Real-Time Biological Early Warning System based on Freshwater Mussels' Valvometry Data

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Abstract. 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 biosensors. Using the valvometry technique, we monitored the valve gaping of FMs and analysed both amplitude and frequency. 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 normalised to give the percentage opening of the FMs. 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 conducted with FMs in two configurations: freely moving on the bed and immobilised on vertical rods. Testing of the immobilised configuration was necessary because the same configuration was used in the field in order to prevent FMs from packing against the downstream wall of the protection cage during floods or from breaking their connection wires. These experiments allowed us to verify that immobilised mussels show similar responses to abrupt changes in flow conditions as free mussels. Moreover, immobilised mussels produced more neat 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 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 the flow. These results highlight the effectiveness of using FMs as biosensors for the timely detection of environmental stressors related to natural floods and emphasise the utility of CWT as a powerful signal-processing tool for the analysis of valvometry data. The study proposes the integration of FM valvometry and CWT for the development of operational real-time Biological Early Warning Systems (BEWS) with the aim of monitoring and protecting aquatic ecosystems. Future research should focus on extending the investigation of the responsiveness of freshwater mussels to specific stressors (e.g., turbidity, temperature, chemicals) and on testing the applications of the proposed BEWS to quantify the impact of both natural stressors (e.g., heat waves, droughts) and anthropogenic stressors (e.g., hydropeaking, reservoir flushing, chemical contamination).

1 Introduction

Sustainable water resource management requires the protection of water-dependent ecosystems, as they play a pivotal role in maintaining the ecological balance and overall health of our water resources (Zieritz et al., 2022; Makanda et al., 2022). This is a challenging task, that is further compounded by the ongoing effects of climate change on water resources, which intensifies conflicts related to water resource allocation. Indeed, besides impacting water availability and quality, climate change influences water demand, thereby affecting the availability of water needed to sustain the ecological functioning of water bodies (Barron et al., 2012; Scanlon et al., 2023). There are several manifestations of climate change impact on water resources, encompassing floods, droughts, rising temperatures, deterioration of water quality, and in general intensification of extreme events (Lewsey et al., 2004; Piccolroaz et al., 2018; Sukanya and Joseph, 2023). These phenomena, combined with anthropogenic alterations of flows and water quality resulting from various activities (e.g., irrigation, hydropower production, aquaculture), can exert profound influences on aquatic ecosystems, causing alterations in their structure, function, and overall ecological balance (Weiskopf et al., 2020; Qu et al., 2020; Antala et al., 2022). Consequently, the establishment of comprehensive monitoring systems and analytical tools is imperative for accurately quantifying these impacts on aquatic ecosystems.

In the field of river monitoring, technological advancements have significantly improved our ability to assess both water quantity and quality. Standard monitoring methods for key variables such as water level, temperature, and quality have been greatly enhanced through the utilisation of real-time sensors (Hernandez-Ramirez et al., 2019; Nawar and Altaleb, 2021), the establishment of cost-effective sensor networks (Meng et al., 2017), the development of more advanced monitoring instruments (Chowdury et al., 2019; Pasika and Gandla, 2020), and the access to remote sensing imagery (Gitelson et al., 1993; Cao et al., 2021), ultimately leading to heightened precision and reliability. However, it is important to emphasise that none of these variables provide a direct quantification of the impact of external stressors, whether they arise from natural or anthropogenic disturbances, on the aquatic ecosystem. Although early-warning indicators based on physical and biological state variables can be used to predict loss of system resilience and the occurrence of critical transitions, these indicators typically operate on long-term time scales (e.g., decades) and require knowing the underlying mechanisms that steer ecosystem transitions to identify the pertinent state variables (Gsell et al., 2016). If the aim is to assess the impact of external disturbances on the time scale of a flood event or management operation, a good level of assessment still requires labour-intensive *in situ* biological sampling with repeated sampling before and after the event (e.g., Metcalfe et al., 2013; Folegot et al., 2021).

A noteworthy source of inspiration can be found in the field of water pollution monitoring, where biotic communities have been used as direct ecosystem indicators for a long time (Cairns et al., 1979; Coker, 1989; Butterworth et al., 2001; Gerhardt et al., 2006; Li et al., 2010; Holt and Miller, 2011; Siddig et al., 2016). In particular, mussels have been used in biomonitoring since the mid-1970s with the establishment of the "Mussel Watch" program (Goldberg, 1975) and since then they have been widely used worldwide as bioaccumulators for the assessment of aquatic pollution (Schöne and Krause Jr, 2016). Going beyond their employment as bioaccumulators, dating from the 1980's, mussels started being explored as potential biological sensors (or biosensors) for biological early-warning systems (BEWS) (see e.g., Bae and Park, 2014) for real-time surface and drinking water pollution monitoring (Guterres et al., 2020; Dvoretsky and Dvoretsky, 2023; Vereycken and Aldridge, 2023). Over 40 years of studies show that the observation and analysis of mussels' behaviour is a reliable tool for water quality monitoring (Sow et al., 2011), because they change their valve opening and closing activity when they perceive a change in environmental conditions, such as toxicants concentrations (Salanki, 1976; Kramer et al., 1989; Tran et al., 2003, 2007; Beggel and Geist, 2015; Hartmann et al., 2016), food quantity and quality (Higgins, 1980), tidal cycles, and salinity (Davenport, 1979, 1981; Akberali and Davenport, 1982). The immediacy of behavioural responses and the development of simple and cost-effective valve measurement (valvometry) methods have stimulated the production of commercial valvometric systems, such as the Mossel Monitor (Kramer et al., 1989) or the Dreissena Monitor (Borcherding, 1992). The interest in using valvometric responses as an alarm signal in real conditions has stimulated technological innovations, such as online data systems equipped with remote control capabilities (Sow et al., 2011) and, more recently, the integration of artificial intelligence for signal interpretation (Swapna et al., 2022).

