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Perna perna Mussels Network as Pollution Biosensors of Oil Spills and Derivatives

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Abstract: Since the availability of petroleum and derivatives has great impact over the world economy, the oil industry lies in both the formation and the maintenance of modern industrial economy. Petroleum exploration, transport, distribution and storage activities can compromise water resources and provide serious consequences to exposed organisms due to the risk of accidental spillage of oil and refinery effluents. *Perna perna* mussels are acknowledged for their sentinel characteristics being affected by slight environmental changes and one of the promising species in the aquaculture world. Thus, this study aimed to demonstrate that the behavior of *Perna perna* mussels is a suitable biomarker of exposure to petroleum and derivatives. The present research proposes the construction of an online aquatic pollution biosensor based on the behavioral analysis of *Perna perna* mussels network. Thirty-nine mussels instrumented with Hall Effect sensors and magnets were exposed to 0 (control), 5%, and 20% of Water-Accommodated Fraction (WAF) of Diesel S-500 for up to 42 hours. The sensors network outputs were used to evaluate the behavioral parameters average amplitude, filtration activity, transition frequency, amount of motion reversals and weighted average of the ten largest Fourier magnitudes after the first 12 hours of experiment using 6-hours intervals. The employment of the behavioral parameters weighted average of the ten largest Fourier magnitudes and transition frequency provided greater efficacy in distinguishing groups of animals exposed to contaminants in relation to the control group with significant differences in at least 80% of the analyzed intervals.

Keywords: Bioresponses, bio-signals analysis and interpretation, biosensors, behavioral analysis, aquatic pollution.

1. INTRODUCTION

The oil industry provides difficult-to-substitute goods in the productive matrix of any country being basis of the mode of operation, consumption and even culture of modern society (Canelas, 2007). However, petroleum exploration, transport, distribution and storage activities can lead to environmental damages due to risk of accidental spillage of oil and refinery effluents. These accidents can compromise water resources and provide serious risks to exposed organisms.

Therefore, it is necessary to develop sensors capable of detecting petroleum and derivatives presence quickly in aquatic environments. Changes in valve-activity responses of bivalve mollusks represent an immediate biological response to the presence of contaminants being a relatively easy and inexpensive way to monitor aquatic environments (Newton and Cope, 2007). These responses can be employed in automatic and online biomonitoring of aquatic environments providing greater sensitivity and ecological relevance to standard toxicity tests (Liao et al., 2009; Gerhardt et al., 2005) .

In this context, bivalve mollusks have characteristics suitable for use as biosensors being sessile, abundant and available throughout the year (Kramer et al., 1989). Among these animals, the *Perna perna* mussels present great socio-economic importance due to its use in mitiliculture (Resgalla Jr et al., 2007). Besides, they were presented by the United Nations Food and Agriculture Organization (FAO) as one of the promising species among others emerging in the aquaculture world (Furlan et al., 2015).

Behavior analysis of bivalve mollusks requires the use of valvometry techniques that allow the acquisition of data regarding the opening amplitude of their shells. Valvometry techniques have been investigated in ecotoxicology for at least 20 years and employed in aquatic pollution biosensors (Tran et al., 2010). Among valvometry techniques, Hall effect sensors present many advantages such as durability, lightness, easy attachment and offer less stress to the animal facilitating the measurement of its movement (Nagai et al., 2006).

The valve-activity responses of mollusks are strongly related to vital activities such as breathing, feeding and excretion, besides to environmental conditions such as the presence of contaminants and predators. Hence, the proper understanding of their behavior can assist in the management of water quality in natural habitats (Hartmann et al., 2016).

The data resulting from the continuous monitoring of bivalve organisms are absolutely complex due to their nonlinear behavior and its statistically challenging analysis (Sow et al., 2011; Hartmann et al., 2016; Bae and Park, 2014). Since animals present specific and rhythmic movement patterns, they should be monitored for a minimum of 24 to 36 hours using a minimum frequency of 2 Hz (Markich, 1995).

Toxicologically evaluating mixtures with petroleum and derivatives is a complex task. There is substantial variability in the collection, interpretation and use of toxicological data arising from the use of petroleum, derived products and their mixture with dispersants. Hence, the comparison of results is debatable even when differences between experiments are considered (Singer et al., 2000). Methodological variability and lack of standardization affect data reproducibility.

