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IMPACT OF NAPHTHALENE EXPOSURE ON MARKER ENZYMES OF GREEN MUSSEL *Perna viridis*

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Backgrounds: Environmental contamination has been increased in recent decades as a result of the fast expansion of anthropogenic activities, particularly in the aquatic ecosystem. Under a range of severe environmental circumstances, marine organisms can be susceptible to oxidative stress and results in the changes in the biochemical components which can be assessed to know the health status of organisms.
Aim of the Works: The aim of this study is to observe the impact of Naphthalene on bivalve mussels and to employ a large number of biomaker to discover distinct and unique patterns. For this the green mussels *Perna viridis* exposed to naphthalene, in order to understand the changes in marker enzymes in *P. viridis* haemolymph, gill, and digestive gland.
Methodology: Green mussels were exposed to naphthalene for 28 days in order to assess changes in biomarker. Acid phosphatase (ACP) and alkaline phosphatase (ALP), alanine transaminase (ALT), and aspartate transaminase activity (AST) were examined in the haemolymph, gill, and digestive gland of mussels *P. viridis*.
Results: The haemolymph has considerably higher in all marker enzymes after 28 days of naphthalene exposure. Marker enzymes were inhibited in the gill and digestive gland, and in most cases, all of the marker enzymes in the haemolymph, gill, and digestive gland were concentration dependent. The observed changes in marker enzymes which detected in the haemolymph and the other two tissues of *P. viridis* were statistically significant.
Conclusions: The present study showed a significant association between the entire biomarkers tested in mussels exposed to naphthalene. Overall, the results indicate that haemolypmh is the most vulnerable component to naphthalene exposure when compared to tissues, and it may be employed as a bioindicator of organic pollution exposure.

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1. INTRODUCTION

Human activities are anticipated to release roughly 228 000 tonnes of polyaromatic hydrocarbons (PAHs) into the aquatic system each year. Petroleum spills and leaks are the leading causes of this loading, which totals around 170 000 tonnes. The other significant contributions are industrial waste water effluents, sewage effluents, and run-offs, which amount 50 000 tonnes, and the residual masses of PAH are provided by atmospheric fallout. Sublethal PAH concentrations frequently have an impact on the physiology and biochemistry of aquatic species. The primary cause of PAH toxicity is that they attach to the hydrophobic regions of macromolecules in the cell, causing molecular and cellular damage and so disrupting normal physiological function. The primary cause of PAH toxicity is that they attach to the hydrophobic regions of macromolecules in the cell, causing molecular and cellular damage and so disrupting normal physiological function.

PAHs in the aquatic environment are primarily derived from four sources: petrogenic fuels, incomplete combustion (pyrogenic), organic metabolism (biogenic), and diagenetic transformation in sediments. Petrogenic and pyrogenic sources are the most significant contributors of PAHs to aquatic contamination. Further, PAHs are a major organic contaminant in the marine environment [1,2] including naphthalene, and have a molecular structure that comprises two or more fused aromatic rings and neighbouring rings that share two or more carbon atoms. Naphthalene is a key component of petroleum hydrocarbons oil water soluble fractions. Naphthalene is one of the most poisonous oil fractions to marine creatures and has the most narcotic properties of any polycyclic aromatic hydrocarbon. The potential environmental and biota impact of naphthalene has been studied [3].

In temperate regions of the world, mytilid mussels have long been employed in marine pollution monitoring programmes, since its widespread subtropical representatives species and hence it has been used to monitor the impacts of pollution in Southeast Asia. *Perna viridis* (Linnaeus) (Class: Bivalvia; Family: Mytilidae; Order: Mytiloida; Suborder Mytilicae) is found in coastal waters on India's east and west coastlines. It easily absorbs contaminants and exhibits a variety of physiological and biochemical reactions, offering insight into the general state of pollution in the coastal environment as well as the animal's own health. Indeed, *P. viridis* has been employed as an environmental bioindicator in toxicity investigations. Several studies reported that this mussel has been used to monitor the degree of pollution in the environment using a number of biomarkers [4-8]. The majority of *P. viridis* toxicity research has focused on the process of bio-accumulation in this species and the organs that aid in metal bio-accumulation. There has been no research on the impact of naphthalene on mussel health biomarkers to our knowledge. As a result, this is the first study to look at marker enzymes in *P. viridis* exposed to PAH over a long period of time.

