

Groundwater microbiomes balance resilience and vulnerability to hydroclimatic extremes

Corresponding Author: Professor Kirsten Küsel

This file contains all editorial decision letters in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Decision Letter:

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Dear Professor Küsel,

Your manuscript titled "Balancing Act: Groundwater microbiome's resilience and vulnerability to hydroclimatic extremes" has now been seen by 3 reviewers, whose comments are appended below. You will see that they find your work of some potential interest. However, they have raised quite substantial concerns that must be addressed. In light of these comments, we cannot accept the manuscript for publication, but would be interested in considering a revised version that fully addresses these serious concerns.

We hope you will find the reviewers' comments useful as you decide how to proceed. **Additionally, we want you to work on the following editorial thresholds for further consideration in Communications Earth & Environment:**

*** Provide sufficiently novel insights into resilience and vulnerability of groundwater microbes to hydroclimatic extremes**

*** All the conclusions should be well supported by data and not beyond it. Any assumptions made should be clearly mentioned as assumptions and the rationale behind those.**

*** Fully describe the transparency and reproducibility in methodology.**

Should additional work allow you to address these criticisms, we would be happy to look at a substantially revised manuscript. If you choose to take up this option, please either highlight all changes in the manuscript text file, or provide a list of the changes to the manuscript with your responses to the reviewers.

When resubmitting, please provide a point-by-point response to the reviewers' comments. Please submit your responses as a separate file, distinct from your cover letter where you can add responses to the Editors' comments that you do not want to be made available to the reviewers. Word files are preferred. We recommend that any figures, tables or graphs that are included in the response to reviewers are also included in the main article or Supplementary Information.

Please bear in mind that we will be reluctant to approach the reviewers again in the absence of substantial revisions.

If the revision process takes significantly longer than three months, we will be happy to reconsider your paper at a later date, as long as nothing similar has been accepted for publication at Communications Earth & Environment or published elsewhere in the meantime.

We are committed to providing a fair and constructive peer-review process. Please do not hesitate to contact us if you wish to discuss the revision in more detail.

Please use the following link to submit your revised manuscript, point-by-point response to the reviewers' comments with a list of your changes to the manuscript text (which should be in a separate document to any cover letter), a tracked-changes version of the manuscript (as a PDF file) and any completed checklist:

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Please do not hesitate to contact us if you have any questions or would like to discuss the required revisions further. Thank you for the opportunity to review your work.

Best regards,

**Temitope Sogbanmu, PhD
Editorial Board Member
Communications Earth & Environment
orcid.org/0000-0002-8913-267X**

**Somaparna Ghosh, PhD
Associate Editor - Communications Earth & Environment
Consulting Editor- Communications Sustainability**

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REVIEWER COMMENTS:

Reviewer #1 (Remarks to the Author):

Please find the attachment

Reviewer #2 (Remarks to the Author):

Recommendation: Major Revision

The manuscript is a potentially impactful contribution to the field, especially due to its unique temporal depth and multidisciplinary scope. However, major clarifications and revisions are needed to strengthen the conceptual framework, improve data interpretation, and ensure methodological transparency.

General Assessment:

This manuscript presents a highly comprehensive, decade-long investigation into the temporal dynamics of groundwater microbiomes in carbonate aquifers, focusing on their resilience and vulnerability to hydroclimatic extremes. The authors integrate microbial, metabolomic, and hydrochemical datasets in an exemplary fashion. The scope, temporal scale, and interdisciplinary integration are impressive and represent a major contribution to groundwater ecology. However, despite the strength of the dataset and analysis, there are several substantial issues that must be addressed before the manuscript can be accepted. These include concerns related to conceptual clarity, methodological justification, the robustness of some interpretations, and presentation clarity.

1. Several conclusions, such as the link between metabolomic shifts and microbial community changes or between seepage-associated bacteria and groundwater levels, are based solely on correlation. These should be framed more cautiously. Consider alternative explanations and clarify whether causal relationships are truly supported by

the data.

2. While PICRUSt2 provides useful predictive insights, the reliance on inferred gene functions from 16S rRNA gene sequences should be acknowledged more clearly, particularly in the discussion of "functional stability." Emphasize that these are predictions, not direct measurements of metabolic activity, and consider whether metatranscriptomic or metaproteomic validation is planned or possible.
3. The study claims sinusoidal/seasonal variation in microbiome dynamics, yet the methods mention monthly sampling without showing whether the sampling frequency is sufficient to resolve seasonality across all years. Please clarify temporal gaps (if any), sampling regularity, and how these may affect the robustness of inferred patterns.
4. The definitions of resilience and resistance, while briefly mentioned, require clearer operationalization. The metrics used to quantify them (e.g., turnover rates, Bray-Curtis similarity) are not clearly linked to established definitions in microbial ecology. A schematic or expanded explanation in the methods would greatly help.
5. The study touches on aquifers' vulnerability to surface disturbances but does not sufficiently explore the potential influence of land use (e.g., agricultural or forested areas), which is mentioned in methods but not analyzed. Please elaborate on whether land use was considered as a variable or if future analysis will explore this.
6. There is substantial overlap and repetition in the reporting of DOM and metabolomic results. Consider reorganizing or consolidating these sections to improve readability and avoid redundancy, particularly in the Results and Discussion.
7. Several figures (e.g., Fig. 1–3) are overloaded and difficult to interpret without more context. Please ensure all axes, labels, and statistical elements are fully explained in figure legends. Additionally, indicate sample sizes where comparisons are made.
8. Terms like "hydrological connectivity," "resilience," "core microbiome," and "functional redundancy" are used frequently but are not always defined or consistently applied. Ensure consistency and clarity for interdisciplinary readers.
9. Although seepage-associated microorganisms were shown to contribute a small fraction (~4%) to groundwater microbiomes, their biological/ecological significance is unclear. The authors should elaborate on whether these organisms are functionally relevant or merely transient.
10. The comparison with ocean microbiomes, while interesting, feels somewhat speculative and distracts from the core groundwater-focused findings. Consider moving such comparisons to a dedicated "Outlook" subsection or trimming them.
11. Abstract: The abstract is dense. Consider breaking up long sentences and more clearly highlighting the major findings.
12. Line 67: Spelling error in "recovery" → should be "recover".
13. Supplementary Materials: Ensure they are cross-referenced consistently throughout the text.

