

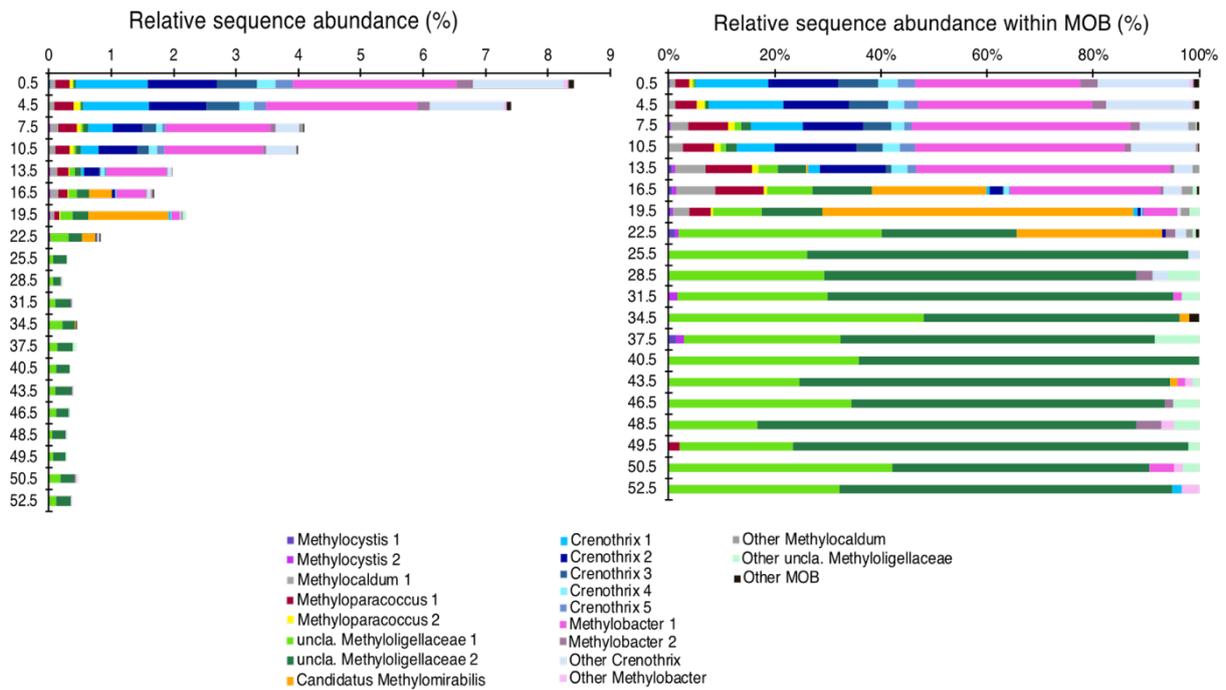
**This manuscript explores whether past and present eutrophication affects microbial community structure in lake sediments, with a special emphasis on methane-cycling microbial communities. The authors characterized the sediment geochemistry and performed 16S rRNA gene amplicon sequencing at high resolution in sediment cores collected from the Swiss lake Lake Joux, which has a well documented history of human activity and the resulting effects on nutrient inputs and ecological changes. This context is very well outlined in the manuscript. In general, I think this manuscript is well-written and methodologically sound.**

We thank the reviewer for their positive evaluation of our manuscript and for acknowledging the clarity of our contextual framework and methodological approach. We appreciate their supportive comments on the quality and soundness of our study.

**My main comment concerns the relatively superficial nature of the authors analyses of their amplicon data. In line 444, the authors state that aerobic methanotrophs were mainly represented by the genera *Methylobacter* and *Crenothrix*, both showing notable abundances. However, there is no information on how many ASVs were affiliated with these genera and whether there were depth-related differences in their abundance that could point to niche differentiation such as observed in other lacustrine systems. Such analyses would provide more detailed insights into community structure, especially within the upper sediment layers where chemical gradients are steepest.**

We thank the reviewer for this valuable suggestion. The diversity within *Methylomonadaceae* is relatively limited, including *Methylobacter* [4 ASVs], *Crenothrix* [17 ASVs] and three additional ASVs belonging to other genera. Among these, five ASVs are dominant. The relative proportion of the single abundant *Methylomonas* ASV increased notably with depth (from 30% to 70% of all the MOB-related sequences), while its relative abundance in relation to the total microbial community decreased with depth. In contrast, in relation to all MOB sequences, the relative abundance of the three most abundant *Chrenothrix*-related ASVs remained relatively stable across depth, while likewise decreasing in relation to the total microbial community. A supplementary figure was added (see below) and this information was added to the discussion (see below).

