

Dear Reviewer #1,

we thank you for the positive assessment of our manuscript and for providing constructive comments and useful suggestions that we will include in the revised version of the manuscript. In the following pages, we respond (in black) to each of the Reviewer's comments (in *blue*). Please notice that pages and lines in this document refer to the original manuscript present in open discussion.

CI. In the Abstract, I think that you could mention what was done in a more logical order, as it was the laboratory experiments that helped inform what was best to do in the following field experiment – i.e. include the sentence “The experimental results demonstrate that stuck mussels produce signals...” before “Subsequently, we examined the response of...”.

A1. We thank the Reviewer for the suggestion. The abstract has been updated accordingly, and in general it has been rewritten in order to clarify the background and objectives of the study. The new abstract is provided below:

Quantifying the effects of external climatic and anthropogenic stressors on aquatic ecosystems is an important task for scientific purposes and management progress in the field of water resources. In this study, we propose an innovative use of biotic communities as real-time indicators, which offers a promising solution for directly quantifying the impact of these external stressors on the aquatic ecosystem health. Specifically, we investigated the influence of natural river floods on riverine biotic communities using freshwater mussels (FMs) as reliable bioindicators. Using a well-established valvometry technique, we monitored the valve opening behaviour of FMs by measuring both amplitude and frequency of the FM valve gaping. The valve movement of the FMs was tracked by installing a magnet on one valve and a Hall effect sensor on the other valve. The magnetic field between the magnet and the sensor was recorded using an Arduino board, and its changes over time were rescaled to the distance between the two valves. The recorded data was then analysed using the Continuous Wavelet Transform (CWT) analysis to study the time-dependent frequency of the signals. The experiments were carried out both in a laboratory flume and in the River Paglia (Italy). The laboratory experiments were carried out with FMs in two configurations: freely moving or immobilised on vertical bars. Testing the immobilised configuration was necessary because, in the field, the FMs cannot be allowed to move freely to prevent them from packing against the downstream wall of the protection cage during floods. These experiments allowed us to verify that immobilised mussels show similar responses to abrupt increases in flow conditions as free mussels. Moreover, immobilised mussels produced more consistent and interpretable signals than free-moving mussels due to the reduced number of features resulting from movement constraints. We then analysed the response of thirteen immobilised mussels in real river conditions during a moderate flood on 31 March 2022. The FMs in the field showed a rapid and significant change in valve gap frequency as the flood escalated, confirming the general behaviour observed in the laboratory results in the presence of an abrupt

increase in flow. These results highlight the effectiveness of using FMs as bioindicators for assessing flood impacts on aquatic ecosystems, and emphasise the utility of CWT as a powerful signal processing tool for analysing valvometric time series, with potential applications in other monitoring contexts where FMs are used. The study proposes the integration of FM valvometry and CWT for the development of operational real-time Biological Early Warning Systems (BEWS) aimed to monitor and protect aquatic ecosystems.

C2. I would also recommend changing the word “stuck” to “immobilized” throughout the manuscript, as the former sounds too colloquial.

A2. Thank you for pointing this out. We have changed the word “stuck” to “immobilised” throughout the manuscript.

C3. It seems that the Materials and Method section could be written more concisely. Make sure that when you first refer to the field site, you also then call it the “Enterprising site” (as it appears in Fig. 1), and consistently use a single term for the site throughout, as it seems strange to switch between multiple terms for the same thing later in the manuscript. You could also perhaps give a grid reference of the site. It may also be sensible to use a different shade for Italy in Fig. 1, as it seems too similar in shade to that of the Lakes.

A3. The field site is now referred to as “Field monitoring site” throughout the manuscript. The Figure has been updated as suggested by the Reviewer. We revised the Materials and Methods section in order to make it more concise and to the point.

C4. The letters seem to be missing from Fig. 2. I recommend you label each animal as it is numbered – i.e. say “FM4 did...” rather than “the FM numbered 4 did...”. As you effectively used two cohorts of FMs, 8 in the laboratory and 13 in the field, I would recommend each having its own number or letter – perhaps call animals in laboratory FMa to FMh and animals in field FMI to FM13.

A4. We thank the Reviewer for this suggestion. We have referred to the animals in the text as FMx (rather than FM number x). We have also referred to them using letters for the laboratory and numbers for the field, after clarifying that the animals used in both cases were of the same species, so as not to confuse the reader.

