MS No.: egusphere-2025-4143

Stability of stream biofilm community composition to transient shifts in dissolved organic carbon characteristics

Dear editor and reviewer,

We thank the reviewer for their constructive comments, our manuscript has improved from the feedback provided. Our responses to each comment are provided in purple. We have followed the format 1) reviewer comment, 2) our response and 3) proposed change(s) to the manuscript (given in ‘’) where provided.

Overall assessment

The manuscript by Lines et al. investigates the response of stream biofilm microbial communities to storm-event-driven pulses of dissolved organic carbon (DOC) in a sub-alpine peatland catchment. This is a relevant topic, given the projected increase in storm intensity under climate change and the critical role of biofilms in aquatic carbon cycling. The study employs a comprehensive multi-method approach that combines detailed hydrology, sophisticated DOC characterisation, and high-throughput sequencing of algal, bacterial, and fungal communities. The central finding – that biofilm community composition remained largely stable following short-term DOC pulses and was instead more strongly influenced by site-specific factors – is noteworthy and contributes a valuable perspective to the field. The manuscript is generally well-written, and the data appear robust. However, several points require clarification and further discussion to strengthen the interpretation of the results and the overall impact of the study before it can be considered for publication.

We thank the reviewer for their thoughtful feedback and encouraging assessment of our manuscript. Below we address each point raised by the reviewer to improve the interpretation of our results and the clarity of our manuscript.

Please note all referenced line numbers refer to the preprint version of the manuscript.

Major Comments

1. **Temporal Resolution of Biofilm Sampling:** Biofilms were sampled only “within 24 hours prior to zero storm time” and “12–16 days after each storm event.” This gap misses short-term (e.g., 1–7 days post-storm) community dynamics, which could reveal transient shifts that recover by 12–16 days. For example, if biofilms temporarily respond to DOC pulses but revert to pre-storm composition by the post-storm sampling, the current design would mischaracterise “stability” as absence of change rather than resilience. If retrospective sampling is not possible, discuss this limitation explicitly and propose future studies with intermediate time points (e.g., 3, 7 days post-storm) to capture potential short-term responses.

This is a valid observation, and we agree that the sampling interval (11days between pre- and post-storm) represents a potential limitation of our study that needs to be discussed. It is possible that the biofilm community composition could have shifted and returned to pre-storm composition during the sampling interval. It is not possible to retrospectively sample, however in the discussion, we address this as a possible study limitation and propose a future study in paragraph 1 of Section 4.2:

'Most of the stream biofilm microbial communities did not show a significant shift in composition following the storm-event-driven pulses of DOC, despite the changes in DOC composition over the course of each pulse (Fig. 4; Table S12). It is important to consider that the biofilm communities may have responded within the Before and After sampling interval, or the biofilm communities may have responded metabolically (O'Donnell and Hotchkiss 2022; Hallberg et al., 2024) without any significant shifts to composition (Battin et al., 2016), leading to the observed stability following a storm event. Future studies could address these gaps by sampling three and or seven days post storm event and include meta-transcriptomic (RNA) analyses to compare differences in gene expression between biofilm communities before and after a storm event. Despite these study limitations, there are several other more compelling reasons that could explain the observed biofilm stability following a storm event.'

Reference added:

- Battin et al., 2016 (already in reference list)
- Hallberg, L., Bernal, S., & Bieroza, M. (2024). Seasonal variation in flow and metabolic activity drive nitrate and carbon supply and demand in a temperate agricultural stream. *Journal of Geophysical Research: Biogeosciences*, *129*(11), e2024JG008308.
- O'Donnell, B., & Hotchkiss, E. R. (2022). Resistance and resilience of stream metabolism to high flow disturbances. *Biogeosciences*, *19*(4), 1111-1134.

2. **Interpretation of the PERMANOVA Results:** The significant three-way interaction (Event × Site × Storm-time) is reported, but the subsequent analysis and discussion focus heavily on the overwhelming effect of 'Site'. The PERMANOVA model uses Type I (sequential) sums of squares, meaning the variance explained by 'Site' (entered first as a random factor?) is removed before testing the other factors. The authors should clarify the order of factors in the model and consider whether the significance of the three-way interaction is largely an artefact of the strong site effect, as they allude to. A more detailed presentation of the variance explained by each term (e.g., via a more complete table than S10) would be helpful. Furthermore, for the pairwise tests following the significant interaction, the use of Monte-Carlo p-values due to low permutation numbers should be explicitly stated in the main text methods, not just the supplement.

Thank you for this feedback. We have clarified in the methods that type 1 and type 3 sums of squares are mathematically equivalent in a balanced design and added that the order of fit of terms was checked and did not change the p-value significance of the terms. We have also updated the methods to better clarify the use of the Monte-Carlo p-values. Additionally, we can see that the wording about the contribution of Site and where it was introduced in the results was confusing and detracted from the interaction effect of Storm-time*Site*Event. Our intention was to highlight the strong contribution of the factor Site to variation in biofilm community composition. We have removed the description of the effect of from section 3.3.3 lines 426 – 431 and modified paragraph 1 of section 3.3.4. Lastly, we updated table S10 and its description to include estimates of the components of variation.

