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INDUCTION OF OXIDATIVE STRESS AND MODULATION OF ANTIOXIDANT DEFENSE IN THE HAEMOLYMPH OF GREEN MUSSEL *Perna viridis* EXPOSED TO NAPHTHALENE

J. GOPI [a], K. RUPA NATHIYA [a], R. SANGEETHA [a] AND R. KRISHNAMURTHY [a#*]

[a] PG and Research Department of Zoology& Aquaculture, Government Arts College for men (Autonomous), (Affiliated to University of Madras), Chennai-600 035 India.

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: In recent decades, environmental pollution has occurred from the rapid increase in anthropogenic activities, notably in the aquatic ecosystem. Marine organisms can be subjected to oxidative stress under a variety of severe environmental conditions.
Aim of the Works: The aim of this study is to use a large number of biomarkers to discover individual and unique patterns of *Perna viridis* responses to naphthalene, in order to better understand the changes in oxidative stress and antioxidant defence that occur in the bivalve *P. viridis* after exposure to naphthalene.
Methodology: The mussels were exposed to naphthalene for 14 days in order to evaluate changes in oxidative stress and antioxidant defence. Lipid peroxidation (LPO) and Carbonyls protein (CP) levels were measured for oxidative stress indicators. The antioxidant defence enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were measured as part of the research.
Results: The haemolymph contains significantly higher carbonyls protein and lipid peroxidation following 14 days of naphthalene exposure. A series of antioxidant defence enzymes, including superoxide dismutase, catalase, and glutathione peroxidase were modulated in the haemolymph of mussels exposed to very low levels of naphthalene over 14 days. The activities of antioxidant defense were altered when exposed to naphthalene.
Conclusions: In mussels exposed to naphthalene, the current study discovered a substantial relationship between oxidative stress and antioxidant defences. Overall, the findings suggest that haemolypmh is the most susceptible component to oxidative damage, meaning that antioxidant enzyme activities in *P. viridis* might be used as a bioindicator of organic pollution exposure.

[#]Assistant Professor;
**Corresponding author: Email: professorkrishnamurthy@gmail.com;*

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1. INTRODUCTION

Anthropogenic chemicals from the industrial, agricultural, and household sectors are released into the aquatic environment, causing a variety of reactions in species with a dose-effect relationship [1]. Any incomplete combustion of organic material produces polycyclic aromatic hydrocarbons (PAHs), which are present worldwide as a result of anthropogenic activities. PAHs are becoming more frequent in estuarine and coastal areas as a result of human activities, and they are often found in marine sediment, water, and the tissues of marine species, affecting the normal function of life in marine organisms [2]. PAHs, particularly naphthalene are prevalent environmental contaminants that are of great concern due to their toxicity, mutagenicity, and carcinogenicity [3].

Reactive oxygen species (ROS) may be produced during polycyclic aromatic hydrocarbons (PAH) metabolism [4]. Protein oxidation, enzyme inactivation, and DNA damage are some of the principal consequences of ROS generation in biological systems [5]. The balance between production and breakdown is the steady-state level of ROS, and an imbalance in these activities in favour of the former is referred to as "oxidative stress" [6,7]. In aquatic animals, increased generation of ROS is regarded to be a major mechanism of pollutant-mediated toxicity [8]. Many cellular functions can be disrupted and damaged by oxidative stress, which can lead to cell death. Both molecular and enzymatic (different antioxidant enzymes) antioxidant defences have emerged to combat the potential risks of ROS. The enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) have been identified as a critical interacting line of defence against ROS and their assault products [6]. Antioxidant enzymes are so important for cell homeostasis, and their induction has been proposed as a biomarker of contaminant-mediated oxidative stress in a range of marine organisms, with their induction reflecting a unique response to pollutants [9].