Since a single biomarker may not reflect an individual's overall health, it has been suggested that a variety of biomarkers be used to assess the impact of naphthalene exposure. Hence the current study used several marker enzymes including phosphatase and transaminase markers to assess the impact of naphthalene exposure on bivalve mussels and this investigation is the first to deal with marker enzymes in *Perna viridis* exposed to sublethal concentrations and environmental relevant levels of naphthalene. The results obtained in this study shows that this research will be utilised to examine the current state and trends in chemical contamination reactions. The biomarkers utilised in this work can be used in the field to quantify organic pollution levels in the coastal zone.

2. MATERIALS AND METHODS

Green mussels *Perna viridis* weighing 60-100 g were collected and transported to the laboratory from the sea mouth of the Pulicat Lake near Chennai, Tamil Nadu, India. The mussels were kept in a big glass aquaria (60 cm X 30 cm X 45 cm) filled with seawater and fed a fresh Chlorella phytoplankton culture as mentioned in our previous manuscript [9]. For a week, mussels were acclimatised to laboratory conditions and a feeding regimen by changing the water every day [9]. Dissolved oxygen (5–7 mg/L), salinity (34±1 ppt), temperature (28±1^{o}C), and pH (8.1±0.1) were the tank holding conditions. A 14:10 h light: dark cycle was used to produce illumination.

2.1 Toxicity Test for Biomarkers

To analyse the changes in biomarkers when mussels were exposed to naphthalene, mussels were separated into five groups of 10 specimens each. Group I mussels were reared in normal saltwater. Mussels in Group II were reared in a solvent control. Mussels in groups III–V were exposed to seawater containing 1, 2, and 4 mg L^{-1} naphthalene, which were the sublethal

concentrations at which 0% death occurred after 96 hours [9]. The experiments were carried out in glass aquaria (60 cm X 30 cm X 45 cm), with three chambers for each concentration. The test water and seawater were replaced every day during the experiment, and the mussels were fed with Chlorella following standard toxicological protocol. After 28 days of exposure, the experiment was terminated, and the animals were sacrificed.

Mussels' haemolymph was collected from the posterior adductor muscle using a 23-G needle coupled to a sterile plastic syringe filled with ice-cold TBS (50 mM tris, 370 mM NaCl; pH 8.4; 840 mOsm). About 200 µl haemolymph was collected in one ml of iso-osmotic TBS. To avoid haemocyte clumping, haemolymph was promptly mixed with the buffer. For the experiment, the mussel haemolymph samples were not pooled. The organs including gill and digestive gland of the mussels were carefully dissected and kept at -20^{o}C for further enzymes and other biochemical analyses. Protein estimation in all the tissues and haemolymph was detected following the method of Lowry et al. [10].

2.2 Marker Enzymes

2.2.1 Acid phosphatase activity

The activity of acid phosphatase in the haemolymph and tissue samples was measured using a spectrophotometric technique with minor modifications [11]. In a 37 ^{o}C water bath, a tube containing 0.1 ml sample, 0.5 ml 0.1 M sodium citrate buffer (pH 4.8), and 0.2 ml 10 mM p-nitrophenol phosphate was incubated for 30 minutes. To stop the reaction, 0.5 ml of 1 M NaOH was given to each tube and the absorbance was measured at 405 nm with an Ultrospec 2100 pro UV/visible spectrophotometer. Under the test conditions, one unit of enzyme specific activity was defined as the quantity of enzyme capable of releasing 1 M p-nitrophenol per minute per mg protein.

2.2.2 Alkaline phosphatase activity

The activity of alkaline phosphatase was measured using p-nitrophenyl phosphate as the substrate, with minor modifications [12]. The reaction mixture of 0.1 ml sample, 1.9 ml of 0.1 M carbonate (Na_2CO_3/ $NaHCO_3$) buffer (pH 8.5), and 0.2 ml of 20 mM substrate was incubated for 30 minutes at 37^{o}C. To stop the reaction, 1 ml of 1 M NaOH was added and the absorbance was measured at 405 nm. Under the test conditions, one unit of enzyme specific activity was defined as the quantity of enzyme capable of releasing 1 M p-nitrophenol per minute per mg protein.