C5. I think there is no need to include Table 1, as these details are clear enough in the text, and are not so extensive as to necessitate a table.

A5. We agree with the Reviewer’s opinion and we removed Table 1.

C6. Make sure you refer to the discharge in L/s, with a space between the number and unit – i.e. at L157, 158, 159, 161.

A6. We thank the Reviewer for detecting these typos. In the new version of the manuscript, all of them were corrected.

C7. It would probably be best to consistently use the term, “vertical rods”, rather than changing to “bars” occasionally.

A7. We agree with the suggestion and we corrected it in the revised version.

C8. When mentioning the probe, I think it is unnecessary to provide the resolution and accuracy.

A8. We agree with the suggestion. Since this information is not fundamental here, we removed it from the revised version of the manuscript.

C9. I find L173-188 needlessly long. I think the important details could easily be said more succinctly. I also think it is unnecessary to include the equation for what is essentially only converting values to percentage opening using the minimum and maximum voltage readings. I think it is important to make clear that the scale was assumed to be linear.

A9. According to the Reviewer’s comment, we removed Eq. 1 and shortened L173-188.

C10. Could you provide a little more detail on the Matlab function/mean (L194) – I presume it is a running mean calculated within an individual over a certain time frame?

A10. We modified the sentence as follows:

Abrupt change points in the mean of the opening signals were identified using the Matlab function `findchangepts`, an iterative procedure that detects significant transitions in time-series data through adaptive segmentation of the original time series.

C11. Perhaps you could label where the multiparametric probe is positioned in Fig. 3.

A11. We agree with the Reviewer and added a label for the position of the multiparametric probe in Fig. 3.

C12. I also wonder whether you could include a little more detail and justification for detrending and removal of step changes, otherwise it could sound arbitrary.

A12. To prevent the introduction of artifacts into the results, as detailed in L227-232, the removal of step changes was deemed necessary. In fact, when decomposed step changes are characterized by a mix of high-frequency and lower-frequency components in the signal, which are spurious and may obscure the real signal characteristics. Likewise, detrending, involving the removal of mean

and linear trend, is a standard signal processing procedure employed to eliminate low-frequency components without affecting the high-frequency content of the original signal. The paragraph at L225-233 has been revised for enhanced clarity.

In this study, CWT has been computed by applying the Matlab cwt function using the Morse wavelet as the mother wavelet to the time series signal of each FMs, after removal of abrupt changes in the mean of the opening signal. Identifying and removing step changes in the mean of the signal was necessary to avoid introducing spurious results. In fact, the CWT decomposition of a signal featuring an abrupt step change will result into a combination of high-frequency components, which encapsulate the abrupt transition, along with lower-frequency components of the signal, delineating the signal's smoother and gradual variations, across the entire spectrum of frequencies. This would generate an artifact in the resulting scalograms and pseudo-frequency-averaged wavelet spectra, possibly hindering the interpretation of the informative features of the signal. Step change removal was achieved by detrending the segments of the signal between two successive step changes, i.e. by subtracting the mean and removing the linear trend, without altering the high frequency content of the original signal.

CI3. I think you should include key dates, such as when you collected specimens, when you attached sensors, how long after attachment of sensors that you started work, as it is important to know the time frames. I also think you should include temperatures the specimens were maintained at for completeness.

A13. We added this sentence to the Materials and method: *A preliminary survey of the river revealed that the native species of the area, *Unio mancus* (Lamarck, 1819) is locally extirpated. Therefore, specimens of the same species were collected from the neighboring Lake Montepulciano, Siena Province, Tuscany, Italy (Figure 1) on March 29, 2022, and they were maintained in a tank filled with lake's water. The mussels were divided into two groups: a group was installed at the field monitoring site on March 30, 2022, while the other group was sent to the Hydraulics Laboratory of the University of Trento (Italy) for the flume experiments. The details of the laboratory and field installation are provided in Section 2.3.*

CI4. I think the Materials and Method section should be slightly restructured to provide details in a more logical way and avoid repetition as far as possible. I think you can start as you have with the site choice, how that dictated choice of mussel species for investigating both in the laboratory and in the field. Then give the details of the Hall sensor method. Then I think you should give the details of the conversion of the mV readings, then highlight that you were particularly interested in the opening amplitude – that you extracted the median opening for multiple specimens and looked at the mean within each specimen, with Matlab determining abrupt changes – and the frequency, using CWT, including an explanation of that. Then describe the laboratory work, explain its purpose was to determine if there were differences between free and immobilized mussels because only immobilized mussels could realistically be used in the field. Then give details

of what the work in the river entailed. I think you need to make it clear that you deliberately recorded behavior in the field during a flood event here too.