PAH interacts with aquatic species, whether it comes from natural sources or manmade activity. PAHs have been the subject of risk assessments and toxicological investigations by a number of academics [10,11,12,13]. The mechanisms of PAH toxicity in aquatic creatures, on the other hand, are not well understood. Oxidative stress is a well-known mechanism of PAH toxicity [8,14,15,16], and several studies have shown that PAHs change antioxidant activity in mussels [8,14,15,16]. Though, studies on oxidative stress and antioxidant defences in mollusc have been reviewed, the toxicity of naphthalene and its effect on antioxidant enzyme regulation in marine molluscs have been ignored till now.

The objective of this study is to use a large set of biomarkers to identify specific and distinctive patterns of responses of *Perna viridis* to naphthalene to further characterize the changes occurring in several indicators of oxidative stress during naphthalene exposure in bivalve P. *viridis.* Carbonyl protein and lipid peroxidation as indicators of the oxidative modification of lipids in the haemolymph have been investigated. In response to oxidative stress, activities of selected antioxidant response including catalase, superoxide dismutase and glutathione peroxidase were assessed in the haemolymph of mussel exposed to naphthalene.

2. MATERIALS AND METHODS

Green mussels *Perna viridis* weighing 60-10 g were gathered and transported to the laboratory from the sea mouth of the Pulicat Lake near Chennai, Tamil Nadu, India. The mussels were kept in big glass aquaria filled with seawater (60 X 30 X 45 cm) and fed a fresh Chlorella phytoplankton culture. For a week, mussels were acclimatised to laboratory settings and a feeding regimen by changing the water every day. 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.

By using the static renewal method (EPA/ROC, 1998), an acute toxicity (96-hour) study was conducted to estimate the lethal (LC_{100}), median lethal (LC_{50}), and sublethal (LC_0) levels of Naphthalene to *P. viridis*. Naphthalene was prepared as a stock solution in acetone at a concentration of 1 part per thousand (PPT) (HPLC grade). The concentrations of 1, 5, 10, 20, 40 mg L^{-1} were prepared from this stock solution. In 10L glass aquaria and seawater, ten mussels were placed as a group into each concentration, and the test chemical was replaced every day. During the 96-hour bioassay test period, no food was provided. Any dead mussels were removed from the test medium right away. For each concentration, duplicates were carried out. After 96 hours, the percentage mortality of mussels was recorded, and the percent mortality was calculated using probit regression to determine the 96-hour LC_{50} value, as stated by Finney [17].

2.1 Toxicity Test for Biomarkers

Mussels were divided into five groups of ten specimens each to assess changes in biomarkers when

mussels exposed to naphthalene. The mussels in Group I were raised in regular seawater. Mussels in Group II were raised in solvent control. Mussels in groups III to V were exposed to seawater containing 1, 2 and 4 mg L^{-1} Naphthalene, which were the sublethal values at which 0 percent mortality occurred after 96 hours. The studies were conducted in glass aquaria (30" X 25" X 20") with triplicate chambers for each concentration. During the experiment, the test solution and seawater were replaced daily, and the mussels were fed Chlorella. The experiment was ended after 14 days of exposure, and the animals were sacrificed. Using a 23-G needle attached to a sterile plastic syringe with ice-cold TBS, mussels' haemolymph was extracted from the posterior adductor muscle (50 mM tris, 370 mM NaCl; pH 8.4; 840 mOsm). In one ml of iso-osmotic TBS, about 200 µl haemolymph was collected. Haemolymph was quickly mixed with the buffer to avoid haemocyte clumping. The mussel haemolymph samples were not pooled for the experiment.

The carbonyl protein (CP) content was measured using the 2,4-dinitrophenylhydrazine (DNPH) as described by Lushchak et al., [18]. CP was measured spectrophotometrically at 370 nm with a molar extinction value of 10^3 M^{-1} cm^{-1}. Nano moles per unit protein were used to calculate the amounts of CP in the guanidine chloride solution. LPO was determined using Devasagayam and Tarachand's [19] method. The sample's malondialdehyde (MDA) concentration was reported as nmol of MDA created/unit protein, and the colour produced was measured at 532 nm.

