et al., 2012; Wegener et al., 2015). In marine environments where sulfate concentrations are high, sulfate-AOM is the dominant process (Jørgensen et al., 2001; Wegener and Boetius, 2009). In contrast, the electron acceptors sulfate, nitrate/nitrite, humic substances, and diverse metal oxides contribute in various degrees to AOM in freshwater sediments (Chen et al., 2023; Deutzmann and Schink, 2011; Martinez-Cruz et al., 2018; Zhao et al., 2024).

Recently, it has been proposed that eutrophication induced by anthropogenic nutrient inputs (e.g., nitrates and phosphates) into lake ecosystems influences methanogen and methanotroph community structure by altering organic matter quality and quantity (Beaulieu

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et al., 2019; Yang et al., 2021; Yang et al., 2019; Yang et al., 2020; Zhu et al., 2022). The influx of organic carbon from phytoplankton blooms enhances organic matter mineralization in lake bottom waters and sediments, depleting electron acceptors such as oxygen (O_2), nitrate (NO_3^-), sulfate (SO_4^{2-}), and metal oxides (Fe(III), Mn(IV)). The decomposition of phytoplankton biomass also releases significant amounts of methyl-sulfur compounds, favoring methylotrophic CH_4 production (Penger et al., 2012; Tebbe et al., 2023; Tsola et al., 2021; Yan et al., 2017; Zhou et al., 2022). Anaerobic conditions, combined with increased organic matter availability, are expected to boost methanogenesis, resulting in elevated CH_4 release following eutrophication (Fiskal et al., 2019; Sanches et al., 2019; Zhou et al., 2022).

Conversely, nutrient addition can stimulate microbial CH_4 oxidation (Yang et al., 2019). Some aerobic MOB can oxidize CH_4 while respiring NO_3^- (denitrification), and a growing body of evidence supports the widespread occurrence and activity of these bacteria in O_2-limited environments (Almog et al., 2024; Kits et al., 2015; Reis et al., 2024; Schorn et al., 2024). Importantly, MOB exhibit niche partitioning along O_2–CH_4 and nutrients gradients with Gammaproteobacteria, Alphaproteobacteria, and nitrite-dependent taxa that produce O_2 intracellularly, such as *Candidatus Methylomirabilis,* occupying distinct layers (Mayr et al., 2020; Reis et al., 2020). Gammaproteobacterial MOB are generally associated with fast-growing life strategies in resource-rich conditions, whereas alphaproteobacterial MOB are adapted to resource-limited or stable environments (Ho et al., 2013). Indeed, P and N enrichment, for instance, can increase CH_4 oxidation rates and favor Gammaproteobacteria over Alphaproteobacteria MOB (Nijman et al., 2022; Veraart et al., 2015). Together, these findings highlight that nutrients modulate both CH_4 production and consumption, adding complexity to how eutrophication shapes lacustrine CH_4 dynamics (Nijman et al., 2022; Reis et al., 2020; Veraart et al., 2015; Wei et al., 2022).

Eutrophication of lakes in Switzerland reached critical levels during the mid-20th century, particularly in the 1970s, due to rapid industrialization, urbanization, and agricultural intensification. Public outcry and scientific research prompted the introduction of wastewater treatment plants and stricter regulations on phosphate detergents, leading to significant improvements in water quality by the late 20th century. Nevertheless, sediments retain a legacy of this eutrophication in the form of increased organic matter content (Fiskal et al., 2019), which continues to shape microbial community structure (Han, 2020) long after lake waters recovered. Some studies have reported clear vertical zonation of methanogenic and methanotrophic communities in relation to trophic history and electron acceptor distributions (Rissanen et al., 2023; Van Grinsven et al., 2022), while others found little to no stratification

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(Meier et al., 2024). Consequently, the cascading impacts of anthropogenic nutrient inputs on the balance of CH_4 production and oxidation in lake sediments remain poorly constrained.

To address this knowledge gap, we focused on the sedimentary record of Lake Joux (Vaud, Switzerland), a site with a well-documented history of trophic regime shifts and phytoplankton bloom deposits (Lavrieux et al., 2017; Monchamp et al., 2021). We combined microbial community sequencing with isotopic analyses of different carbon pools in lake sediments dating back to the 16th century. The isotopic behavior of the two stable carbon isotopes, ^{12}C and ^{13}C, plays a critical role in understanding microbial CH_4 production and consumption, as ^{12}C is preferentially used over ^{13}C due to its lighter mass and greater reactivity. During methanogenesis, CH_4 produced is typically depleted in ^{13}C relative to precursor substrates such as organic matter or CO_2, resulting in isotopically lighter CH_4 (Conrad, 2005). Conversely, methanotrophs preferentially oxidize $^{12}CH_4$, enriching the residual CH_4 pool in ^{13}C and simultaneously generating isotopically lighter dissolved inorganic carbon ($\delta^{13}C_{DIC}$) (Barker and Fritz, 1981; Jahnke et al., 1999; Templeton et al., 2006; Whiticar, 1999). Thus, this carbon isotope fractionation provides key insights into the microbial processes driving CH_4 cycling, enabling inference of microbial activity even without direct rate measurements (Blaser and Conrad, 2016; Conrad, 2005, 2007; Valentine et al., 2004).

2. Materials and methods

2.1 Study Area

Lake Joux is a perialpine lake in the Joux Valley in the Swiss Jura Mountains (Fig. 1). The valley developed in a Jura syncline, marked by glacial erosion and Quaternary deposits, and it lies mainly on Upper Jurassic and Tertiary limestones. The lake has an average depth of 32 m, a surface area of approximately 9 km^2 (maximum 9 km in length and 1 km in width), and a watershed covering around 211 km^2. Situated at an altitude of 1,183 meters, the lake is subject to intense seasonal variations and meteorological events, which drive the runoff of both natural and anthropogenic materials.

Human activity in the watershed dates back over 6,850 years (Lavrieux et al., 2017; Mitchell et al., 2001; Monchamp et al., 2021). By the 16th century, the area around Lake Joux became more densely populated, leading to land drainage and deforestation for livestock farming (Piguet, 1946). Frequent crop failures and food shortages during the late 17th century spurred the growth of glassmaking and lapidary industries. Horology, introduced in the 18th century, became the region's dominant economic activity by the 19th century. This period of

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industrialization resulted in a transition from cultivated farmland to pastures and fallow fields (Lavrieux et al., 2017).