The extensive and successful use of mussels as reliable biosensors for real-time detection of water quality-related disturbances, suggests that mussels' valvometry can be a suitable technique also for the automated assessment of the effects of physical stresses, such as the occurrence of floods and droughts or the anthropogenic alteration of flow patterns, on the aquatic ecosystem. These and further hydrological perturbations are increasing in frequency and intensity due to climate change. The extension of mussels' valvometry beyond its initial use in ecotoxicological monitoring of water quality can indeed enhance the importance and highlight the unique insight of this approach. Recent laboratory tests (Modesto et al., 2023; Termini et al., 2023) were performed on different freshwater mussels' (FMs) populations to investigate the variation of mussels' valve gaping (i.e., the act of partially opening their shells for respiration, filter-feeding, and moving) under different flow discharge and sediment transport scenarios. Valve gaping frequency (Hz) and opening amplitude (%) were used to analyse mussels' behaviour, according to behaviour classifications such as the one proposed by Hartmann et al. (2016).Two distinct kinds of behaviour were identified in non-stressed mussels: normal activity and resting. Regular valve movements related to feeding and moving characterise normal behavioural activities. Valves constantly opened for filtration/respiration characterise the resting behaviour. Three types of behaviour characterised the mussels' response to stress: transition, adaptation, and avoidance. Transition behaviour can be identified by rapid cycles of abduction (valve-opening) and adduction (valve-closing). The gradual reduction in gaping frequency/amplitude after the transition period can be interpreted as adaptation, i.e. the reduction of responsiveness to ambient stimulation levels through the adjustment of sensitivity. Avoidance behaviour is identified by the steady closure of valves. The above-referred experimental results fostered the possibility of using the FMs for assessing the impacts of flow discharge variation on riverine biotic communities, paving the way for their application in natural river settings.

The present study, conducted within the framework of the Enterprising PRIN Project (2017), funded by the Ministry of Education, University and Research (MIUR) of Italy, aims to explore the use of mussels as an effective real-time BEWS in rivers, with a particular focus on assessing the response of aquatic communities to a change of flow intensity during natural floods. In this regard, this work marks the next phase following the aforementioned laboratory tests. It addresses both the technical challenges related to the installation of live organisms in the field and the interpretation of the data obtained within the complexity of real-world conditions. The transition from laboratory-controlled conditions to the field represents a challenge in the development of monitoring methodologies and protocols. First, the installation of a monitoring system to assess the effects of discharge dynamics on FMs' behaviour necessitated securing the mussels using cages and/or anchoring systems to prevent them from being displaced by the flow. Secondly, to prevent the packing of FMs against the downstream wall of the cage during high discharge, we deemed it advisable to secure the FMs to steel rods that are anchored *in situ* rather than allowing them to move freely in the substrate as done in the laboratory tests (Modesto et al., 2023; Termini et al., 2023). The use of steel rods to anchor the FMs was required also considering that the river bottom of the field monitoring site was characterised by bedrock, which is not an ideal substrate for FMs. The need to immobilise the mussels in an unnatural position, as is commonly practised to monitor the quality of aquatic environments (Kramer et al., 1989; Nagai et al., 2006; Robson et al., 2009), may alter the behavioural responses compared to those measured in the laboratory where mussels can freely move within the substrate. This aspect should be carefully considered when analysing the results.

With the overall aim of proposing the operational use of FMs as a real-time BEWS for hydrological disturbances in rivers, in this study we address three main challenges to: i) define a robust signal processing methodology to analyse the valvometric data and assess the FMs' behaviour, ii) compare the behaviour of free to move and immobilised FMs in the laboratory in presence of discharge perturbances, iii) transfer the experience acquired from laboratory-controlled experiments to applications in real river conditions.

The manuscript is structured as follows. Section 2 provides an overview of the field monitoring site location and the area where the FMs were collected. It also describes the laboratory and field installation, signal recording and analysis approaches. Section 3 presents the results of laboratory experiments and field monitoring. Finally, in Section 4 we discuss the results of the work and draw the final conclusions.

2 Materials and method

2.1 Field monitoring site and mussels' collection

The field monitoring site is located along the River Paglia (Italy). The River Paglia (Figure 1a) originates in the southeastern region of Tuscany, specifically from Mt. Amiata (1738 meters above sea level). It is located in the central part of Italy and is one of the primary right-side tributaries of the River Tiber. The River Paglia has a length of 86 km and its basin covers an area of approximately 1320 km^2. A painting of J.M.W. Turner (1775–1851), made during his journey to Rome in 1828, shows landmarks that remind us snapshots of our fieldwork site: the towering city of Orvieto, the arches of a bridge, a flowing stream (presumably a tributary of River Paglia) and people at work with their hands in the water (Figure 1b). The monitoring system based on FMs was installed in the River Paglia at Orvieto city, under the Adunata Bridge, at the right bank of the river where the riverbed is rocky. A gauging station was available at this site for monitoring water level and discharge.

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 neighbouring Lake Montepulciano, Siena Province, Tuscany, Italy (Figure 1) on March 29, 2022, and they were maintained in a tank filled with lake water. The mussels were divided into two groups, a group was installed at the field monitoring site in the afternoon of March 30, 2022, while the other group was sent to the Hydraulics Laboratory of the University of Trento (Italy) for the flume experiments. On arrival at the laboratory, the animals were acclimated for two weeks in a 500 L recirculating flow-through aquarium with aerated water and gravel-sand substrate, and fed with a mixed culture of natural algae. Details of the laboratory and field installation are given in Section 2.2.

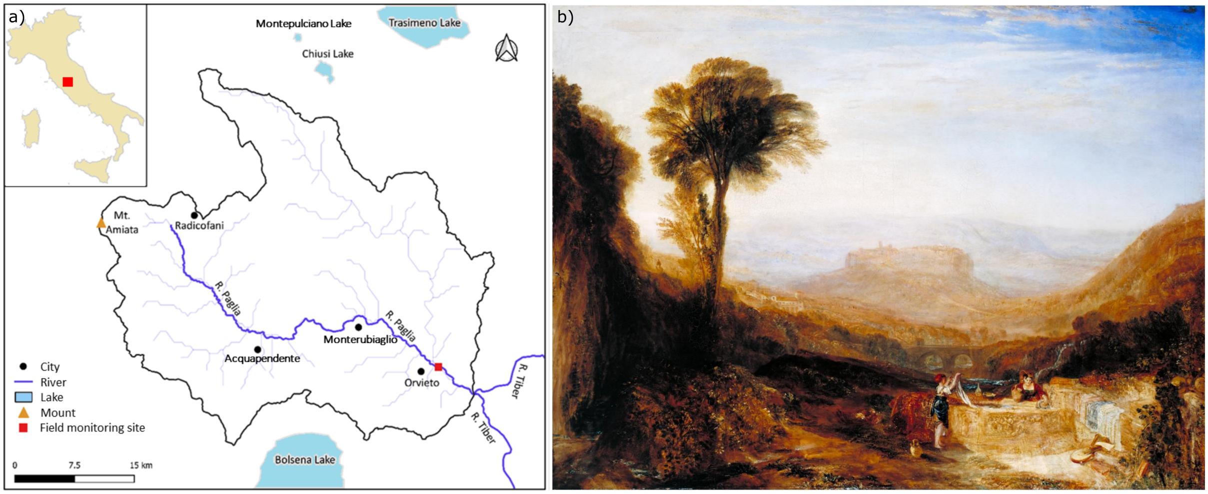

Figure 1. a) Map of the River Paglia and its catchment, showing the location of the field monitoring site and of Lake Montepulciano, where the FMs were collected; b) Joseph Mallord William Turner (1775–1851), *View of Orvieto, Painted in Rome* (1828, reworked 1830), © Photo: Tate, CC-BY-NC-ND 3.0, https://www.tate.org.uk/art/artworks/turner-view-of-orvieto-painted-in-rome-n00511.