The Sinha [20] method was used to calculate CAT activity. When heated in the presence of H_2O_2, dichromate in acetic acid was converted to chromic acetate, with perchromic acid forming as an unstable intermediate. At 570 nm, colorimetric analysis of chromatic acetate was performed. The reaction was allowed to run for various amounts of time before being halted using a dichromate acetic acid solution. Colorimetric measurements of chromic acetate were used to assess how much H_2O_2 was left. The amount of H_2O_2 used per minute per ml of protein was measured. Marklund and Marklund's [21] method was used to determine SOD activity as the degree of inhibition of pyrogallol auto-oxidation at an alkaline pH. The amount of enzyme that suppresses the oxidation reaction by 50% of maximum inhibition is defined as one unit of SOD activity. According to Rotruck et al., [22] method's GPx activity was measured by counting the amount of reduced glutathione (GSH) consumed in the reaction mixture.

2.2 Statistical Analysis

SPSS Version 20.0 software was used to do statistical comparisons. In a summary, duplicates of six mussels were collected for each group, and the results were reported as the mean S.E. of six individuals per group. Bartlett's test was used to assess the data for normality and homogeneity. 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. Significant was defined as a p-value of less than 0.05. The statistical difference between each treatment groups was determined using the Tukey's multiple comparison post hoc test.

3. RESULTS

During an acute naphthalene exposure, survivability of mussels was recorded. Survivability decreased as the concentration of the exposure media increased. At 40 mg/L, about 20% of mussels survived after 96 h, however at 80 mg/L, no mussels survived. The 96-hour LC_{50} value and 95 percent confidence level was calculated using probit analysis (Fig. 1). Mussels subjected to naphthalene had an LC_{50} value of 26.625 mg/L with a 95% confidence limit ranged between 21.22-32.29 mg/L.

After 96 hours of exposure, no mortality was found at 5 mg/L, hence the concentration was used as a sublethal concentration. A long-term exposure was carried out at 1 mg, 2 mg, and 4 mg/L for 14 days. Furthermore, since there is no significant difference between the solvent control and normal seawater control during the biochemical analysis, the normal control data were not used for any comparison. Solvent control was represented as control.

3.1 Carbonyl Protein

Concentrations of Carbonyl Protein (CP) varied among the different concentration of the naphthalene (Fig. 2). CP was present predominantly high in haemolymph of mussel exposed to the highest naphthalene concentration. The CP content in haemolymph was greatest at 4 mg L^{-1} and 2 mg L^{-1} of naphthalene exposure while 1 mg L^{-1} exhibited the least concentration of CP in haemolymph. Both 2 and 4 mg L^{-1} of naphthalene exposure significantly ($p<0.01$) increased the CP content in the haemolymph than the control group.

3.2 Lipid Peroxidation

Exposure to naphthalene resulted in statistically significant oxidative stress as determined by concentrations of lipid peroxidation level (LPO) in haemolymph of mussels (Fig. 3). LPO of haemolymph was greater in mussels exposed to naphthalene after 14 d. The magnitudes of

haemolymph were greater in mussels exposed at 4 mg L^{-1}, 2 mg L^{-1} and 1 mg L^{-1}. All the three exposed concentration showed statistical significant. The increase in LPO level was concentration dependent.

3.3 Superoxide Dismutase

The primary antioxidant and associated enzymes such as CAT, SOD and GPx in the haemolymph of mussels changed after 14 d of naphthalene exposure when compared to control (Figs. 4-6). SOD activities decreased significantly when the mussels exposed to the highest concentration of naphthalene (4 mg L^{-1}). The level of SOD in control mussels was greater after 14 days of exposure compared with other exposure group and the same decreased nearly 50% at the highest concentration of naphthalene exposure (Fig. 4). The decrease in SOD levels were concentration dependent.