Agricultural intensification and urban expansion during the 20th century significantly increased nutrient inputs to Lake Joux, resulting in pronounced eutrophication. Phosphorus levels peaked at 35 µg/L in 1979 (Lods-Crozet et al., 2006), triggering major ecological changes, including rapid shifts in phytoplankton communities as eutrophication-adapted taxa outcompeted the lake's original species (Monchamp et al., 2021). A re-oligotrophication phase began in 1988–1989 following improved nutrient management and mitigation efforts. However, despite these reductions in external nutrient loading, the lake has not returned to its pre-eutrophication conditions. More than 70 years after the documented episode of eutrophication, the water column remains altered, suggesting that the system has shifted to an alternative stable state with a biological configuration resistant to reversal (Lods-Crozet et al., 2006; Monchamp et al., 2021).

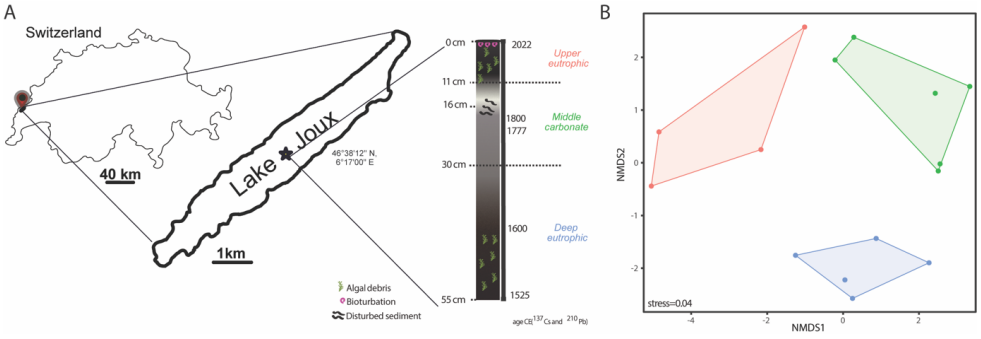

Figure 1 - Schematic representation and geographic coordinates of where the sediment cores from Lake Joux, Switzerland, were sampled in 2023. The 55 cm sedimentary profile has three distinct stratigraphic layers: deeper eutrophic, middle carbonate, and upper eutrophic.

2.2 Sampling

In 2023, three gravity cores (45-55 cm long) were recovered from Lake Joux's lakebed using a Uwitec gravity corer. The cores were taken from one of the deepest parts of the lake (46°38'12" N, 6°17'00" E; 28 m depth) and sealed with rubber caps. One core was pre-drilled and taped at 3 cm intervals to facilitate rapid CH_4 sampling using 3 ml syringes on shore. The other two cores, one for porewater and the other for sediment chemistry and microbiome analyses, were processed in the laboratory within 24 h. Porewater was extracted via N_2 flushed syringes attached to 0.2 µm Rhizons (Rhizosphere), inserted every 3 cm along the core, stored

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at 4°C and analyzed within 48 h. The third sediment core was opened with a handheld saw and sectioned every 3 cm. Each sample was split into an acid-cleaned vial and a sterile vial, then frozen at -20°C.

2.3 Porewater chemistry

Samples for dissolved anions (PO_4^{3-}, NO_3^-, SO_4^{2-}) were transferred to plastic vials while flushing with N_2, capped, and analyzed using an ion chromatograph (DX-ICS-1000, DIONEX) equipped with an AS11-HC column. Dissolved inorganic carbon (DIC) porewater samples were filled into 1.5 ml borosilicate vials and capped without headspace to prevent CO_2 degassing. To measure $\delta^{13}C_{DIC}$, samples were transferred to helium-flushed Exetainers containing 200 µL of 85% phosphoric acid, converting all DIC to CO_2. The CO_2 in the headspace was then analyzed using a GasBench II (ThermoFisher Scientific) coupled with an isotope ratio mass spectrometer (Delta V, ThermoFisher Scientific). Carbon isotopes are reported in the conventional delta notation relative to the Vienna Pee Dee Belemnite (VPDB) standard.

For dissolved sulfide analysis, porewater was fixed with 0.05 M Zn-acetate ($Zn(CH_3COO)_2 \cdot 2H_2O$) solution at a 1:2 ratio immediately after extraction, and dissolved sulfide was quantified photometrically using the methylene blue method (Cline, 1969).

2.4 Sediment description and chemistry

Sediment from the opened core was visually assessed (using standard charts) for color and granulometry based on observable differences in particle size, texture, and sorting within the sediment layers.

For total phosphorus (P), ~1 g of wet sediment was digested in 9 ml of 4:2 HNO_3:HCl using an Anton Paar microwave system, filtered (0.45 µm glass fiber), and analyzed by ICP-OES (Agilent 5800). Calibration used a multi-element standard, with certified reference materials yielding 85–102% recovery.

Elemental C, N, H, and S were measured on 1–3 mg of freeze-dried sediment using a UNICUBE (Elementar®) at EPFL's ISIC-MSEAP. TOC and TIC were estimated via loss on ignition (500°C and 1200°C). $\delta^{13}C_{org}$ was determined by EA-Isolink IRMS (Thermo Fisher) after 48 h treatment with 6 N HCl to remove carbonates. Results are reported in delta notation (VPDB), with a reproducibility better than 0.2‰.

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Acid-volatile sulfur (AVS) and chromium-reducible sulfur (CRS) were extracted from 1–2 g of frozen sediment as per Spangenberg and Bosco-Santos (2024). Sulfide in AVS and CRS fractions was measured colorimetrically (Cline, 1969) and CRS sulfur isotopic composition ($\delta^{34}S_{crs}$) by IRMS (Spangenberg and Bosco-Santos, 2024). These measurements help distinguish easily mobilized sulfide pools (AVS) from more stable sulfur forms (CRS) in sediments.

2.5 Dissolved oxygen and methane

Oxygen concentrations were measured using a 200 µm-tip glass microsensor (Unisense) after 2-point calibration in Na-dithionite and air-saturated water. Vertical profiles were recorded at 250 µm steps with a motorized controller and Field Multimeter (Unisense).

For CH_4 analysis, 3 cm^3 of sediment was transferred into 100 ml serum bottles with 5 ml of 10% NaOH, sealed, and homogenized. Dissolved CH_4 was extracted by headspace displacement and quantified via gas chromatography (Joint Analytical Systems) at Eawag. $\delta^{13}C_{CH4}$ was measured using GCC-IRMS (Agilent 6890N with Thermo Finnigan IRMS) and analyzed with IonVantage software (Khatun et al., 2024). Results are reported in delta notation relative to VPDB with an analytical error <1.1‰.