In order to ensure that the toxicological data are directly comparable across laboratories and usable in decision making when oil enters the aquatic environment, it is necessary the standardization of experimental aspects and processes. Among the toxicological aspects considered most necessary for standardization are the preparation of the exposure medium, its analytical inspection and the exposure method (Singer et al., 2000). The Water-accommodated Fraction (WAF) is a standardized exposure media that corresponds to a laboratory prepared medium resulting from the low energy blending (non vortex formation) of a poorly soluble material (such as petroleum and derivatives) essentially without particles (Singer et al., 2000).

Among petroleum derivatives, diesel oil is an important fuel used in diesel cycle engines in road, rail and marine vehicles and one of the most common aquatic contaminants (Nogueira et al., 2011). The effects of diesel fuel spills on aquatic organisms include mortality, reproductive impairment, altered growth rates and greater susceptibility to diseases (Lloyd and Cackette, 2001).

This work presents the development of a aquatic pollution biosensor based on the behavioral analysis of *Perna perna* mussels network. For this objective, it proposes a controlled exposure of *P. perna* mussels to different WAF concentrations of diesel S-500 being this network instrumented with Hall effect sensors and neodymium magnets. The present research also proposes evaluating the valve activity responses according to the behavioral parameters average amplitude, filtration activity, transition frequency, amount of motion reversals and weighted average of the ten largest Fourier magnitudes.

2. METHODOLOGY

2.1 Animals

Perna perna mussels were acquired from a mariculture farm situated at a southwestern region of Santa Catarina Island - SC, Brazil. Animals were immediately transported to the laboratory facilities at the Federal University of Rio Grande on which the experiments were conducted. For approximately 15 days, mussels were maintained at constant conditions (temperature 20 °C, salinity 30 and photoperiod 12L:12D). Water was renewed every 2 days and feeding occured afterwards using *Isochrysis galbana, Chaetoceros muelleri, Nannochloropsis sp* and *Conticribra weissfloggi* microalgae.

2.2 Water-Accomodated Fraction of Diesel S-500

Diesel S-500 was purchased from a local gas station and used for the preparation of the WAF, according to the protocol of Singer et al. (2000). Briefly, 1L of Diesel S-500 was diluted in 9L of seawater (salinity 30) in two 5L glass flaskes. The mixture was then left for a 24h agitation (without vortex). To minimize evaporation of diesel-oil components, flasks were sealed and protected from light. After the 24h agitation period, the mixture was left to stand for 1h. The water phase which contained the water soluble part of diesel oil was withdrawn and reserved.

2.3 Experimental Design: a network of mussels

Mussels (N=39) were placed in 9 tanks of 5L with aerated seawater (salinity 30) and maintained at constant conditions as described previously. The experiment was conducted in triplicates (three aquaria for each treatment) and three experimental groups were performed: control (0% of WAF), 5% of WAF and 20% of WAF. The two concentrations of diesel WAF were prepared through dilutions of the WAF with the control seawater.

The animals network was instrumented with UGN3503 Hall effect sensors and neodymium magnets (10 mm diameter by 4 mm high). The instrumentation process consisted on the fixation of Hall effect sensor and neodymium magnet at opposite ends of the middle ventral edges of these mussels' shells (Fig. 1). These components were attached onto the shells with a cyanoacrylate glue and the sensors were waterproofed using epoxy resin (Basti et al., 2009; Nagai et al., 2006).

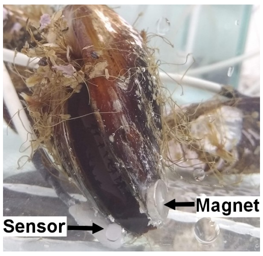

Fig. 1. *Perna perna* mussel instrumented with Hall effect sensor and neodymium magnet

The Hall effects sensors were connected to a data acquisition board in order to generate a CSV (Comma-Separated Values) file with the measures of valve-activity responses acquired at a 2Hz frequency for 45 hours. The data acquisition board was composed of a 16-channel multiplexer, and a SD card module. It operated along with an Arduino Mega prototyping device whose analog to digital converter has 10 bits of resolution. Fig. 2 illustrates the experiment layout.

2.4 Behavioral Analysis

The construction of the aquatic pollution biosensor predicts the exposure of *Perna perna* mussels to different levels of diesel WAF. The analysis of opening and closing data is necessary to search for significant behavioral changes resulting from this exposure. Since mussels were attached to an equipment, their bahavior was monitored for four days before the presence of the diesel WAF. Once acclimated, experiments were initialized.