Reviewer #3 (Remarks to the Author):

In their article „Balancing Act: Groundwater microbiome’s resilience and vulnerability to hydroclimatic extremes“ He Wang and colleagues provide a good overview analysis of an amazing dataset containing a 10-year time series of groundwater well microbial communities and organic matter. The authors show little influence of seepage microbes in the well groundwater communities and stability/resistance patterns driven by connectivity and environmental fluctuations. Overall, the manuscript is well structured and prepared. It would however benefit from some clarifications and, owing to the journal’s style, some placing of the results in the environmental context in the results section. I outline my minor concerns and comments below.

Introduction:

II. 60-68: It is not quite clear to me what is the difference between hydrologically connected aquifers and hydrologically isolated aquifers if both receive surface recharge (l. 63, l. 66). Is there a threshold, how is it usually defined?

I. 76: I believe the statement that the combination of two compositional, semi-quantitative datasets and a prediction tool results in “quantitative” information is an exaggeration.

I. 86: Do you mean “old organic carbon” sources? Not sure this is a meaningful number with bulk organic carbon age quickly approaching this number, what organic carbon age do you find in the other sites? Compare e.g. Shi et al. 2020: “the SOC in subsoils (30–100 cm) is on average 8.3 kyr old”.

Results:

II. 109-110: It would be nice if there was a bit more detailed description of the patterns at the beginning of the section in addition to “alternating greater and lesser similarity”, especially if the next sentences all start with “this pattern” or “this behavior” - starting with the info given in . 115 ff.

II. 112-115: This sentence seems to be missing a word...

II. 120-128: I suggest to start this section by incorporating what is the assumed difference in dissimilarity analysis and phylogenetic signal here, especially since the type of article has the methods section in the end. Also, since the introduction is tuned towards well-connected vs. less connected aquifers - to state which well belongs to which group, so the reader can interpret results with this knowledge. At this point, the reader has no idea about the different wells and their numbers.

I. 137: This section is really only about the degree of variation, not on how the variation manifests. I think the wordings suggests the latter.

I. 138: Should be Bray-Curtis dissimilarity or similarity, never distance.

II. 154ff: Could you add in also your definition for resistance, as was done for resilience in I. 156?

II. 184-186: Isn't this basically the same information as given in II. 167-169 with some addition?

II. 256ff: It does not become totally clear to me why the time interval May 2020 to Oct 2021 was analyzed in detail. Was it the only time interval changes like this could be observed, is it just an example, is it representative of multiple such events?

Discussion:

II. 320 ff. There is always something to be learned across biomes, I appreciate the comparison. What are the degrees of variability in environmental parameters experienced by groundwater microbes, surface ocean microbes, and deep ocean microbes? I major change I could imagine is that surface ocean microbes likely never experience anoxia, but this may happen to groundwater communities. Comparing open ocean to groundwater communities includes on the one hand only a quite similar setting, while groundwater microbes affected by recharge seem to me rather comparable to maybe nearshore waters with significant, episodic input from a terrestrial system, but just speculating here. You state “This suggests that groundwater systems are more vulnerable and less resilient than marine systems”. Can you offer any hypothesis why this may be the case?

Conclusion:

The conclusion states, building on the last part of the of the discussion based on hypotheses, that the manuscript findings underscore importance of innovative integrated management strategies to guard groundwater resources. While that is very likely not a wrong statement, it is not supported by the previous data and analysis thereof. Surface recharge affects the deep microbial communities, but it is now shown how that would have a negative impact - we believe a stable microbial community is likely better, but we cannot be sure a different community or new ecological balances wouldn't provide the same (or better) ecosystem services.

Figures:

Figure 1: The sinusoidal pattern are difficult to see in some of the panels (may be due to the resolution of the file, not sure). There may be a better way to show these prominently discussed patterns. Potentially zoom in on y axis, or show one well in detail, and for all others just a combined figure of the monthly aggregate. Different panels, at least in a), are not explained as “per well”. If I cannot decipher the color tones (e.g. the reds are very similar), is it even meaningful to give this information?

Fig. 2b: only one bar is sufficient since the two bars add up to 100% per definition, correct?

Figure 4: a) Using colors here for microbial classes becomes quite meaningless, not readable.

Methods:

I. 454: What does “preferential” mean here?

I. 467: What is the temperature of the aquifers, would cold storage in the dark even change anything compared to natural conditions, especially considering bacterial cells may be small in the environment and pass through a 0.7 µm filter in significant numbers?

I. 506: Using the term “clusters” for compound classes (I assume) took me a minute to understand, was looking for additional clusters. Suggest to change compound groups.

I. 548 and elsewhere: Some use of environmental parameters remains unclear. “15 hydrochemical parameters, including water level, water temperature, specific electrical conductivity, dissolved oxygen concentration, redox potential, acidity, alkalinity, total inorganic carbon, element and ion concentration (Cl⁻, S, Ca, K, Na, and Mg).” I count 14 parameters here. If yes, how was recession/recharge integrated in analyses, categorical or as some value? How was a correlation to precipitation established (l. 179)?