Carbon isotopic fractionation factors (α) between C_{org} (substrate) and CH_4 (product) were as: $\alpha = (\delta^{13}C_{org} + 1000)/(\delta^{13}C_{CH_4} + 1000)$. The corresponding isotopic fractionation (ε, ‰) was then determined by the relationship $\varepsilon = (\alpha - 1) \times 1000$, allowing interpretation of trends in dominant methanogenic pathways.

2.6 DNA extraction and 16S rRNA gene amplicon analysis

DNA was extracted from Lake Joux sediments using the PowerSoil Pro Kit (Qiagen). Extraction, sequencing, and raw data processing were conducted at the Joint Microbiome Facility (Medical University of Vienna and University of Vienna; project ID JMF-2310-14). The V4 hypervariable region of the 16S rRNA gene was amplified and sequenced to assess the total microbial diversity in the collected samples. Amplification was performed with linker-modified 515F and 806R (Apprill et al., 2015; Parada et al., 2016) primers, and amplicons were barcoded, multiplexed, sequenced on an Illumina MiSeq (v3 chemistry, 2x 300 bp), and extracted from the raw sequencing data as described in detail in Pjevac et al. (2021). Amplicon Sequence Variants (ASVs) were inferred using the DADA2 R package v1.42 (Callahan et al., 2016b), applying the recommended workflow (Callahan et al., 2016a). FASTQ reads 1 and 2

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were trimmed at 220 nt and 150 nt with allowed expected errors of 2. ASV sequences were subsequently classified using DADA2 and the SILVA database SSU Ref NR 99 release 138.1 (Quast et al., 2012; Mclaren and Callahan, 2021) using a confidence threshold of 0.5. ASVs without classification or classified as eukaryotes, mitochondria, or chloroplasts, as well as well-known buffer contaminations, were removed. After filtering, only samples with at least 7000 read pairs were kept for further analyses. The relative abundance of chloroplast sequences was examined separately to assess phytoplankton debris across the sediment profile.

Downstream analyses were performed using R v4.3.2 and Bioconductor v3.16 packages SummarizedExperiment v1.32, SingleCellExperiment v1.24, TreeSummarizedExperiment v2.8 (Huang et al., 2021), mia v1.8 (https://github.com/microbiome/mia), LMdist (Hoops and Knights, 2023), vegan 2.6-8, phyloseq v1.44 (Mcmurdie and Holmes, 2013) (Vegan R package; phyloseq R package), microbiome v1.22 (http://microbiome.github.io), microViz v0.10.8 (Barnett et al., 2021), and corrplot (Wei, 2024). In order to determine sediment zonation by environmental variables, we performed non-metric multidimensional scaling (NMDS) on a Euclidean distance matrix of z-scored environmental data for samples between 0.5 and 43.5 cm sediment depth, using the function metaMDS() in the R package vegan. The NMDS stress value was 0.04. Microbial community alpha diversity indices were calculated on rarified 16S rRNA gene amplicon data using R packages vegan and mia. For community dissimilarity analysis, microbial 16S rRNA gene amplicon sequence count data was centered log ratio (CLR) transformed, a pairwise Aitchison distances matrix was computed, and oversaturated distances in the dissimilarity matrix were corrected and smoothed using LMdist with default settings prior to ordination using principal coordinates analysis (PCoA). To identify the environmental variables that significantly contributed to the variation in microbial community structure, correlations between microbial community composition and environmental variables were assessed using Mantel tests, based on Euclidean distances calculated from Z-score standardized environmental variables and LMdist corrected and smoothed Aitchison distances of 16S rRNA gene amplicon sequencing data. Prior to correlation analysis, five samples from the deep eutrophic layer without corresponding environmental data were excluded. Mantel tests were performed using Spearman's rank correlation as implemented in the R package vegan. The resulting p-values were adjusted for multiple testing using the false discovery rate (FDR) method. Highly correlated environmental variables (Spearman's $r>0.8$, Figure S1), as assessed by the function cor() in the R package corrplot, were removed before the Mantel tests.

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3. Results

3.1 Sediment description

The 55-cm deep sediment record of Lake Joux could be classified into three main intervals based on distinct lithological and chemical features (Fig. 1 and Supplementary Fig. 1). The 'deep eutrophic' interval, from 55 cm to 30 cm, comprises black and silty sediments, indicating a period of higher lake productivity and low oxygen conditions. Occasional fine sand and organic fibers are also present. In the 'middle carbonate' interval, from 30 cm to 11 cm, the sediments transition from a murky gray with heterogeneous brownish features, suggesting changes in organic matter quality and oxidation states (Fig. 1) to whitish silty-sandy sediments, with the contribution of shells above 13.5 cm, indicating the dominant deposition of carbonates (Fig. 1). The 'upper eutrophic' interval, from 11 cm to 0 cm, contains intensely black sediment with frequent plant debris, reflecting recent environmental changes.

To assign approximate ages to our sedimentary profile, we correlated our lithological intervals to other Lake Joux sedimentary sequences previously published and dated using ^{237}Cs and ^{210}Pb (Lavrieux et al., 2017; Magny et al., 2008) (Fig. 1). This correlation places the base of our core, the deep eutrophic interval, in the late 16th century (middle-upper U3 of Lavrieux et al., 2017). Reported sedimentation rates were 0.04 - 0.11 cm/yr until the end of the middle carbonate interval, when they increased to a short-lived peak of ~ 0.83 cm/yr near the late 18th century, and then declined to an average of 0.18 cm/yr over the past 60 years in the upper eutrophic interval (Lavrieux et al., 2017).