Update to methods section 2.7.2 from line 294 to 301:

'[…]. Multivariate variation was partitioned using Type 1 (sequential) sums of squares (SS). In a balanced design, Type 1 SS is mathematically equivalent to Type 3 (marginal) SS (Anderson et al., 2008). The order of fit of model terms was checked and there were no changes in the statistical significance of the terms based on order of fit. […]. Follow up pairwise comparisons of biofilm communities between the Storm-time levels 'Before' and 'After' within each level of Event and Site were conducted using the same parameters and significance level as the main PERMANOVA test; however, due to the limited number of unique permutations (10 per comparison), Monte-Carlo p-value estimates were used to assess the statistical significance of each comparison. Homogeneity of variance amongst groups was tested using the PERMDISP routine from PRIMERv7.'

Modification to section 3.3.4 from line 462 from line 462 to 466:

'An unexpected finding from this study was that Site contributed the greatest amount of variation to biofilm microbial community composition within the Watchbed Creek catchment. Estimates of the components of variation for each term within the PERMANOVA model indicated that Site was the largest contributor to variation in community composition amongst biofilm samples relative to all other terms (Table S10). The importance of Site on biofilm community composition is also observed in Fig. 4a where, following grouping by replicate sample, biofilm community composition strongly clustered by Site. These results suggest that biofilm community composition may be strongly influenced by Site. On average […]'

Update to Table S10 and its description:

'[…]. Type 1 sum of squares was used to partition variation. Site was treated as a random factor (four levels), and Event and Storm-time were treated as fixed factors (2 levels each). The order of fit did not change the statistical significance of terms within the model. Additionally, the square root of the estimates of the components of variation (ECV) in units of Bray-Curtis dissimilarity, are provided for each term in the model. The square root of ECV provides an estimate of the variation attributed to each term in the model in the units of the distance measure chosen for the test.'

Source of Variation	**df**	**SS**	**Pseudo F score**	**P value (Perm)**	**Sq. root ECV**
Storm-time	1	1674.7	1.21	0.3778	3.488
Site	3	34459	17.2	**0.0001**	30.03
Event	1	7132.2	2.72	0.0718	13.71
Storm-time *Site	3	4148.1	2.07	**0.0001**	10.92
Storm-time *Event	1	1920.3	1.76	0.2106	8.325
Site*Event	3	7869.5	3.93	**0.0001**	18.05
Storm-time *Site*Event	3	3266	1.63	**0.0022**	11.84

Residual	32	21373			25.84
Total	47	81843			

Reference added:

- Anderson MJ, Gorley RN & Clarke KR. (2008). "*PERMANOVA+ for PRIMER: Guide to Software and Statistical Methods.*" PRIMER-E Ltd: Plymouth, UK.

3. **Linking DOC Change to Biofilm Function:** The study expertly characterises DOC composition but does not link these changes to any functional response of the biofilms (e.g., metabolic activity, extracellular enzyme assays). The authors hypothesise that the biofilm matrix may buffer against change, but without concurrent data on microbial function or carbon utilisation, this remains speculative. Whilst a comprehensive functional analysis may be beyond the scope of this paper, the discussion on *why* the communities are stable (Section 4.2) would be strengthened by acknowledging this gap and suggesting that functional stability (or plasticity) could occur without compositional shifts. Are the observed communities functionally redundant?

Thank you for this feedback. We agree that the communities could have responded metabolically and rather than compositionally. This is a limitation of DNA metabarcoding; we gain information on what microbes are present, but we do not receive any information about their metabolic function and activity. We address this study limitation in response to major comment #1 (this reviewer).

4. **Climate Change Implications**: The manuscript concludes that biofilm stability "suggests that with the predicted changes in the frequency and intensity of storm events under a warming climate, these communities may be resilient" (p. 29). However, it does not address whether repeated storms (e.g., the three events between SE1 and SE2, p. 12) could exhaust resilience over time. For example, if each storm depletes EPS stores, successive pulses might eventually trigger community shifts. Discuss the limitations of extrapolating from two storms to long-term climate change, and propose future studies on the effects of repeated DOC pulses on biofilm resilience.

We thank the reviewer for their comments. We agree that only having data from two storm events is somewhat limited, although the stability was observed over the four sites (except for sites C and D during SE2). While it would be extremely informative to study the effects of multiple storm events on biofilm resilience, this can be difficult to achieve in a natural setting where storm events are unpredictable. We have proposed future study directions within section 5.

Addition to section 5 line 632:

'[…]. Future studies could build on this work by investigating how quickly a storm event driven DOC pulse passes stationary points within a stream and calculate exposure time as the length of time between when a change in DOC composition is detected to when the compositional components of interest return to pre-storm conditions;

and compare this to changes in biofilm metabolism or community composition. Additionally, future studies could investigate the stability of stream biofilms to repeat storm events that could lead to a shift in community composition. However, the feasibility of this type of experiment is entirely dependent upon receiving a series of storms where this can be tested within any given study period. […]

Minor Comments

1. **Introduction:** The introduction is comprehensive but could be more sharply focused. The link between DOC characteristics, bioavailability, and *expected* microbial community shifts could be articulated more directly early on to set a clearer hypothesis.

