Dear Prof. Cynthia Maan,

We would like to thank you for taking the time to review our manuscript and provide valuable comments. We are delighted that our work has attracted your attention. We greatly appreciate the insights you have provided, which will undoubtedly enhance the quality of our research. In the following pages, we reply (in black) to each of the comments (*in blue*).

C1. Figure 4: Why does the median opening of the free mussels suddenly decrease at 6 hours after the start, without stimulus? Why is the median opening of the stuck mussels increasing clearly before the end of the discharge peak, and why is the median opening of the free mussels not reacting to the end of the stimulus? The response of the frequency is more straight forward/ convincing.

A1. The fact that the median opening shows a decrease after 6 h is not linked to a systematic decrease across the four free FMs. Indeed, Figure S1, which reports the signal of all mussels separately, shows that before the stimulus the signals from the free FMs are different, according to their independent activities. This is also evident in Figure 4, where the 25th-75th percentile confidence bound is wide before the stimulus but shrinks as the discharge increases, indicating a coherent response across the four free FMs. Generally, we will emphasize in the revised manuscript that the use of the opening alone limits understanding of the mussel's behavior. Instead, our analysis clearly shows the value of analyzing the frequency response for a deeper interpretation and use of the signal. In the revised manuscript, we will also emphasize the reference to Figure S1, which will facilitate the reading of the median behavior and avoid confusion. In addition, we note that the frequency response is more evident because the amplitude is only variable within the range determined by the length of the adductor muscle. This varies with mussel size and, to a lesser extent, with intraspecific variation in shell shape and muscle insertion location. Therefore, amplitude is considered a secondary parameter concerning frequency variations, except in cases where the animal is completely closed (avoidance) or moribund (muscle relaxation before death).

C2. Figure 5: could you indicate the time-span of the missing data (10-12h) in the figure? The response to the stimulus in this experiment seems different than the response in the laboratory experiment in the way that there is no clear 'recovery' back to the original values. Based on the laboratory experiment, would you not expect a faster recovery (compared with the measurements in the river) of the (stuck) community?

A2. We will highlight in the Figure the missing data from 10 to 12 h caused by some technical issue. Thank you for the suggestion.

As for the recovery to pre-stimulus animal conditions, an in depth analysis of this specific aspect needs further verification. Contrary to laboratory conditions, in the field the environmental conditions may not completely return to the pre-stimulus state. The recession stage of a flood, by instance, is quite longer than the physiological recovery time the mussels showed in the laboratory runs. This involves other aspects, such as the skill of mussels to resist prolonged external variations (see also our reply A8, below). Furthermore, in order to extrapolate laboratory results to natural conditions, it is necessary to consider the greater variability of boundary conditions with respect to the parameter being examined. 1) The response of the mussels is an expression of a reaction to changes in multiple conditions that can only be controlled and restored to previous conditions in the laboratory. 2) Mussels that are immobilised undergo an unnatural constriction that alters their ability to return to pre-stress conditions. For these reasons, further field experiments should be conducted in order to investigate the method's limitations and to develop protocols for addressing them adequately. Also, we note that in the field high discharge conditions lasted for a long time after the initial peak and it is not excluded that within the time window analyzed here, the FMs were constantly stressed by hydrodynamic conditions. We will add specific comments to the Discussion section of the revised manuscript.

C3. Line 10: The stuck mussels produce signals that are 'more consistent' with flood occurrence. I wonder if this is true because for the free mussels the frequency is increased over the full period of enhanced stress, whereas the frequency of the stuck mussels falls back to lower frequencies well before the period of enhanced discharge/ stimuli (figure 4). Also, such consistency would be beneficial if the aim was to measure hydrodynamic conditions. However, the impact on biotic communities might be overestimated due to the ' larger consistency'. If the link between the stimuli/ stress factors and the free mussels (so the 'reality') is weaker (?), can the response of the stuck mussels still be an indicator for free-community behavior or stress?

A3. The term "consistent" was misused or misinterpreted here. What we mean here is that signals from immobilized FMs show a higher level of consistency between each other, rather than with the occurrence of floods. We adjusted the sentence as follows: "Moreover, immobilised mussels produced more interpretable signals than free-moving mussels due to the reduced number of features resulting from movement constraints." The key point is that the signals from immobilised mussels are easier to interpret since they are cleaner (fewer features due to restricted movement). In addition, our laboratory experiments demonstrated that immobilised mussels (required in the field for logistical reasons, as commented in the manuscript) can be used to detect when the community is stressed by external stimuli, similar to the use of free mussels. In this case, the aim is to introduce a real-time biological early warning system (BEWS). An alarm system needs to detect a fault in a timely manner (see also A5) and overestimation is always preferable to underestimation. The precautionary approach commonly applied to monitoring and defining toxicity thresholds is adopted precisely to avoid underestimating the risk. According to the same principle, to make real-time warning systems more effective, the most sensitive species that are or could be present in the target environment are used.

C4. Line 250-251 : is "whereas" the right word to use in this sentence?