2.2 Laboratory experiments and *in situ* installation

The *in situ* testing of FMs as a possible BEWS for flood events poses a number of challenges ranging from the selection of the field monitoring site to the choice of the most suitable system for FM installation. The exposure of the animals to the parameters to be monitored is one of the main operational challenges in natural environments. While for the monitoring of chemical contamination it is possible to install the animals in lateral derivations of the watercourse or water pipes, for monitoring the responses of FMs to hydrological stresses (i.e., water velocity, turbulence, sediment transport) it is essential to expose the animals in the main channel of the watercourse. This requires installing the FMs in structures that are as transparent as possible to the flow, ensuring they do not substantially affect the natural flow pattern, and sufficiently stable to guarantee the integrity of both the FMs and the installation throughout the exposure (e.g., Kramer and Foekema, 2001; Sow et al., 2011). For the sake of logistical convenience, the FMs are commonly fixed to solid structures such as steel rods, thus limiting their ability to move. Furthermore, it is important to note that FMs are frequently used in environmental conditions that may differ from their natural habitat, such as when using FMs accustomed to lentic waters in river environments (e.g., Martel et al., 2003), as is the case in the present study.

To assess the extent to which limiting FMs movement affects their behavioural response to environmental stress, a laboratory experimental comparison was carried out, analysing and comparing valve movements in both freely moving and immobilised animals on vertical rods. These experiments were conducted at the Hydraulics Laboratory of the University of Trento (Italy). The FMs were exposed to the same external conditions for 24 hours (Figure 2). FMs from 1 to 4 were free to move, while the others i.e., from 5 to 8, were immobilised on vertical rods by glueing one valve to the rod, that was hung vertically from the top of a 10 m long and 40 cm wide flume (Figure 2b). Free and immobilised mussels were positioned in the middle of the flume, sufficiently far from the upward and downward boundary conditions. After 10 hours of continuous discharge at a constant rate of 5.3 L/s with 10 cm substratum (and without sediment transport), the discharge was instantaneously increased to 22 L/s and maintained at this high value for 2 hours, before returning to the initial baseline value (5.3 L/s). This baseline discharge has been kept constant during the rest of the experiment, i.e., for the following 12 hours. Additional information we sought during the experiments relates to sediment transport under different discharge configurations, as previous research has shown that FMs are particularly affected by the presence of sediment transport (Modesto et al., 2023; Termini et al., 2023). As reported in Modesto et al. (2023), who conducted experiments using the same flume and discharge settings as our study, the baseline discharge of 5.3 L/s is characterised by negligible sediment transport, whereas the higher discharge of 22 L/s involves both bedload and suspended sediment transport. In the same previous study, the critical discharge, i.e. the value corresponding to the onset of sediment transport (also referred to as the incipient condition), was found to be around 14 L/s. Specifically, this is the discharge value capable of entraining the finest grains (~ 0.06 mm in this case) from the mobile bed mixture into the flow.

At the River Paglia field monitoring site, thirteen FMs were fixed to vertical rods of the same type as those tested in the laboratory and installed in a cage secured at the riverbank. The use of steel rods was deemed necessary to mitigate the risk of FMs being displaced during flood events and due to the unsuitable bedrock substrate at the installation site. The cage was necessary to prevent damage to FMs and electronics. The cage has been designed to ensure robustness while minimising interaction with the flow. To achieve this, a thin steel frame covered by a coarse metal grid was used. The electronics for the valvometry recordings (see Section 2.3) were installed on the bridge above the riverbank where the cage was positioned. An overview of the installation is provided in Figure 3. A multi-parameter probe (OTT PLS-C) was installed at the FM cage site to measure water level, temperature and conductivity every 10 minutes. The FMs and multiparametric probe were in operation during a flood on 31 March 2022, which is the event analysed in this study.

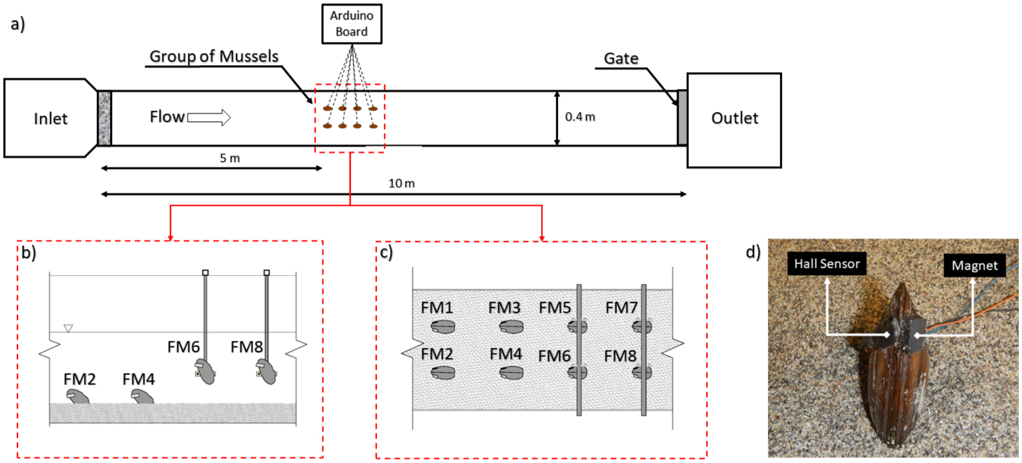

Figure 2. a) Experimental setup in the laboratory; b) and c) side and plan views of mussels' arrangement in the flume; d) an example of FM equipped with a Hall sensor and a magnet.