Supplement:

Reference to Supplementary Figure 4 not found in text.

Table S2: Mantel test environmental parameters are the same 15 environmental parameters used in dbRDA? Can be easily listed in the supplement.

Table S5: Add information here which literature the compound class limits were taken from.

Supplementary Figure 11 could be put in landscape format quite easily with one pathway over all samples, would be easier to read and compare between wells. But ok, since supplemental figure.

I appreciate the good data plus code availability!

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Decision Letter:

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Dear Professor Küsel,

Your manuscript titled "Balancing Act: Groundwater microbiomes' resilience and vulnerability to hydroclimatic extremes" has now been seen by our reviewers, whose comments appear below. In light of their advice we are delighted to say that we are happy, in principle, to publish a suitably revised version in Communications Earth & Environment.

We therefore invite you to revise your paper one last time to address the remaining concerns of our reviewers. At the same time we ask that you edit your manuscript to comply with our format requirements and to maximise the accessibility and therefore the impact of your work.

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We hope to hear from you within two weeks; please let us know if you need more time.

Best regards,

Somaparna Ghosh, PhD
Associate Editor,
Communications Earth & Environment
Consulting Editor,
Communications Sustainability

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

I am satisfied with the authors' responses and the revisions made, which have substantially improved the manuscript. The clarifications provided are scientifically sound and thoughtfully integrated. I recommend acceptance pending minor revisions, specifically a careful review for grammatical consistency and uniformity in citation formatting.

Reviewer #3 (Remarks to the Author):

I appreciate the effort that was put into the revised version of the manuscript by the authors, clarifying methods and their limitations, and offering additional interpretations e.g. of environmental factors given in response to a question raised by reviewer 1. I would have liked to see a deeper dive into the comparison between different aquatic biomes, which was removed in the revision process, but also recognize that the strengths of this work lie elsewhere. I have no further comments on the manuscript.

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href="http://www.nature.com/authors">www.nature.com/authors for information about policies, services and author benefits**

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Overall Comments:

This manuscript presents a highly insightful and comprehensive analysis of groundwater microbiomes in the context of climate change, providing valuable contributions to our understanding of microbial dynamics in carbonate aquifers. The study's integration of diverse datasets—including 16S rRNA gene sequences, DOM profiles, metabolomics, and seepage microbiomes—is commendable, and the long-term nature of the study provides an invaluable perspective on microbial resilience and stability under changing environmental conditions.

That being said, there are several important aspects of the study that would benefit from further clarification and/or elaboration to strengthen the manuscript. Below are my main concerns:

1. The study utilizes Source Tracker to investigate the seepage of surface-derived microbes into groundwater systems. However, given the long-term time series, it is important to consider that not only the groundwater microbial communities but also the surface-derived microbial communities (source communities) would have changed over the study period. I would suggest addressing whether and how these temporal changes in source communities might impact the results from the Source Tracker analysis.
2. The results indicating the relatively stable microbiomes in connected groundwater systems and the significant changes in isolated groundwater systems are intriguing. The observed Bray-Curtis dissimilarity suggesting that the microbiomes in isolated groundwater are completely different after 10 years is compelling. I would be interested in further elaborating on the potential driving forces behind these significant variations, particularly in the isolated groundwater systems. What environmental factors or microbial interactions are contributing to these changes?
3. The manuscript notes a decrease in *Nitrospira* spp. and an increase in Planctomycetota in wells H52 and H53 over the 10-year sampling period. Both of these groups are involved in nitrogen metabolism, with *Nitrospira* primarily playing a role in nitrite oxidation and Planctomycetota generally acting as ammonia oxidizers. The observed shift could indicate changes in nitrogen cycling dynamics. Specifically, it would be helpful to explore whether ammonium availability or other environmental factors (such as oxygen levels or redox conditions) may have increased the prevalence of ammonia-oxidizing Planctomycetota. If possible, comparing these microbial shifts with available nitrogen concentration data would provide more context to the observed changes.
4. The use of LC-HRMS to analyze the molecular characteristics of DOM is an excellent approach, especially given the long sampling period. However, I would appreciate clarification regarding the storage and analysis of the PPL extracts. Were all the DOM samples analyzed at the same time, or were they analyzed in batches over the course of the study? Additionally, how long were the PPL extracts stored at -80°C prior to analysis? These details would help ensure reproducibility and transparency in the methods.
5. The manuscript mentions temporal shifts in groundwater levels, with an observed high groundwater table in May 2018 and a very low groundwater table in May 2017. I am curious whether any extreme climate events (e.g., droughts or floods) occurred during the study period and how these events might have impacted the microbiomes, particularly in the connected groundwater systems. A discussion of how extreme climate events might have influenced microbiome dynamics would add important context to the findings.

Minor Concerns:

Lines 148-151: It would be helpful to include the results of statistical tests for comparisons, especially when discussing significant differences. This would enhance the rigor of the results.

Line 180: The reported correlation value, $r = 0.04$, is quite low. Could this level of correlation still be considered significant? I would recommend addressing this concern or discussing the potential implications of such a low correlation in the context of the study.

We sincerely thank the reviewers for their thoughtful and constructive feedback. The questions and suggestions raised were highly valuable, and we believe that addressing these points has significantly improved the clarity and quality of our study.

