Reviewer Note: I want to personally apologize to the authors for the delayed review. A family member experienced a significant medical emergency and required care, which impacted my ability to submit this review in a timely manner.

General Assessment: This manuscript analyzes sediment cores from Lake Joux to determine the long-term impact of eutrophication on methane (CH_4) cycling. By pairing a historical deposition record dating back to the 16th century with high-resolution geochemical profiles and 16S rRNA gene amplicon sequencing, the authors aim to link sediment history to modern benthic biogeochemistry. The study identifies 3 depth specific clusters, which correlate with sediment geochemistry and shifts in the methanogens/methanotrophs. Deep sediments with a geochemical record of eutrophic deposition exhibited a distinct niche for methylotrophic methanogenic taxa. Enrichment of cyanobacteria amplicon sequencing variants (ASVs) and increased sulfur compounds relative to the middle carbonate layer provided indirect evidence for methylated compounds in these deep eutrophic sediments. The authors conclude differences in historical sediment deposition influence modern biogeochemical cycling.

We thank the reviewer for the careful reading and helpful suggestions, and we appreciate the note regarding the delay. We have revised the manuscript to clarify the aims, separate Results from Discussion, strengthen the statistical analysis, and refine the interpretations. Below we respond point-by-point, indicating where we added text in the revised, line-numbered manuscript.

The manuscript provides robust data to support the conclusions. However, I have comments concerning the interpretation of the geochemical and 16S rRNA gene amplicon sequencing variant (ASV) data. Given the issues listed below, care should be taken to update the manuscript to improve clarity.

Major Comments:

1. **Rewrite final paragraph of Introduction: The final paragraph of the Introduction currently includes an overview of carbon isotopes. While this background is relevant, placing it in the closing paragraph distracts from the study objectives and findings. Consider moving this text to the Discussion. Please revise the final paragraph to focus on the study objectives and the significance of linking the Lake Joux depositional record to modern methane cycling.**

   We thank the reviewer and agree. We rewrote the final paragraph of the Introduction to state the study objectives and significance, and we moved the isotope background to the Discussion.

   Introduction **LINES 149-158**: Here, we test whether historical eutrophication has left a sedimentary legacy that structures contemporary methane-cycling communities in 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). Using a ~400-year, 55-cm sediment archive, we combine porewater and solid-phase geochemistry, 16S rRNA gene amplicon profiling, and stable-carbon-isotope measurements to resolve depth zonation of methanogens and methanotrophs, and link community shifts to organic-matter sources, lithology, and redox conditions. By explicitly coupling the depositional record to present microbial community structure and isotope compositions of different carbon pools , we provide process-level constraints relevant for forecasting benthic CH_4 in eutrophying and recovering lakes.

2. Statistical testing of depth specific clusters: The text describes three distinct depth-specific clusters derived from center-log ratio (CLR) transformed 16S rRNA data in Section 3.3.4 and supplemental figure 2. While distinct groupings are visually observed along the first principal component (explaining 95.1% of the variance), the differences between the clusters could be interpreted as subjective. Please justify the differences among clusters with appropriate statistical tests to confirm significant differences between groups.

We apologize for omitting the statistical test results; we performed a PERMANOVA and found that the depth-defined clusters are statistically supported for both sediment geochemistry and community structure. Results have been amended as follows:

LINES 563-569: Microbial community composition was more similar within sedimentary intervals than between them (Fig. 3D). The separation of samples into three depth clusters based on sediment biogeochemistry was statistically supported (PERMANOVA: $F = 25.05$, $R^2 = 0.81$, $p < 0.001$, Figure 3D). Significant differences in microbial community composition between the three depth clusters were also observed (PERMANOVA: methanogens - $F = 10.57$, $R^2 = 0.64$, $p < 0.001$; MOB - $F = 13.32$, $R^2 = 0.69$, $p < 0.001$; Cyanobacteria - $F = 6.57$, $R^2 = 0.52$, $p < 0.001$; Chloroplasts - $F = 8.4$, $R^2 = 0.58$, $p < 0.001$; Fig. S4).

We also included the following sentence in the methods:

LINES 378-380: Differences among depth-defined clusters based on environmental variables were tested using a permutational multivariate analysis of variance (PERMANOVA) on smoothed Euclidean distance matrices.

LINES 416-419: Differences in 16S rRNA gene amplicon community composition among three depth-defined clusters were tested using permutational multivariate analysis of variance (PERMANOVA) on an Aitchison distance-based dissimilarity matrix.

