Discussion of "Sediment characteristics regulate anaerobic oxidation of methane coupled with nitrate and nitrite in the hyporheic zone"

Responses to the Comments from the Reviewer 1

Note: The reviewers' comments are shown in black, and our responses to the comments are shown in blue.

General answer

We would like to express our sincere gratitude to Reviewer 1 for the careful evaluation of our manuscript and for the detailed and constructive feedback. We particularly appreciate the reviewer's recognition of the scientific relevance of methane source–sink dynamics in rivers, the value of our field dataset, and the integrative approach combining porewater chemistry, stable isotopes, microbial community analysis, and reactive transport modelling.

Reviewer 1 raised important concerns regarding the clarity, structure, and scientific rigor of the manuscript. In particular, the reviewer noted that the Results and Discussion are occasionally repetitive, and that some interpretations extend beyond what is sufficiently supported by the available evidence.

We fully appreciate these concerns and will substantially revise the manuscript accordingly. We will separate the Results and Discussion into distinct sections, improve their organization, readability, and logical flow, reduce repetition, and adopt a more cautious interpretation of the data. We will also critically reassess the corresponding interpretations and conclusions to ensure that they are supported by the available data and model results. We sincerely appreciate the reviewer's detailed comments and believe that the proposed revisions will substantially improve the clarity and scientific rigor of the manuscript.

Major comments

On overall writing

1. The Moosach River and its connected branches, and even DAMO processes, have been studied previously, in part also by the authors' own group. The Introduction should therefore state precisely what is new in this study and properly summarize the relevant prior findings on the same field site. In addition, the motivation for investigating this topic, and the specific hypotheses being tested, should also be made explicit. Moreover, the scope of the study needs to be more clearly defined. Only two sites (B and C) are discussed in detail, some conclusions may be specific to this case study, and such limitations should be better acknowledged.

   Thank you for this suggestion. We agree that the present study should contain a summary of the previous study by Michaelis et al. (2022), which was also conducted in the Moosach River and published in this journal. In this context, we have clarified the scientific motivation, research hypothesis, novel aspects, and scope of the present study. We have therefore revised the Introduction accordingly. The revised text reads as follows:

   Previous work by Michaelis et al. (2022) investigated methane cycling processes in fine-grained, organic-rich sediments in the hyporheic zone of the Moosach River. Stable carbon isotope signatures and microbial community distributions indicated hydrogenotrophic and H_2-dependent methylotrophic methanogenesis, while hydrochemical and $\delta^{13}C–CH_4$ profiles provided evidence for biological methane oxidation. Microbiological analyses and PROFILE modelling showed both aerobic methane oxidation and anaerobic methane oxidation coupled with denitrification (N-DAMO) as plausible pathways. However, as the oxygen and nitrate reduction zones were in very close proximity, no single electron acceptor could be identified for biological methane oxidation.

   In the present study, methane cycling was investigated in a contrasting gravel-dominated riverbed to improve the process-level understanding of how sediment characteristics are associated with biogeochemical dynamics in the hyporheic zone. To achieve this objective, high-resolution porewater

hydrochemistry, stable carbon isotope analyses, microbial community characterization and 1D reactive transport modelling were combined as complementary approaches. Reactive transport modelling, combined with uncertainty and sensitivity analysis, was applied to quantitatively evaluate the plausible relative contributions of alternative methane oxidation pathways by linking field observations with process-based reaction scenarios and assessing the robustness of the inferred pathway partitioning.

We hypothesize that differences in sediment characteristics, particularly sediment permeability, affect the transport of dissolved electron acceptors and thereby the shape of hydrochemical gradients and the distribution of microbial communities within the hyporheic zone. These differences are expected to result in contrasting redox zonation, thereby potentially affecting the occurrence of biogeochemical processes.

The process-level interpretations derived from this study are specific to the investigated sedimentary and hydrogeochemical conditions and should not be directly generalized to other hyporheic environments.

2. I recommend a clearer separation of the results from the discussion. Even if the authors choose to combine them, it would be much easier to read if the results were always presented first within each paragraph, followed by the discussion.

Thank you for this suggestion. We agree that this change improves the readability of the manuscript. Following the reviewer's recommendation, we will substantially revise the manuscript by separating the previously combined Results and Discussion section into two independent sections. In the revised structure, all observational and modelling results will be presented first, followed by a separate Discussion section.