The revised manuscript includes substantial additions and clarifications aimed at enhancing conceptual clarity, methodological transparency, and the robustness of key interpretations, particularly in relation to the novel insights into resilience and vulnerability of groundwater microorganisms to hydroclimatic extremes. Below we provide a summary of the major changes:

1. **Methods section expansion:** We have substantially expanded the methods section to include detailed descriptions of DOM characterization via LC-HRMS and DI-HRMS, the Sourcetracker approach, sampling resolution, the definitions of key ecological terms (e.g., resilience, resistance, temporal stability, and hydrological connections) to ensure methodological transparency and reproductivity.
2. **Evidence-based interpretation:** We have carefully revised our results and discussions to ensure all interpretations are directly supported by the data, avoiding any overstatements.
3. **Figures and visual clarity:** Figures have been updated with improved, more intuitive visualization and revised captions to enhance clarity and interpretability.

Referees' comments:

Reviewer #1

Overall Comments:

This manuscript presents a highly insightful and comprehensive analysis of groundwater microbiomes in the context of climate change, providing valuable contributions to our understanding of microbial dynamics in carbonate aquifers. The study's integration of diverse datasets—including 16S rRNA gene sequences, DOM profiles, metabolomics, and seepage microbiomes—is commendable, and the long-term nature of the study provides an invaluable perspective on microbial resilience and stability under changing environmental conditions.

That being said, there are several important aspects of the study that would benefit from further clarification and/or elaboration to strengthen the manuscript. Below are my main concerns:

1. The study utilizes Source Tracker to investigate the seepage of surface-derived microbes into groundwater systems. However, given the long-term time series, it is important to consider that not only the groundwater microbial communities but also the surface-derived microbial communities (source communities) would have changed over the study period. I would suggest addressing whether and how these temporal changes in source communities might impact the results from the Source Tracker analysis.

You are correct. We were aware that seepage microbial communities exhibit both temporal and spatial heterogeneity. That is why we pooled all seepage samples as source communities, covering a seepage sampling time period from June 2016 to February 2018, which represents 17% of the total observation period. This approach ensures that even if certain taxa were absent at specific time points or locations, they may remain represented in the analysis. It also helps to account for differences in sampling sizes between seepage and groundwater, and for variability in microbial survival and travel times from surface to subsurface, which can be influenced by local heterogeneities and precipitation patterns.

We have revised the methods (l 675-679) and added a sentence about the limitations of our approach in the discussion section (l 336-339).

2. The results indicating the relatively stable microbiomes in connected groundwater systems and the significant changes in isolated groundwater systems are intriguing. The observed Bray-Curtis dissimilarity suggesting that the microbiomes in isolated groundwater are completely different after 10 years is compelling. I would be interested in further elaborating on the potential driving forces behind these significant variations, particularly in the isolated groundwater systems. What environmental factors or microbial interactions are contributing to these changes?

Thank you for this comment, which encouraged us to give more details about the environmental factors shaping the microbiome. But first of all, we would like to highlight that we do not consider the communities completely different after 10 years, as core ASVs (present in $\geq 80\%$ of samples) still accounted for 55-65% of total abundance in the more isolated groundwater. Although microbiome turnover rate was high, this turnover mainly occurred at ASV level rather than the class level, as shown in Fig. 1. This shift is likely driven by environmental changes linked to declining groundwater levels (associated with 14-21% microbiome variation) and to certain hydrochemical parameters. Concentrations of dissolved oxygen, nitrate, sulfate, and ammonium were significantly associated with microbiome variation: oxygen (2-5%), nitrate (3-9%), sulfate (4%), ammonium (7-13%). These results suggest the availability of both electron acceptors and donors likely contributes to the observed long-term changes in microbial composition in hydrologically more isolated aquifers. We added this information to the text in the results (l 220-224) and discussion (l 400-412).

Another possible driver of the high microbiome turnover rate observed at more isolated groundwater might be the high relative abundance of Patescibacteria (constituting mean of 36-41% of the total abundance). Previous studies on CPR lineages have shown that CPR bacteria may act as microbial parasites, controlling host population dynamics. We included also this statement (l 412-416).

3. The manuscript notes a decrease in *Nitrospira* spp. and an increase in Planctomycetota in wells H52 and H53 over the 10-year sampling period. Both of these groups are involved in nitrogen metabolism, with *Nitrospira* primarily playing a role in nitrite oxidation and Planctomycetota generally acting as ammonia oxidizers. The observed shift could indicate changes in nitrogen cycling dynamics. Specifically, it would be helpful to explore whether ammonium availability or other environmental factors (such as oxygen levels or redox conditions) may have increased the prevalence of ammonia-oxidizing Planctomycetota. If possible, comparing these microbial shifts with available nitrogen concentration data would provide more context to the observed changes.

We thank the reviewer for this insightful comment. Indeed, we can observe a significant decrease in relative abundance of *Nitrospira* over time at well H52, and a significant increase at well H53 (linear regression, $P < 0.05$). In contrast, Planctomycetota showed a consistent increase in relative abundance at both wells (linear regression, $P < 0.05$).

To further explore potential links to nitrogen cycling, we assessed Pearson correlations between the relative abundances of *Nitrospira* (relating both ammonia oxidation (comammox-*Nitrospira*) and nitrite oxidation) and Brocadiae (a class within Planctomycetota associated with anammox) and the concentrations of ammonium, nitrate, and dissolved oxygen. At well H52, *Nitrospira* showed a significant positive correlation with ammonium, while Brocadiae showed a significant negative

correlation (both $P < 0.05$). No significant correlations were found with nitrate or oxygen, nor were similar patterns observed at well H53.

Nonetheless, these findings are only based on relative abundance, not absolute abundance or activity, leaving the direction of a relationship unclear (i. e., increased ammonium concentration could stimulate both aerobic and anaerobic ammonia oxidizers but might also be an indication of reduced consumption, that is less activity by these organisms). Given the complexity and site-specific nature of nitrogen transformations in the groundwater at our study site, we caution against overinterpreting these patterns. Thus, we only added information in the discussion (l. 411-412).