3. Interspersed Results and Discussion sections: There are several instances where results are presented in the Discussion and findings are interpreted in the Results. For example, the correlation between methanotrophic taxa and nitrate/phosphate (Fig. 5) was presented in the Discussion, when it belongs in the Results section. Conversely, interpretative text and literature citations were included when describing the high abundance of Nanoarchaeota in lines 384-386 of the Results. I recommend the authors review the manuscript to separate the description of data in Results from interpretation in Discussion.

We revised the manuscript to clearly separate observations from interpretation. Specifically, (i) all statistical relationships and descriptive trends are now reported in the **Results** only; (ii) mechanistic explanations, literature context, and implications are confined to the **Discussion**; and (iii) instances where interpretive language appeared in the Results (e.g., taxon notes) were removed or relocated. We also audited the full text to ensure consistent sectioning throughout.

4. Describing Methanomassiliicoccales ASVs as strict methylotrophic methanogens: Methanomassiliicoccales taxa are dependent on methylated compounds for growth and methane production. However, the growth rate of Methanomassiliicoccus isolates on methanol increase when utilizing hydrogen (H_2) as an electron donor [1–3]. A recent metagenomic survey of Methanomassiliicoccales identified the genes required for the hydrogen dependent reduction of methanol to methane across all analyzed metagenome-assembled genomes (MAGs) [4]. Consider identifying Methanomassiliicoccales ASVs as hydrogen-dependent methylotrophic methanogens.

We thank the reviewer and agree with the wording. The same issue was flagged by the other reviewer. *Methanomassiliicoccales* are hydrogen-dependent methylotrophic methanogens: they reduce methylated one-carbon substrates (e.g., methanol, methylamines, methyl-S compounds) using H_2 as the electron donor, rather than reducing CO_2. Cultures show faster growth on methanol in the presence of H_2, and genomic surveys indicate the genes for H_2-dependent methyl reduction are widespread in this clade (e.g., Speth & Orphan, 2018; Borrel et al., 2023). Our interpretation therefore emphasizes that methylated-substrate availability—consistent with eutrophication legacies—selects for this metabolism at depth, while recognizing the obligate role of H_2 as the reductant. We revised the text to improve clarity and added additional references.

LINES 695 - 706: Deep eutrophic sediments, characterized by the highest CH_4 concentrations, are dominated by Methanomassiliicoccales, which are hydrogen-dependent methylotrophic methanogens that use H_2 as the electron donor and methylated one-carbon compounds (e.g., methanol, methylamines, methylated S compounds) as electron acceptors, rather than reducing CO_2 (Bueno De Mesquita et al., 2023; Ellenbogen et al., 2024; Söllinger and Urich, 2019; Sun et al., 2019; Wang and Lee, 1994).

LINES 727 -743: It is important to note that methylotroph distributions could also be influenced by competition for H_2 with CO_2-reducing hydrogenotrophs. In sulfate-poor anoxic sediments, H_2 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_2 thresholds and are predicted to outcompete hydrogenotrophs for H_2 when methyl groups are available. Thus, their activity is primarily considered to be limited by the availability of methylated substrates (Borrel et al., 2023; Bueno De Mesquita et al., 2023; Feldewert et al., 2020; Söllinger and Urich, 2019; Speth and Orphan, 2018). Given the dominance of *Methanomassiliicoccales* at depth, we infer that methylated-substrate supply rather than H_2 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 $\delta^{13}C_{DIC}$ in the deep eutrophic layer and a shift to lighter $\delta^{13}C_{DIC}$ above ~30 cm where the relative abundance of CO_2-reducing hydrogenotrophic methanogens increased (Fig. 2F).

Minor Comment:

1. **Functional Assignment of nitric oxide dismutase (NOD) from 16S rRNA data: The authors assign the presence of nitric oxide dismutase (NOD) to specific Bacteroidota taxa in line 581 of the Discussion. However, the NOD annotation method was not described. The distribution of NOD to specific taxa appears to be inferred by matching the identified ASVs with the NOD database presented in Ruff et al. 2024 [5]. Please describe the NOD annotation method with a reference to the database or remove the description of NOD containing taxa.**

We agree that assigning NOD to specific taxa from 16S rRNA gene amplicons is not warranted. Our earlier wording was based on genus-level taxonomic matching to genera reported to harbor NOD-encoding members in Ruff et al. (2024), without gene-based detection or phylogenetic placement. To avoid over-interpretation, we removed the taxa-specific NOD assignments and the abundance estimate from the Discussion and now present NOD strictly as a potential mechanism supported by the literature. We also clarified in the Discussion that our 16S data cannot resolve the presence/absence of *nod*.