2.2.3 Alanine and aspartate transaminases

The modified Wooten [13] method was used to quantify transaminases such as alanine transaminase (ALT) and aspartate transaminase (AST) utilising 2,4-dinitrophenyl hydrazine (DNPH) as a standard. In brief, 0.1 ml of sample was mixed with 1 ml of AST substrate, and the reaction mixture was incubated at 37^{o}C for 60 minutes for the AST test and 30 minutes for the ALT assay before being stopped with 1 ml of DNPH and left for 20 minutes at room temperature. The standard tube was filled with standard pyruvate at a concentration of 0.2 micromoles. After that, 5 ml of 0.4 N NaOH was added to each test tube. A spectrophotometer was used to determine the quantity of pyruvate at 520 nm. The results were represented in milligram of pyruvate per milligram of protein per hour.

2.3 Statistical Analysis

Statistical analysis was made using SPSS software (version 20.0). In brief, each group received duplicates of six mussels, and the findings were given as the mean ± S.E. of six individuals per group. The data were tested for normality and homogeneity using Bartlett's test. Because all of the data was normally distributed, a one way analysis of variance (ANOVA) was used to check if any differences existed between the groups. A p-value < 0.05 was considered significant. The Tukey's multiple comparison post hoc tests' was used to establish the statistical difference between each treatment group.

3. RESULTS

3.1 Impact of Naphthalene Exposure on Acid Phosphatase Activity in *P. viridis*

Acid phosphatise (ACP) activity in the haemolymph, gills, and digestive gland were considerably altered after 28 days of naphthalene exposure (Fig. 1). The greatest concentration (4 mg/L) of naphthalene exposure produced the highest ACP activity in the haemolymph of mussels. The increase of ACP activity in haemolymph was up to 10 folds when the mussels exposed to 4mg/L of naphthalene. The concentration-dependent increase in ACP activity was observed when the compared to the control group. The lowest level of naphthalene exposure increased ACP activity; however this increase was not statistically significant.

After 28 days of naphthalene exposure, ACP activity in mussel gills reduced significantly in all treatment groups (Fig. 1). ACP activity in the mussels exposed to 1 mg/L naphthalene reduced significantly. Similar to haemolymph, gill and digestive gland showed concentration-dependent ACP activity, however in digestive gland and gill it was decreasing trend. Mussels exposed to high sub-lethal concentration of naphthalene (4 mg/L), the ACP activity reduced considerably compared to the respective control group. ACP activity in the digestive gland decreased similarly to that of the gills. The Tukey multiple comparison test demonstrated that there is a statistically significant difference between the treatment and control groups in haemolymph, gills, and digestive gland (Fig. 1).

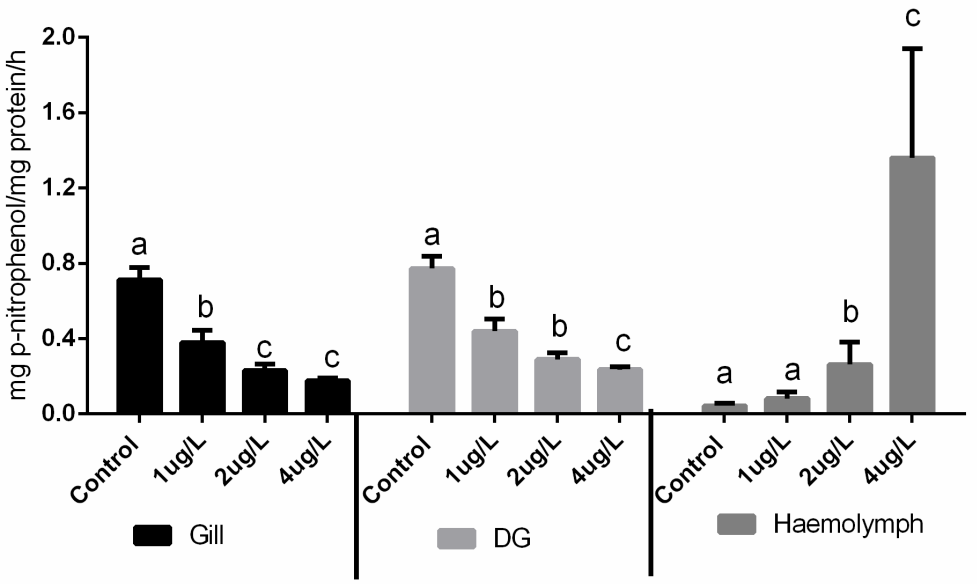

Fig. 1. Acid phosphatase activity in the haemolymph, gill and digestive gland (DG) of mussels exposed to different concentration of naphthalene. Each bar represents mean ± standard deviation of six individuals. Two-way analysis of variance followed by Tukey's post hoc test was used. The same letters (a, b, c) indicate no significant difference between the exposure groups whereas different letters indicate statistically significant differences ($p < 0.05$) between different groups