A14. According to the Reviewer's suggestion we re-organized the Materials and Methods section introducing the case study and the collection of the FMs, presenting the laboratory and in-situ installation, describing the valvometry data collection and, finally, describing the signal processing technique. Also, we removed unnecessary repetitions.

C15. L250 "The difference..." – this sentence explains the observation, which is a point that should really be saved for the Discussion section.

A15. We agree that the sentence highlighted by the Reviewer is an interpretation of the results that could fit into the Discussion. We prefer to leave the sentence where it is, as it helps to understand the results. The same concept is recalled and expanded in the Discussion.

C16. Make sure you consistently use either "pseudo-frequency" or "pseudofrequency" but not both.

A16. Corrected in the revised version.

C17. In Fig.s 4 and 5, it would be best to have a little more detail than just "Magnitude" for the scalograms.

A17. We revised the captions of the figures specifying that these plots refer to "scalogram showing the median normalised magnitude of the continuous wavelet transform over the". Lso, in the main text we specified that "[scalograms are] constructed by considering the absolute value (or magnitude) of the complex wavelet coefficients"

C18. I think in the Results section, the observation that the immobilized laboratory mussels returned to baseline behavior quicker should be highlighted but inferring that this suggests they adapted quicker (and what this suggests) should be kept for the Discussion.

A18. Similarly to above, we prefer to keep this sentence in the Results as it helps to understand what is shown in the figure. The same concept is recalled and expanded in the discussion.

C19. L273 – I wonder if you should say consistent "responsiveness" rather than "responses", as the actual response of free and immobilized were only similar through CWT analysis (though of differing duration) and rather different in terms of opening amplitude.

A19. We agree with the Reviewer's recommendation.

C20. Also, I recommend saying it “supported the suitability” rather than “possibility” (L274). I think the dates should be mentioned in the Materials and Method section rather than here, although it is obviously fine to mention the missing data/technical issue here.

A20. Thank you, we corrected the sentence. Also, we reported the relevant dates and timing (e.g., the installation date) in the Materials and Method section.

C21. Also, probably best to refer to the shift as “marked” rather than “significant” unless significance tests were done.

A21. The changes were detected using the *findchangepts* procedure described above, which required setting a threshold to identify change points. We therefore agree that the term "marked" is better, as no significance tests are performed by default.

C22. I would avoid classifying the behavior and explaining the observations (“state of resting”, “regular valve movements as expected during respiration and filtration”, “displayed avoidance behavior” and “trying to restore their normal activity”) and save explanation and classification of behaviour for the Discussion. I also think that it would be best to slightly change the structure, i.e. to first mention the observations in the hydrodynamic conditions, then mention the behavioral observations, and then to tie the observations within both together, rather than giving details sporadically.

A22. According to the Reviewer’s suggestion, we partially moved the comment on the FMs behaviour to the Discussion section. However, we prefer to leave the following sentence in the Results section as it is required to correctly interpret the Results for FM3 and FM12:

“On the other side, the sensors installed on FM3 and FM12 were operating normally but the FMs were already closed before the flood event (likely because they were in the state of resting, see Introduction), hence not displaying any additional closure but a minor and progressive opening and gaping. All the other ten FMs were characterized by normal behaviour before the flood event, with their valves open and characterized by regular valve movements as expected during respiration and filtration.”

C23. The details of which mussel(s) were excluded from Fig. 4 b-d should be mentioned in the caption for that figure.

A23. No HMs were excluded from the figure. We have noted this in the caption by saying that the plots represent the results for all free and all immobilised FMs.

C24. In the Discussion, make sure you highlight all the key findings and explain observations where appropriate – first from the laboratory, then the field. As mentioned before, I think “responsiveness” may be more appropriate than “responses” (in L325). Details of method/calculation of the incipient discharge (L345-359) should be included in the Materials and

Method section, and the outcome of the calculation included in the Results, with only its implications mentioned here. I think the details about logistical challenges (L360-366) should be mentioned in brief, and only after the findings. After that I would include a reiteration of the particular value of the findings, specifically how it extends the use of valvometry for hydrodynamic factors (including ideas mentioned in Introduction). Then you can include the bullet points.