On hydrology and environment

1. The manuscript uses many hydrological and hydraulic terms, but none of the key parameters are actually properly described in the main text. Basic hydrological data (stream order, discharge, temperature, permeability, hydraulic conductivity, hydraulic gradient in the sediment, and related parameters) should be reported in numbers in the main text. Whether site C is diffusion-limited needs to be better defined.

Thank you for this suggestion. We agree that the manuscript should provide the key hydrological and hydraulic characteristics necessary for a comprehensive characterization of the study sites and for supporting the interpretation of the observed biogeochemical processes.

Following your recommendation, we will revise the manuscript to include the relevant hydrological and hydraulic parameters in the main text rather than only in the Supplement. Specifically, key parameters such as stream order, discharge, stream water temperature and other relevant site characteristics will be incorporated into Section 2.1 (Study Site Characterization). In addition, sediment permeability and hydraulic conductivity that are directly relevant to the interpretation of the results and discussion will be included in Section 3 (Results) to provide additional support for the associated inferences and conclusions.

We also agree that the previous characterization of Location C as diffusion-limited was too definitive. Because the available field observations do not provide a quantitative characterization of the transport regime, we will describe diffusion-limited transport at Location C and advective(-dispersive) transport at Location B as process interpretations inferred from the combined sediment characteristics and hydrochemical profiles, rather than as directly demonstrated transport regimes.

2. Ebullition is also a primary methane transport pathway and introduced by the authors. It can export more methane to the atmosphere than diffusion, with much of the bubble-transported methane escaping microbial oxidation at the sediment surface. However, its role is not mentioned after the Introduction. Given that the authors report methane supersaturation and infer free-gas formation at site C, the potential contribution of ebullition should be discussed.

Thank you for this valuable comment. We agree that ebullition is an important methane transport pathway and should be considered in the interpretation of our results. In the revised Discussion, we will include a short discussion of its potential role at location C, where methane supersaturation at some depths suggests the potential for free-gas formation. Ebullition could facilitate methane transport from the hyporheic zone to the atmosphere while reducing its exposure to microbial oxidation within the sediment. However, because ebullition was not directly measured, methane supersaturation alone cannot demonstrate bubble-

mediated transport, and we will therefore discuss ebullition as a plausible transport pathway rather than as an observed process.

On microbiology

1. Function and activity (rates) should not be inferred from 16S relative abundance. The DAMO conclusion leans on the relative abundances of Methylomirabilis and Crenothrix, but amplicon abundance reflects presence more than activity. The authors already note this for aerobic methanotrophs, so the same caution applies also to NO_2-DAMO paragraphs, and the limitation should be discussed explicitly. To strengthen the functional interpretation, the authors could add absolute-abundance data from qPCR targeting functional genes (e.g. *mcrA* for methanogens, *pmoA*, or *Methylomirabilis*-specific 16S/*pmoCABC or* assays). Alternatively, a $^{13}CH_4$ tracer incubation with the same sediments would also be helpful for an activity story.

   We thank the reviewer for this valuable and constructive comment. We fully agree that relative abundances derived from 16S rRNA gene amplicon sequencing should not be used to directly infer microbial function, activity, or process rates. The presence of taxa such as *Methylomirabilis* and *Crenothrix* may indicate the potential presence of methane-oxidizing functional groups, but does not demonstrate their metabolic activity.

   In the revised manuscript, we will apply this limitation consistently, particularly in the sections discussing NO_2-DAMO, and clearly distinguish taxonomic evidence from functional interpretation. Microbial community data will be interpreted as indicating the potential presence of functional groups, while process interpretations will be based on the consistency among the available microbial, hydrochemical, and modelling results rather than attributed to individual taxa.

   We also appreciate the reviewer's suggestions to complement the microbial community analyses with quantitative functional gene measurements (e.g., *mcrA, pmoA*, or *Methylomirabilis*-specific markers) or with $^{13}CH_4$ tracer incubation experiments. We agree that these approaches would substantially strengthen the functional interpretation by providing direct information on microbial abundance and methane oxidation activity. However, these analyses were not included in the original experimental design and are therefore beyond the scope of the present study. We will explicitly acknowledge this limitation in the revised manuscript and highlight targeted qPCR analyses and stable-isotope tracer incubation experiments as important directions for future research.