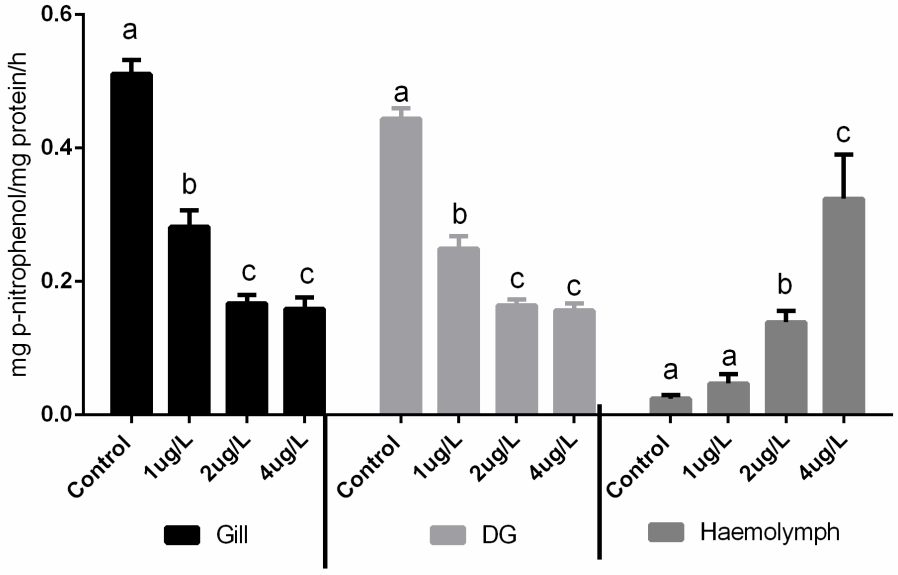

Fig. 2. Alkaline phosphatase activity in the haemolymph, gill and digestive gland (DG) of mussels exposed to different concentration of naphthalene. Each bar represents mean ± standard deviation of six individuals. Two-way analysis of variance followed by Tukey's post hoc test was used. The same letters (a,

b, c,d) indicate no significant difference between the exposure groups whereas different letters indicate statistically significant differences ($p < 0.05$) between different groups

3.2 Impact of Naphthalene Exposure on Alkaline Phosphatase Activity in *P. viridis*

After 28 days of naphthalene exposure, alkaline phosphatise (ALP) activity increased in the haemolymph component, as did ACP activity (Fig. 2). ALP activity was greater in the haemolymph of mussels exposed to highest naphthalene concentration. There is no significant difference between the control and the lowest tested concentration of naphthalene (1 mg/L). At the highest concentration of naphthalene produced up to a fourfold induction. Tukey's multiple comparison tests revealed that there was difference between the 4 mg/L, 2 mg/L treatment groups. Similarly, no difference was seen in the multiple comparison tests between the control and lowest concentration of naphthalene exposed groups (Fig. 2).

ALP activity decreased considerably in all treatment groups in the gills and digestive gland of mussels after 28 days of naphthalene exposure (Fig. 2). In mussel gills exposed to different concentration of naphthalene showed drop in ALP activity and such drop was statistically significant. Similarly the digestive gland showed substantial decrease in ALP activity. Highest concentration of naphthalene tested inhibited the ALP up to three folds and the ALP activity inhibited in digestive gland was concentration dependant.

3.3 Impact of Naphthalene Exposure on Alanine Transaminase Activity in *P. viridis*

After chronic exposure (28 days) of naphthalene to *P. viridis*, the activity of alanine transaminase (ALT) in the haemolymph increased significantly (Fig. 3). ALT activity was greater in the haemolymph of mussels exposed to 4 mg/L of naphthalene. At the lowest concentration tested, ALT does not differ with control group. The increase in ALT in haemolypmh was concentration dependent. ANOVA results revealed that there are significant differences between the groups (Fig. 3).