Thank you for highlighting this. We have re-arranged paragraph 2 of the introduction to state the relationship between DOC characteristics, bioavailability and potential microbial community shifts earlier. This now reads:

'DOC is a complex mixture of organic compounds, encompassing molecules of various sizes, structures and chemical characteristics (Hope et al., 1994). The composition and characteristics of DOC is strongly influenced by its origin (e.g., source vegetation, peat soil characteristics) and any biogeochemical transformations it may have undergone (Hope et al., 1994; Jones et al., 2016; Pinsonneault et al., 2016; Nelson et al., 1992). The chemical characteristics of DOC can affect its bioavailability for microbial metabolic processes (Berggren et al., 2010; Demars et al., 2020; Guillemette and Del Giorgio, 2011; Boulton et al., 2014). Therefore, it could be expected that microbial community composition may shift in response to the mobilisation and delivery of different characteristic DOC, particularly during storm cycles. Studies have shown that aquatic microbial communities respond to DOC of differing concentrations and characteristics (Fasching et al., 2020; Demars et al., 2020; Berggren and Del Giorgio, 2015; Findlay et al., 2003; Roiha et al., 2016; Olapade and Leff, 2005; Covert and Moran, 2001), potentially driven by the differing bioavailabilities of DOC to microbial organisms (Guillemette and Del Giorgio, 2011; Creed et al., 2018). In stream systems, DOC can originate from in-stream biological production or terrestrially derived organic matter (Hope et al., 1994). The composition (and characteristics) of DOC in streams is highly dependent on hydrological connections between the stream and different source pools of DOC, which can vary with changes in water flow paths (Worrall et al., 2002; Jones et al., 2016; Broder et al., 2017; Limpens et al., 2008). High intensity storm events can raise water tables, increase water discharge, and create transient, surficial flow paths for water, all of which can facilitate the mobilisation DOC from different pools of carbon and lead to changes in DOC composition within the receiving stream (Clark et al., 2007; Branfireun and Roulet, 1998; Schiff et al., 1998; Wagner et al., 2019; Silvester et al., 2021).'

Reference added:

- Silvester et al., 2021 (already in ref list).

2. **Methods (Section 2.7.1):** The use of a correlation cut-off ($r \geq 0.7$) to remove variables from the PCA is a valid approach to reduce multicollinearity. However, the choice of the cut-off is somewhat arbitrary. A brief justification for this threshold would be helpful. Furthermore, stating that DOC is "best represented by Electrical Conductivity" is an overinterpretation; it is *correlated with*, not a direct proxy for.

Thank you for this feedback. The value was chosen as a value of 0.7 is widely considered to be a strong linear correlation between two variables. We have updated the methods to address this. Also, thank you for the correction of descriptor, it more clearly defines the relationships between the variables; we have adopted the feedback provided.

Update to methods section 2.7.1 DOC characteristics analyses, lines 260 - 270:

'[…] package caret (Kuhn, 2008). The cut-off threshold of 0.7 was chosen as variables with a linear correlation of 0.7 or higher are widely considered strongly correlated (Mukaka 2012; Schober and Boer 2018). The 'findCorrelation' function […]. Importantly, this process identified bulk DOC concentration as strongly correlated (r >0.7) with several parameters in the water chemistry dataset. Of the parameters retained for the PCA analysis, DOC was most strongly correlated with Electrical Conductivity (conductivity, r = 0.76).'

Added reference:

- Mukaka, M. M. (2012). A guide to appropriate use of correlation coefficient in medical research. *Malawi medical journal: the journal of Medical Association of Malawi*, *24*(3), 69.
- Schober, P., Boer, C., & Schwarte, L. A. (2018). Correlation coefficients: appropriate use and interpretation. *Anesthesia & analgesia*, *126*(5), 1763-1768.

3. **Discussion (Section 4.2):** The argument that short water residency times limited community response is compelling. Could the authors provide a rough estimate of water travel times during peak flow vs. baseflow for their sites to quantitatively support this line of reasoning?

We thank the reviewer for their feedback and appreciate the question about water residency times. We respectfully respond that to calculate a reasonable travel time/distance we would need to calculate how fast the DOC pulse (and its compositional components) travel within the stream. To do this we need to make several assumptions about stream morphology, roughness etc., all of which are beyond the scope of this study. Rather, the focus of this study is on the response of stream biofilm communities to a short pulse of DOC generated by a storm event, where the concentration and characteristics of the DOC changed over the pulse. The period of exposure is well defined by the length of the DOC pulse. However, we have added in the conclusion future study directions addressing water residency times and community responses. We propose these future study directions in response to major comment #4 (this reviewer).

4. **Language and Typography:** The manuscript is well-written but would benefit from a final proofread for minor grammatical inconsistencies (e.g., consistent use of present/past tense). Ensure all acronyms are defined at first use.

Thank you for pointing this out. All co-authors will proofread the final version of the manuscript.

I recommend major revisions.