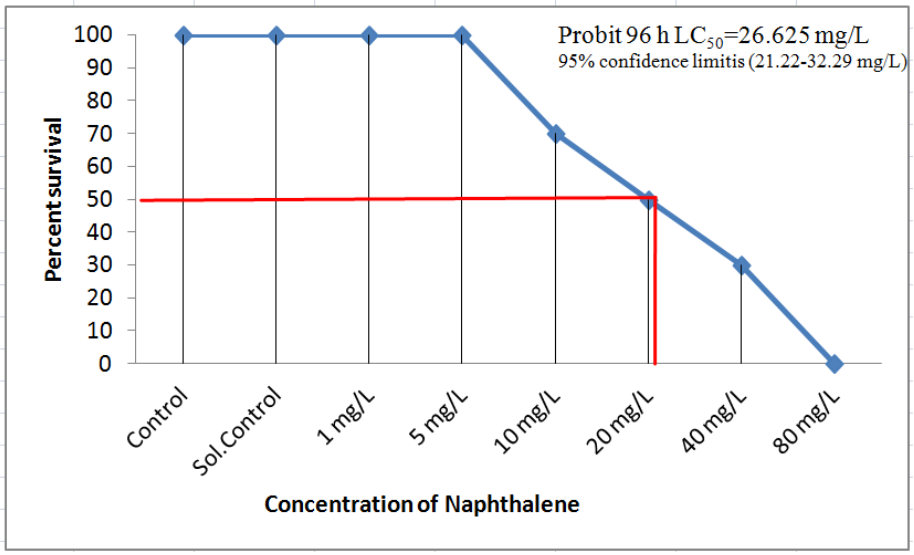

Fig. 1. Percent survival when the mussels exposed to different concentration of naphthalene

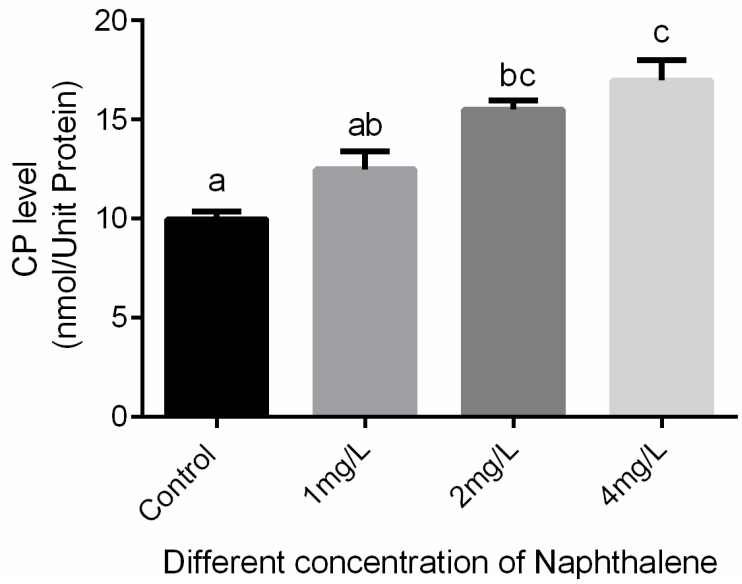

Fig. 2. Carbonyl Protein content in mussels exposed to different concentration of naphthalene. Each bar represents mean ± standard deviation of six individuals. Two-way analysis of variance followed by Tukey's post hoc test was used. The same letters (a, b, c,d) indicate no significant difference between the exposure groups whereas different letters indicate statistically significant differences ($p < 0.05$) between different groups