ALT activity in gill of mussels was significantly inhibited in all treatment groups after 28 days of naphthalene exposure, with the exception of mussels exposed to 1 mg/L of naphthalene concentration (Fig. 3). The decline in ALT activity in mussel gills exposed to 2 mg/L and 4 mg/L was statistically significant. Digestive gland of mussels shows similar trends as observed for the gills. But the ALT activity was predominant in digestive gland compare to gill of mussels; also the level of inhibition was greater in digestive gland compare to gill following naphthalene exposure. It was interesting to see the lowest concentration of naphthalene doesn't show any significant change in ALT activity with respective control group (Fig. 3).

3.4 Impact of Naphthalene Exposure on Aspartate Transaminase Activity in *P. viridis*

Aspartate transaminase (AST) activity in the haemolymph component rose considerably after 28 days of naphthalene exposure (Fig. 4). The haemolymph of mussels exposed to 4 mg/L of naphthalene had higher AST activity than other exposed groups. Up to a six folds induction was detected at the highest concentration (4 mg/L) examined. However, there was no statistically significant difference in AST activity between the control and 1 mg/L naphthalene exposure. Tukey's multi-comparison results show that there are significant differences between the different exposure groups (Fig. 4).

AST activity in gill was decreased considerably in all treatment groups after 28 days of naphthalene exposure (Fig. 4). When the mussels were exposed to 1 mg/L of naphthalene showed decrease AST activity and such decreased was significantly different from control group. In mussel gills exposed to highest concentration (4 mg/L) of naphthalene showed greater inhibition compared with respective control group. Digestive gland of mussels exposed naphthalene showed a strong inhibition of AST activity in all the exposed concentration except 1 mg/L of naphthalene concentration. The inhibition was greater and similar in both the highest concentration test (2 and 4 mg/L) of naphthalene (Fig. 4).

4. DISCUSSION

Phosphatase is a non-specific pH-specific phospho-monoesterase that hydrolyzes different phosphate esters and releases phosphate from the digestive gland's stored matrix under varied physiological conditions [14]. Because acid phosphatase is a lysosomal enzyme that hydrolyzes phosphorus esters in an acidic environment, it seems plausible that this enzyme is functionally hydrolyzed and works as one of multiple acid hydrolases during cell autolysis following apoptosis. Alkaline phosphatase is a brush border enzyme that cleaves different phosphorus esters at alkaline pH. Alkaline phosphatases are widely recognised for their roles in glucose

metabolism, development and differentiation, protein synthesis, enzyme manufacture, secretory activity, and transport to phosphorylated intermediates across cell membranes [15].

Xenobiotic components have been proven to influence phosphate activity. In this study, mussels exposed to naphthalene demonstrated increased haemolymph phosphatase activity as well as reduced digestive and gill functions. The method utilised to maintain a balanced pool of free amino acids during protein synthesis involves transaminases, i.e., transamination involving two kinds of transaminases, alanine amino-transaminase and aspartate amino-transaminase and these enzyme catalyses the inter-conversion of amino acids and keto acids by transferring amino groups. In the amino-transfer process, the -ketoglutarate/L-glutarate pair functions as amino-group acceptors and donors. ALT catalyses the transfer of the amino group from alanine to ketoglutarate to generate glutarate and pyruvate, whereas AST catalyses the transfer of the ammo group from α ketoglutarate to form glutarate and oxaloacetate [16].

