Response to Reviewers – *Biogeosciences*

Liquid Polymer Enhances Methanogenesis and Restructures Prokaryotic Communities in Freshwater Sediments (Feckler et al.; egusphere-2026-1216)

We thank the Editor and the Reviewers for their efforts and comments that greatly improved the quality of the manuscript. We have addressed all comments in a point-by-point manner below (in blue) and hope that the manuscript is now suitable for publication in *Biogeosciences*. Please find the detailed changes and corresponding line numbers in the revised manuscript as well as our responses to the comments below.

Reviewer 1

I very much appreciate the motivation behind this paper and think that the introduction reads quite nicely. However, the paper only contains 2 main text figures, which I do think is insufficiently short to tell a compelling mechanistic story as the authors are intending to do per the discussion. While I certainly see value in this experiment, I do not think the paper shows enough as written – to me, it reads as an introduction to a list of hypotheses instead of actually a demonstration mechanism, and the discussion/conclusion emphasizes those hypotheses over analysis of the data in hand. I think follow up experimentation/laboratory analyses on these reactors could be extremely beneficial to help support the ideas that are put forth in the discussion, to strengthen the merits of the study on its own accord. The bones of a good experimental paper are here, but as it currently stands, I do not think there is sufficient data or analysis to warrant publication currently.

We thank the reviewer for their generally positive feedback and the constructive comments that helped improving the quality of the manuscript. We have addressed all points of concern below.

Specific comments:

Introduction: I think this section reads very well!

We thank the reviewer for this very positive feedback.

Methods:

In section 2.1, what is the rationale for supplementing your sediment with extra carbon? Further, why were leaves from another site used to do so? Are these standard practice for sediment/pond water incubation, or are your sediments naturally too low carbon to see a strong effect? Please explain and justify the method here more for clarity.

The addition of organic substrates is a standard procedure in studies that mechanistically address methane production (Bollinger et al., 2021, 2024, 2025; already cited in the manuscript; but see also Bhullar et al., 2013). The purpose of the amendment is to ensure sufficient organic carbon availability as a resource for microbial metabolism (including methanogenesis) during

the incubation. In a previous application of this approach, Bollinger et al. (2021) quantified that the added leaf material contributed approximately 12% of the total sediment organic carbon, indicating that the amendment supplements, rather than dominates, the native sediment carbon pool while helping to avoid carbon limitation during the experiment. We acknowledge that the leaves were collected from a different site than the sediment source. This was necessary because leaf material was not available in sufficient quantities at the sediment collection site. The leaf material was used solely as a carbon source and was processed identically across all treatments; therefore, we do not expect its origin to have influenced the treatment comparisons. To clarify these points, we have added the following text to lines 71-76:

"Adding organic substrates (e.g., leaf litter and glucose) to the test system is common practice when mechanistically addressing methane production (e.g., Bhullar et al., 2013; Bollinger et al., 2025) to ensure sufficient substrate availability for microbial metabolism (including methanogenesis) during incubation. In a previous study using the same approach, the added leaf material contributed approximately 12% of the total sediment organic carbon (Bollinger et al., 2021). Due to limited leaf availability at the sediment source site, leaves were collected from a different site near Landau, Germany (49°12′ N, 8°08′ E), dried at 60 °C for 24 h, and ground into a fine powder using an electric mill prior to sediment amendment."

Bhullar, G. S., Edwards, P. J., and Olde Venterink, H.: Variation in the plant-mediated methane transport and its importance for methane emission from intact wetland peat mesocosms, Journal of Plant Ecology, 6, 298–304, https://doi.org/10.1093/jpe/rts045, 2013.

Looking at table 1, your measured PVP concentrations are all fairly off from your expected. Nothing is far off enough to likely affect your experiment or matter for your conclusions since you are within the right order of magnitude, but please comment on the discrepancy in manuscript somewhere (i.e. where do you think the error is coming from).

We agree that the measured PVP concentrations deviated from the nominal concentrations, particularly during the later stages of the experiment. Although these deviations did not change the order of magnitude of the applied treatments and therefore do not affect the interpretation of our results, we agree that a discussion of their potential causes is warranted. We have therefore amended the manuscript in lines 101-107 reading as:

"The initial deviation between nominal and measured PVP concentrations may have resulted from adsorption of PVP to the glass walls of the storage vials, as PVP is known to adsorb to silica-based surfaces (Al-Harbi et al., 2016). The more pronounced decline in measured concentrations during later stages of the experiment may additionally reflect interactions of PVP with sediment constituents, including organic matter, as PVP has been shown to associate with humic and fulvic substances (Watanabe & Kuwatsuka, 1992). Because initial measured concentrations remained within the intended treatment range and did not alter the interpretation of treatment effects, nominal concentrations are used throughout the manuscript for ease of presentation."