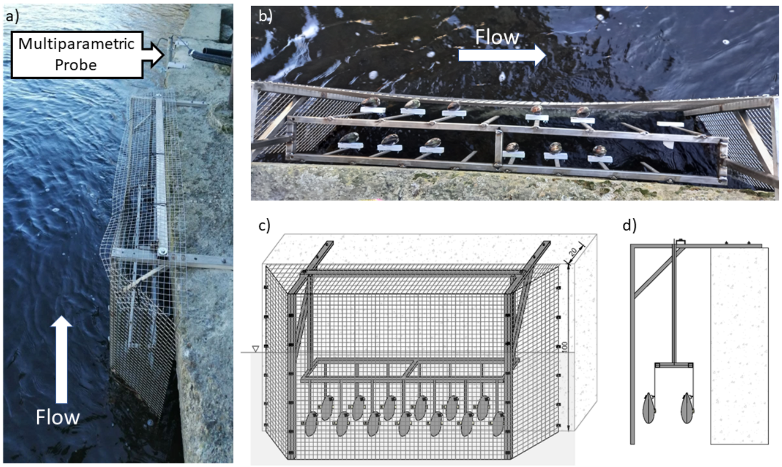

Figure 3. Field Installation - Enterprising pilot site, Orvieto City, Italy: a) overview of the FM cage, location of the multiparametric probe and flow direction; b) top view of the FM cage; c) front view (schematic); d) side view (schematic).

2.3 Valvometry data collection

In order to monitor the frequency and intensity of FMs gaping, different valvometry methods have been proposed for over one century (reviewed in Vereycken and Aldridge, 2023). In his pioneering work, Marceau (1909) first used a kymograph (i.e., a rotating drum or moving paper strip onto which data is drawn as a function of time) to track the valve movement of mussels by attaching a balanced arm equipped with a scribe to one valve of the mussel. Electromagnetic induction to measure the valve displacement was first used by Schuring and Geense (1972) and then further developed thanks to technological advancements. Wilson et al. (2002) introduced the use of the Hall effect to record the valve movement of mussels. This approach requires installing a magnet on one valve and a Hall effect sensor on the other valve. The Hall effect sensor measures the magnetic field between the magnet and the sensor itself, which changes according to the distance between the two valves. In this way, both the frequency and intensity of valve gaping can be measured. When the mussel is closed, the magnetic field around the sensor is at its maximum, and when the mussel is fully opened the magnetic field strength around the sensor decreases due to the increased distance between the magnet and the sensor.

In this study, a Hall sensor (Honeywell SS495A1, 13 × 10.5 mm, 1.1 g weight) was glued on one side of the mussels' shell, a magnet (12 × 10 mm, 1.8 g weight) on the opposite side of the shell (see the right plot in Figure 2). An Arduino board (Mega 2560) was used to record the response of the Hall effect sensor in mV, and then, by knowing the minimum and maximum values, the output was normalised and turned to percentage opening (see Section 2.4). An SD card connected to the Arduino was used to store the voltage values. In laboratory experiments, each mussel provided data at a frequency of 1 Hz, while in the field, due to a different set-up of the recording system, a frequency of 2 Hz was used.

2.4 Signal processing

Describing the behaviour of FMs in terms of the frequency and intensity of gaping using raw data expressed in mV may not be straightforward, because of inherent physiological variations among FMs, primarily influenced by their size and shape, as well as the nonuniform attachment of magnets and sensors to the individual FMs. For this reason, in order to have a common frame of response among all mussels, the opening signals were normalised between 0 to $100\ \%$, employing linear scaling based on the minimum and maximum values recorded for each FM. Accordingly, 0% indicates that the mussel's valves are fully closed, and 100% that the mussel's valves are fully open. Before normalising the signal, possible outliers due to occasional acquisition artefacts have been removed. In this context, outliers have been defined using the 0.1 and 99.9 percentiles as the lower and upper threshold bounds, respectively. The removed points were subsequently reconstructed through interpolation. It should be noted that in order to effectively normalise a signal, the signal duration must be long enough to include both the fully closed and fully open periods of the FM.

The resulting FM signals were analysed with the aim of identifying the occurrence of change points in the FMs' behaviour. As discussed in the Introduction, these changes may be linked to the normal behaviour of non-stressed FMs, but also to the response of these organisms to external perturbations. The monitoring of a sufficiently large number of FMs allowed us to discriminate between the specific behaviour of individual FMs driven by their own activity, and a systematic response of the FM community to external disturbances. 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.

Parallel to abrupt changes in behaviour characterised by step-like discontinuities in the opening signal, it has been observed that when FMs are subject to stress they exhibit marked changes in both the frequency and intensity of their gaping (Modesto et al., 2023; Termini et al., 2023). Here, the statistical analysis of the FM gaping frequencies was carried out using the Continuous Wavelet Transform (CWT), a mathematical technique that decomposes a signal into different frequency components. CWT is particularly useful when dealing with non-stationary signals. Indeed, unlike traditional Fourier analysis, CWT can capture both high and low-frequency variations in time-series data, making it especially effective for analysing signals that exhibit dynamic changes in frequency and amplitude over time (Meyers et al., 1993; Rhif et al., 2019).

The CWT analysis is based on the convolution of a signal $f(t)$ with a set of functions $\psi_{ab}(t)$, known as wavelets, derived from translations and dilations of a so-called mother wavelet $\psi(t)$:

$$\psi_{ab}(t) = \frac{\psi}{\sqrt{a}}\left(\frac{t-b}{a}\right) \qquad a,b \in R, a > 0 \tag{1}$$

where a is known as the scale factor and b defines a shift in time. Different mother wavelets can be used to decompose a signal, all of which must meet specific conditions (see e.g., Meyers et al., 1993). The convolution of the signal $f(t)$ with the set of wavelets is the wavelet transform:

$$T_{\psi}(a,b) = \frac{1}{\sqrt{a}} \int\limits_{-\infty}^{+\infty} \psi^{*}\left(\frac{t-b}{a}\right) f(t)dt \tag{2}$$

where the superscript * denotes the complex conjugate and $T_{\psi}(a,b)$ is the wavelet coefficient (which, for the sake of completeness, depends not only on a and b but also on the choice of the mother wavelet ψ). In this way, the signal $f(t)$ is analysed by comparing it to a set of wavelet functions ψ_{ab} characterised by continuously varying scale a and shift b. Unlike sinusoidal functions in Fourier analysis, these wavelet functions do not have a fixed frequency. Rather, they are versatile mathematical functions inherently flexible in both time and frequency domains, which adapt to the non-stationary characteristics of the signal being analysed. The scale factor a is inversely related to frequencies: smaller scales correspond to more "compressed" wavelets (thus higher pseudo-frequencies), and capture details in the signal at shorter time scales, while larger scales correspond to more "stretched" wavelets (thus lower pseudo-frequencies), capturing broader features at longer time scales. Note that the term pseudo-frequencies is often used to emphasise that these values should not be confused with the fixed frequencies associated with sinusoidal waves.