LINES 855 - 864: One potential source of *in-situ* O_2 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 ~0.54 ± 0.2% of the microbial community in the upper eutrophic sediments, suggesting this pathway may contribute to localized O_2 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_2 production is nevertheless consistent with our geochemical context: porewater NO_2^- remained near detection limit with no subsurface maximum (Fig. S2), indicating rapid NOx turnover typical of energy-limited sediments.

Line specific comments by section:

Methods

Line 152: The text cites Fig 1 for the site location, but the figure also contains an NMDS plot. Please redefine the citation as Fig 1A.

Done

Line 199-206: The method for measuring δ^{13}CDIC is described, but the method for determining the DIC concentration has been omitted. Please clarify how DIC concentrations were determined.

Thank you for pointing it out. The paragraph was corrected:

LINES 254 - 267: For dissolved inorganic carbon (DIC) porewater samples were filled into 1.5 ml borosilicate vials and capped headspace-free to prevent CO_2 degassing. DIC concentration (mmol L^{-1}) was obtained from the CO_2 yield after acid conversion of aliquots transferred to helium-flushed Exetainers containing 200 µL of 99% H_3PO_4, and the resulting CO_2 peak areas were quantified on a GasBench II (Thermo Fisher Scientific). A response factor (µmol CO_2 per peak-area unit) was derived from identically processed Carrara Marble (CM) standards and applied to the second CO_2 peak of each sample; moles of CO_2 were converted to DIC using the injected sample volume. External uncertainty on DIC concentration, based on CM reproducibility, was <7%. For $\delta^{13}C_{DIC}$, the headspace CO_2 was analyzed by GasBench II coupled to a Delta V Plus IRMS; each sample was measured six times, and we report the mean with 1σ (typically <0.10‰). Values were normalized to the in-house CM standard ($\delta^{13}C$ = +2.05‰) calibrated against NBS-19 and NBS-18; external reproducibility from CM replicates (n = 12) was <0.05‰ (1σ). Carbon isotopes are reported in delta (δ) notation relative to the Vienna Pee Dee Belemnite (VPDB) standard, defined as $\delta = ((R_{sample} \backslash R_{standard}) - 1) \times 1000$ ‰, with *R* the $^{13}C/^{12}C$ ratio; positive δ indicates enrichment in ^{13}C relative to Vienna Pee Dee Belemnite (VPDB) standard.

Line 236-239: Please specify the gas chromatography detector used to measure bulk CH4.

LINES 351 - 353: Dissolved CH_4 was extracted by headspace displacement and quantified via gas chromatography (Joint Analytical Systems) equipped with an FID at the Eawag (Khatun et al., 2024).

Line 270-273: The method for generating the geochemical NMDS (Fig 1B) is in the section describing 16S rRNA gene amplicon sequence analysis. I suggest the description of the method be moved to the end of Section 2.5 for clarity.

Thanks for the suggestion. The following sentence was moved to Section 2.5:

LINES 275-280: 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.

Results

Line 303-304: Data supporting the correlation with previously dated (137Cs/210Pb) cores was not presented. Consider showing or explaining the stratigraphic alignment.

We agree and now provide a detailed textual description of the stratigraphic alignment to the dated Lake Joux record of Lavrieux et al. (2017). In the Results, we specify the tie-points (carbonate interval and black organic-rich unit, including the ~16–11 cm pale boundary) and the transferred age markers (^{137}Cs ~1954/1963), and we state the inferred age of our deep eutrophic base (late 16th–early 17th century). We also added a sentence in Methods clarifying the correlation procedure and that ages were transferred from Lavrieux's $^{210}Pb/^{137}Cs$ model.

RESULTS LINES 451 - 461: To assign approximate ages to our core, we correlated its lithological boundaries to the dated Lake Joux sequence of Lavrieux et al. (2017) ($^{210}Pb/^{137}Cs$). The middle carbonate unit (30–11 cm) in our core aligns with their U3–U4 carbonate interval, including the distinctive pale "white" boundary at ~16–11 cm. The overlying upper eutrophic black, organic-rich sediments (11–0 cm) correspond to U5, which spans the 20th-century eutrophication phase and includes the ^{137}Cs markers at ~1954 and ~1963 in the Lavrieux record. By transfer of their age–depth model, the base of our deep eutrophic interval (below 30 cm) falls in the late 16th–early 17th century. Reported sedimentation rates in Lavrieux (≈0.04–0.11 cm yr^{-1} before the 18th century, a short-lived peak ≈0.83 cm yr^{-1} in the late 18th century, and ≈0.18 cm yr^{-1} over recent decades) are consistent with the thicknesses of our corresponding units.