While we did explore correlations with further environmental factors and constrained ordinations, but found that due to the small number of ASVs related to MOB and the relatively stable community composition across depth, further diversity or redundancy analyses did not provide any novel insights into the environmental drivers of MOB community composition.



**Figure S1.** Depth profiles of methane-oxidizing bacteria (MOB). (A) MOB relative sequence abundance in the total microbial community. (B) MOB composition expressed as relative sequence abundance within the MOB fraction (16S rRNA gene amplicons). Shades of the same color denote the same genera; distinct colors indicate different genera.

**LINES 611 - 622:** The 16S rRNA gene sequences of aerobic MOB 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, with two genera prevalent near the surface: *Crenothrix* and *Methylobacter* (Fig. 4B). At the resolution available with V4-region 16S rRNA gene amplicon analyses, limited within-group diversity is detected, as 4 ASVs affiliated with *Methylobacter*, 17 ASVs with *Crenothrix*, and a small number of ASVs assigned to other Methylococcales genera, including a single abundant *Methylomonas* ASV (Figure S1), were recovered. Within the MOB community, the fraction of the *Methylomonas* ASV increased with depth ( $\approx 30\%$  to  $\approx 70\%$ ). By contrast, the fraction of the three most abundant *Crenothrix* ASVs within the MOB was stable across depth. The abundance of all individual MOB ASVs decreased with depth, relative to the total community (Figure S1).

**Integrating a phylogenetic analysis of the ASVs affiliated with the *Methylobacter* and *Crenothrix* could help to better resolve their niche partitioning and environmental roles. This would also strengthen the statement in line 591.**

We thank the reviewer for their suggestion, but respectfully disagree. 16S rRNA gene amplicon sequencing for this study, as commonly done in microbial ecology, was performed using broad-capture but low-resolution V4-targeted primers. Phylogenetic placement of amplicons of only  $\sim 240$  bp length comes with high uncertainty and does not provide reliable information beyond genus-level classification. Thus we fail to recognize the value such an analysis could add, as genus level classification already is achieved and reported.

**Secondly, the authors attribute the predominance of Methanomassiliicoccales in the deepest, eutrophic sediment layers to selection by past eutrophic conditions (see lines 493 and following). However, the prevailing understanding is that Methanomassiliicoccales are hydrogen-dependent methanogens.**

We agree and have revised our wording throughout to specify that Methanomassiliicoccales are hydrogen-dependent methylotrophic methanogens that use H<sub>2</sub> as the electron donor and methylated one-carbon compounds (e.g., methanol, methylamines, methylated S compounds) as electron acceptors, rather than reducing CO<sub>2</sub>. We acknowledge that the phrasing in the manuscript was unclear, but would like to clarify that this physiology aligns with our interpretation (selection by methylated substrates derived from degradation of eutrophication-driven biomass) and with current understanding of methanogen community structuring. We also explicitly note that methylated substrates were not measured here and frame this as an inference supported by convergent lines of evidence. We rephrased their definition to improve clarity.

**LINES 682 - 693:** Deep eutrophic sediments, characterized by the highest CH<sub>4</sub> concentrations, are dominated by Methanomassiliicoccales, which are hydrogen-dependent methylotrophic methanogens that use H<sub>2</sub> as the electron donor and methylated one-carbon compounds (e.g., methanol, methylamines, methylated S compounds) as electron acceptors, rather than reducing CO<sub>2</sub> (Bueno De Mesquita et al., 2023; Ellenbogen et al., 2024; Söllinger and Urich, 2019; Sun et al., 2019; Wang and Lee, 1994).