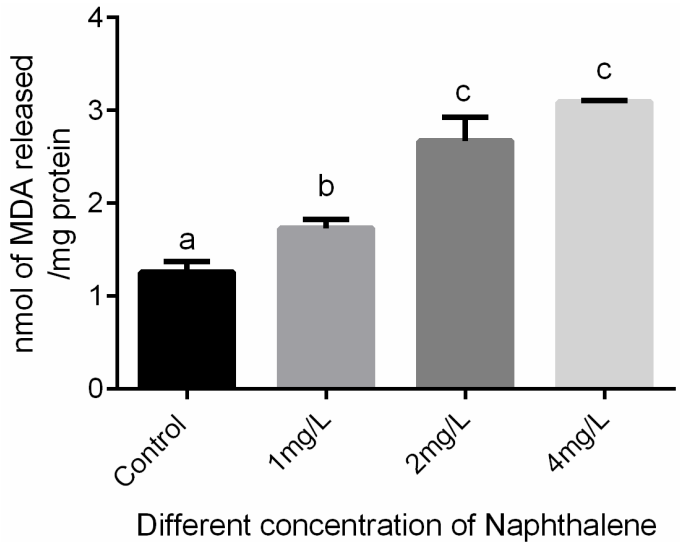

Fig. 3. Lipid peroxidation level in the mussels exposed to different concentration of naphthalene. Each bar represents mean ± standard deviation of six individuals. Two-way analysis of variance followed by Tukey's post hoc test was used. The same letters (a, b, c,d) indicate no significant difference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

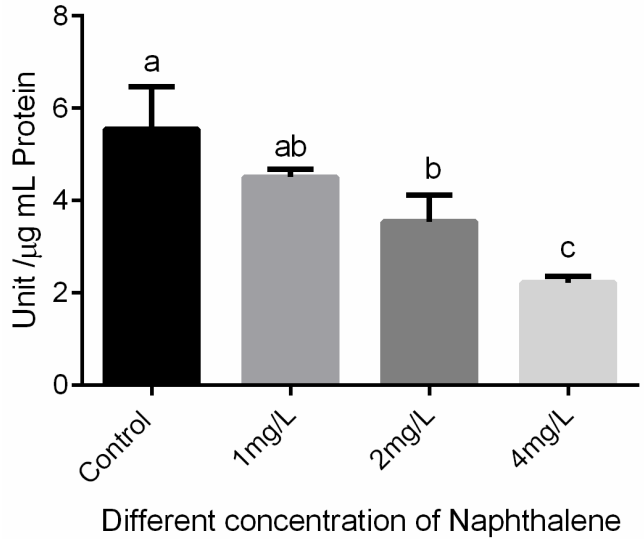

Fig. 4. Super oxide dismutase activity in the mussels exposed to different concentration of naphthalene. Each bar represents mean ± standard deviation of six individuals. Two-way analysis of variance followed by Tukey's post hoc test was used. The same letters (a, b, c,d) indicate no significant difference between the exposure groups whereas different letters indicate statistically significant differences ($p < 0.05$) between different groups

3.4 Catalase

Similar decreasing trend was observed for CAT activity when the mussels were exposed to naphthalene for 14 days. The lowest tested concentration (1 mg L^{-1}) of naphthalene does not show any significant change with respective control group, however the highest concentration decrease the CAT activity predominantly in the haemolymph of mussels when compared to control group (Fig. 5).

3.5 Glutathione Peroxidase Activity

GPx activity significantly reduced in haemolymph of mussels exposed to 4 mg L^{-1} of naphthalene

concentration and the level of GPx activity in haemolymph of mussels decreased gradually as the concentration of naphthalene increased in seawater (Fig. 6).

The Pearson correlation analysis was calculated and given in Table 1. The correlation between individual parameters showed significant (p<0.01; p<0.05) association between the parameters studied. There were good correlations between haemolymph oxidative stress and corresponding antioxidant response (SOD, CAT and GPx) with CP and LPO levels in the mussels exposed to naphthalene. The coefficients of correlation in most cases were greater than 0.850.