4. The use of LC-HRMS to analyze the molecular characteristics of DOM is an excellent approach, especially given the long sampling period. However, I would appreciate clarification regarding the storage and analysis of the PPL extracts. Were all the DOM samples analyzed at the same time, or were they analyzed in batches over the course of the study? Additionally, how long were the PPL extracts stored at -80°C prior to analysis? These details would help ensure reproducibility and transparency in the methods.

Thanks for asking. We added the following details to the Methods to ensure reproducibility and transparency: (l. 585-586, l. 592-595, l. 614-616)

For DOM measured by DI-HRMS: After extraction on PPL columns, the methanol DOM extracts were stored at -80°C until the DI-HRMS analysis, which followed within a year. Thus, all the DOM extracts prepared throughout ten years of observations were analysed in batches over the course of the study. An in-house groundwater DOM extract was used as a standard with the analysis of each batch to evaluate the stability of measurements. An evaluation of this standard is available in Schroeter et al. (2025).

For DOM measured by LC-HRMS: Based on operational reasons, the time between sampling and LC-HRMS injection varied; meanwhile, the THF:methanol-dissolved extracts were kept at -20°C , for a median storage time of 94 days (25/75% quantile 33 and 560 days; min/max 7 and 959 days).

5. The manuscript mentions temporal shifts in groundwater levels, with an observed high groundwater table in May 2018 and a very low groundwater table in May 2017. I am curious whether any extreme climate events (e.g., droughts or floods) occurred during the study period and how these events might have impacted the microbiomes, particularly in the connected groundwater systems. A discussion of how extreme climate events might have influenced microbiome dynamics would add important context to the findings.

Correct! Indeed, annual groundwater recharge peaks varied year to year in response to accumulated precipitation, with notably low and high peaks in May 2017 and May 2018, respectively. Another high peak was observed at most wells in 2013, representing the highest levels observed during the 10-year monitoring period. During the study period, at least one extreme flood event (2013; Grams et al., 2014) and one extreme drought (2018; Hari et al., 2020) were recorded.

Strong precipitation events may have influenced groundwater microbiomes in the short term, likely through the introduction of surface-derived microorganisms via seepage, as suggested by weak but significant correlations between precipitation and groundwater levels at shallow wells, as well as between seepage-associated microorganisms and groundwater levels. Notably, rising groundwater

levels may also mobilize microorganisms attached to rock matrix or residing in the vadose zone, contributing to changes in groundwater microbial communities.

In contrast to the fast response of groundwater to intense precipitation, only well H14 reached its lowest level during the extreme 2018 drought, while most other wells experienced their lowest levels between 2019 and 2021, likely due to cumulative effects of prolonged low precipitation. Although groundwater levels showed similar temporal patterns across wells, absolute levels varied, indicating that groundwater dynamics depend not only on local precipitation but also on site-specific factors such as water retention time and subsurface connectivity. In addition, we observed significant shifts in microbiome composition at class level and predicted degradation pathways at shallowest wells (e.g., H14 and H32) beginning in 2021. These findings suggest that the extended drought may lead to cumulative and delayed shifts in microbiome composition (function), potentially driven by gradual changes in DOM.

We added this information to the text and revised the discussion (L. 381-399).

Minor Concerns:

Lines 148-151: It would be helpful to include the results of statistical tests for comparisons, especially when discussing significant differences. This would enhance the rigor of the results.

A: Added as reviewer suggested (L. 163).

Line 180: The reported correlation value, $r = 0.04$, is quite low. Could this level of correlation still be considered significant? I would recommend addressing this concern or discussing the potential implications of such a low correlation in the context of the study.

A: While the Pearson correlation coefficient between groundwater levels at well H14 and precipitation is indeed low ($r = 0.04$), the association was statistically significant ($P = 0.03$). This suggests that the observed correlation is unlikely to have occurred by chance. However, we fully acknowledge the effect size is small, thus we revised this sentence (L. 191-194).

Reviewer #2

Recommendation: Major Revision

The manuscript is a potentially impactful contribution to the field, especially due to its unique temporal depth and multidisciplinary scope. However, major clarifications and revisions are needed to strengthen the conceptual framework, improve data interpretation, and ensure methodological transparency.

General Assessment:

This manuscript presents a highly comprehensive, decade-long investigation into the temporal dynamics of groundwater microbiomes in carbonate aquifers, focusing on their resilience and vulnerability to hydroclimatic extremes. The authors integrate microbial, metabolomic, and

hydrochemical datasets in an exemplary fashion. The scope, temporal scale, and interdisciplinary integration are impressive and represent a major contribution to groundwater ecology.

Thank you for your positive feedback.

However, despite the strength of the dataset and analysis, there are several substantial issues that must be addressed before the manuscript can be accepted. These include concerns related to conceptual clarity, methodological justification, the robustness of some interpretations, and presentation clarity.

1. Several conclusions, such as the link between metabolomic shifts and microbial community changes or between seepage-associated bacteria and groundwater levels, are based solely on correlation. These should be framed more cautiously. Consider alternative explanations and clarify whether causal relationships are truly supported by the data.

We agree. In the Results section, we now use the phrase “statistically accounted for” to emphasize that the findings refer to the outcomes of statistical tests showing significant relationships between microbiome variation and the parameters, rather than implying ecological causation.