**I wonder whether their distribution is influenced not solely by eutrophic conditions, but also by competition for hydrogen between them and hydrogenotrophic methanogens. I believe this aspect warrants further discussion and a more nuanced interpretation of the data.**

We thank the reviewer for this thoughtful suggestion and agree that the distribution of methylotrophic methanogens may reflect not only eutrophic legacies but also potential competition for H<sub>2</sub> with CO<sub>2</sub>-reducing hydrogenotrophs. It was already proposed that methylotrophic methanogens should outcompete hydrogenotrophic methanogens for hydrogen and that their activity is limited by the availability of methyl groups (Feldwert et al., 2020). We have revised the Discussion to acknowledge this mechanism and to present a more nuanced interpretation. We also clarify that *Methanomassiliicoccales* are hydrogen-dependent methylotrophs, and we explain why methylated-substrate availability—rather than H<sub>2</sub> limitation—likely governs their depth distribution in our sulfate-poor setting. Citations were added as indicated below.

**LINES 714-730:** It is important to note that methylotroph distributions could also be influenced by competition for H<sub>2</sub> with CO<sub>2</sub>-reducing hydrogenotrophs. In sulfate-poor anoxic sediments, H<sub>2</sub> is typically buffered at low steady-state levels by continuous fermentative supply and rapid consumption—reflecting thermodynamic control rather than chronic scarcity (Conrad, 1999; Schütz et al., 1988; Kessler et al., 2019). Obligately methyl-reducing methanogens have very low H<sub>2</sub> thresholds and are predicted to outcompete hydrogenotrophs for H<sub>2</sub> when methyl groups are available. Thus, their activity is primarily considered to be limited by the availability of methylated substrates (Cord-Ruwisch et al., 1988; Feldewert et al., 2020; Borrel et al., 2019; Söllinger & Urich, 2019; Speth & Orphan, 2018; Bruno de Mesquita et al., 2023). Given the dominance of *Methanomassiliicoccales* at depth, we infer

that methylated-substrate supply rather than H<sub>2</sub> limitation is the primary methanogenic community structuring factor in the deep eutrophic interval. This interpretation is consistent with isotope patterns, as we have recorded comparatively heavier  $\delta^{13}\text{C}_{\text{DIC}}$  in the deep eutrophic layer and a shift to lighter  $\delta^{13}\text{C}_{\text{DIC}}$  above ~30 cm where the relative abundance of CO<sub>2</sub>-reducing hydrogenotrophic methanogens increased (Fig. 2F).

**Line-specific comments:**

**Line 184: Please add information on when sampling was conducted.**

Info was added: **LINE 226:** In May 2023, three gravity cores (45-55 cm long) were recovered from the lakebed of Lake Joux's lakebed using a Uwitec gravity corer.

**Line 197: Is there a reason why nitrite was not analyzed or was it not detected? Knowing where nitrite accumulates would help to define where conditions become denitrifying, information that could then be linked to the presence of specific MOB ASVs.**

We thank the reviewer for raising this point. Nitrite was measured, but concentrations were uniformly near the analytical detection limit (range 0.038–0.087  $\mu\text{M}$ , median  $\approx 0.043 \mu\text{M}$ ) and showed no depth-specific accumulation. This pattern is consistent with rapid nitrite turnover in energy-limited sediments where nitrite is transient and quickly reduced further during denitrification. Because there was no resolvable peak or trend to localize a “nitrite maximum,” we focused on nitrate and phosphate—which did vary and correlated with Methylococcales abundance. We now report nitrite quantification methods and results explicitly and provide the nitrite profile in the Supplement.

**LINES 241 - 243:** Porewater samples for dissolved anions ( $\text{PO}_4^{3-}$ ,  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{SO}_4^{2-}$ ) were transferred to plastic vials while flushing with N<sub>2</sub>, capped, and analyzed using an ion chromatograph (DX-ICS-1000, DIONEX) equipped with an AS11-HC column.

**LINES 479 - 481:** Nitrite concentrations were close to the detection limit (0.03  $\mu\text{M}$ ) and uniformly low (range 0.038–0.087  $\mu\text{M}$ , median  $\approx 0.043 \mu\text{M}$ ) with no systematic depth trend (Fig. S2).