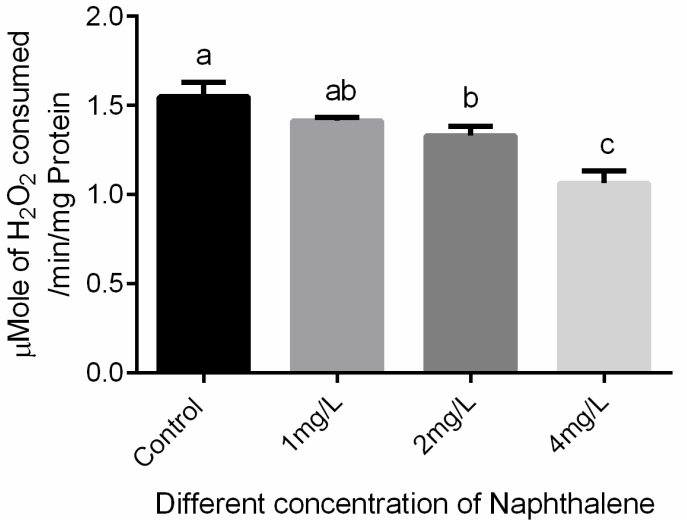

Fig. 5. Catalase activity in the mussels exposed to different concentration of naphthalene. Each bar represents mean ± standard deviation of six individuals. Two-way analysis of variance followed by Tukey's post hoc test was used. The same letters (a, b, c,d) indicate no significant difference between the exposure groups whereas different letters indicate statistically significant differences (p < 0.05) between different groups

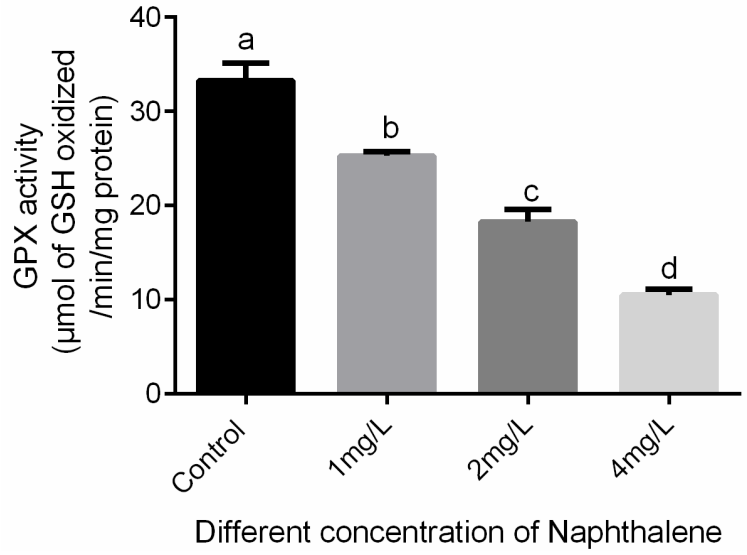

Fig. 6. Glutathione peroxidase activity in the mussels exposed to different concentration of naphthalene. Each bar represents mean ± standard deviation of six individuals. Two-way analysis of variance followed by Tukey's post hoc test was used. The same letters (a, b, c,d) indicate no significant difference between the exposure groups whereas different letters indicate statistically significant differences (p < 0.05) between different groups

Table 1. Correlation analysis between oxidative stress and antioxidant defenses in haemolymph of *P. viridis* naphthalene

	CAT	SOD	GPx	LPO	CP
CAT	1.00	947"	911"	-.867"*	-.839"
SOD		1.00	884"	-.943	-.898"
GPx			1.00	-.902	-.890
LPO				1.00	917"
CP					1.00

4. DISCUSSION

The selected organic pollutant naphthalene has been reported in the coastal region of Chennai and the environmental concentrations of naphthalene in the local coastal area was around 4 μg/L in the surface water and the concentrations used in this study are probably close to environmental relevant levels and such higher concentrations used in the animals tested are quite possibly caused by bioaccumulation. It is obvious from the present study that exposure of mussels to sublethal concentration of naphthalene not only influence the oxidative stress parameters but also the antioxidant enzymes in haemolymph of mussels. The present study agrees with previous study by Vijayavel et al., [23] that naphthalene produce oxidative stress and antioxidant defence

Protein carbonylation causes protein oxidation. When a protein is damaged by reactive oxygen species (ROS), it generates carbonyl, which is irreversible and causes a conformational shift [24]. Enzyme catalytic activity will be reduced as a result of the change, resulting in protein breakdown [25]. Protein carbonyl levels of 3–10 nmol carbonyl/mg protein were found to be greater in the current investigation than those seen in mammals under normal, non-disease conditions, which are in the range of 1–4nmol carbonyl/mg protein [26]. Severely oxidised proteins can be stabilized by aggregation, cross-linking, and decreased solubility [27]. The most severe of the possible effects is likely to be a rise. However, the extent to which such levels are unlikely to have a negative impact on mussel survival must be determined. Several investigations have shown that LPO levels can rise in a variety of tissues of organisms exposed to various environmental circumstances [28]. The increased quantity of LPO in the haemolymph of mussels exposed to OA corresponds to a lower pH. Malonaldehyde, a product of the LPO, has been shown to be extremely reactive and a key mediator of DNA damage.