2. While PICRUSt2 provides useful predictive insights, the reliance on inferred gene functions from 16S rRNA gene sequences should be acknowledged more clearly, particularly in the discussion of "functional stability." Emphasize that these are predictions, not direct measurements of metabolic activity, and consider whether metatranscriptomic or metaproteomic validation is planned or possible.

We agree. As suggested, we have revised the terminology from “functional potential” to “predicted functional profiles” to better reflect the nature of PICRUSt2-based inferences. While not all predicted functions are necessarily expressed, we already have clear evidence for key biogeochemical functions such as nitrification, anammox and CO₂ fixation through metatranscriptomic and activity-based studies at the same field site (Wegner et al., 2019; Krüger et al., 2023; Kumar et al., 2017; Overholt et al., 2022). In the revised version, we point to these previous results more explicitly (l. 343-349).

3. The study claims sinusoidal/seasonal variation in microbiome dynamics, yet the methods mention monthly sampling without showing whether the sampling frequency is sufficient to resolve seasonality across all years. Please clarify temporal gaps (if any), sampling regularity, and how these may affect the robustness of inferred patterns.

Correct. As temporal inconsistencies would affect the robustness of the patterns, we insisted on regular sampling campaigns (every 28 days). Sampling gaps were rare. Since December 2021, the sampling interval was extended to every 42 days, and a detailed sampling schedule is provided in the figure S12 and table S5 in the Supplementary material. The sampling information was clarified in the method part (l. 539-546).

Seasonal patterns were inferred from pairwise comparisons of community composition across time intervals, ranging from short to long-term lags. For example, we calculated 3916 pairwise comparisons across time intervals up to 91 months, and 7750 comparisons across intervals up to 121 months at well H32. With this resolution, recurring seasonal patterns clearly emerged. Furthermore, the seasonal patterns we refer to here are implied by annual repeating trends, which require sampling over multiple years for robustness. Our approach is in line with other studies in aquatic systems (Ferrera et al., 2024; Fuhrman et al., 2015; Yeh & Fuhrman, 2022). We revised the text accordingly (l. 657-660).

4. The definitions of resilience and resistance, while briefly mentioned, require clearer operationalization. The metrics used to quantify them (e.g., turnover rates, Bray-Curtis similarity) are

not clearly linked to established definitions in microbial ecology. A schematic or expanded explanation in the methods would greatly help.

As suggested, details about the definition and quantification methods of temporal stability, resilience, and resistance have been added (l.646-657). Briefly, **temporal stability**, refers to the consistency of a microbiome's composition and function over time (Shade et al., 2012; Yeh & Fuhrman, 2022) We define **resistance** as a community's ability to withstand environmental changes(Shade et al., 2012). We define **resilience** as the ability of groundwater microbiomes to maintain their composition and predicted functional profiles over time despite ongoing environmental changes (Shade et al., 2012; Fuhrman et al., 2015).

5. The study touches on aquifers' vulnerability to surface disturbances but does not sufficiently explore the potential influence of land use (e.g., agricultural or forested areas), which is mentioned in methods but not analyzed. Please elaborate on whether land use was considered as a variable or if future analysis will explore this.

As all the groundwater wells receive at least partially recharge formed under forest (Lehmann & Totsche, 2020), we did not include the land use as a direct variable. We provided this additional information in the methods section (l.531-533).

6. There is substantial overlap and repetition in the reporting of DOM and metabolomic results. Consider reorganizing or consolidating these sections to improve readability and avoid redundancy, particularly in the Results and Discussion.

We have restructured the results section as per the reviewer's recommendation (l.236-290). For the discussion, we could not identify an emerging redundancy, as we consider it a necessity to address the differences in patterns observed between the respective acquisition methods.

7. Several figures (e.g., Fig. 1–3) are overloaded and difficult to interpret without more context. Please ensure all axes, labels, and statistical elements are fully explained in figure legends. Additionally, indicate sample sizes where comparisons are made.

As suggested, we have revised the figures and updated the captions to fully explain the figures. We also added information about sample size.

8. Terms like “hydrological connectivity,” “resilience,” “core microbiome,” and “functional redundancy” are used frequently but are not always defined or consistently applied. Ensure consistency and clarity for interdisciplinary readers.

We have ensured the terms to be defined and applied consistently. We added the definitions of these terms in lines 60-62; 170-173; 161-162; and 350-351 and into the methods sections (l.646-657).

9. Although seepage-associated microorganisms were shown to contribute a small fraction (~4%) to groundwater microbiomes, their biological/ecological significance is unclear. The authors should elaborate on whether these organisms are functionally relevant or merely transient.

A: Great question! Although seepage-associated microorganisms contribute a small fraction (~4%) to the total groundwater microbiome at any given time, their high contribution to groundwater core ASVs (up to 12.5%) suggests they might not merely be transient. This persistence likely results from recurrent immigration over long timescales, enabling them to establish and potentially play active roles in nitrogen, sulfur, and iron cycling. We elaborated more on these findings (l. 333-339).

10. The comparison with ocean microbiomes, while interesting, feels somewhat speculative and distracts from the core groundwater-focused findings. Consider moving such comparisons to a dedicated “Outlook” subsection or trimming them.

Good point. To improve the narrative of our study, we decided to delete the comparison with the ocean microbiome.

11. Abstract: The abstract is dense. Consider breaking up long sentences and more clearly highlighting the major findings.

A: As suggested, we have broken up long sentences.

12. Line 67: Spelling error in “recovery” → should be “recover”.

A: Corrected (l.67).

13. Supplementary Materials: Ensure they are cross-referenced consistently throughout the text.

A: We have ensured the consistent cross-referencing through the manuscript.