The results of the CWT analysis can be effectively visualised through the use of scalograms and pseudo-frequency (or scale)-averaged wavelet spectra. The scalogram is a graphical representation of signal power distribution across various pseudo-frequencies and through time. It is constructed by considering the absolute value (or magnitude) of the complex wavelet coefficients introduced in Equation (2) and allows for a comprehensive examination of how different pseudo-frequencies and times contribute to the overall power of the signal. The pseudo-frequency-averaged wavelet spectrum provides a summary of the signal's energy distribution across multiple scales over time, offering insights into both localised and broad-frequency features present in the signal over time. The pseudo-frequency-averaged wavelet spectrum is obtained by scale-averaging the magnitude-squared scalogram over all scales.

In this study, CWT was computed by applying the Matlab *cwt* function using the Morse wavelet as the mother wavelet to the time series signal of each FM, 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, when a CWT decomposition is performed on a signal with an abrupt step change, the result is a mixture of high-frequency components that capture the abrupt transition and lower-frequency components that describe the smoother and more gradual changes in the signal, across the entire frequency spectrum. The presence of abrupt changes would generate an artefact 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 (identified as discussed above), i.e. by subtracting the mean and removing the linear trend, hence without altering the informative, high-frequency content of the original signal.

Similar to the signal pre-processing described above, in order to get a consistent frame of reference, the scalogram of each FM was normalised between minimum and maximum values after removal of outliers. This allows us to effectively appreciate the existence of coherent features across FMs and characterise them in terms of dominant pseudo-frequencies and position in time. In order to obtain a synthetic summary of the results, the normalized scalograms obtained from the wavelet analysis of all FMs were combined into one, corresponding to the median scalogram. The summary pseudo-frequency-averaged wavelet spectra was obtained from the median scalogram.

3 Results

3.1 Laboratory results

The median opening signal of the FMs measured during the 24-hour laboratory experiment along with the 25th and 75th percentiles are shown in Figure 4a, for free and immobilised mussels separately. The evolution of discharge in time is also shown in the second y-axis. The figure clearly shows that both groups of FMs responded to the discharge increase, 10 hours after the start of the experiment, with a sharp and localised change in the median opening (thick coloured lines). By examining the shaded area of this plot (representing the 25th and 75th percentiles), it becomes evident that, in general, the signals from free FMs exhibit a more complex and varied behaviour compared to that of immobilised FMs. Indeed, the shaded area for the free FMs is much ticker compared to that of immobilised FMs, if we analyse the periods away from the discharge perturbation. This is explained by the fact that the former group displayed a larger number of features, most of which did not appear to be directly related to hydrodynamic changes. By instance, the decrease in the median opening of free FMs after 6 h after the start of the experiment is only apparent and not related to a consistent response across the four free FMs, but rather to independent and uncorrelated activities of the organisms. This is further evidenced in Figure S1 in the Supplementary Material, which illustrates the distinctive behavioural patterns exhibited by each FM and the major discontinuities in the mean of the signal. However, during the perturbation (from 10 hours to 12 hours after the start of the experiment), the width of the shaded area (25th-75th percentiles) shrinks and matches that of the immobilised FMs, indicating a coherent response across the four free FMs.

The distinction between the two groups of mussels arises from the restricted mobility of immobilised FMs in contrast to free FMs, as behaviours such as walking and drifting are not possible, resulting in a more straightforward signal. Notably, all

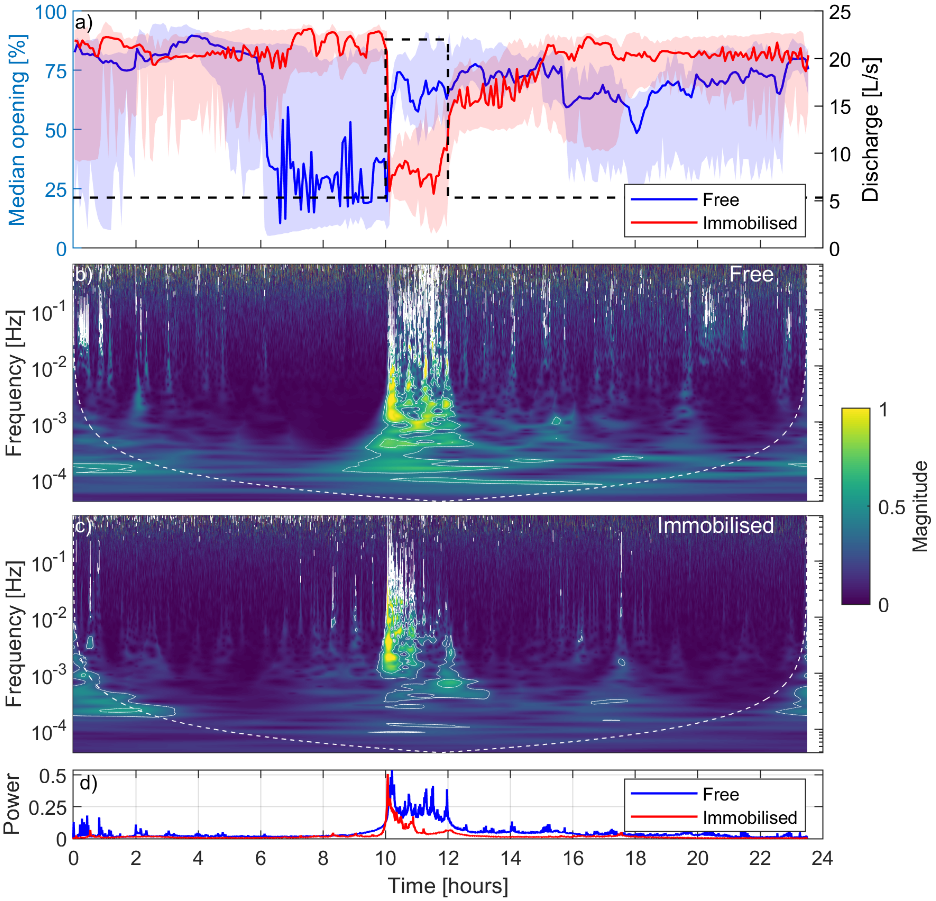

Figure 4. Laboratory experiment: a) left y-axis: median valve opening signals of free and immobilised mussels with 25th and 75th percentiles indicated by the shaded area; right y-axis: discharge (dashed, black line); b) scalogram showing the median normalised magnitude of the continuous wavelet transform over all the free FMs; c) scalogram showing the median normalised magnitude of the continuous wavelet transform over all the immobilised FMs; d) pseudo-frequency-averaged wavelet spectrum. White contours in b) and c) represent the 95th and 99th percentiles of the CWT coefficient.

immobilised FMs responded by closing their valves when the discharge increased, while two out of the four free FMs responded with an increase of the opening (FM1 and FM2) and one with a decrease (FM4; see Figure S1). Apart from exhibiting some noise, likely arising from electrical issues, which, however, did not significantly impact the results, the free FM3 displayed a behaviour similar to that of FM4. While both free and immobilised FMs responded clearly and promptly to the rapid increase 280 in discharge, as evidenced by the change in the mean valve opening discussed above, only immobilised mussels displayed a similar response upon the re-establishment of the base flow (12 hours after the start of the experiment), albeit to a lesser extent compared to the signal variation the mussels exhibited when the flow increased (Figure 4a and Figure S1).