METHODS LINES 275 - 278: Lithological boundaries in our core were aligned to the dated Lake Joux record of Lavrieux et al. (2017) using their carbonate sediment interval (whiter sediments) as reference. Ages were transferred from their ^{210}Pb/^{137}Cs model; uncertainties are those reported therein.

Line 320-322: sulfate and sulfide exhibit clear inverse gradients in the upper eutrophic sediment (< 7.5 cm), suggesting active sulfate reduction. This trend is described in lines 485-487 of the Discussion, but not presented in the Results. Please update the Results to describe these coupled profiles.

Thank you for this helpful suggestion. We have updated the results:

LINES 483 - 488: In the upper eutrophic interval (0 – 11 cm), opposing gradients of SO_4^{2-} and H_2S from the surface to 7.5 cm are evidence of sulfate reduction (Fig. 2A, B). Below this depth, sulfate is absent, but a broad H_2S maximum in the deep eutrophic sediments (~40 cm) could be associated with organic sulfur degradation.

Line 327: The citation to Fig. 2E refers to the DIC concentration profile, but the text suggests a reference to the δ^{13}CDIC profile in Fig 2F. Please update the text or reference accordingly.

Updated

Line 381-383: The text states diversity is lowest in the deep eutrophic layer. However, Figure 3C shows the upper eutrophic layer has lower species richness and evenness. Please verify or correct the text.

Thanks for noticing the error. We corrected it to the “upper eutrophic interval”.

Line 438-440: The text discusses Methylomirabilota (NC10) abundance, but the abundance data is not presented in Figure 4B. Please add NC10 abundance to Fig 4B or remove the discussion of their abundance from the Results.

We agree and have revised Figure 4 to display NC10 (Candidatus *Methylomirabilis*) 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.