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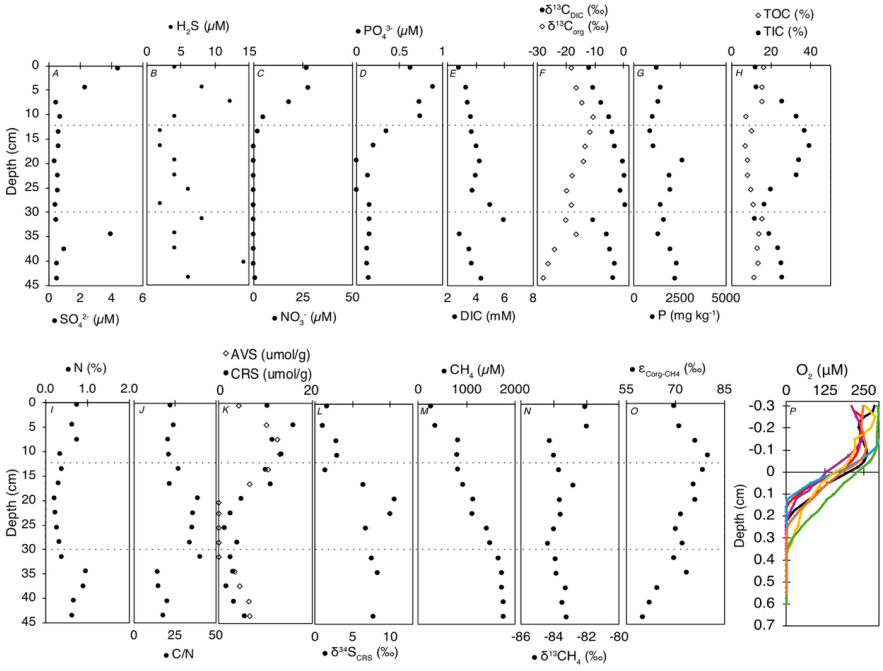

Figure 2 - Geochemical profiles of porewater, solid-phase compounds, and dissolved gases in Lake Joux sediments. Dashed lines represent the interpreted intervals corresponding to *'deep eutrophic'* from 55 to 30.5 cm; *'middle carbonate'* from 28.5 to 11 cm; and *'upper eutrophic'* from 11 to 0 cm. All data are available in Supplementary Material Table 1 (panels A to N) and Table 2 (panel O).

3.2 Porewater chemistry

Sulfate (SO_4^{2-}, between 0.35 µM and 4.5 µM) and dissolved sulfide (H_2S, between 2 µM and 14.3 µM) were measurable throughout the entire sedimentary profile, with peak concentrations in deep eutrophic sediments around 40 cm and again in the upper eutrophic sediments around 7.5 cm depth (Fig. 2A and 2B).

Dissolved nitrate (NO_3^-) and phosphate (PO_4^{3-}) first appeared at 19.5 cm and 16.5 cm, respectively, with concentrations progressively increasing toward the surface, reaching maximum values of 27 µM for NO_3^- and 0.62 µM for PO_4^{3-} (Fig. 2C and 2D).

Dissolved inorganic carbon (DIC) concentrations with isotopically heavier composition ($\delta^{13}C_{DIC}$) were highest in the middle carbonate interval between 16.5 cm and 31.5 cm (Fig. 2E). Above 7.5 cm depth, $\delta^{13}C_{DIC}$ values progressively became lighter toward the surface, reaching a minimum of -12‰ (Fig. 2F).

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3.3 Sediment chemistry

3.3.1 Phosphorous and organic matter characterization

Total phosphorus (P) content, ranging from 860 to 2612 mg kg^{-1}, was generally higher in the deeper sediments and progressively decreased towards the surface, except for a sharp peak at 19.5 cm depth (Fig. 2G). Organic carbon (TOC) exhibited higher concentrations in both the deep eutrophic and upper eutrophic sediments, contrasting with TIC content, which peaked in the middle carbonate interval (Fig. 2H). The $\delta^{13}C_{org}$ was lightest in the deep eutrophic sediments (-28.22‰ at 43.5 cm) and heaviest in the upper eutrophic sediments (-10.76‰ at 10.5 cm) (Fig. 2F).

Nitrogen content followed the same pattern as TOC, with higher N in the deep and upper eutrophic sediments compared to the middle carbonate region (Fig. 2I). The ratio between C and N, a qualitative parameter of organic matter source (Meyers, 1994), exhibited relatively lower values in the deep eutrophic sediments, increasing in the middle carbonate sediments and decreasing again in the upper eutrophic sediments (Fig. 2J).

3.3.2 Solid-phase sulfides

Acid volatile sulfides (AVS) were measurable in the deep eutrophic sediments between 43.5 and 34.5 cm and within the upper eutrophic sediments above 19.5 cm depth. The maximum concentrations of AVS in the upper eutrophic sediments (around 418 µg kg^{-1} at 10.5 cm) were about twice as high as in deep eutrophic sediments (200 µg kg^{-1} at 40.5 cm) (Fig. 2K). Chromium reducible sulfur (CRS) also exhibited higher concentrations in the shallower sediments, becoming more prominent from 16.5 cm depth to the surface. CRS concentrations were more variable than AVS, varying from 23 µg kg^{-1} to 510 µg kg^{-1} (Fig. 2K). The isotopic composition $\delta^{34}S$ of CRS was positive throughout the profile, ranging from ~1‰ near the surface to a maximum of 10.5‰ at 19.5 cm depth. Values remained elevated in the middle carbonate and deep eutrophic zones (e.g., 8.3‰ at 34.5 cm and 7.7‰ at 43.5 cm), indicating that the reduced sulfur pool is isotopically enriched in ^{34}S across the sediment column (Fig. 2L).

3.3.3 Dissolved oxygen and methane

Methane (CH_4) concentrations were highest in the deep eutrophic sediments, with a maximum of approximately 1760 µM at 45 cm depth. From 31.5 cm depth, CH_4 exhibited a

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clear decreasing trend, reaching the lowest concentration (253 μM) at the surface (Fig. 2M). The most significant drop in CH_4 concentrations occurred between 7.5 and 4.5 cm depth, where the concentrations decreased by half (Fig. 2M). The $\delta^{13}C_{CH_4}$ exhibited minimal variation along the profile, averaging -83.0 ± 0.7‰. The most pronounced isotopic shift (>2.2‰) towards heavier values occurred at the same depth as the sharp decline in CH_4 concentration (Fig. 2N).

Fractionation factors (α) between C_{org} and CH_4 ranged from 1.069 to 1.080 across sediment depths, corresponding to carbon isotope fractionations (ε) of 69‰ to 80‰ (Fig. 2O). These values reflect the measurable discrimination between ^{13}C and ^{12}C during CH_4 production from organic substrates, which arises from the enzymatic pathways and substrates utilized. Lower ε values were consistently observed in deeper sediments compared to shallower layers.

Oxygen concentrations were measured across seven different profiles, and free O_2 was detectable only in the uppermost sediments, between 0.165 cm and 0.365 cm depth (Fig. 2P). Below 0.4 cm, sediments were consistently anoxic. The heterogeneous penetration of O_2 into the sediments is attributed to bioturbation, which was confirmed by visual observations of worm castings.