2. Dissimilatory nitrate reduction to ammonium (DNRA) is not considered at all. Organic matter mineralization is a plausible ammonium source, but in the nitrate-reducing interval DNRA is a competing sink that should be addressed. This matters because the balance between DNRA and denitrification changes the nitrogen fate and nitrogen budget.

   Thank you for this valuable comment. We agree that dissimilatory nitrate reduction to ammonium (DNRA) represents a plausible competing nitrate reduction pathway under nitrate-reducing conditions and should be considered when interpreting nitrogen cycling in the hyporheic zone. As the reviewer correctly points out, the relative importance of DNRA and denitrification has important implications for nitrogen fate and the overall nitrogen budget.

   However, our study did not include measurements that would allow us to distinguish DNRA from denitrification, such as isotope-based analyses or functional gene characterization. Therefore, the available data do not permit an assessment of the relative contribution of these two pathways. In the revised Discussion, we will explicitly acknowledge DNRA as a plausible alternative nitrate reduction pathway and discuss its potential implications for nitrogen cycling and the nitrogen budget. At the same time, we will clarify that the available field observations do not allow this process to be resolved, and we will avoid overinterpreting the observed NH_4^+ dynamics.

3. There is an internal contradiction regarding aerobic methane oxidation at site C. The microbial composition is interpreted as favouring methanotrophs (eg, Line 569: "This spatial relationship provides compelling microbiological evidence for active MOx in the shallow sediment"), yet the PHREEQC model assigns MOx no contribution. This discrepancy needs to be explained.

Thank you for this valuable comment. We agree that the original manuscript contains an inconsistency between the interpretation of the microbial community data and the reactive transport modelling results at location C. This inconsistency requires clarification.

To address this issue, we will revise both, the microbial part and modelling sections of the manuscript. As discussed in our response to Comment 1 on microbiology, we will interpret the microbial community data more cautiously, treating them as indicating the presence and distribution of potentially relevant functional groups rather than as direct evidence of their activity or quantitative process contributions.

In parallel, as described in our responses to Comments 3 and 4 on modelling, we will revise the PHREEQC model structure, evaluate alternative reaction scenarios, and perform uncertainty and sensitivity analysis (UASA) to assess the robustness of the inferred methane oxidation pathway contributions within reasonable parameter ranges. Based on these revisions, both the microbial and modelling sections will be updated to provide a consistent process interpretation while ensuring that each line of evidence is interpreted within the limits of the available data.

4. The authors state that they "assume heterotrophic nitrate reduction… in which OC may serve as the electron donor." Porewater DOC was not measured, OC reactivity was not characterized, and no 15N data. Heterotrophic denitrification is therefore one possibility among several and should be presented as a hypothesis rather than asserted as established. Similarly, no sulfide was measured, there are no sulfur isotope data, and no rates are reported. A decrease in sulfate can also result from dilution or mixing (have you tried to calculate SO_4^{2-}/Cl^- ratio?). In short, the nitrogen and sulfur mechanistic claims should either be much more softened or supported with more

   We thank the reviewer for this valuable and constructive comment. We agree that the available geochemical data do not uniquely constrain the specific nitrogen and sulfur transformation pathways. Accordingly, we will revise the manuscript to distinguish more clearly between geochemical observations and mechanistic interpretations and will avoid presenting individual pathways as established processes where direct evidence is lacking.

   Regarding nitrate reduction, we agree that the available data do not constrain the identity of the electron donor. Because porewater DOC, organic matter reactivity, and nitrogen isotope data were not determined, we will no longer attribute nitrate reduction specifically to heterotrophic denitrification. Instead, the revised manuscript will refer more generally to nitrate reduction and discuss heterotrophic denitrification as one plausible pathway among several possible nitrate reduction processes.

   Following the reviewer's suggestion, we will additionally calculate NO_3^-/Cl^- and SO_4^{2-}/Cl^- ratios for all porewater profiles to evaluate the potential influence of conservative mixing. These ratio profiles will be included in the Supplement and referenced in the revised Discussion. The results will be used to assess whether conservative mixing or dilution contributes to the observed concentration patterns or whether deviations from conservative behavior indicate the potential contribution of additional biogeochemical processes. However, these analyses cannot uniquely identify the underlying reaction pathways, and the manuscript will be revised accordingly.