Section 2.3, you only cite flushing the headspace of your incubations for 30S and say this will generate anoxic conditions. These methods are insufficient for producing true anoxia – the oxygen in the headspace may be displaced, but the pond water and sediment are not noted as being collected/handled in an anaerobic fashion. Did you sparge the water/sediment slurry with nitrogen as well, or are you depending on oxygen respiring organisms to deplete the oxygen present in the bottles and hope the methanogens persist until then? Please explain the methods here more.

We agree that flushing only the headspace does not immediately establish fully anoxic conditions throughout the sediment slurry. We did not sparge the sediment-water mixture directly with N_2 prior to incubation. Instead, following headspace flushing and sealing, we relied on aerobic microbial respiration to consume the residual dissolved oxygen present in the microcosms. Evidence for this initial oxygen-consuming phase is provided by the increase in CO_2 concentrations during the first days of incubation (Fig. S5), from which oxygen depletion was inferred to occur after approximately seven days. Subsequently, substantial CH_4 production (a strictly anaerobic process) was observed and progressed into a clear logistic phase, indicating that methanogenic communities persisted through the initial transition period and became active once anoxic conditions were established. After all, with the N_2 flow rates used, the headspace was replaced roughly 100 times and the residual O_2 not detrimental. To clarify this aspect of the experimental design, we have revised the Methods section (lines 114-118) as follows:

"We flushed the headspace of each microcosm with nitrogen for 30 s and subsequently hermetically sealed the vessels. This procedure removed oxygen from the headspace but did not immediately eliminate dissolved oxygen present in the sediment slurry. Consequently, fully anoxic conditions developed progressively during the initial incubation period as residual oxygen was consumed by aerobic microbial respiration. This initial oxygen-consuming phase is supported by the observed increase in CO_2 concentrations (Fig. S1), with oxygen depletion inferred to occur after approximately seven days."

Section 2.4 confused me some – did you do RNA extraction followed by 16S rRNA sequencing of the cDNA produced from that, versus typical methods of 16S sequencing based on just DNA? This is not typically done for 16S analysis, but I think is quite a cool way to do this if the choice was to effectively get an activity proxy for cheaper than MetaT sequencing. I think the rationale for the way you did this needs clarifying, but also that this method you chose is quite cool and is something you could upsell and discuss more!

We thank the reviewer for this positive comment and agree that the rationale for our approach was not sufficiently explained. Our objective was not to characterize the total prokaryotic community, as would typically be achieved through DNA-based 16S rRNA gene sequencing, but rather to focus on the potentially active microbial community. Therefore, we extracted total RNA and reverse-transcribed it to cDNA prior to amplification and sequencing of 16S rRNA. Because ribosomal RNA abundance is generally associated with metabolic activity and protein synthesis potential, RNA-based community profiling provides insight into the potentially active fraction of the microbial community and is therefore particularly relevant for linking

microbial community composition to ecosystem processes such as methane production. We agree that this rationale should be stated more clearly and have revised the manuscript in lines 153-158:

"To characterize the potentially active sediment-associated prokaryotic community, we used an RNA-based sequencing approach rather than DNA-based 16S rRNA gene sequencing. Specifically, 2 µL of each RNA extract was reverse-transcribed into cDNA using random primers supplied with the iScript cDNA Synthesis Kit (Bio-Rad). The resulting cDNA, representing cellular 16S rRNA transcripts, was subsequently used for amplicon sequencing. This approach targets the potentially active fraction of the microbial community and was therefore selected to better link microbial community composition to observed biogeochemical processes."

However, as the focus of the present study was the effect of PVP on biogeochemical processes in sediments, we refrained from further discussing our metabarcoding approach in the following.

Section 2.6 is quite dense, and honestly hard to read as a non-expert in the equations presented - Is there anything that could be done to help simplify this?

We agree that the original presentation of the statistical analyses was overly technical and difficult to follow. We have therefore revised Section 2.5 (lines 177-186 and 211-213) to improve readability by simplifying the description of the von Bertalanffy model and focusing on the parameters that are most relevant to the interpretation of our results. Specifically, we retained concise definitions of all model parameters appearing in the equation, while placing greater emphasis on the upper asymptote (L) and temporal displacement parameter (τ), which are the parameters discussed in the Results section.

"For the statistical analysis of CH_4 production profiles, we followed the approach by Bollinger et al. (2024) and Middelanis et al. (2025), using a non-linear function to describe methane production dynamics. To characterize temporal dynamics of cumulative CH_4 production over time (t, days), we fitted an extended form of the von Bertalanffy growth function (Eq. 5):

$$CH_4(t) = A + \frac{L-A}{(1+v\,e^{-k(t-\tau)})^{\frac{1}{v}}} \quad (5)$$

where A and L represent the lower and upper asymptotes of the curve, respectively, k is a rate constant controlling the steepness of the increase, τ determines the temporal displacement of the curve (i.e., the location where the incubation reaches its fastest methane production), and v controls the curve's symmetry. In the following, we primarily focus on L, representing the

maximum attainable CH_4 concentration, and τ, describing the timing until the inflection point of the curve is reached."