3.3.4 Microbial community composition and chloroplast relative sequence abundances

The microbial community in Lake Joux sediments was dominated by the phyla Chloroflexota, Nanoarchaeota, and Pseudomonadota (Fig. 3A). In the deep eutrophic sediments (below 30 cm), microbial species richness and evenness (Chao1 and Shannon alpha diversity indices) were significantly lower than in overlying layers (Fig. 3C). In this zone, Nanoarchaeota reached their highest relative sequence abundances (>10%), decreasing to ~7% in the shallower sediments. These elevated abundances, also reported in freshwater (Chen et al., 2023; Xie et al., 2024) and marine environments (Brick et al., 2025), likely reflect their wide environmental tolerance and host associations (Jarett et al., 2018) (Fig. 3A).

In the middle carbonate-rich interval (30–11 cm), microbial diversity increased, and Bacteroidota appeared, consistently representing >5% of the microbial community. Reduced relative abundances of chloroplast sequences in this layer (Fig. 3B) also indicate limited input from photosynthetic organisms during this depositional phase. Cyanobacteria-related ASVs displayed similar depth trends to chloroplast sequences, but with lower overall abundance, reaching a maximum of 2% at 4.5 cm depth (Fig. 3B). In the upper eutrophic sediments (11–0 cm), Pseudomonadota became more abundant (>20%) and chloroplast sequences markedly increased, reflecting enhanced sedimentation of photosynthetic organisms.

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Microbial community composition was more similar within sedimentary intervals than between them (Fig. 3D). Stratification was especially pronounced for cyanobacterial and chloroplast sequences, which formed three distinct depth-specific clusters corresponding to the eutrophic, carbonate, and deep eutrophic intervals (Fig. S2). The methanotrophic community separated into two main groups, upper and deep eutrophic, while samples from the carbonate layer did not form a distinct cluster (Fig. S2). Methanogens, however, displayed clearer depth partitioning, with methylotrophic Methanomassiliicoccales dominating in the deep eutrophic interval and hydrogenotrophic Methanobacteriales increasing toward the surface (Fig. 4). Notably, depth patterns in methanogens and methanotrophs, as well as cyanobacterial and chloroplast-related sequences, tracked the same environmental gradients, with CH_4, NO_3^-, and CRS showing the strongest correlations and AVS, $\delta^{13}C_{org}$, and sedimentary P as secondary correlates (Fig. 3E; Fig. S2).

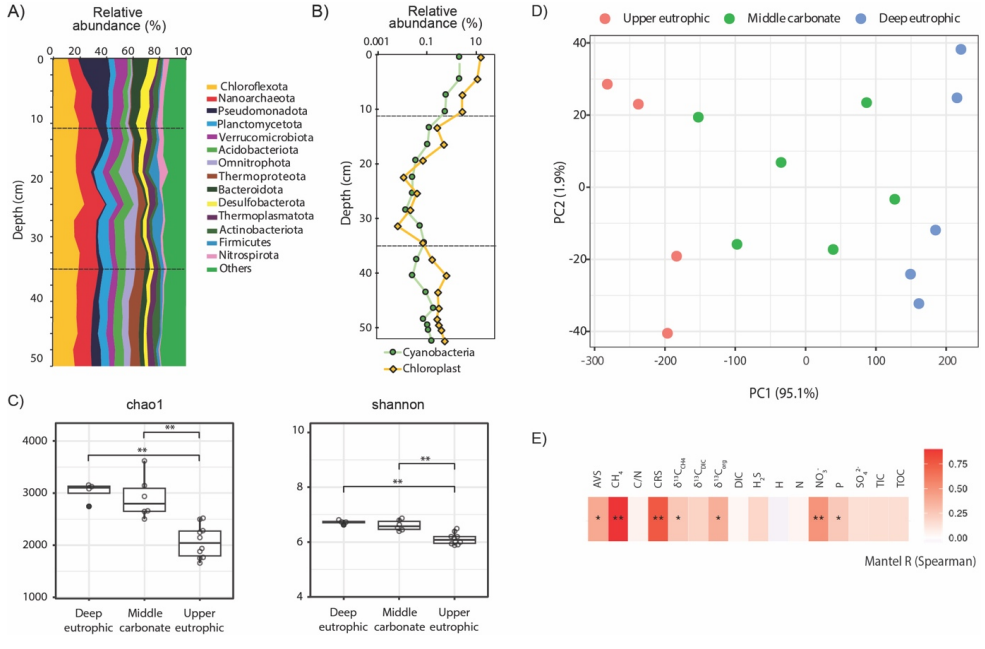

Figure 3 - (A) Relative 16S rRNA gene amplicon sequence abundances of bacterial and archaeal phyla, depicted as depth distribution in the 55 cm sedimentary profile of Lake Joux distribution; (B) Relative abundance of cyanobacteria and chloroplasts (algae and plants) affiliated 16S rRNA gene amplicon sequences in the 55 cm sedimentary profile of Lake Joux; (C) Alpha diversity (chao1 richness and Shannon diversity) of methanogens and methanotrophs. (D) Principal component ordination of centered log ratio (CLR) transformed 16S rRNA gene amplicon data, based on an Aitchison distance for which oversaturated distances were corrected and smoothed using LMdist. (E) Mantel tests results (Spearman's rank correlation) of community dissimilarity (corrected and smoothed Aitchison distance) and

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environmental parameters (z-scored). P-values were adjusted for multiple testing using the false discovery rate (FDR) method. ** p <= 0.01; * p <= 0.05.

3.3.4.1 Methanogenic and methanotrophic microbial communities

The relative sequence abundance of archaeal methanogens consistently accounted for more than 1% of the microbial community across all sampled depths (Fig. 4A). Methanomassiliicoccales were the dominant methanogenic group in the deep eutrophic sediments, accounting for 1.4% of the microbial community at a depth of 37.5 cm (Fig. 4A). In contrast, Methanomicrobiales was the most abundant methanogen group in the middle carbonate interval, while Methanobacteriales sequences were most abundant in the upper eutrophic sediments (11–0 cm, Fig. 4A). Sequences affiliated with Methanosarciniales and Methylacidiphilales were rare throughout the profile (<0.01%).

In contrast, methanotrophic taxa were not correlated with sediment intervals but rather with dissolved P and N. In the deep and middle sediments below 19.5 cm, Rhizobiales-affiliated methylotrophs (e.g., Methylocystis, Methylocapsa, Methyloligellaceae, (Tamas et al., 2014; Vekeman et al., 2016) were the dominant putative methanotrophs, although they represented a modest portion of the community (max. 0.6%) (Fig. 4B). Anaerobic methanotrophs from ANME archaeal groups were not identified in the sedimentary profile of Lake Joux. Still, between 23 and 16 cm, Methylomirabilota NC10 bacteria capable of nitrite-dependent methane oxidation with intracellularly produced O2 under anoxic conditions (Ettwig et al., 2010) were detected at a relative abundance of 0.2–1.3%.