SOD is an important antioxidant enzyme and the first to scavenge superoxide radicals (O_2-), whereas CAT is in charge of detoxifying large amounts of H_2O_2 generated as a result of the SOD-catalyzed process. The reduced SOD and CAT activity seen in this study could be due to ROS produced during PAH metabolism. The increased formation of superoxide anion radical, which has been shown to impede CAT activity, can also be blamed for the decrease in CAT [29].

Oxyradicals paired with H_2O_2 can create hydroxyl radicals, resulting in a rise in LPO, according to the Haber–Weiss reaction. These greater levels of oxidative stress markers compensate for higher antioxidant levels in mussel haemolymph, lowering oxidative stress. Reduced levels of antioxidant enzymes like SOD, CAT, and GPx in haemolymph could reflect the animal's adaptive response to oxidative stress caused by Naphthalene exposure. Due to oxidative stress caused by naphthalene, glutathione depletion is common in the haemolymph. Reduced glutathione levels could be due to direct interactions with PAH compounds or increased glutathione consumption as a ROS scavenger. The activity of GPx was reduced in mussels exposed to naphthalene for 14 days in the present study reveals that the mussels are under stress due to naphthalene. GPx and GSH are essential cellular antioxidants that help protect cell components from ROS/RNS-mediated damage. As a result, suppression or depletion of such antioxidant defences in mussels exposed to naphthalene may enhance their sensitivity to lipid peroxidation. Furthermore, following 14 days of naphthalene exposure, there was a significant decrease in GPx activity with a commensurate increase in MDA levels.

Some research has looked into the effects of xenobiotic exposure on antioxidant enzyme activity in marine invertebrates [30,31,32,33]. These investigations found that antioxidant enzymes can rise in low toxicant concentrations, but can decline or even be inhibited when time is extended or dosage is increased. In our research, we discovered that at all concentrations, the antioxidant enzyme activity in the haemolymph of mussels studied decreased, indicating an increase in the formation of oxygen free radicals (O_2^- and H_2O_2). Antioxidant enzyme activity was also significantly varied in the haemolymph examined, implying that naphthalene has diverse physiological functions and reactions.

During the last decade, the importance of studying the consequences of xenobiotic stress in aquatic organisms has grown, particularly in species raised for human consumption. Indeed, studying stress responses in aquatic animals, particularly those related to oxidative stress (antioxidant defence systems), can provide valuable information that can be used to assess environmental quality. After 14 continuous exposures to a sublethal quantity of naphthalene, our findings show that oxidative stress and regulation of antioxidant and related enzymes such as GPx, SOD, and CAT occur in the haemolymph of *P. viridis.* As a result, employing biomarkers assessed in haemolymph, the bivalve *P. viridis* could be utilised as a bioindicator to assess PAH pollution.

5. CONCLUSION

In conclusion, invertebrates' haemolymph component and features are important indicators of health and stress because they have representative cells (haemocytes) that play an important role in internal defence. The measurement of haemolymph function could reveal information about the health of the host. As a consequence of this research, we can conclude that exposing *P. viridis* to naphthalene significantly increases reactive oxygen species in mussels, which may have an indirect influence on scavenging antioxidant activities, as indicated by a significant increase in all oxidative stress indicators. Inducing oxidative stress in the host may also result in the creation of free radicals, which may have an impact on the host's health when confronted with pathogenic microbes. Overall, our findings back with the theory that naphthalene is the main cause of oxidative stress in mussel haemolymph.

ETHICAL APPROVAL

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

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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