   We agree that sulfate concentration profiles alone do not constitute direct evidence for microbial sulfate reduction. Likewise, increases in sulfate concentration cannot be uniquely attributed to sulfide reoxidation because sulfide concentrations, sulfur isotope data, and process rate measurements are not available. Therefore, in the revised manuscript, sulfate reduction and sulfide reoxidation will both be discussed only as plausible hypotheses that are consistent with the observed concentration patterns, rather than as demonstrated processes. Overall, the revised manuscript will consistently distinguish between geochemical observations and process interpretations, ensuring that all proposed nitrogen and sulfur transformation pathways are presented at a level of confidence supported by the available data.

On modelling

1. The rationale for using two models, and how their respective results enhance the study findings, needs to be further

Thank you for this valuable comment. We agree that the respective purposes of PROFILE and PHREEQC and their contributions to the study findings were not sufficiently clear in the original manuscript. We have therefore reassessed the role of each modelling approach in relation to the objectives of this study.

PROFILE was originally applied as an inverse modelling approach to identify net production and consumption zones and estimate corresponding reaction rates from measured porewater concentration profiles. Sediment characteristics and hydrochemical profiles are consistent with a stronger influence of advective-dispersive transport at Location B and diffusion-dominated transport at Location C. This inferred transport regime at Location B is not adequately represented by the diffusion-based PROFILE configuration, therefore, we will remove the PROFILE results for Location B. The methodological implications are discussed in detail in our response to Comment 2 on modelling. At Location C, the transport interpretation is compatible with PROFILE, allowing reaction zones and rates to be estimated. However, the strong spatial overlap of the O_2 and NO_3^- reduction zones prevents identification of the dominant electron acceptor associated with methane oxidation. PROFILE therefore provides limited further constraints beyond the reaction patterns already indicated by the hydrochemical and stable-isotope profiles. Based on these considerations, we ultimately decided to remove the PROFILE modelling from the revised manuscript.

PHREEQC will be retained and further extended because it accounts for diffusion, advection, and dispersion together with the considered biogeochemical reactions. The model will be used to quantitatively evaluate the plausible contributions of different biological methane oxidation pathways by linking the observed hydrochemical profiles with process-based reaction scenarios. Uncertainty and sensitivity analyses will additionally be incorporated to assess the robustness of the inferred pathway partitioning within reasonable parameter ranges. The revised modelling section will therefore focus on PHREEQC. Further details on the revised PHREEQC modelling approach are provided in our responses to Comments 3 and 4 on modelling.

2. I am not a modeler, but as I understand it, the PHREEQC model includes advection, whereas PROFILE considers only diffusion (with bioturbation and irrigation set to zero). At site B, however, advection matters, and PROFILE's neglect of it is the most likely origin of the alternating nitrate and sulfate "source" terms. This and whether it is legit to claim “two models ‘agree’ with each other” needs to be clarified.

Thank you for pointing out this important methodological issue. We have reassessed the applicability of PROFILE at Location B in relation to the transport processes suggested from our field observations. Under our model configuration, solute transport in PROFILE was represented by molecular diffusion, with bioturbation and irrigation set to zero. Sediment characteristics and hydrochemical profiles are consistent with a substantial influence of advective transport at Location B, indicating that non-diffusive transport cannot be neglected. This transport regime is therefore not adequately represented by our diffusion-based PROFILE configuration. Under these conditions, PROFILE may still provide a mathematical fit to the measured concentration profiles, but transport processes not represented by the model may be incorporated into the inferred source–sink terms. Consequently, the resulting reaction zones and rates cannot be unambiguously attributed to biogeochemical transformations. We will therefore remove the PROFILE results and their corresponding interpretations from the revised manuscript.

PHREEQC explicitly accounts for diffusion, advection, and dispersion. At Location C, sediment characteristics and hydrochemical profiles suggest predominantly diffusion-dominated transport. We will therefore constrain the relevant transport parameters within reasonable ranges so that the model setup remains consistent with this interpretation based on the measured sediment characteristics and hydrochemical profiles. The uncertainty associated with these parameters will additionally be considered through uncertainty and sensitivity analyses.