The 16S rRNA gene sequences of aerobic MOBs represented between 0.3% and 8.7% of the microbial community throughout the sediment profile and were especially numerous (>1%) above a depth of 19.5 cm (Fig. 3C). The most abundant methanotrophs from 19.5 cm depth to the surface were members of the order Methylococcales, mainly represented by two genera of MOB: *Crenothrix* and *Methylobacter* (Fig. 4B). At 0.5 cm depth, these two genera were notably abundant, accounting for 5% and 3% of 16S rRNA gene sequences, respectively (Fig. 4).

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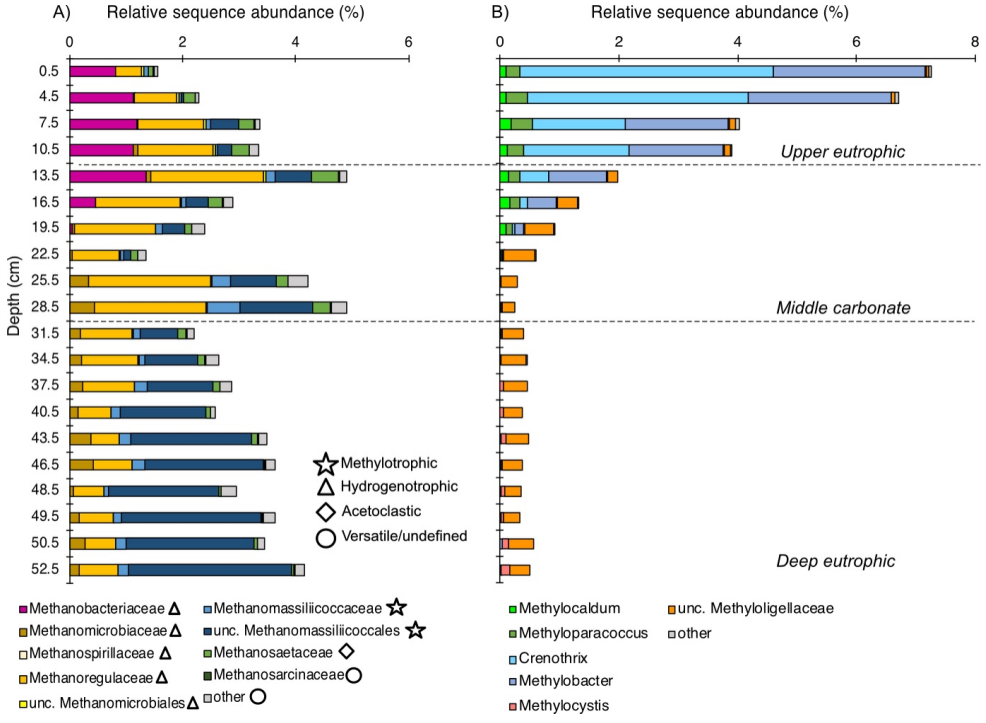

Figure 4 - Relative sequence abundance of (A) main methanogenic archaea taxa at family taxonomic level and their pathways (B) and aerobic methanotrophic bacteria at genus taxonomic level in the microbial community throughout 55 cm of the sedimentary profile of Lake Joux.

4. Discussion

4.1 Tracing historical land use, industrialization, and eutrophication

The intensely black sediments abundant in chloroplast-related sequences and elevated TOC content with low C:N ratios and light $\delta^{13}C_{org}$ in the deep eutrophic interval (55–30 cm, Fig. 1) attest to a predominantly autochthonous organic matter, derived from phytoplankton blooms (Lamb et al., 2006; Morales-Williams et al., 2017). Similar patterns recorded in other Lake Joux sediment profiles (Dubois, 2016; Lavrieux et al., 2017; Magny et al., 2008) correspond to a period of intensified deforestation and settlement expansion between 1525 and 1790 CE (Dubois, 2016; Lavrieux et al., 2017; Magny et al., 2008). While no official records confirm eutrophication during this period, these anthropogenic activities likely led to increased erosion and sediment/nutrient transport, stimulating phytoplankton productivity (Fig. 1). Notably, these sediments also exhibit high relative H_2S and AVS concentrations (Fig. 2B and 2K), which

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are characteristic of late sediment diagenesis under eutrophic depositional conditions (Holmer and Storkholm, 2001).

The middle carbonate layer (30–11 cm depth) reflects a shift towards more oligotrophic conditions, likely linked to the abandonment of land-intensive activities and the switch to manufacturing in the 18th century. In addition, the 1777 construction of a dike between Lake Joux and Lake Brenet lowered the lake level by 3.6 m, mobilizing limestone-rich sediments (high TIC) and terrestrial plant material with heavier $\delta^{13}C_{org}$ and higher C/N ratios (Lavrieux et al., 2017; Magny et al., 2008; Monchamp et al., 2021) (Fig. 2). The sedimentological transition that marks the beginning of this interval at 30 cm depth aligns with a shift from methylotrophic to hydrogenotrophic methanogens (Fig. 4), likely responsible for the lighter $\delta^{13}C_{DIC}$ values (Fig. 2F). Furthermore, the white-colored boundary of the middle carbonate layer (16–11 cm) coincides with warmer post-Little Ice Age conditions, promoting calcium carbonate precipitation and TIC enrichment (Lavrieux et al., 2017).

The upper eutrophic sedimentary interval (11–0 cm) consists of black sediments rich in TOC, lighter $\delta^{13}C_{org}$ values, low C/N ratios, and abundant chloroplast- and cyanobacteria-related sequences reflecting a well-documented 20th century eutrophication phase (Lavrieux et al., 2017; Magny et al., 2008; Monchamp et al., 2021) (Fig. 2, Fig. 3B). Elevated nutrient levels in this interval could result from external nutrient inputs trapped in porewater or from organic matter remineralization. Porewaters are strongly reducing, reflected by elevated H_2S and CRS, and the absence of O_2 below 0.5 cm. Upward diffusion of SO_4^{2-} meets downward-diffusing CH_4, and in the 7.5-0 cm horizon, CH_4 concentrations fall sharply while $\delta^{13}C_{CH4}$ becomes heavier and $\delta^{13}C_{DIC}$ lighter (Fig. 2A, B and K, M). Together with the dominance of MOB and the absence of ANME-related 16S rRNA gene amplicon sequences, these paired isotopic shifts indicate methanotrophy dominated by MOB as the main CH_4 sink in these anoxic, nutrient-replete surface sediments, whether sustained by micro-oxic niches or alternative oxidants (as discussed in further detail in 4.3).