A24. We have restructured the Discussion section as suggested by the Reviewer, moving some sentences from the Results to the Discussion, moving the logistical challenges after the presentation of the results, and adding some comments on the possible extensions of the valvometry technique. As for this last point, we added the following text before the bullet points:

The results obtained pave the way for the utilization of the valvometry technique and of the signal processing framework presented here in operational BEWS in different contexts. Freshwater mussels can serve as indicators to quantify the impact of both natural stressors (e.g. heat waves, droughts) and anthropogenic stressors (e.g. hydropeaking, reservoir flushing, chemical contamination) on the aquatic ecosystem. As such, they can be instrumental in reporting the impacts of climate change on water resources and in the management and permitting processes implemented by local authorities. Future research should focus on extending the investigation of the responsiveness of freshwater mussels to other stressors (e.g. turbidity, temperature, chemicals) and on verifying the effectiveness of the signal processing technique presented here in identifying possible synthetic indicators related to different stressors.

As for the incipient discharge evaluation, since this is a corollary comment, during the revision of the manuscript we will consider if it is better to keep the definition of the well-known formula for the Shields parameter in the Discussion section or anticipate it in the Material and Method. Also, we note that we found an error in the value of the Shields parameter reported in the text. Although the message does not change, we will amend this error. Specifically, we found that the critical discharge is not 30 m³/s, as reported in the original submission, but about 4 m³/s. As a courtesy to the Reviewer, we have reported here the distribution of the simulated Shields parameter at Q=160 m³/s and Q=4 m³/s.