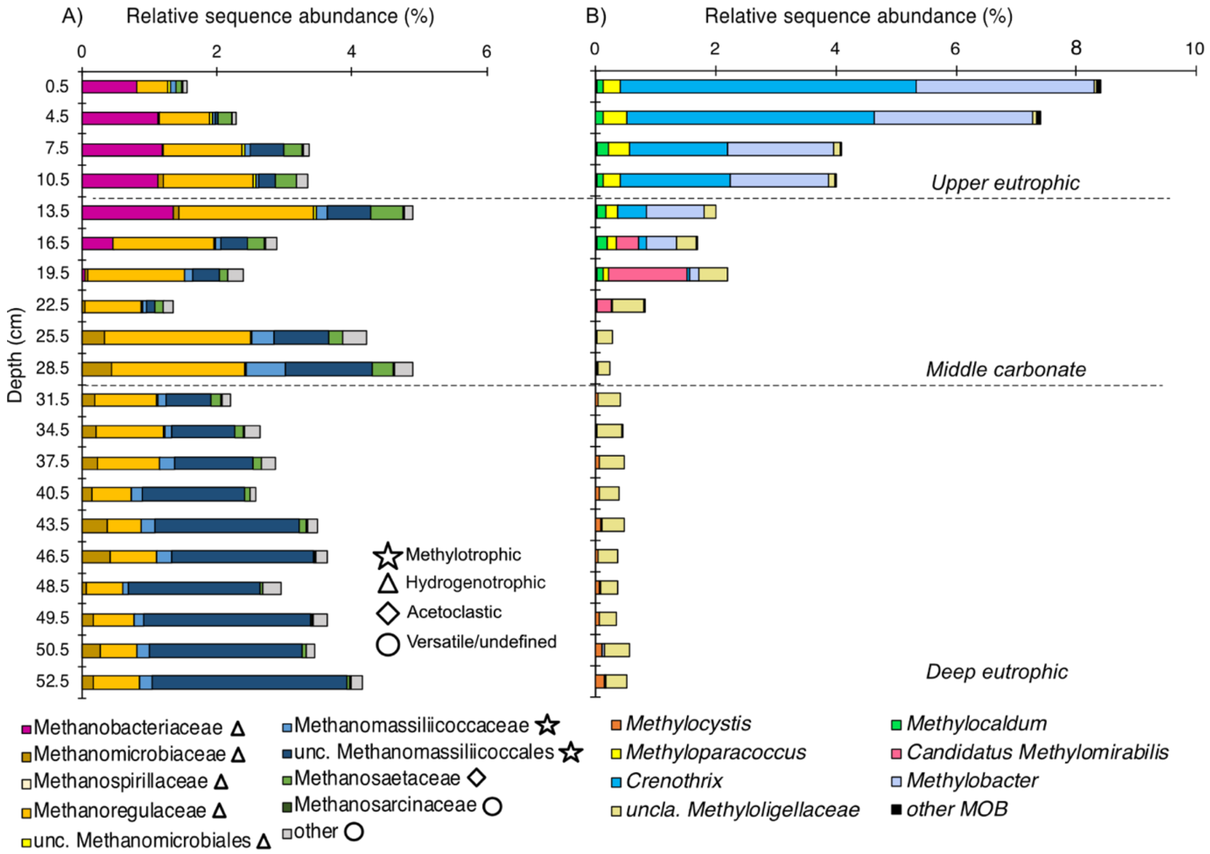


Figure 4. Depth-resolved composition of methanotrophic and methanogenic taxa in Lake Joux sediments. **(A)** Methanogenic archaea clustered by family/order (relative sequence abundance of total community), and grouped by inferred pathways (methylotrophic, hydrogenotrophic, acetoclastic, versatile/undefined).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

Line 443: The text discusses methanotroph abundance but cites Fig 3C, which refers to the alpha diversity metrics of the sediment layers. Please correct the figure reference.

We updated the Figure citation to Figure 4B.

Discussion

Lines 474-475: A transition from methylotrophic to hydrogenotrophic methanogenesis is a point in the Discussion. However, families are introduced only by taxonomic name in the Results. Please explicitly assign the inferred substrate preferences of taxa when introduced in the Results section to improve clarity.

We updated the results to add the substrate preferences:

LINES 610 - 621: The relative sequence abundance of methanogens consistently accounted for more than 1% of the microbial community across all sampled depths (Fig. 4A).

Methanomassiliicoccales, which are H_2 dependent methylotrophs, 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 (hydrogenotrophs) was the most abundant methanogen group in the middle carbonate interval, while Methanobacteriales (hydrogenotrophs) sequences were most abundant in the upper eutrophic sediments (11–0 cm, Fig. 4A). Sequences affiliated with Methanosarciniales (metabolic versatile) were rare throughout the profile (<0.01%).

Lines 475-476: The link between the taxonomic shift of methanogens and the δ^{13}CDIC discontinuity at 32 cm is correct, but the explaination is complex. I suggest adding a brief explanation to aid readers unfamiliar with stable isotopes. Consider including information from the last paragraph of the introduction here.

We agree with the reviewer and have added a deeper explanation between lines 618 -623 when discussing the transition between dominant methanogens and $\delta^{13}C_{DIC}$ changes:

LINES 762-767: 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). Methanogenesis discriminates against the heavier ^{13}C isotope (Conrad, 2005). In theory, when CH_4 is produced from CO_2 + H_2 (hydrogenotrophy), microbes selectively withdraw ^{12}C from the DIC pool, leaving residual DIC relatively ^{13}C-enriched (less negative $\delta^{13}C_{DIC}$); when methylotrophy dominates, CH_4 carbon is drawn from methyl pools and $\delta^{13}C_{DIC}$ is affected less.

Line 528: The decline of methylotrophic methanogens is attributed to a decrease in the availability of methylated compounds. However, these compounds were not measured. Please revise the text to clarify that the decrease was inferred.

We agree and updated the text to clarify it.

LINES 757 - 761: Above ~28.5 cm, concurrent with a shift toward more terrestrial organic matter, methylotrophic methanogens decline and hydrogenotrophs progressively dominate (Fig. 4A). We interpret this pattern as consistent with a reduced supply of methylated substrates, typically derived from algal organic matter although these compounds were not directly measured.

Line 550: This statement implies anaerobic methane oxidation (AOM) is dominant CH4 oxidation pathway across all sediment types. However, aerobic methane oxidation can be the dominant oxidation pathway in aerobic terrestrial sediments. Please update the text to reflect the specific environmental context of AOM.

We agree and revised the sentence to specify that AOM is commonly dominant in anoxic lacustrine sediments.