Moving beyond the analysis of mean valve opening, however, a distinct signature of the discharge perturbation is discernible in both free and immobilised FMs through the occurrence of broad-frequency features localised over time around the perturbation period (see the period between 10 and 12 hours in Figure S1 and in Figure S2, the latter showing the signals after detrending and removal of changes in the mean valve opening). Such features are more clearly appreciable when looking at the scalograms of free (Figure 4b) and immobilised (Figure 4c) FMs. Prior to the increase in discharge (from the beginning of the experiment to 10 hours), the FMs' gaping was characterised by low pseudo-frequencies (below 10^{-3} Hz) with less energy. However, following the increase in discharge, they promptly began responding across the whole range of pseudo-frequencies (up to 1 Hz), displaying higher energy levels (as indicated by the more yellowish regions). The white contours on the scalogram plot represent the 95th and 99th percentiles of the CWT coefficient and were used to emphasise the energy-rich areas. The response is similar between free and immobilised FMs, although immobilised FMs adapted quicker to the new discharge conditions compared to free FMs. This is evident also looking at Figure 4d, which shows the pseudo-frequency-averaged wavelet spectrum for both FM classes: immobilised mussels' power returned normal an hour after the discharge increase, followed by free mussels who responded with high power until the end of the event (two hours after the discharge increase). In both cases, the dominant pseudo-frequencies, after the discharge variations, reset to values consistent with those characterising the first part of the experiment.

3.2 Field results

Because laboratory experiments showed overall consistent responsiveness between free and immobilised FMs in the presence of hydrodynamic stresses, this supported the suitability of installing immobilised FMs in the field. Figure 5a illustrates the signals of the thirteen immobilised FMs at the field monitoring site along the River Paglia (see Figures 1 and 3) from 10 PM on March 30, 2022 until midnight on April 1, 2022. It should be mentioned that there is a gap of an hour and a half in the data for all FMs, with missing signals from mussels between 10.5 and 12 hours since the start of the signal (i.e., between 8:30 AM and 10:00 AM on March 31) due to unspecified technical issues. According to this plot, all FMs except for mussels numbered 2, 3, and 12 experienced a marked shift in the mean valve opening with a generalised closing of the valves approximately 5.5 hours after the start of the time series, specifically around 3:30 AM on March 31, coinciding with a flood event in the river. As can be observed in the data, the sensor installed on FM2 experienced technical issues, preventing its use in the analysis. 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 characterised by normal behaviour before the flood event, with their valves open and characterised by regular valve movements as expected during respiration and filtration.

In general, in terms of change in the mean valve opening, the mussels responded similarly to changes in hydrodynamic conditions as observed in the flume experiment and illustrated in Figure 5b, which depicts the median valve opening signal of the FMs (all individuals except FM2) in relation to the changes in water level measured by the multiparametric sensor every 10 minutes (see also Figure S3). The median valve opening shows two main discontinuities, which coincide with marked changes in the water level line. The first discontinuity is evident at 5.5 hours from the start of the time series (i.e., at 3:30 AM), when the

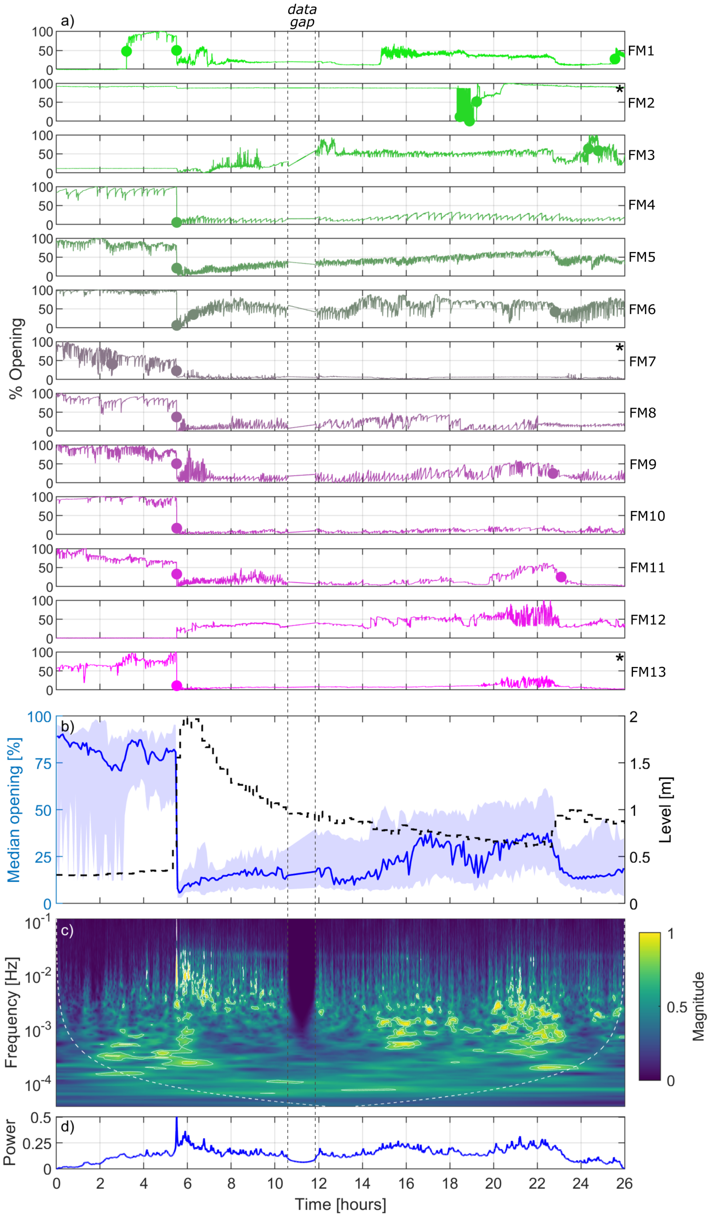

Figure 5. Results from the thirteen FMs deployed at the River Paglia field monitoring site during the flood on March 31, 2022; a) valve opening signals for the individual FMs (dots indicate abrupt change points in the mean of the opening signals when the mean opening changes by more than 25%; the asterisk * depicts FMs that are excluded from the wavelet transform analysis); b) left y-axis: median valve opening signals with 25th and 75th percentiles indicated by the shaded area (all FMs except FM2); right y-axis: water level (dashed, black line); c) scalogram showing the median normalised magnitude of the continuous wavelet transform over the FMs; d) pseudo-frequency-averaged wavelet spectrum. White contours in c) represent the 95th and 99th percentiles of the CWT coefficient.