**LINES 842 - 851:** One potential source of *in-situ* O<sub>2</sub> is nitric oxide dismutation catalyzed by the nitric oxide dismutase (NOD) enzyme, which has been recently attributed to multiple bacterial lineages, including several families within the phylum Bacteroidota (Ruff et al., 2024). In Lake Joux, putatively NOD-containing Bacteroidota account for  $\sim 0.54 \pm 0.2\%$  of the microbial community in the upper eutrophic sediments, suggesting this pathway may contribute to localized O<sub>2</sub> production. However, as NOD is not encoded by all representatives of these taxa, we can not perform further reliable abundance estimates of NOD based on the available 16S rRNA gene amplicon data. The mechanism of O<sub>2</sub> production is nevertheless consistent with our geochemical context: porewater NO<sub>2</sub><sup>-</sup> remained near detection limit with no subsurface maximum (Fig. S2), indicating rapid NO<sub>x</sub> turnover typical of energy-limited sediments.

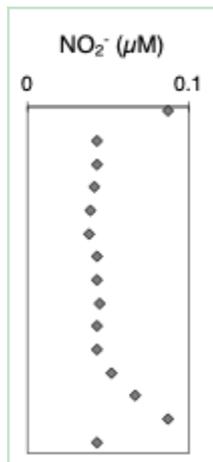


Figure S2. Porewater nitrite ( $\text{NO}_2^-$ ) concentrations versus depth in Lake Joux sediments.

**Line 246:** Could you add here information on how relative abundances were calculated and does it refer to relative abundance of bacteria and archaea together?

As the V4 targeted primers capture both bacteria and archaea, after the described filtering of off-target amplicons (see below), relative abundance in relation to both bacterial and archaeal reads was examined. Chloroplast sequences, not considered to be a part of active sedimentary microbiomes, were assessed separately, reflecting a plant/algae/cyanobacteria deposition and burial signal.

**LINES 389-393:** After filtering, only samples with at least 7000 read pairs were kept for further analyses, and relative abundances of ASVs grouped at higher taxonomic levels were calculated in relation to all remaining data. The relative abundance of chloroplast sequences, which were removed from the microbial community dataset, was examined separately to assess phytoplankton debris abundance across the sediment profile.

**Line 498:** Methanol is also a common substrate for them and could be produced during the breakdown of organics.

The sentence was changed according to other reviewers' comments:

**LINES 682-693:** Deep eutrophic sediments, characterized by the highest  $\text{CH}_4$  concentrations, are dominated by Methanomassiliicoccales, which are hydrogen-dependent methylotrophic methanogens that use  $\text{H}_2$  as the electron donor and methylated one-carbon compounds (e.g., methanol, methylamines, methylated S compounds) as electron acceptors, rather than reducing  $\text{CO}_2$ .

**Line 525:** Again here, could competition for hydrogen influence the depth distribution?

Please see our response to the comment above, and the referenced literature therein.