We also streamlined the description of the CLR transformation by providing more concise parameter definitions while retaining all information required to interpret the equation.

"where RA_i is the relative abundance of ASV i, RA_j is the relative abundance of ASV j, D is the total number of ASVs in the sample, and ε (= 10^{-6}) is a pseudocount added to avoid zero values. CLR differences between treatment and control therefore reflect deviations in taxon abundance relative to the overall community composition."

The explanation of the phases of methanogenesis is also unclear to me – do you mean these are typical phases in a closed system? Same uncertainty in line 211.

Thank you for pointing this out. We agree that our previous wording could be interpreted as describing universal phases of methanogenesis. Our intention was only to motivate the use of a sigmoidal growth model for cumulative CH_4 production in our incubation system, which is broadly used throughout many studies and backed up by sufficient theory. Nevertheless, because the discussion of these phases was not essential for understanding the statistical analysis and may have caused confusion, we removed it from the manuscript. The revised text now directly introduces the von Bertalanffy growth model used to characterize temporal CH_4 production dynamics.

Results:

Figure 1 – it's not clear to me what the inflection point is. It is somewhat defined in section 2.6, but in a technically challenging fashion for the average reader to understand. I think a more casual definition in the results would help readability here.

We thank the reviewer for pointing out this difficulty and have repeated the revised definition for the inflection point froim the methods in lines 228-230:

"Specifically, at all PVP concentrations, the time until the inflection point was reached (τ; the location where the incubation reaches its fastest methane production) was 2 days earlier (BF = 28.6–Inf), indicating a faster transition from the initial to the exponential phase."

Figure 1 – this isn't clearly discussed, but the 0.5 mg per L treatment seems to buck the otherwise linear response trend in your data in 2 of 3 metrics measured. I think this for sure needs discussion/analysis in the manuscript.

Thank you for spoting that the non-linear response was not properly addressed and have therefore revised the Discussion in lines 263-274:

"Notably, the response magnitude was relatively modest compared with the large PVP concentration range tested. Moreover, the response did not follow a simple concentration-

dependent pattern. A substantial proportion of the observed effect occurred already at the environmentally relevant concentration of 0.5 mg L^{-1}, whereas further increases in PVP concentration caused comparatively small additional stimulation. This pattern was particularly evident for τ and r_{max}, indicating that the lowest exposure treatment already accounted for a considerable fraction of the total response observed across the entire concentration gradient. The absence of a proportional increase in methanogenesis across four orders of magnitude in PVP concentration suggests that the affected process became saturated at relatively low exposure levels. Rather than reflecting a continuously increasing effect of PVP concentration, the observed response appears more consistent with a threshold-like alleviation of a constraint on methanogenesis. Once this constraint was sufficiently reduced, additional PVP may have produced only limited further stimulation. Although the identity of this constraint remains unknown, the findings indicate that environmentally realistic concentrations may already be sufficient to induce measurable changes in methane-production dynamics."

Typo on line 230 – "ASVs o average"

We deleted the "o" as pointed out by the Reviewer.

Table 2 – I don't understand the two groups of taxa as phrased, since Euryarchaeota *are* The grouping/organization needs renaming here for sake of accuracy and clarity.

Thank you for pointing this out. We agree that the previous wording could be confusing, as Euryarchaeota are a subset of the prokaryotic community rather than an independent group. To clarify this relationship, we revised the caption of Table 2 to explicitly distinguish between analyses of the complete prokaryotic community and the Euryarchaeota subset:

"Table 2. PERMANOVA results for community compositions of all prokaryotic taxa and the Euryarchaeota subset. Statistical significance is highlighted in bold." (lines 255-256)

We also clarified the rationale for this analytical separation in the Methods section (lines 202-205), which now reads:

"For metabarcoding analyses, we first analyzed the complete prokaryotic community and subsequently examined Euryarchaeota separately, as this phylum comprises the methanogenic Archaea responsible for CH_4 production. This hierarchical approach allowed us to distinguish broad shifts in prokaryotic community composition from responses specifically associated with methanogenic microorganisms."

I think Figure S6 would be a reasonable main text figure instead of a supplemental – that is data I wanted to see to be able to understand/assess Figure 2, so it is definitely important data.

We have moved the figure from the Supporting Information to the main maunscript, as suggested by the Reviewer.

Discussion

As an overall comment, I don't think there is much analysis of your own data here, versus review of literature. I think this paper really would benefit from more specific analysis of the figures before the theorizing on mechanism comes in.