Considering the above, the present study proposes the use of statistical methods in conjunction with the behavioral analysis parameters average amplitude, filtration activity, transition frequency and amount of motion reversals. Moreover, in order to analyze the behavior of bivalve animals in frequency domain, this work proposes to use the weighted average of the ten largest Fourier magnitudes as parameter. For this purpose, the Fourier transform was performed through the fast Fourier transform algorithm available in Matlab [1] software. One way to perform the Fourier transform is by defining an function $F(k) = \sqrt{2\pi} c(k)$ in (1).

$$F(k) = 1/(\sqrt{2\pi}) \int_{-\infty}^{+\infty} f(x) e^{-ikx} dx \tag{1}$$

On which $f(x)$ represents the time domain signal with index x and $F(k)$ corresponds to its Fourier transform with angular frequency k (Butkov, 1988).

- Filtration Activity - The filtration activity was measured as the fraction of time on which the bivalve shell was open and considered to be filtering. The status of each animal was determined as open or closed according to mean voltage over the experimental period (Hartmann et al., 2016). If the Hall effect sensor output was greater than this value, the mussel was considered open (filtering), otherwise its status was closed (not filtering).
- Transition Frequency - The transition frequency parameter was evaluated as the number of observations on which the mussel state changed from closed to open and vice versa.
- Amount of Motion Reversals - The amount of motion reversals was measured as the number of times the animal's shells were opening and changed its state of movement to closing.
- Weighted Average of the Ten Largest Fourier Magnitudes - The frequency of each magnitude was used as weight. In such a manner, the higher the frequency of the analyzed magnitudes, the greater was its influence on the average value.

2.5 Statistical Analysis

The behavioral parameters were applied to the valve-activity responses considering sample windows of one hour. The resulting data were grouped into 6-hour analysis periods for further statistical analysis. This process was performed to normalized behavioral data generated after the first 12 hours of experiment. The normalization of

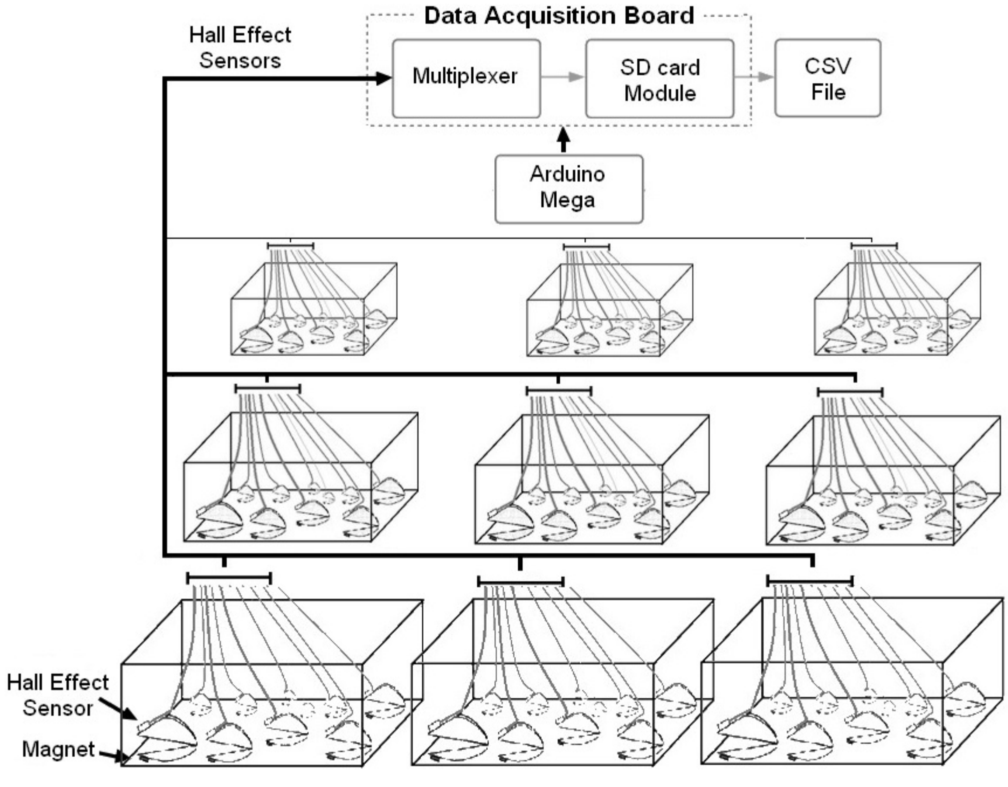

Fig. 2. Experiment layout on which 39 instrumented mussels (shell length from 50 to 70 mm) were equally-distibuted between the control, WAF 5% and WAF 20% groups. The experiment was carried in triplicates and the sensors outputs were connected to a data aquisition board that along with an Arduino Mega generates a CSV file containing the behavioral data