4.2 Methylotrophic methanogens selected by past eutrophication

Changes in organic matter sources to Lake Joux over the last four centuries appear closely tied to shifts in dominant methanogenic groups within its sediments. Deep eutrophic sediments, characterized by the highest CH_4 concentrations, are dominated by Methanomassiliicoccales, which are methanogens known to utilize methylated substrates such as dimethyl sulfide (DMS) and methylamines (Bueno De Mesquita et al., 2023; Ellenbogen et

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al., 2024; Söllinger and Urich, 2019; Sun et al., 2019; Wang and Lee, 1994). The decomposition of algal and cyanobacterial biomass can release methylated sulfur compounds (including DMS and dimethylsulfoxide) and methylated amines, which stimulate methylotrophic methanogenesis in laboratory experiments and natural environments (Bose et al., 2008; Chistoserdova, 2011; Chistoserdova et al., 2009; Huang et al., 2018; Singh et al., 2005; Tebbe et al., 2023; Whiticar, 1999; Zhou et al., 2022).

Indirect evidence for the presence of methylated sulfur compounds comes from relatively higher concentrations of H_2S, AVS, and CRS at depth (Fig. 2B, 2K), indicating active sulfur cycling despite limited SO_4^{2-} availability. Furthermore, $\delta^{34}S$ values measured in CRS (primarily pyrite) consistently show positive isotopic signatures (7‰ to 10‰) in both the deep eutrophic and middle carbonate zones. While microbial SO_4^{2-} reduction typically produces ^{34}S-depleted sulfides ($\delta^{34}S$ < 0‰) under open-system or moderately sulfate-limited conditions (Bradley et al., 2016; Canfield, 2001; Habicht and Canfield, 1997), the isotopic enrichment observed here is more consistent with either the degradation of sulfurized organic matter or methylated sulfur compounds (Phillips et al., 2022; Raven et al., 2019; Werne et al., 2004). These could simultaneously fuel methylotrophic methanogenesis and pyrite formation. This interpretation warrants confirmation through direct measurements of methylated sulfur species in future studies. Alternatively, the ^{34}S enrichment could reflect near complete consumption of a limited SO_4^{2-} pool so that ^{34}S sulfide reflects positive values of the original sulfate (Bernasconi et al., 2017)(Fig. 2).

Methylotrophic methanogenesis is typically a minor pathway in freshwater sediments because methylated substrates are scarce (Borrel et al., 2011; Bueno De Mesquita et al., 2023). However, in the deep eutrophic layer, prolonged algal biomass degradation likely generated a reservoir of recalcitrant methylated compounds (Achtnich et al., 1995; Rissanen et al., 2018), favoring methylotrophic methanogens. In contrast, hydrogenotrophic (using CO_2 + H_2) and acetoclastic (using acetate) methanogens primarily depend on fresh, labile organic matter, which rapidly becomes limited with burial (Achtnich et al., 1995; Meier et al., 2024; Rissanen et al., 2023; Rissanen et al., 2018). Thus, methylotrophs gain a selective advantage in these older, more refractory sediments. Above ~28.5 cm, as sediment inputs shift toward terrestrial organic matter and methylated substrate availability diminishes, methylotrophic methanogens decline and hydrogenotrophs progressively dominate (Fig. 4A).

To further support the interpretation of distinct methanogenic pathways, we analyzed the $\varepsilon_{Corg-CH4}$, reflecting the isotopic discrimination during CH_4 formation from C_{org} (Fig. 2O). We observed lower ε values in deeper eutrophic sediments compared to shallower zones.

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Although interpreting specific metabolic pathways from isotopic fractionation is challenging in mixed microbial communities, the contrast in $\varepsilon_{Corg-CH4}$ (Fig. 2O) indicates distinct CH_4-producing processes dominating at different sediment depths.

Our results support the view that eutrophication leaves a distinct imprint on methanogen stratification in sediments. In contrast to earlier studies that reported either weak vertical structuring (Meier et al., 2024) or only subtle shifts in methanogen dominance (Rissanen et al., 2023), we found clear zonation with Methanomassiliicoccales prevailing in the deepest eutrophic interval, Methanomicrobiaceae in the carbonate-rich middle section, and Methanobacteriaceae dominating the upper eutrophic sediments. Considered alongside these previous observations in other lakes, our findings question the usefulness of broad generalizations and suggest that methanogen communities are primarily shaped by habitat-specific conditions—such as lithology, organic-matter quality, and redox context—rather than exhibiting universal hydrogenotroph dominance. By comparison, a pronounced vertical structuring of methane-oxidizing bacteria appears more consistent across systems (Mayr et al., 2020; Rissanen et al., 2018; Van Grinsven et al., 2022).

4.3 Aerobic methanotrophs are selected by nutrient availability

Within sediments, CH_4 is typically oxidized anaerobically (Borrel et al., 2011; Martinez-Cruz et al., 2018). Interestingly, in the anoxic sediments of Lake Joux, anaerobic methanotrophic archaea are not detectable. Sequences related to *Candidatus Methylomirabilis*—capable of intracellular O_2 production to fuel methane monooxygenase activity—occur in notable relative sequence abundances but are confined to 16–23 cm depth within the middle carbonate interval, but are relatively scarce compared to their aerobic counterparts. Namely, gammaproteobacterial MOB 16S rRNA gene sequences recovered from Lake Joux sediments are highly abundant (1- 9%) from 19.5 cm upward, despite prevailing anoxic conditions (Fig. 4, 5). The MOB 16S rRNA gene sequences primarily affiliate with Methylococcales, notably the genera *Crenothrix* and *Methylobacter* (Fig. 5). The dominance of Methylococcales associated MOB in the methane-oxidation zone suggests that serve as the dominant CH_4 sink in these nutrient-replete but anoxic surface sediments (Fig. 2N, Fig. 5).

How these aerobic methanotrophs meet their O_2 demand below 0.4 cm—where no O_2 could be detected, remains unresolved. Nanomolar O_2 cannot be excluded, but diffusive supply from the sediment–water interface to higher sediment depths is implausible. While some Methylococcales respire alternative electron acceptors (e.g., nitrate, Fe(III)) at low O_2 levels (Li et al., 2023; Van Grinsven et al., 2020; Yang et al., 2025), methane monooxygenase remains

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O_2-dependent for the oxidation of CH_4 to methanol. Three microbial mechanisms could generate microscale O_2 at depth within the sediment ("dark O_2"): methanobactin-mediated water splitting (Dershwitz et al., 2021), chlorite (ClO_2^-) dismutation by (per)chlorate-respiring bacteria (Xu and Logan, 2003), and nitric-oxide dismutation as described for NC10 bacteria (Ettwig et al., 2010).