**Figures:**

**Fig 1. Please add information/description on panel B.**

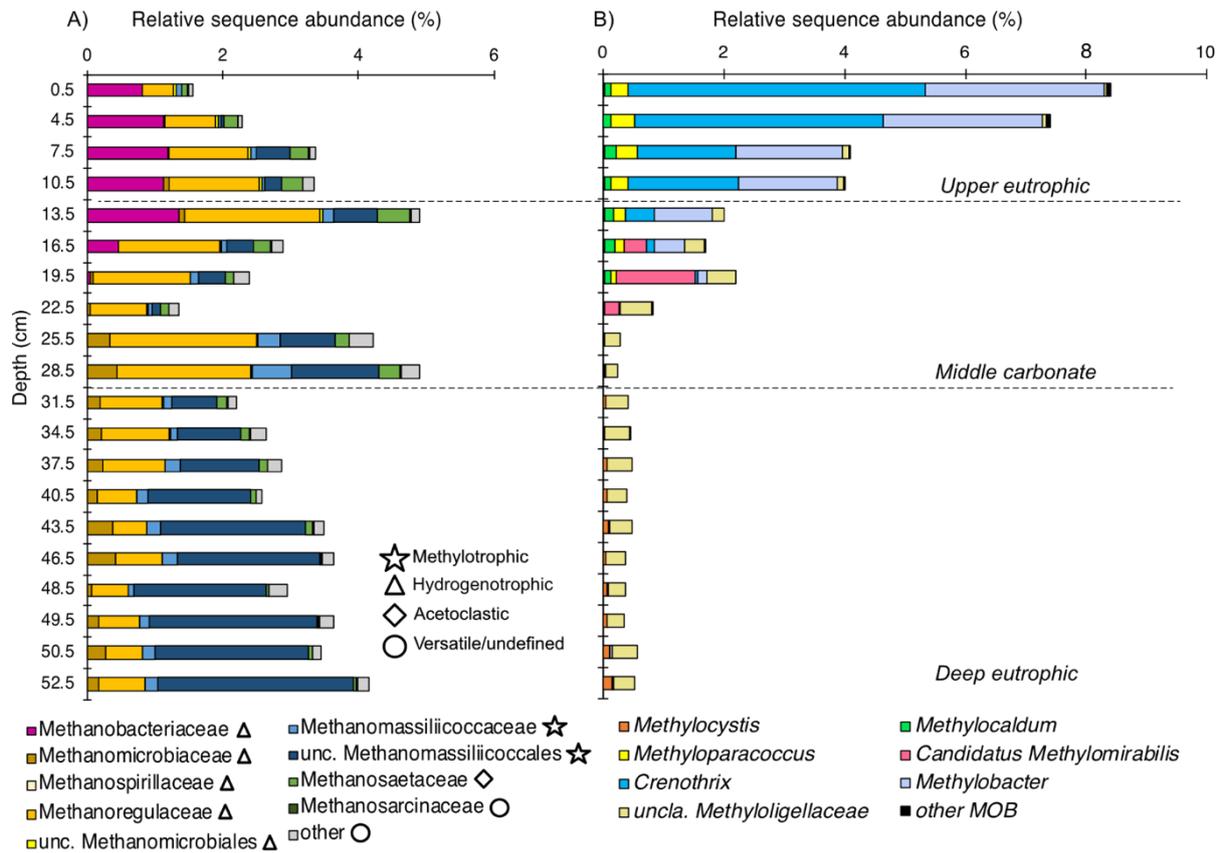
**New caption Figure 1.:** (A) Location of Lake Joux (Switzerland; sampling in 2023) and 55 cm core photograph with the three stratigraphic intervals identified from lithology and age markers: deep eutrophic, middle carbonate, and upper eutrophic. (B) Non-metric multidimensional scaling (NMDS) of the porewater and solid-phase geochemical dataset, showing separation of samples by stratigraphic interval (upper eutrophic = red, middle carbonate = green, deep eutrophic = blue). Points are individual depth samples; colored polygons outline the convex hull for each interval and symbols mark group centroids. Ordination was performed on z-scored variables using Bray–Curtis dissimilarities.

**Fig 2 and supplementary table 2: The different oxygen profiles, are these repeated measurements of the same core or are these obtained from different cores?**

We added the information: **LINES 349-351:** Seven vertical profiles from the same core were recorded at 250  $\mu\text{m}$  steps with a motorized controller and Field Multimeter (Unisense).

**Fig 4. Is there a reason to not show the distribution of NC10 in figure 4? I suggest to show NC10 here as well, either combined with the Methylococcales or in a separate panel.**

We agree and have revised Figure 4 to display NC10 (*Candidatus Methyloirabilis*) alongside Methylococcales in panel B. The legend and caption now explicitly list NC10, and colors were adjusted for clarity. NC10 occurs mainly between ~23–16 cm at low relative abundance, consistent with our text (see updated Fig. 4B). We also provide an ASV-level breakdown of methanotrophs in Figure S1.



**Figure 4.** Depth-resolved composition of methanotrophic and methanogenic taxa in Lake Joux sediments. **(A)** Methanogenic archaea clustered by family/order (relative sequence abundance of total community), and grouped by inferred pathways (methylophilic, hydrogenotrophic, acetoclastic, versatile/undefined). **(B)** Methanotrophic bacteria, including canonical MOB and *Candidatus Methyloirabilis* (NC10), were expressed as relative sequence abundance of the total community.