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[1] https://www.mathworks.com/products/matlab.html

data from 0 to 1 for each mussel aimed to avoid possible to variations from one sensor to another due to the instrumentation process (Comeau et al., 2018; Tran et al., 2010).

Analysis of Variance (ANOVA) was used to test for significant effects of the contamination level on the behavioral parameters considering each analysis period. For this purpose, seven mussels of each treatment were randomly selected for each inspected period. Before using ANOVA, the Kolmogorov–Smirnov test was performed to evaluate the normality of data. If this assumption was not verified, the Box-Cox transformation was used as defined in (2).

$$\begin{aligned} Y^* &= (Y^\lambda - 1)/\lambda, \quad for\ \lambda \neq 0 \\ Y^* &= ln(Y), \quad for\ \lambda = 0 \end{aligned} \tag{2}$$

On wich Y and Y^* are the original and the transformed data, respectively. λ is the Box-Cox parameter that maximizes the normality of the transformed variable. If after performing the Box-Cox transformation the normality criterion was not yet verified, the non-parametric Kruskal-Wallis test were employed. If the null hypothesis was rejected, the Tukey-Kramer test was applied to determine significant differences among groups. For all statistical tests the significance level was set at 5% ($p < 0.05$). Data were expressed as *mean* ± *standard deviation* and the MATLAB software was used.

3. RESULTS

The *Perna perna* mussels network has demonstrated idiosyncratic behavior patterns which illustrates the challenges in behavior data analysis. Figure 3 illustrates examples of valve-activity responses of animals from control, WAF 5% and WAF 20% groups. Mortality and Hall effect sensor failure were experienced during the experiment. All affected data were removed from the analysis. Statistical results were expressed as *mean* ± *standard deviation* and different letters indicate statistical differences ($p < 0.05$).

3.1 Average Amplitude

No significant differences were evidenced between the groups average amplitudes in the periods of 12 to 42 hours of experiment, except for the interval of 30 to 36 hours. In this interval the control and the WAF 5% groups presented significantly different average amplitude as demonstrated in Fig. 4.

3.2 Filtration Activity

Except for the interval of 24 to 30 hours, no significant differences were observed in filtration activities of the groups. In this period (Fig. 5), the methodology has identified significant changes in the opening duration between the control and WAF 20 % groups. The control group filtration activity was on average 1.09 and 1.52 times higher compared to the WAF 5% and WAF 20% groups, respectively.

3.3 Transition Frequency

Considering the transition frequency parameter, Figure 6 presents the resulting data expressed as *mean* ±

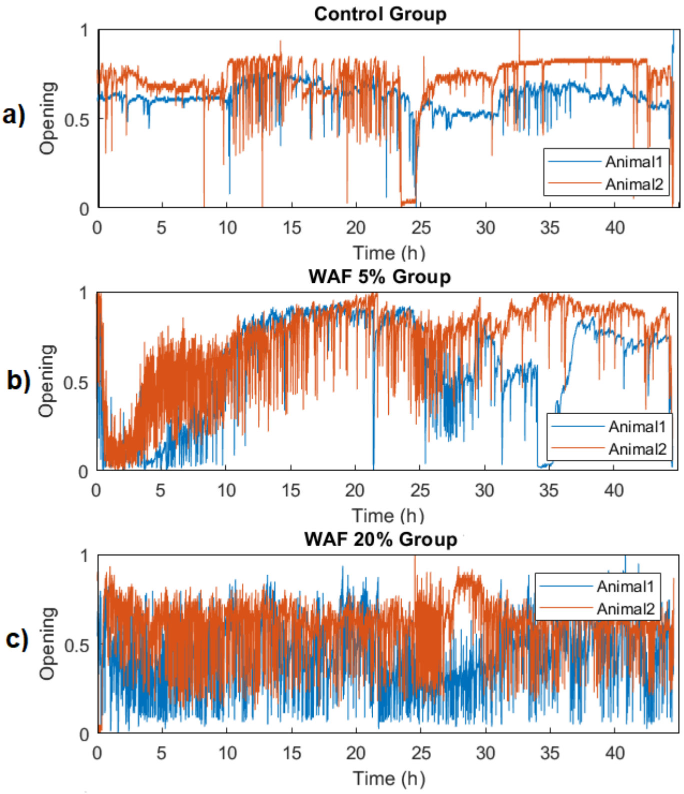

Fig. 3. Example of valve-activity responses of the control (a), WAF 5% (b) and WAF 20% (c) groups