Since PROFILE will be removed from the revised manuscript, we will also remove the joint interpretation and comparison of the two models, including statements that their results “agree”. Instead, the revised modelling section will focus on PHREEQC and its application to evaluating plausible methane oxidation pathways. Further details on the revised PHREEQC modelling approach are provided in our responses to Comments 3 and 4 on modelling.

3. Nitrite concentrations below the detection limit could suggest efficient and consistent complete denitrification (nitrite simply not accumulating) or with a detection limit that is too high. Interpreting below detection limit nitrite as evidence for rapid nitrite turnover, and then feeding this interpretation into

a model whose result depends on rapid nitrite turnover, is like a circle. The concentration data do not independently support the model, and the text presents it as though they do.

We thank the reviewer for this thoughtful and important comment. We agree that, in the previous version of the manuscript, the wording could be interpreted as using the non-detection of nitrite both as a premise for rapid nitrite turnover and as support for the resulting model interpretation, thereby giving the impression of circular reasoning. We also agree that nitrite concentrations below the analytical detection limit do not uniquely constrain the underlying nitrogen transformation processes.

To address this concern, we substantially revised the modelling framework to account for alternative conceptual scenarios that are compatible with the available observations, rather than relying on a single interpretation of nitrite dynamics.

In the first conceptual scenario, nitrite is represented explicitly as an aqueous intermediate. Unlike the original formulation, nitrite is no longer partially allocated directly to the NO_2^--DAMO pathway through the predefined allocation factor (fNO_2). Instead, all nitrite produced by the representative nitrate reduction pathway first enters a common porewater nitrite pool, from which it may accumulate, be transported, or be rapidly consumed by the explicitly represented NO_2^--DAMO pathway together with a lumped background nitrite sink. Consequently, low aqueous nitrite concentrations are no longer imposed by the model structure but emerge from the interaction between transport and reaction kinetics. The only observational constraint imposed on simulated nitrite concentrations is consistency with the analytical observations (i.e., remaining below the analytical detection limit). In this conceptual scenario, heterotrophic nitrate reduction is used as a representative source of nitrite. This choice does not imply that heterotrophic nitrate reduction is the only or dominant nitrite-producing pathway. Rather, given the available hydrochemical observations and the absence of microbial or isotopic evidence supporting alternative nitrite-producing pathways, it was selected as a representative process to evaluate whether an explicit nitrite intermediate can consistently explain the observed hydrochemical profiles. We will explicitly acknowledge this limitation in the Methods, Results, and Discussion sections of the revised manuscript.

The second conceptual scenario explores the alternative possibility that nitrate reduction proceeds without measurable accumulation of dissolved nitrite. Accordingly, nitrite is not introduced as an explicit aqueous intermediate. Instead, nitrate is consumed through a lumped background nitrate reduction process that does not generate an explicit dissolved nitrite pool. This representation is not intended to describe one specific nitrate reduction pathway, but rather to account for the possibility that nitrate may be reduced without measurable nitrite accumulation in porewater. Model performance is therefore evaluated based on its consistency with the remaining hydrochemical observations (e.g., CH_4, NO_3^-, and O_2).

We acknowledge that the available observations do not allow us to determine objectively which of these conceptual scenarios more closely represents the actual subsurface processes. Therefore, rather than interpreting the absence of detectable nitrite as evidence for rapid nitrite turnover or for a dominant NO_2^--DAMO pathway, the revised modelling framework evaluates multiple plausible scenarios that are all compatible with the available observations and assesses which provides the most consistent explanation of the complete hydrochemical dataset. In this way, nitrite concentrations below the analytical detection limit are treated solely as an observational constraint rather than as independent evidence for a particular nitrogen transformation pathway.

4. The dominance of NO2-DAMO (>99% of methane oxidation) appears to be dependent on parameters the authors chose, rather than completely demanded by the data. NO2-DAMO was assigned a high methane affinity (K_s,CH_4 = 0.005 mmol kg^{-1} w), a value transplanted from a wastewater study. It was supplied with nitrite through a allocation parameter (f_NO_2 = 0.50), but it was assigned the smallest biomass of any guild (Table S7). Such a parameter choice needs to be justified.