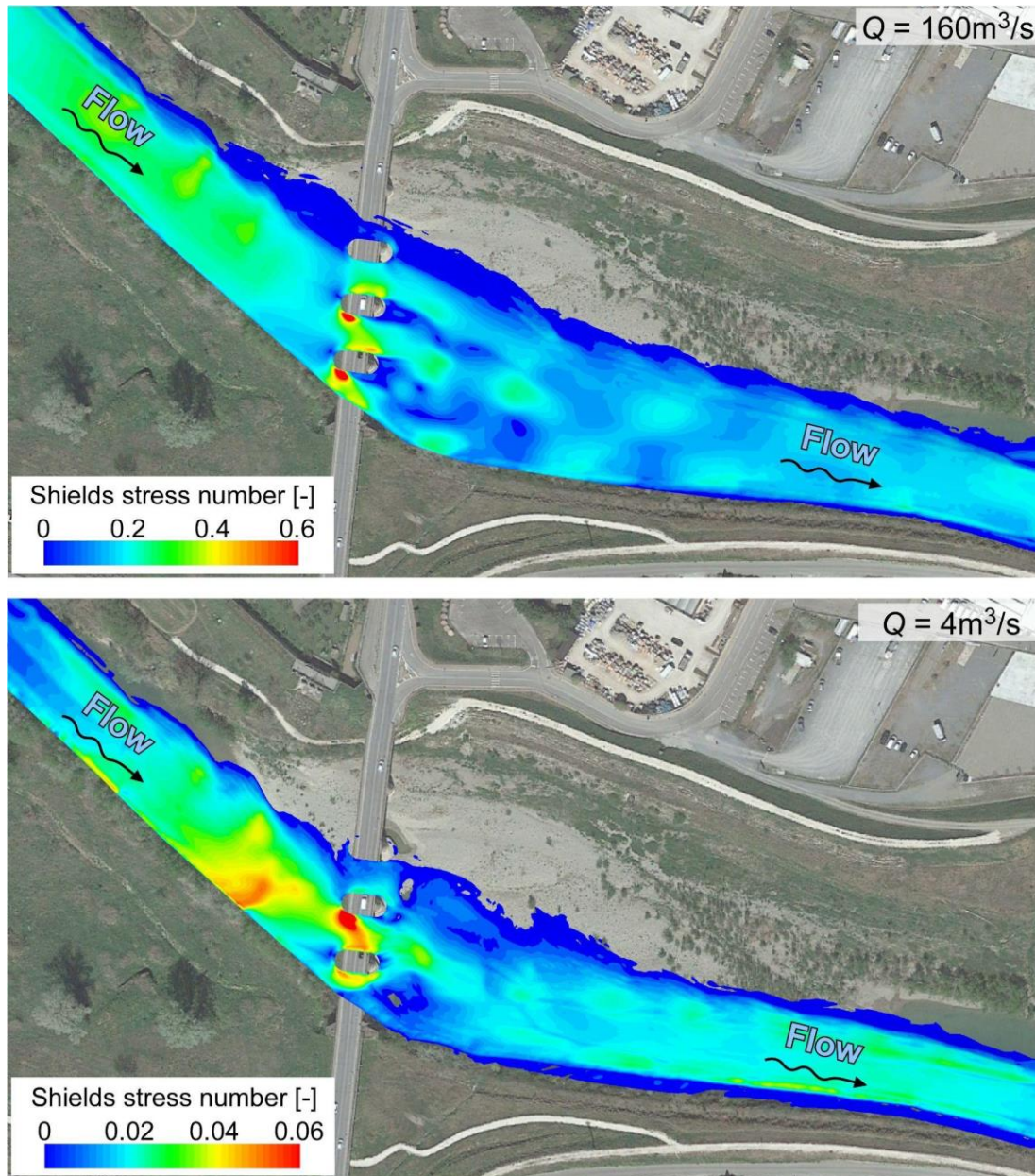


Figure R1: spatial distribution of the Shields parameter for 160 m³/s and 4 m³/s.

C25. I think the Supplementary Figures should all have been referred to in the Results section and no figures should really be referred to in the Discussion.

A25. Following the Reviewer's suggestion, we have referenced all figures in the Results section for the first time (some are referenced in the Discussion where appropriate).

C26. I presume it should read as “d90” in L351, and I presume it should not read “0.2[-]” in L354. I presume that a separate bullet point starts at “continuous wavelet transform proves...” in L388.

A26. We corrected d₉₀ into d_{90} and removed [-] (meaning dimensionless).

C27. In the Supplementary Figures, I think the plots should have labels for the FM from which the data was taken (as in Fig. 5a). I presume it would be better for the captions of Figs S3 and S4 to refer to the “removal of step changes”. I also think it might be better for what appears as Fig. S2 to instead appear after what is currently Fig. S3, and for the supplementary figures to all be mentioned in the Results section.

A27. In Figure S4, we have added the * symbol to FMs that are not considered in the signal processing, as in Figure 5. We have changed the caption of Figures S3 and S4 as suggested by the Reviewer. All figures are now mentioned for the first time in the Results section. We have kept the same order for the figures in the Supporting Information as this is consistent with the order in which they are referenced in the main text.

C28. Make sure that you use a consistent version of English. I noticed that most words used US English spelling, but a few used the British version of “behaviour” and “set-up” too. The manuscript is generally easy to understand. It may be beneficial for a native English speaker to read through it to make minor changes.

A28. Thank you for highlighting this aspect, we revised the manuscript carefully and used only words with UK English spelling.

C29. Here are some possible changes that would make it easier to read as written English:

“FMs” should appear as “FMs” in L3,5, in L189 (second occurrence), in L262 (second occurrence), and as “FM” in L15,135,169, in L189 (first occurrence), and L193,197,226,249,268

“have been” should be changed to “were” in L4,234, and “has been” should be changed to “was” in L109,225,236,239,373,374

L12 – change “thus confirming” to “consistent with”

L34 – format of source seems to be inconsistent

L40 – change “temporal horizons” to “time frames”

L42 – little unsure of what you mean by “event-time-scale” (For Sebastiano)

L55 – reference order should be 1979 then 1981

L60 – remove the extra bracket on the reference

L65-66 – change the end of the sentence (“become important and unique”) to something that does not suggest its use before was neither of these things, such as by saying “enhance the importance and highlight the unique insight of this approach”.

L69-70 – change “was” to “were”

L71 – remove “be”

L76 – change “was” to “is”

L93 – remove “is”

L95 – change “final” to “overall”, so it’s clear that this is the main aim of the work, not the last in a list of aims

L114 – add comma after “(Figure 1)” and change to “for the laboratory and in situ installation”

L126 – presume should read “Honeywell”

Make sure that when giving dimensions, you use a consistent format in spacing, i.e. L126 and 127

L148 – insert “considered” before “necessary”, as it was a conscious decision

L164 – change “was necessary” to “was deemed necessary”

L244 – remove “occurred” and put a comma before and after “10 hours after the start of the experiment”

L247 – change “various” to “varied” and “those from” to “that of”

L248 – change “are not” to “appear not to be” as it is speculative

L263 – change “prompted” to “promptly”

L264 – change “pseudo-frequency” to “pseudo-frequencies”

A29. Thank you for your comments. All points have been addressed.