We thank the reviewer for this constructive comment and agree that the original Discussion placed too much emphasis on hypothesized mechanisms relative to the direct interpretation of our results. We have therefore substantially revised the Discussion. In particular, we now place greater emphasis on the observed methane-production dynamics, the non-linear concentration-response relationship, and the temporal patterns in microbial community composition before discussing potential mechanisms. Furthermore, we introduced a new subsection entitled "Implications and outlook", in which we clearly separate data-supported interpretations from mechanistic hypotheses and future research needs. As the revised Discussion is too extensive to be pasted in full here, we refer the reviewer to lines 260-353 of the revised manuscript and to our responses to the specific comments below. Finally, we also revised the Abstract according to the new Discussion structure (lines 15-23).

Line 244: how meaningful is a 2-hour difference in reaching this inflection point? Is this an ecologically significant difference?

We believe there may be a misunderstanding regarding the reported effect size. The manuscript (lines 228-230) states that the inflection point (τ) was reached approximately 2 days earlier, not 2 hours earlier, in the PVP treatments compared to the control. We consider this shift ecologically meaningful because it reflects an earlier onset of the period of fastest methane accumulation. As the effect size is already reported in days and the biological interpretation of τ is provided in the text, we did not revise the manuscript further.

Lines 244-245, also stated above, the 0.5 mg per L treatment does not follow the trend you are purporting in the paper of a maybe linear increase in the metrics with PVP dosing, so I think that really needs to be discussed.

As highlighted in our response to a similar comment of the Reviewer above, we have revised lines 263-274 of the manuscript.

Lines 249-251 – I am not following the basis of this assumption. Do you have organisms in your community that are specifically known to produce EPS in response to plastic? Is this a taxa-specific response? The theorizing here feels too general to me.

We agree with the reviewer that the original discussion of EPS production was overly speculative and not directly supported by our data. EPS production was not quantified in the present study, nor did we identify taxa for which an EPS response could be demonstrated. We therefore substantially revised this section. Rather than discussing EPS as a likely explanation for the observed effects, we now present it in the newly introduced "Implications and outlook" section (lines 332-335) as one of several hypotheses that may warrant investigation in future

studies. The revised wording clarifies that the relevance of EPS production cannot be evaluated with the available data.

As with the EPS idea, the discussion of TEAs and iron feels unsubstantiated to me in the current manuscript. Could you do a ferrozine assay or something to measure iron in the incubations to support this idea for your own experiment? Or if you do not have any more sample material, could you at least look for iron-cycling taxa in your data? Is iron relevant to your specific sediments? There is a lot of discussion of iron metabolism specifically here without clear linkage to your data and material.

We agree that the original Discussion placed too much emphasis on an iron-mediated mechanism relative to the supporting evidence available. Unfortunately, no sediment material remained available for additional geochemical analyses such as iron speciation or Fe(III)-reduction assays. We therefore revisited this aspect of the manuscript and screened the microbial dataset for known iron-cycling taxa. These taxa did not exhibit consistent responses across treatments. Consequently, we substantially shortened the Discussion of iron-mediated effects and moved it to the new "Implications and outlook" subsection (lines 327-331), where it is presented as a mechanistically plausible but currently untested hypothesis. We now explicitly state that the relevance of iron-mediated pathways remains unresolved and requires direct investigation in future studies.

Line 283 – please change kingdom to phylum

Given the substantial revision of the Discussion, this comment does not apply anymore.

You talk about the need for explicit mechanism testing in a follow up paper, but I don't quite understand why that data is lacking from this manuscript e.g. I think this current work needs more data to be able to say much to most of what you say in the discussion section. I fully understand that there might not be stored/extra experimental material to do more chemical analysis on to look for iron/TEAs, EPS production, etc, but at least the microbial data in hand could be used to do more to get at addressing the ideas brought up in the paper.

We thank the reviewer for this thoughtful comment. We recognize that the original Discussion could give the impression that the study aimed to identify specific mechanisms underlying the observed responses. However, the primary objective of the present work was to serve as a proof-of-concept study and establish whether a widely used soluble synthetic polymer can influence sediment methanogenesis and microbial community composition at all. We agree that this distinction was not sufficiently clear in the original manuscript. In response, we substantially revised the Discussion to focus more strongly on the observed methane-production dynamics and microbial community responses, while clearly separating data-supported findings from mechanistic hypotheses. We also conducted additional inspection of the microbial dataset to evaluate whether the proposed mechanisms were reflected in the community data. This resulted in greater emphasis on the observed responses of fermentative bacterial taxa and a reduced emphasis on unsupported mechanisms such as iron cycling and

EPS production. Finally, we now explicitly state that the present study was designed as a proof-of-concept experiment and that resolving the underlying mechanisms will require future studies combining microbial, geochemical, functional, and process-based measurements.