We thank the reviewer for this valuable comment. We agree that the inferred contribution of NO_2^--DAMO should not rely on a single parameterization and that the uncertainty associated with model parameters should be evaluated explicitly.

As described in our response to the previous comment, we have substantially revised the modelling framework to consider multiple conceptual scenarios that are compatible with the available observations. Within each conceptual scenario, the model is parameterized using literature-derived Monod kinetic

parameters wherever possible. However, we acknowledge that these parameters were not determined for hyporheic zone sediments and that uncertainties remain regarding their transferability to the investigated system. We will therefore further review the available literature and, where possible, select kinetic parameters derived from environmental conditions and microbial groups that are more representative of the investigated hyporheic system.

Therefore, within each conceptual scenario, we will first establish a baseline parameterization based on published and available kinetic parameters. We will then perform an uncertainty and sensitivity analysis (UASA) by varying the key model parameters within plausible literature-based ranges to evaluate how parameter uncertainty influences the inferred methane oxidation pathways. The purpose of this analysis is not to identify a unique parameter set, but rather to assess the robustness of the model interpretation with respect to parameter uncertainty.

Accordingly, the interpretation of methane oxidation pathways will be revised based on the complete set of modelling results. If multiple parameterizations provide similarly acceptable fits to the observations, this uncertainty will be explicitly acknowledged. Rather than presenting NO_2^--DAMO as a uniquely constrained dominant pathway, the revised manuscript will discuss it as one plausible methane oxidation scenario that is compatible with the available observations.

Other comments

1. Is plant material present in the streambed? Methanol derived from pectin in terrestrial plant material could be a key substrate supporting methane production, which may be relevant to the methanogenic pathway inferred here.

   Thank you for this valuable comment. We agree that plant-derived methanol represents a plausible substrate that could support methanogenesis, and we appreciate the reviewer for highlighting this possibility. Vegetation was present to varying degrees around all four sampling locations; however, we deliberately selected the sampling sites to minimize the direct influence of plant material. As plant-derived substrates, including methanol, were not investigated in this study, we have no direct evidence to evaluate their contribution to methanogenesis. Moreover, the influence of vegetation was not the focus of this study. Therefore, we will briefly acknowledge this possibility in the Discussion but avoid an extensive discussion that is not supported by our data.

2. The use of methane carbon isotopes should be introduced in the Introduction.

   Thank you for this helpful suggestion. We agree that the role of methane carbon isotopes should be introduced to better explain their contribution to the study. Therefore, we will add the following sentence in the Introduction: In addition, stable carbon isotope compositions of methane ($\delta^{13}C$-CH_4) were determined to provide independent evidence for microbial methane production and oxidation processes along the sediment profiles.

3. The terminology for depth zones should be more consistent. Terms such as "shallow," "deep," and "reactive" should be defined and used consistently throughout. Please introduce clearly how many zones are defined, how the depth intervals are separated, and apply this scheme uniformly.

   Thank you for this valuable suggestion. We agree that the terminology describing the different sediment depth zones should be more clearly defined and applied consistently throughout the manuscript.

   In the revised manuscript, we will introduce the depth-zone classification at the beginning of the Results section, clearly defining the number of zones, their corresponding depth intervals, and the terminology used for each zone. We will then revise the manuscript to apply this classification consistently throughout the Results and Discussion, avoiding the interchangeable use of terms such as “shallow”, “deep”, and “reactive”.

4. Methanoperedens / ANME-2d, the canonical NO_3-DAMO archaeon cited in the Introduction (eg, Line 44–46), is never reported in the Results. Was it absent from the sequencing data? This should be stated clearly.

Thank you for this comment. Methanoperedens (ANME-2d) was not detected in our 16S rRNA gene sequencing dataset. We will now explicitly state this in the revised manuscript to avoid ambiguity.

5. Line 47: What kind of evidence is referred to here on *Crenothrix*, and in what type of river was the hyporheic zone studied?