water level, as measured by the multiparametric sensor, rapidly increased from 0.3 m (corresponding to about 4 m^3/s) to 2 m (corresponding to about 140 m^3/s, based on the data from the official gauging station, available every 30 minutes). This rise in water level marked the onset of the flood event, which was characterised by a sharply rising front. The associated discontinuity in the valve opening signal is clearly visible when examining both the individual FM signals (Figure 5a) and the median signal (Figure 5b). The second discontinuity clearly emerges only by looking at the median signal (Figure 5b), while it is hindered in the individual time series. It occurred at about 23 hours after the start of the time series (i.e., at 9 PM), when the water level rose from 0.6 m (approximately 18 m^3/s) to 1 m (approximately 45 m^3/s), thus interrupting the previously gradual decrease in water level following the initial peak.

The FMs response to the hydrodynamic disturbance, as reflected in changes in the frequency of gaping, is shown in the scalogram of Figure 5c. This plot is obtained excluding FM2, which was affected by the technical issues discussed above, and for the sake of clarity also FM7 and FM13, respectively characterised by intense activity before the flood and a significantly damped response after the flood, to an extent that was not in alignment with the behaviour of the other FMs. These mussels are labelled with * in Figure 5a (and in the corresponding Figure S4 showing the signals after detrending and removal of changes in the mean valve opening). As indicated by the scalogram, the mussels exhibited responses at a low frequency (below $10^{-4} - 10^{-3}$ Hz) before the peak of the flood, displaying signals of lower energy. As soon as the level raised, parallel to the sudden closure of the valves seen in Figures 5a and b, the FMs showed a generalised response shifted towards higher frequencies (up to 0.1 Hz) and characterised by higher energy levels (yellowish regions). This response gradually diminished as the discharge decreased, resulting in lower frequencies and reduced energy levels. Between 15 to 23 hours from the beginning of the time series, the water level, and consequently the discharge, transitioned into the latter phase of the flood event, characterised by minor fluctuations over time. Additionally, water temperature and conductivity steadily returned to higher values and stabilised (see Figure S3). During this time window, the FMs underwent a slight opening of the valves (Figure 5b) and intensified their gaping around frequencies of 10^{-3} Hz (Figure 5c). Following the occurrence of the second, smaller peak in water level (approximately 23 hours after the start of the time series), all FMs closed their valve once more (Figure 5b) and the majority of them exhibited a significant decrease in the frequency of gaping (Figure 5a and c). The overall picture is summarised also in the pseudo-frequency-averaged wavelet spectrum shown in Figure 5d, which clearly indicates the instantaneous evident response of the FMs to the main perturbance, similar to what was observed in the laboratory experiments.

4 Discussion and conclusion

In recent research, based on laboratory experiments Modesto et al. (2023) and Termini et al. (2023) proposed FMs as effective BEWS for assessing the impact of hydrodynamic stresses on the aquatic ecosystem. In this study, for the first time, FMs were deployed and evaluated in real river conditions, in the River Paglia at Orvieto, Italy. The initial challenge we faced was securing the mussels in place and preventing them from being carried away by the river's current. In consideration of other *in situ* exposure methods (e.g., Kramer and Foekema, 2001; Sow et al., 2011), we opted to glue the mussels to vertical rods anchored at the riverbank. To ensure that the use of immobilised mussels did not significantly alter their behavioural responsiveness, we conducted controlled laboratory experiments to confirm that immobilised and free mussels exhibit consistent reactions under the same hydrodynamic stressors.

The results demonstrated that immobilised and free mussels behave in a consistent way during a hydrodynamic perturbation, but the signals of the immobilised mussels exhibit less complexity, primarily due to the reduced number of features resulting from movement constraints (Figure 4). Consequently, these signals can be more easily interpreted and associated with perturbations in external conditions. In fact, immobilised mussels have limited means of response, primarily relying on gaping, as they are unable to engage in actions such as escaping or burying themselves more deeply. It is worth noting that concerning mussel behaviour classification, both free and immobilised mussels showed avoidance behaviour by immediately closing their valves and changing their gaping frequency at the onset of the higher flow levels. However, immobilised mussels showed a faster adaptation in response to a prolonged stimulus, facilitating the faster restoration of pre-event gaping frequencies. This could be explained by the positive correlation between the intensity of the stimulus and the degree of adaptation (Capraro et al., 1979; Hollins et al., 1990). In fact, immobilised mussels experience a stronger stimulus than free mussels due to the impossibility of actively searching for shelter at the time of the event, and as a result they have a shorter adaptation period most likely because they get tired sooner. Overall, based on the laboratory comparison, we could confidently assert that the installation of immobilised mussels in real river conditions ensures both site stability during floods and the representativeness of the results. This is particularly true in the context of using FMs as real-time BEWS, which requires timely detection of significant changes, while other information such as response type and duration may be of interest for deeper biological interpretation, but is not the primary concern.

Field data acquired at the field monitoring site in the River Paglia provided a solid validation of the effectiveness and reliability of using immobilised mussels as part of a real-time BEWS. All the FMs exhibited a synchronised and highly distinct response to the flood event that occurred during the monitoring campaign, promptly transitioning their behaviour in terms of both mean valve opening and gaping frequency. Of paramount importance for practical applications is the observation that these mussels responded immediately as the discharge increased, effectively detecting the flood at its very onset. These results confirm and validate what has been observed in the laboratory. The sharp and rapid mussel reactions can be explained by the almost abrupt rise in water levels and discharges of the flood, and also by the fact that, as in the flume experiments (see Section 2.2), the discharge values around the peak triggered sediment transport and turbidity. To establish for the River Paglia an incipient (alias critical) discharge value, we exploited the shear-stress distribution relative to the flood on May 31, 2022, determined by a 3D Reynolds-Averaged Navier-Stokes (3D-RANS) model described in Bahmanpouri et al. (2023), and some bed material samples we collected from the bed surface just upstream and downstream of the Adunata Bridge site. With a constant flow discharge corresponding to that observed at the flood peak, the numeric shear stress distribution reached a maximum of about 100 Pa under the bridge, and smaller values away from the bridge. Looking at the numerical results, a reference value of 30 Pa is exerted over a large part of the flow domain. From the bed material sample, we found that the d_{90} (i.e., diameter corresponding to the 90^{th} percentile of the granulometric distribution) of the sediments forming the substrate of the bed is about 0.01 m. With these values, the well-known dimensionless Shields stress number $\theta = \tau / \left[\left(\rho_s - \rho_w \right) g d \right]$ (where τ is the bottom shear stress, $\rho_s \left(\rho_w \right)$ is the sediment (water) density, g the gravitational constant, and d the characteristic particle diameter), reached a value of about 0.6 at the discharge peak, well above the critical one, which can be assumed equal to about 0.06, as in many references (see e.g., Pähtz and Durán, 2018). According to the 3D-RANS model, a critical discharge for the initiation of bedload in this reach of River Paglia (Adunata Bridge) corresponds to about 4 m^3/s. The ratio, in terms of peak flood-discharge over the critical value, is therefore about 35 (i.e., 140/4), whereas in the flume experiments the mussels experienced a discharge about 1.6 times larger than the critical one (i.e., 22/14), as from the data reported in Section 2.2. Our laboratory and field results show that the responsiveness of the mussels is efficient in a wide range of peak/critical discharge ratios.