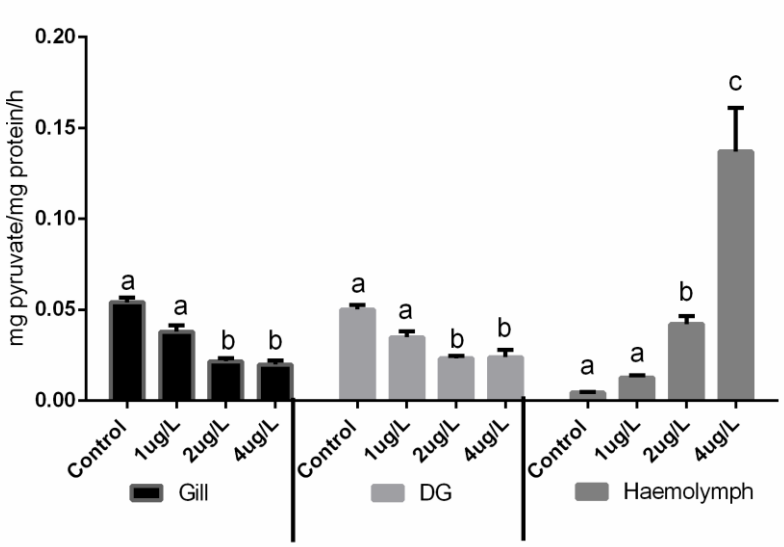

Fig. 3. Alanine transaminase activity in the haemolymph, gill and digestive gland (DG) of mussels exposed to different concentration of naphthalene. Each bar represents mean ± standard deviation of six individuals. Two-way analysis of variance followed by Tukey's post hoc test was used. The same letters (a, b, c) indicate no significant difference between the exposure groups whereas different letters indicate statistically significant differences ($p < 0.05$) between different groups

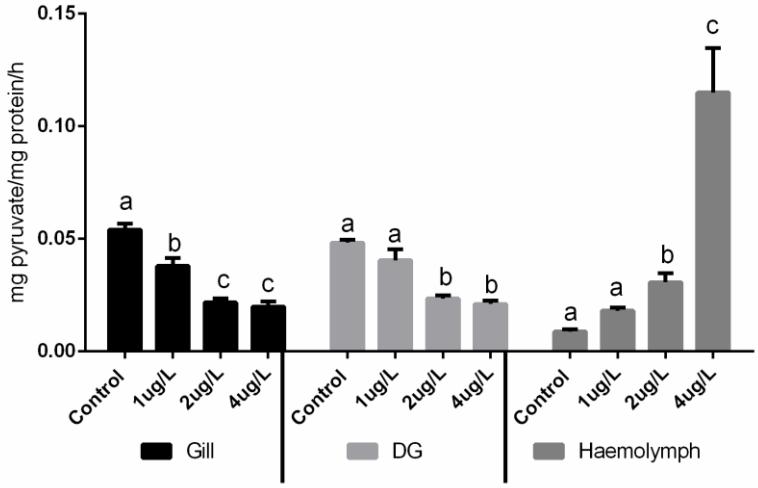

Fig. 4. Aspartate transaminase activity in the haemolymph, gill and digestive gland (DG) of mussels exposed to different concentration of naphthalene. Each bar represents mean ± standard deviation of six individuals. Two-way analysis of variance followed by Tukey's post hoc test was used. The same letters (a,

b, c) indicate no significant difference between the exposure groups whereas different letters indicate statistically significant differences ($p < 0.05$) between different groups

It is well acknowledged that an increase in enzyme activity in extracellular fluid or plasma is a sensitive sign of even mild cellular injury, since the levels of these enzymes grow above normal levels in extracellular fluids [16,17]. As a result, measuring transaminase activity in circulating fluid is commonly employed as a diagnostic technique in water contamination research [18]. The current study found that alanine and aspartate transaminase activity reduced in the digestive gland and gills of mussels exposed to naphthalene while increasing in the haemolymph. The shift in alanine and aspartate transaminase activity might be attributed to changes in protein metabolism in tissues caused by naphthalene stress. The current study's findings are consistent with those of Reddy et al. [19] and Casillas et al. [20], who revealed enzymatic alterations in aquatic animals as a result of diverse chemical stressors.

Because of naphthalene's toxicity, the levels of marker enzymes reduced dramatically in most tissues, demonstrating the substance's severe toxico-pathological impact on mussels. With regard to PAH, marker enzymes are a reliable predictor of stress conditions. Low amounts of naphthalene in the environment can induce stress and even can leads to death in marine biota. It is clear that mechanistic research analysing naphthalene metabolism and disposal should continue in order to gain a better knowledge of its bioavailability for recreational and commercial fisheries, as well as the marine food chain.

5. CONCLUSION

In conclusion the current study demonstrated the time-dependent chronic effects of naphthalene (PAH) on *Perna viridis* haematological and serum biochemical levels. The findings indicated that reactions were both exposure- and dose-specific, and that naphthalene toxicity was harmful to mussels. Analyses of all marker enzyme data revealed that haemolymph biochemical characteristics were more important in determining the effects of naphthalene than tissue-specific factors. Long-term naphthalene exposure resulted in greater negative effects. As a result, these reactions might be considered as a biomarker for monitoring PAH contamination, particularly naphthalene, in aquatic ecosystems.

ETHICAL APPROVAL

For the care and use of animals, all applicable international, national, and/or institutional guidelines were followed.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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