   Thank you for this helpful comment. We agree that the original sentence was too vague regarding both the type of evidence and the study system. Oswald et al. (2017) investigated *Crenothrix spp.* in the water columns of two stratified freshwater lakes. Their conclusions were based on stable-isotope labeling experiments combined with NanoSIMS analyses, which demonstrated methane-dependent growth of *Crenothrix* with oxygen as well as under oxygen-deficient, nitrate-amended conditions, together with metagenomic evidence for pathways involved in methane oxidation, aerobic respiration, and nitrate reduction. We will revise the text accordingly to describe both the evidence and the environmental setting more precisely. The revises text as follows: Oswald et al. (2017) provided isotopic and genomic evidence that *Crenothrix spp.* can oxidize methane aerobically and may also couple methane oxidation to nitrate reduction under oxygen-deficient conditions in stratified freshwater lakes.

6. Line 80: The authors should also justify why the hyporheic zone is defined as the top 40 cm.

   Thank you for this comment. We agree that the previous wording could be interpreted as defining the hyporheic zone as the upper 40 cm of the streambed, although the lower boundary of the hyporheic zone was not independently determined in this study. The 0–40 cm interval represents the depth range covered by the peeper measurements and sediment sampling rather than a hydrologically defined extent of the hyporheic zone.

   We will therefore revise the wording throughout the manuscript to refer to the investigated 0–40 cm streambed sediment interval or the upper 40 cm of hyporheic sediment sampled in this study, rather than defining the hyporheic zone itself as 40 cm deep.

7. Line 210: The version of the SILVA database used for taxonomic assignment must be specified.

   Thank you for pointing this out. We will specify the SILVA database version used for taxonomic assignment in the revised Methods section. Taxonomic assignment was performed using SILVA database version 138.2.

8. Line 295: Does the absence of detectable methane necessarily indicate non-methanogenic conditions, or could it reflect the limited sampling depth? Please clarify.

   Thank you for this important comment. We agree that the absence of detectable methane does not necessarily indicate non-methanogenic conditions and may also reflect the limited sampling depth. As also addressed in our response to Reviewer 2, Comment 5, we will revise the manuscript to distinguish the absence of detectable methane accumulation from the absence of methanogenesis. We will clarify that methane did not accumulate to detectable concentrations within the investigated sediment interval, while methanogenesis at greater depths or methane production followed by removal before accumulation cannot be excluded.

9. Line 300-301: The claim that the OC is "aged" needs support. The statement that greater exchange coincides with less disturbance also appears contradictory and should be clarified.

   We thank the reviewer for this valuable comment. We agree that the original wording was speculative, as the available data do not provide information on the age or reactivity of the organic carbon. We also agree that linking enhanced surface water–hyporheic exchange with long-term undisturbed conditions was unclear and potentially contradictory. We will therefore remove the interpretation that the organic carbon is relatively “aged” and less reactive, as well as the statement regarding long-term undisturbed conditions.

10. Line 300: If permeability and OC content are used so extensively to interpret the results, their actual values should be reported in the main text rather than left in the Supplement.

Thank you for this helpful suggestion. We agree that sediment permeability and OC content are important parameters for interpreting our results. We will therefore provide the relevant values more explicitly in the main text.

11. Line 358: Please state clearly whether $^{13}C\text{-}CH_4$ becomes more depleted or more enriched with depth. It is a bit confusing here.

    Thank you for this helpful comment. We agree that the wording could be interpreted as describing a depth-dependent isotopic trend. Our intention, however, was not to describe whether $\delta^{13}C\text{-}CH_4$ becomes more enriched or depleted with depth, but rather to indicate that the measured $\delta^{13}C\text{-}CH_4$ values in this interval fall within the isotopic range commonly associated with hydrogenotrophic and methylotrophic methanogenesis under anaerobic conditions. To avoid this misunderstanding, we will revise the text to make this interpretation clearer. The $\delta^{13}C\text{-}CH_4$ values in this deeper interval have a mean of −72.45 ‰, which falls within the isotopic range commonly associated with hydrogenotrophic and methylotrophic methanogenesis under anaerobic conditions (Liu et al., 2013; Penger et al., 2012).

12. Figure 3: The sampling locations should be labelled directly on the panels for clarity.

    Thank you for this helpful suggestion. We agree that directly labelling the sampling locations on the figure panels will improve clarity. Accordingly, we will revise Figure 3 to include the corresponding sampling location labels on each panel.