To improve the interpretation of behavioural signals, we proposed a statistical analysis based on the combined identification of abrupt change points in the mean of the opening signals and application of continuous wavelet transform to the detrended time series after removal of these discontinuities (refer to Figures S2 and S4 to view the cleaned signals). This approach has proven to be a robust and reliable tool for FMs' signal processing, able to effectively identify the animals' response to external stressors. This identification can be achieved both visually, through the examination of scalogram plots, and quantitatively, through the generation of associated pseudo-frequency-averaged wavelet spectrum plots. For the sake of comparability, the scalogram of each FM was normalised between minimum and maximum values after the removal of outliers. Then, the median scalogram was calculated and used to identify dominant pseudo-frequencies and their temporal positions. This approach enables comparisons within the same frame of reference between different FMs and between median scalograms obtained under various conditions (e.g., in the laboratory and in the river). Similarly, the pseudo-frequency-averaged wavelet spectra derived from the median scalograms can be fairly compared to discern shared patterns and distinctions across the various setups. Notably, results from both the laboratory and the field indicate that during external stress events (i.e., an increase in discharge), the power of the spectrum reached and exceeded a value of approximately 0.25. While further data collection from additional sites is needed before proposing a threshold value as a simple indicator of aquatic ecosystem stress, these findings underscore the effectiveness of combining FM valvometry and CWT processing towards the establishment of real-time operational BEWS.

The utilisation of FMs in real riverine conditions did present logistical challenges. While the monitoring station at the River Paglia was operational for several months, the data acquired only covered the occurrence of a flood. The lack of significant events during the monitoring period and the mortality of some FMs prevented the acquisition of additional data that would have been valuable for the analysis. Additionally, during the monitoring activity after the flood event occurred on March 30, 2022, it happened that metal particles occasionally suspended in the water, likely originating from an upstream mine, accumulated on the magnets installed to the FMs, thus altering the valvometric signal. In addition, the extrapolation of laboratory results to natural conditions should take into account the greater variability of boundary conditions concerning the parameter being monitored. The response of the mussels is an expression of their reaction to changes in multiple conditions that can only be controlled and restored to previous conditions in the laboratory (and even then not completely). In this respect, the increased activity in terms of energy content at the highest frequencies observed at the end of the descending phase of the flood observed in the River Paglia (see Figure 5c and d, between 20-23 hours) cannot be explained based on the available measurements. For example, the water temperature (see Figure S3) should not be influential, since it was always below the tolerance threshold of the eurythermal generalist species we used and showed minor changes during the period analysed (about 1.5 °C). It is likely that other environmental variables, such as turbidity or specific FM behaviour after a long disturbance, were involved. For these reasons, efforts to expand the dataset for a more comprehensive analysis are planned and underway, and further studies should be conducted to deepen the behavioural characteristics of the FMs in the field, investigate the limitations of the method, and develop protocols addressing them appropriately.

The results obtained pave the way for the utilisation 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.

In summary, based on the above insights the following conclusions can be drawn:

- both free and immobilised freshwater mussels can serve as effective ecosystem warning indicators in aquatic environments, with the choice between them depending on the riverbed and flow rate conditions;

- immobilising the mussels to support constrains their behaviour, but this results in sharper event detection compared to free mussels and easier interpretation of the signals;

- continuous wavelet transform proves to be a valuable tool for interpreting the FMs signals. It is effective in identifying pseudo-frequency features present in the signal over time and describing the response of FMs to external perturbations, providing more informative results than only looking at discontinuities in the opening time series;

- laboratory and field experiments with immobilised mussels demonstrate their response to hydrodynamic stresses within a frequency of valve gaping ranging from 10^{-3} Hz to 1 Hz. This frequency range is larger than the background frequency range during normal behaviour (around $10^{-4} - 10^{-3}$ Hz when taking the median across multiple individuals). These frequency values correspond to conditions that indicate the presence of stressful conditions for the FMs, thus underscoring the potential use of FMs as real-time BEWS for identifying potential threats to the aquatic ecosystem;

- the comparison between pseudo-frequency-averaged wavelet spectra obtained in the laboratory and in the field suggests the potential introduction (subject to further data acquisition) of a simple indicator based on power values to detect disturbances in the aquatic environment.

Data availability. The data and code that support the study are available from the corresponding author upon request.

Author contributions. **Ashkan Pilbala:** Investigation, Methodology, Formal Analysis, Data Curation, Writing - Original Draft. **Nicoletta Riccardi:** Conceptualization, Investigation, Methodology, Resources, Supervision, Writing - Original Draft. **Nina Benistati:** Investigation, Writing - Review & Editing. **Vanessa Modesto:** Investigation, Resources, Writing - Review & Editing. **Donatella Termini:** Funding acquisition, Conceptualization, Investigation, Writing - Review & Editing. **Dario Manca:** Data Curation. **Augusto Benigni:** Data Curation. **Cristiano Corradini:** Data Curation. **Tommaso Lazzarin:** Data Curation, Writing - Review & Editing. **Tommaso Moramarco:** Funding acquisition, Project administration, Conceptualization, Investigation, Writing - Review & Editing. **Luigi Fraccarollo:** Conceptualization, Investigation, Methodology, Supervision, Funding acquisition, Writing - review & editing. **Sebastiano Piccolroaz:** Conceptualization, Investigation, Methodology, Software, Formal Analysis, Data Curation, Visualization, Supervision, Writing Original Draft.

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