Water lysis appears energetically unfavorable in natural systems and is associated with Alphaproteobacterial methanotrophs, which are not prevalent in Lake Joux (Dershwitz et al., 2021). Chlorite dismutation, catalyzed by chlorite dismutase found in over 60 genera across 13 phyla (Barnum and Coates, 2023), could be a source of O_2, as Pseudomonadota and Actinobacteria are abundant in these sediments (Fig. 3A). However, environmental levels of (per)chlorate are likely too low to support this pathway at significant levels (Lv et al., 2019; Miller et al., 2014; Wang et al., 2024).

A more plausible mechanism is NO dismutation via the nitric oxide dismutase (NOD) enzyme, which has been recently attributed to several families within the phylum Bacteroidota (Ruff et al., 2024). In Lake Joux, putatively NOD-containing Bacteroidota account for ~0.54 ± 0.2% of the microbial community in the upper eutrophic sediments, suggesting this pathway may contribute to localized O_2 production. Notably, while gammaproteobacterial methanotrophs, including *Crenothrix* and *Methylobacter*, possess genes for respiratory NO_3^- reduction (Almog et al., 2024; He et al., 2022; Martinez-Cruz et al., 2017; Milucka et al., 2012; Schorn et al., 2024), active NO_3^- respiration has only been demonstrated experimentally for *Methylomonas denitrificans* cultures (Kits et al., 2015) and indirectly by denitrification gene expression by MOB in Lake Zug (Schorn et al., 2024). The latter study revealed that *Crenothrix* and *Methylobacter* related microorganisms continue CH_4 oxidation in hypoxic and anoxic regions of the water column by performing denitrification or fermentation-based methanotrophy (Schorn et al., 2024). In Lake Joux, these same MOB taxa dominate the highly reducing, upper eutrophic sediments (Fig. 4B).

Interestingly, we observed strong positive correlations ($p < 0.05$) between the relative sequence abundances of *Crenothrix* and *Methylobacter* and porewater NO_3^- and PO_4^{3-} (Fig. 5). These nutrients exhibited moderate correlation with each other ($R^2 = 0.72$). While the source of NO_3^- cannot be resolved here, possible mechanisms of NO_3^- generation include oxidation of NH_4^+ by Mn(IV) or Fe(III) oxides. Importantly, the MOB–nutrient correlations may also reflect a shared response to favorable near-surface conditions (e.g., sustained inputs of labile organic matter or higher porosity), rather than direct nutrient control. Nevertheless, it has been experimentally demonstrated that PO_4^{3-}, NO_3^-, and NH_4^+ additions can directly enhance CH_4

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oxidation rates by MOB and, in particular, *Methylobacter* (Almog et al., 2024; Kits et al., 2015; Nijman et al., 2022; Xia et al., 2021; Yang et al., 2025). Taken together, these observations suggest that nutrient availability may play a direct role in shaping the structure and activity of MOB communities.

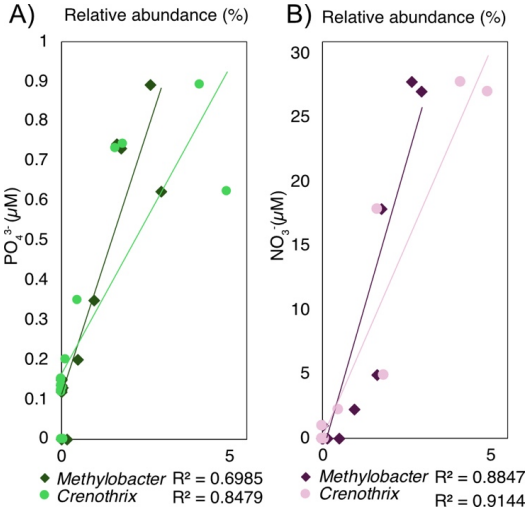

Figure 5 - (A) Correlation between PO_4^{3-} concentration and the relative sequence abundance of *Crenothrix* and *Methylobacter*. (B) Correlation between NO_3^- concentration and the relative sequence abundance of *Crenothrix* and *Methylobacter*.

5. Conclusion

Our results show that historical eutrophication left a lasting sedimentary legacy that structures contemporary methane-cycling microbial communities, selecting methylotrophic methanogens. In upper eutrophic, anoxic sediments, the surprisingly high relative sequence abundances of MOB (up to ~9%) specifically Methylococcales-affiliated Gammaproteobacteria, co-vary with elevated NO_3^- and PO_4^{3-} concentrations. This suggests that eutrophication can simultaneously stimulate CH_4 production and enhance its oxidation by shaping microbial assemblages.

As eutrophication continues to alter freshwater systems globally, understanding nutrient- and substrate-driven shifts in CH_4-cycling communities becomes increasingly important. Future studies should focus on elucidating the in situ activity of aerobic methanotrophs and molecular mechanism of methane oxidation under anoxic conditions, as presumably aerobic MOB have been widely reported in anoxic sediments (Almog et al., 2024; Ruff et al., 2024; Schorn et al., 2024). Combining molecular, isotopic, and geochemical

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approaches will be essential to better constrain methane fluxes in lakes undergoing or recovering from eutrophication.

Data availability

All geochemical data are included in this published article and its supplementary information files. The 16S rRNA gene amplicon sequencing data has been deposited at the Sequence Read Archive under the BioProject accession PRJNA1207472.

Author contribution

ABS contributed to conceptualization, data curation, formal analysis, funding acquisition, methodology, supervision, validation, visualization, writing original draft preparation, review, and editing. ERBB contributed to data curation, formal analysis, and manuscript editing; SK contributed to data curation, formal analysis, and manuscript editing; MEM contributed to resources and manuscript editing; JS contributed to data curation, formal analysis, methodology, validation, visualization, designing and implementing computer codes, and writing original draft preparation, review, and editing. PP contributed to conceptualization, formal analysis, funding acquisition, supervision, validation, visualization, writing original draft preparation, review, and editing. JSB contributed to conceptualization, formal analysis, funding acquisition, supervision, validation, visualization, writing original draft preparation, review, and editing.

Competing interests

The authors declare that they have no conflict of interest.

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