13. Figure 3: Many Deltaproteobacteria are associated with sulfur cycling. Does the abundance of this group support the sulfate-reduction interpretation? Please comment.

    Thank you for this valuable comment. We agree that many members of the Deltaproteobacteria are associated with sulfur cycling. However, Deltaproteobacteria represent a broad taxonomic group, and their abundance alone cannot be specifically attributed to sulfate reduction. In the revised manuscript, we will add a brief discussion of the abundance of Deltaproteobacteria shown in Fig. 3a as part of the overall microbial community composition, while avoiding a direct functional interpretation in terms of sulfate reduction.

    Sulfate-reducing bacteria (SRB) have been examined separately using more specific taxonomic assignments, with their relative abundances and depth distributions presented in Fig. 5e. We will use this more specific analysis when discussing the potential for sulfate reduction, while clarifying that the detection and relative abundance of SRB indicate their presence and may support the potential for sulfate reduction, but do not demonstrate active sulfate reduction.

14. Line 415: Interpreting oxygen utilization at the phylum level is an overinterpretation. Rather than relying on broad taxonomic groups, could the authors identify known functional groups for denitrification or sulfate reduction and check whether their abundances follow the described patterns?

    We thank the reviewer for this valuable comment. We agree that interpreting oxygen utilization based on microbial community patterns at the phylum or class level was an overinterpretation. We will therefore remove this interpretation from the revised manuscript.

    Denitrifiers and sulfate-reducing bacteria (SRB) have already been examined using more specific taxonomic assignments, and their relative abundances and depth distributions are presented in Fig. S4 and Fig. 5e, respectively. Following the reviewer's suggestion, we will use these more specific analyses to reassess the corresponding microbial patterns described in the manuscript. We will also clarify that their taxonomic identification and relative abundance indicate their presence and distribution but do not demonstrate metabolic activity.

15. Line 419: From the figure it is not possible to tell whether methanogenic taxa are present. Please make this clearer.

    We thank the reviewer for this helpful comment. We agree that methanogenic taxa cannot be clearly identified from Fig. 3a, which presents microbial community composition at broad taxonomic levels, and that our original interpretation based on this figure was therefore not sufficiently supported.

Methanogens were examined separately using more specific taxonomic assignments, and their relative abundances and depth distributions, including Methanobacteriales and Methanofastidiosales, are presented in Fig. 5b. In the revised manuscript, we will base the discussion of methanogens on this more specific taxonomic analysis and revise the corresponding interpretation accordingly. We will also clarify that their taxonomic identification and relative abundance indicate their presence and distribution but do not demonstrate methanogenic activity.

16. Line 531: The term "reaction intensity" should be defined.

Thank you for pointing this out. As discussed in our responses to Comments 1 and 2 on modelling, we have decided to remove the PROFILE modelling and the corresponding results and interpretations from the revised manuscript. Consequently, the term “reaction intensity” used in the context of the PROFILE results will also be removed, and a separate definition is therefore no longer required.

17. The NMDS dispersion contrast is interpreted as niche diversity versus homogenization. This should be supported statistically (e.g. PERMANOVA together with a test of multivariate dispersion) rather than resting on the visual spread of the ordination.

We thank the reviewer for this important comment. We agree that differences in microbial community composition and dispersion between Locations B and C should not be inferred solely from the visual spread of samples in the NMDS ordination, and that the interpretation in terms of niche diversity and community homogenization requires statistical support.

We will therefore retain the NMDS ordination but complement it with formal statistical analyses. Following the compositional approach suggested by Reviewer 2 in Comment 9, we will use CLR/Aitchison-based PERMANOVA and PERMDISP to statistically evaluate the community composition and dispersion patterns shown by the NMDS ordination. Further details of the statistical approach are provided in our response to Reviewer 2, Comment 9. Accordingly, the interpretation of niche diversity and community homogenization will be reassessed based on the statistical results rather than on the visual extent of the NMDS ordination alone. The corresponding statements in the Results and Discussion will be revised to reflect the level of statistical support.

18. Table S4: Each parameter should be defined explicitly for readers unfamiliar with these letters.

Thank you for this helpful suggestion. We agree that the parameter abbreviations should be clearly defined. We will add explicit definitions for all parameters in the Supplementary Information to improve clarity and readability.

References

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