Reviewer #3 (Remarks to the Author):

In their article „Balancing Act: Groundwater microbiome’s resilience and vulnerability to hydroclimatic extremes“ He Wang and colleagues provide a good overview analysis of an amazing dataset containing a 10-year time series of groundwater well microbial communities and organic matter. The authors show little influence of seepage microbes in the well groundwater communities and stability/resistance patterns driven by connectivity and environmental fluctuations. Overall, the manuscript is well structured and prepared. It would however benefit from some clarifications and, owing to the journal’s style, some placing of the results in the environmental context in the results section. I outline my minor concerns and comments below.

Thank you so much for this positive feedback.

Introduction:

ll. 60-68: It is not quite clear to me what is the difference between hydrologically connected aquifers and hydrologically isolated aquifers if both receive surface recharge (l. 63, l. 66). Is there a threshold, how is it usually defined?

Good point. Unfortunately, there is no threshold defined that we could use. For a better understanding of the complex hydrology of our system, we revised this part in the introduction (l.60-97) and provided the reader with more details of the hydrologically connected and more isolated aquifers on the Materials and Methods section (l. 509-538).

l. 76: I believe the statement that the combination of two compositional, semi-quantitative datasets and a prediction tool results in “quantitative” information is an exaggeration.

As suggested, we have removed the word “quantitative” in this sentence (l. 75-77).

l. 86: Do you mean “old organic carbon” sources? Not sure this is a meaningful number with bulk organic carbon age quickly approaching this number, what organic carbon age do you find in the other sites? Compare e.g. Shi et al. 2020: “the SOC in subsoils (30–100 cm) is on average 8.3 kyr old”.

We revised the sentence based on Benk et al. (2019), replaced “old” with “older” (l. 92), and added more detailed information about carbon age (~4,500–12,000 years vs. ~1,000 years at more connected wells) in method (l. 527-529).

Results:

ll. 109-110: It would be nice if there was a bit more detailed description of the patterns at the beginning of the section in addition to “alternating greater and lesser similarity”, especially if the next sentences all start with “this pattern” or “this behavior” - starting with the info given in l. 115 ff.

As suggested, we have restructured the paragraph to provide a more detailed description at the beginning of the paragraph (l. 115-127).

ll. 112-115: This sentence seems to be missing a word...

A: Yes! We added “are observed” in this sentence (l. 121).

ll. 120-128: I suggest to start this section by incorporating what is the assumed difference in dissimilarity analysis and phylogenetic signal here, especially since the type of article has the methods section in the end. Also, since the introduction is tuned towards well-connected vs. less connected aquifers - to state which well belongs to which group, so the reader can interpret results with this knowledge. At this point, the reader has no idea about the different wells and their numbers.

A: Thank you for the helpful suggestion! As suggested, we have clarified the distinction between phylogenetic structure and microbiome composition (l. 134-140) and added well group information in the result section (l. 123-125).

l. 137: This section is really only about the degree of variation, not on how the variation manifests. I think the wordings suggests the latter.

Correct. As suggested, we have replaced “contrasting” with “differing degrees of” (l. 149).

l. 138: Should be Bray-Curtis dissimilarity or similarity, never distance.

Correct. We have replaced “Bray-Curtis distance” with “Bray-Curtis dissimilarity” through the manuscript.

ll. 154ff: Could you add in also your definition for resistance, as was done for resilience in l. 156?

We have added the definition for resistance (l. 167-169).

ll. 184-186: Isn't this basically the same information as given in ll. 167-169 with some addition?

Thank you for the observation. While related, the two sections provide distinct information: l. 167-169 (l.180-183) describe the dominant bacterial taxa inhabiting seepage, highlighting contrasts between seepage and groundwater communities, whereas l.184-186 (l.197-200) focus on seepage-associated taxa detected within groundwater. We revised the text to make it clearer (l. 183).

ll. 256ff: It does not become totally clear to me why the time interval May 2020 to Oct 2021 was analyzed in detail. Was it the only time interval changes like this could be observed, is it just an example, is it representative of multiple such events?

Thank you for the question.

Now we renewed the paragraph (l.276-290). During this time, the relative abundance of condensed aromatic structures and unsaturated hydrocarbons significantly increased at shallow wells (e.g., H14 and H32), making it a representative and well-defined event suitable for detailed analysis. The time interval from May 2020 to Oct 2021 was selected because it fully encompasses the grey shaded area in the Supplementary Fig. 11, which is defined by the first data point exceeding the mean plus one standard deviation, following by at least three consecutive values above the mean.

The information was also added in the supplementary figure caption.

Discussion:

ll. 320 ff. There is always something to be learned across biomes, I appreciate the comparison. What are the degrees of variability in environmental parameters experienced by groundwater microbes, surface ocean microbes, and deep ocean microbes? I major change I could imagine is that surface ocean microbes likely never experience anoxia, but this may happen to groundwater communities. Comparing open ocean to groundwater communities includes on the one hand only a quite similar setting, while groundwater microbes affected by recharge seem to me rather comparable to maybe nearshore waters with significant, episodic input from a terrestrial system, but just speculating here. You state “This suggests that groundwater systems are more vulnerable and less resilient than marine systems”. Can you offer any hypothesis why this may be the case?

Thank you for the thoughtful comment. As two reviewers raised concerns about the comparison of these two aquatic biomes, we decided to delete this part.

Conclusion:

The conclusion states, building on the last part of the of the discussion based on hypotheses, that the manuscript findings underscore importance of innovative integrated management strategies to guard groundwater resources. While that is very likely not a wrong statement, it is not supported by the previous data and analysis thereof. Surface recharge affects the deep microbial communities, but it is now shown how that would have a negative impact - we believe a stable microbial community is likely better, but we cannot be sure a different community or new ecological balances wouldn't provide the same (or better) ecosystem services.