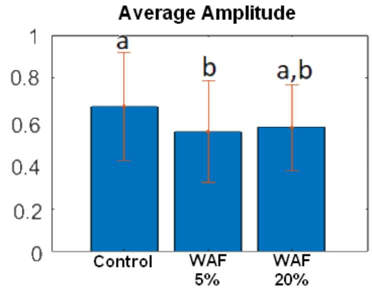

Fig. 4. Average amplitude of *P. perna* mussels network evaluated in the interval of 30 to 36 hours. Values are expressed as the *mean* ± *standard deviation* and different letters indicate statistical differences ($p < 0.05$).

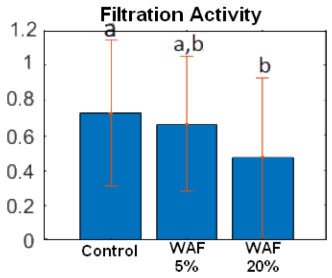

Fig. 5. *P. perna* mussels network filtration activity evaluated in the interval 24 to 30 hours of experiment. Values are expressed as the *mean* ± *standard deviation* and different letters indicate statistical differences ($p < 0.05$).

standard deviation. For all investigated intervals, at least one contaminated group has shown a significant difference in transition frequency compared to the control group at $p < 0.05$.

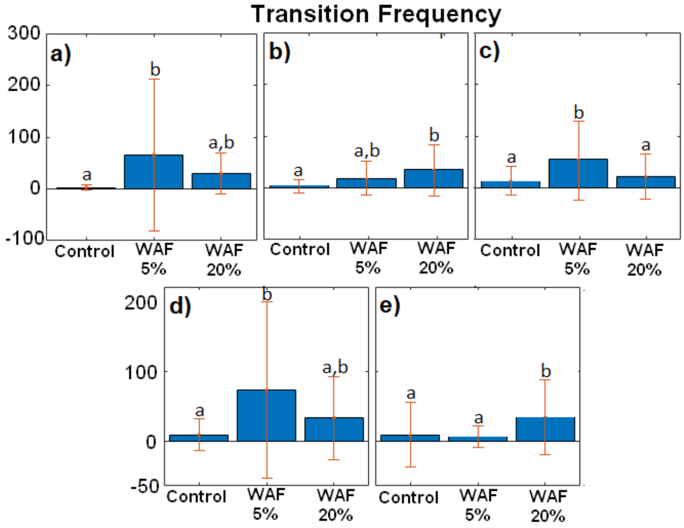

Fig. 6. *P. perna* mussels network transition frequency evaluated in the intervals 12 to 18 (a), 18 and 24 and 24 to 30 (c), 30 to 36 (d) and 36 to 42 (e) hours of experiment. Values are expressed as the *mean* ± *standard deviation* and different letters indicate statistical differences ($p < 0.05$).

In 50% of the evaluated periods (12 to 18, 24 to 30 and 30 to 36 hours), the control group was significantly different from the WAF 5% group with no significant differences between contaminated groups in 66.7% of these cases (intervals from 12 to 18 and 30 to 36 hours).

In the periods from 0 to 6, 18 to 24 and 36 to 42 hours, significant changes in the transition frequency of the WAF 20 % and control groups were evidenced. However, there were no significant differences between contaminated groups during the interval of 18 to 24 hours of experiment.

3.4 Amount of Motion Reversals

Considering the amount of motion reversals, significant differences among groups were evidenced in the period of 24 to 30 hours of experiment. The control group amount of motion reversals was significantly different to the contaminated groups. However, no significant differences were found between the groups exposed to contaminants (Fig. 7).

3.5 Weighted Average of the Ten Largest Fourier Magnitudes

The evaluation of the weighted average of the ten largest Fourier magnitudes among groups demonstrated, except for the 36 to 42 hours interval, significant differences at all investigated cases as illustrated in Fig. 8.