A4. Thank you for pointing this out, in the revised version we corrected the sentence as follows:

"The difference between the two groups of mussels can be attributed to the limited mobility of stuck FMs compared to free FMs. This limited mobility caused by restricting behaviors like walking and drifting leads to a simpler signal for stuck mussels."

C5. Line 273 and line 327: the reactions of stuck and free mussels are not that "consistent": the glued mussels didn't maintain the high frequency over the full period of enhanced stress (falls back to the original frequency before the period of enhanced discharge ends), and there is a difference between the reactions in terms of opening: the opening amplitude of free mussels increased whereas the amplitude of stuck mussels decreased (figure 4).

A5. The aim of this study is to introduce a real-time biological early warning system (BEWS). To this end, firstly, the onset time of changes is critical and then, as further information, the type/duration of response of the mussels to the stimulus can be helpful. The BEWS, based on the wavelet signal processing presented here, has been shown to be successful in identifying when the FMs are undergoing a stress response, when either free or immobilised FMs are considered. In this respect, we can state that the responses of the two types of FMs are consistent (see also Figure 4, and in particular subplot d). Further information, such as the duration of the behaviours, gives the reader a better visualisation and is of interest for deep biological features, but is not the main goal here. In this regard, two other papers published by our group have dealt with the behaviour of mussels to stimuli in the laboratory, focusing on behaviours after the onset of the stimulus, such as adaptation and avoidance (Modesto et al., 2023; Termini et al., 2023).

C6. Line 311: "additionally the temperature .." how does the temperature fit in this story? is it relevant? Could there be an impact of the temperature on the FMs frequency and opening amplitude and/or community ?

A6. FMs can change their gaping behavior in response to fluctuations in environmental conditions such as water depth, light, temperature, and particulate matter (Tran et al., 2003; Ropert-Coudert & Wilson, 2004; Robson et al., 2009). We conducted laboratory experiments which showed that for the species/population used the frequencies increased significantly above 28°C. The variation is much stronger if the temperature variation is rapid because the animal is unable to adapt. The experiments are still under development and the results will be published soon. For the interpretation of the results included in this publication, the temperature should not be influential because it is always below the tolerance threshold of the eurythermal generalist species that we used. We will stress this in the revised manuscript and adjust possible misleading sentences accordingly.

C7. Line 313: "trying to restore their.." They are not nearly close to their original opening amplitude or frequency.

A7. We agree and have removed the comment. As noted in A2, the response of mussels in the field reflects a response to changes in multiple conditions that are difficult to control in real riverine systems. The interpretation of this signal is therefore complex and requires further investigation. However, what is clear is the response at the onset of flooding, which supports the use of FMs and the processing approach to establish a real-time BEWS as discussed in the manuscript.

C8. Line 332 ".. faster adaptation in response to a prolonged stimulus" Can this really be seen as 'adaptation'? Or do the stuck mussels get tired sooner? i.e. would it be beneficial for them to return faster to the lower default frequencies, even when the discharge is still enlarged?

A8. Both hypotheses are probably true: we have verified that there is a tendency towards adaptation in both free and stuck mussels, but it is logical to think that the latter gets tired sooner. All these aspects, little studied in the laboratory and not yet studied in the field, must be studied in depth for the development of an applicable methodology. It is for this reason that we decided to carry out this first attempt at validation in the field in order to clarify aspects that have been neglected until now, although systems based on the use of mussels as alarm sentinels are already marketed (e.g. mosselmonitor). It is worth mentioning here that more data will be needed to fully understand the FMs' behavior in the field and, accordingly, we plan to acquire more data in the future.

References

Modesto, V., Tosato, L., Pilbala, A., Benistati, N., Fraccarollo, L., Termini, D., Manca, D., Moramarco, T., Sousa, R., and Riccardi, N.: Mussel behaviour as a tool to measure the impact of hydrodynamic stressors, Hydrobiologia, 850, 807–820, ISSN 1573-5117, 2023. https://doi.org/10.1007/s10750-022-05126-x

Robson, A. A., G. R. Thomas, C. Garcia de Leaniz & R. P. Wilson: Valve gape and exhalant pumping in bivalves: optimization of measurement. Aquatic Biology 6: 191–200, 2009. https://doi.org/10.3354/ab00128

Ropert-Coudert, Y. & R. P. Wilson: Subjectivity in biologging science: do logged data mislead? Memoirs of National Institute of Polar Research 58: 23–33, 2004. https://doi.org/10.1016/j.anbehav.2003.09.010

Termini, D., Benistati, N., Tosato, L., Pilbala, A., Modesto, V., Fraccarollo, L., Manca, D., Moramarco, T., and Riccardi, N: Identification of hydrodynamic changes in rivers by means of freshwater mussels' behavioural response: an experimental investigation, Ecohydrology, p. e2544, ISSN 1936-0584, 2023. https://doi.org/10.1002/eco.2544

Tran, D., P. Ciret, A. Ciutat, G. Durrieu & J. C. Massabuau: Estimation of potential and limits of bivalve closure response to detect contaminants: application to cadmium.

Environmental Toxicology and Chemistry 22(4): 914–920,2003. https://doi.org/10.1002/etc.5620220432