We have revised the conclusions (l.423-429) to avoid unsupported claims regarding impacts on ecosystem services. The revised text focuses on the observed microbiome responses to hydrological variability and emphasizes the importance of long-term monitoring and integrated research for sustainable groundwater management.

Figures:

Figure 1: The sinusoidal pattern are difficult to see in some of the panels (may be due to the resolution of the file, not sure). There may be a better way to show these prominently discussed patterns. Potentially zoom in on y axis, or show one well in detail, and for all others just a combined figure of the monthly aggregate. Different panels, at least in a), are not explained as “per well”. If I cannot decipher the color tones (e.g. the reds are very similar), is it even meaningful to give this information? As suggested, we clarified that figure 1 show per-well patterns, adjusted the y-axis to better highlight sinusoidal trends, and simplified the color scheme in Fig. 1b to improve visual distinction and interpretability.

Fig. 2b: only one bar is sufficient since the two bars add up to 100% per definition, correct? As suggested, we changed it to one bar.

Figure 4: a) Using colors here for microbial classes becomes quite meaningless, not readable. As suggested, we reduced the number of colored bacterial classes, now showing only those with a maximum fraction of >1%. We retained color coding to allow readers to access more detailed information when zooming in.

Methods:

l. 454: What does “preferential” mean here?

The term 'preferential recharge zone' refers to the presence of outcrop zones of the sloping strata across the large hillslope. Aquifer strata exposed at the surface (outcrops) can receive more direct recharge compared to zones dominated by outcropping aquitards (see detailed information in Kohlhepp et al., 2017). We revised the text to make it clearer (l.533-535).

l. 467: What is the temperature of the aquifers, would cold storage in the dark even change anything compared to natural conditions, especially considering bacterial cells may be small in the environment and pass through a 0.7 µm filter in significant numbers?

This is a standard practice to slow down organic matter transformation/degradation, as organic reactions (with and without microbial catalysis) are accelerated by increased temperatures, and light (particularly UV) may elicit degradation reactions. The groundwater temperature varied most at well H14 between 6.1 and 14.7 and varied at other wells between 8-12°C, while the 4°C corresponds to standard lab cooling room/fridge settings.

l. 506: Using the term “clusters” for compound classes (I assume) took me a minute to understand, was looking for additional clusters. Suggest to change compound groups. Corrected as suggested (l. 595).

l. 548 and elsewhere: Some use of environmental parameters remains unclear. “15 hydrochemical parameters, including water level, water temperature, specific electrical conductivity, dissolved oxygen concentration, redox potential, acidity, alkalinity, total inorganic carbon, element and ion concentration (Cl⁻, S, Ca, K, Na, and Mg).” à I count 14 parameters here. If yes, how was recession/recharge integrated in analyses, categorical or as some value? How was a correlation to precipitation established (l. 179)?

We thank the reviewer for this helpful comment and the opportunity to clarify these points.

Regarding the environmental parameters, the pH value was indeed part of the 15 hydrochemical parameters analyzed but was inadvertently omitted from this sentence (l.642). This has now been added to this sentence.

Recharge and recession phases were included as categorical variables based on groundwater level trends, as described in lines 634-638 and shown in Supplementary Fig. 6. These categories were used to assess the relationship between seepage-associated microbes and groundwater level dynamics.

To assess the correlation between precipitation and groundwater level fluctuations in l. 179, we performed a Person correlation analysis using daily local precipitation and groundwater level data. Although precipitation contributes to groundwater recharge, the recharge/recession phases are influenced by additional hydrological factors including the fractured carbonate aquifer structure, water retention time, precipitation intensity, and multi-directional groundwater flow dynamics. We have also clarified the use of daily resolution data at l. 193 and l. 635 to provide additional context on how the correlation was established.

Supplement:

Reference to Supplementary Figure 4 not found in text.

Supplementary Fig. 4 is cited in the original manuscript (l. 141-142) but splitted across two lines. We have adjusted the formatting in the revised version.

Table S2: Mantel test environmental parameters are the same 15 environmental parameters used in dbRDA? Can be easily listed in the supplement.

Thank you for the suggestion. We have now included the list below the supplementary table.

Table S5: Add information here which literature the compound class limits were taken from.

We have added the corresponding information for Supplementary Tab.S6.

Supplementary Figure 11 could be put in landscape format quite easily with one pathway over all samples, would be easier to read and compare between wells. But ok, since supplemental figure.

Thank you for the comment. We kept individual panels to better highlight well-specific significance area.

I appreciate the good data plus code availability!

Reference:

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Reviewer #1 (Remarks to the Author):

I am satisfied with the authors' responses and the revisions made, which have substantially improved the manuscript. The clarifications provided are scientifically sound and thoughtfully integrated. I recommend acceptance pending minor revisions, specifically a careful review for grammatical consistency and uniformity in citation formatting.

A: Thank you very much for your valuable feedback. We have carefully reviewed the manuscript for grammatical consistency and ensured uniformity in citation formatting.

Reviewer #3 (Remarks to the Author):

I appreciate the effort that was put into the revised version of the manuscript by the authors, clarifying methods and their limitations, and offering additional interpretations e.g. of environmental factors given in response to a question raised by reviewer 1. I would have liked to see a deeper dive into the comparison between different aquatic biomes, which was removed in the revision process, but also recognize that the strengths of this work lie elsewhere. I have no further comments on the manuscript.

A: You are absolutely correct. To highlight the primary strengths of our work and maintain a focused narrative, we chose to concentrate exclusively on groundwater microbiomes. We appreciate your thoughtful review and comments.