Technical Corrections

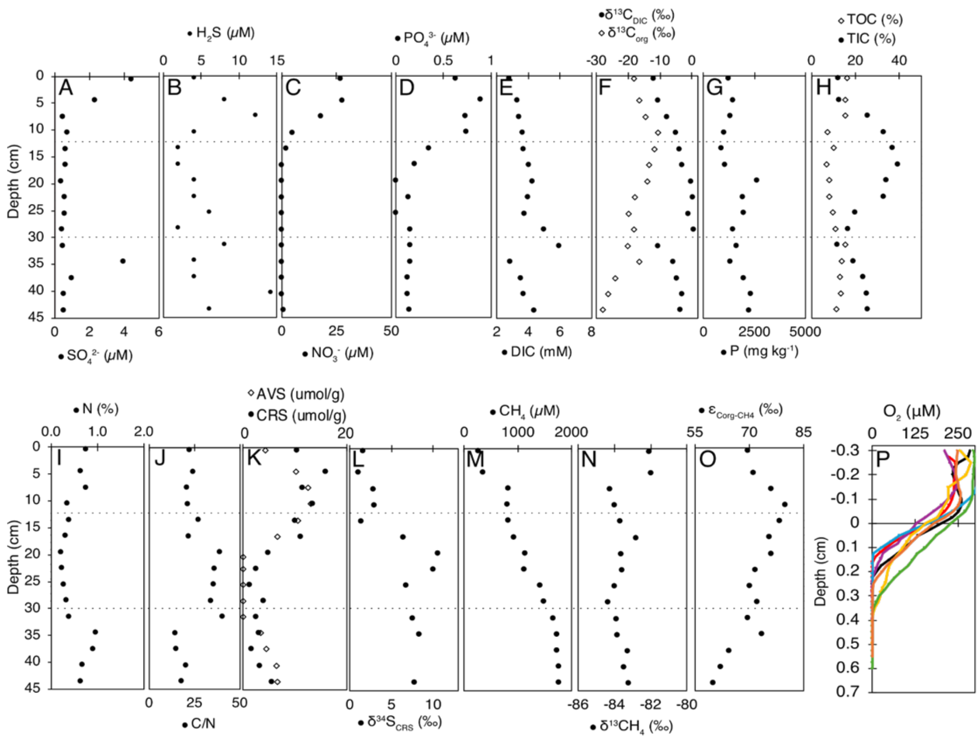
Abbreviations: Define 'DIC', 'TOC', and 'GC-IRMS' at first mention. Explicitly define delta notation (e.g., δ^{13}C-DIC) upon first use.

Thank you. We audited the manuscript for undefined abbreviations and nomenclature and we explicitly define delta notation on first use, including the reference standard (VPDB) and equation:

Example LINE 264-267: Carbon isotopes are reported in delta (δ) notation relative to the Vienna Pee Dee Belemnite (VPDB) standard, defined as $\delta = ((R_{sample} \backslash R_{standard}) - 1) \times 1000$ ‰, with *R* the $^{13}C/^{12}C$ ratio; positive δ indicates enrichment in ^{13}C relative to Vienna Pee Dee Belemnite (VPDB) standard.

Figures: Increase font size of subplot legends in Figure 2 (i.e., increase size of A-P)

Figure updated:



Line 241-242: Complete the sentence "Carbon isotopic fraction factors ... were as".

Updated:
LINES 357 - 374: Carbon isotopic fractionation factors (α) between organic carbon ($\delta^{13}C_{org}$,substrate) and methane ($\delta^{13}C_{CH4}$ (product) were calculated as: $\alpha = (\delta^{13}C_{org} + 1000)/(\ \delta^{13}C_{CH4} + 1000)$. The corresponding isotopic fractionation (ε, ‰) was then determined by the relationship $\varepsilon = (\alpha - 1) \times 1000$, allowing interpretation of trends in dominant methanogenic pathways.

Line 434-435: Close the parenthesis

Done

Line 560: The phrase "CH4-oxidation zone suggests that serve as the dominant" is incomplete.

Thank you. We corrected the incomplete sentence and clarified that Methylococcales-associated MOB are inferred to be the principal CH_4 sink in the anoxic surface sediments.

Line 583: A transition is needed to connect oxygen production via NOD to denitrification by methanotrophic bacteria.

The paragraph was rewritten to attend to another request of the reviewer and now reads:

LINES 852 - 861: One potential source of *in-situ* O_2 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 ~0.54 ± 0.2% of the microbial community in the upper eutrophic sediments, suggesting this pathway may contribute to localized O_2 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_2 production is nevertheless consistent with our geochemical context: porewater NO_2^- remained near detection limit with no subsurface maximum (Fig. S2), indicating rapid NO_x turnover typical of energy-limited sediments.