During the first two evaluated periods, this behavioral parameter was proportional to the level of contamination to which the groups were exposed to. It was 1.29 and 2.49 times higher in the WAF 20% group compared to the WAF 5% and control groups, respectively.

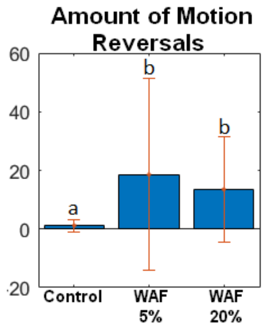

Fig. 7. *P. perna* mussels network amount of motion reversals evaluated in the interval 24 to 30 hours of experiment. Values are expressed as the *mean* ± *standard deviation* and different letters indicate statistical differences ($p < 0.05$).

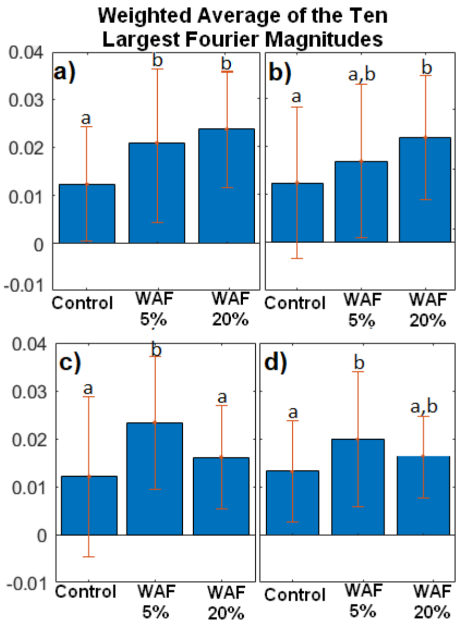

Fig. 8. Weighted average of the highest Fourier magnitudes evaluated in the intervals 12 to 18 (a), 18 to 24 (b), 24 to 30 (c) and 30 to 36 (d) hours of experiment. Values are expressed as the *mean* ± *standard deviation* and different letters indicate statistical differences ($p < 0.05$).

This behavioral parameter was significantly different between the control group and both contaminated groups in the first evaluated interval ($p < 0.05$). For the next 6 hours only the WAF 20% group was considered significantly different from the control group. In cases analyzed after 24 hours of toxicological experiment the group exposed to the highest concentration was not considered significantly different from the control group.

This parameter was able to provide significant behavioral changes between at least one group of exposed animals and the control group in 80% of the inspected cases. In this sense, it seemed to be suitable for detecting behavioral changes resulting from toxicological exposure of *Perna perna* mussels.

4. CONCLUSION

This paper proposed a biosensor network for detection of petroleum and derivatives in aquatic environment using *P. perna* mussels. The proposal was applied and validate using statistical methods to detect significant differences in behavioral parameters among groups of animals exposed to different concentrations of diesel WAF. Although *P. perna* mussels are known for their sentinel characteristics, the idiosyncratic network responses demonstrated the complexity of evaluating behavioral data. Among the investigated parameters, the transition frequency and the weighted average of the ten largest Fourier magnitudes presented better suitability in distinguishing the groups of animals. They were able to provide significant differences in at least 80% of the analyzed intervals.

There is an inherent variability in animal behavior (Basti et al., 2009) which demonstrates the phenotype plasticity for an individual attempt to cope with external stressors (Hartmann et al., 2016). Although it may enhance the complexity of analyzing bivalves behavior as a suitable biomarker of contaminant exposure, the combination of behavioral parameters may improve the statistical separation of groups of animals (Hartmann et al., 2016). According to the results, there were many cases which did not show significant difference between the control and WAF treatments. Since the analyzes were performed after 12 hours of toxicological exposure, it may reflect the adaptive nature of bivalve mollusks to environmental conditions.

The presented methodology was robust enough to deal with the behavioral variability of the analyzed individuals and may be employed in online behavior analysis of mussels. Thus, the present study can contribute to the development of an automatic biosensor for aquatic pollution. Future experiments can be performed to investigate its suitability in natural environments and provide advances along with machine learning algorithms. Moreover, the employability of the proposed biosensor is not restricted to the naturally occurring zones of the *P. perna* mussel as this specie is of great interest in world aquaculture.

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