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OCEAN ACIDIFICATION INDUCED OXIDATIVE STRESS MODULATE TOTAL ANTIOXIDANT CAPACITY IN THE HAEMOLYMPH 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 and Aims: The global chemistry of seawater is changing due to the uptake of carbon dioxide (CO_2) by ocean surface waters. Marine organisms can be exposed to oxidative stress under a variety of severe environmental conditions including Ocean Acidification (OA). The purpose of this investigation is to study the effects of ocean acidification on *Perna viridis* using indicators of oxidative stress and to better understand how oxidative stress affects total antioxidant capacity.
Methodology and Results: Mussels were subjected to acidic seawater at pH levels of 8.1, 7.7, 7.4, and 7.1 for 30 days to assess oxidative stress and total antioxidant capacity (TAC). The protein's carbonyl, malondialdehyde, and thiol groups were evaluated as markers of oxidative stress. Antioxidant defense was evaluated as total antioxidant capacity. Fifteen and thirty days after ocean acidification, mussel blood lymph showed significant induction of carbonyl proteins, lipid peroxides, and thiol groups. After 30 days of exposure to OA, the total antioxidant capacity (TAC) of the mussels haemolymph was significantly modulated.
Conclusion: Current studies have shown a strong association between oxidative stress and total antioxidant capacity (TAC) in mussels exposed to ocean acidification. Overall, the data suggest that haemolypmh is the most susceptible component to oxidative damage, it also suggests that the entire antioxidant capacity of *P. viridis* can be used as a biomarker for ocean acidification (OA).

Keywords: Total antioxidant capacity; oxidative stress; carbonyl protein; haemolymph; ocean acidification and green mussels.

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1. INTRODUCTION

Environmental influences stress whole ecosystems by modifying their structures, functions, and diversity at different levels of organisation, such as the number of species, genetic characteristics, and the complexity of ecosystems and landscapes. Since ecosystems are under pressure from a variety of factors that are not always simple to detect, an assessment of the environmental quality of coastal waters in terms of water chemistry falls short of its objectives. Over the previous two centuries, intensive fossil-fuel burning and deforestation have raised atmospheric CO_2 levels by over 40% beyond preindustrial levels. Ocean acidification has occurred as a result of these CO_2 emissions [1]. Hydrogen ion concentration (pH) is one of the most important determinants of biological response, and it's crucial to understand how pH is changing in the context of current natural variability and future global changes. The partial pressure of CO_2 in the atmosphere rises as it diffuses passively into ocean surface water, as a result of changes in carbonate chemistry, the drops in seawater pH leads to ocean acidification [2]. And thus continuous CO_2 emissions from anthropogenic activities such as fossil fuel combustion and deforestation have resulted in worldwide ocean acidification [3].

Changing ocean conditions are thought to represent a serious hazard to marine species, influencing early development, skeletal growth, and important physiological processes, which can affect animal behaviour and distribution [4,5]. Decreased ocean pH has been shown to affect a variety of calcified organisms such as corals, mollusks and echinoderms, causing a variety of biological responses [6,7]. Ocean acidification can interact with other environmental factors in complicated ways, and it is critical to understand the potential consequences. Furthermore, exposure length and stress level might impact antioxidant enzyme activity [8], and oxidative stress biomarkers in clams (*Mercenaria mercenaria*) and oysters (*Crassostrea virginica*) were significantly affected by varied exposure periods [9]. As a result, mussels may react to environmental influences differently depending on when they are exposed.

When an organism is exposed to environmental stress, a biomarker, also known as a stress indicator, acts as a warning system. In general, biomarkers can be a powerful tool for detecting a wide variety of diseases in organisms, both positive and negative. Biochemical components and enzymes have been studied as potential biomarkers in various animals [10,11]. They are the first detectable/quantifiable responses to environmental changes and can serve as indicators for both exposure and effect in organisms, making it critical to understand how seasonal variation modulates physiological implications in marine and estuarine species.

The imbalance in the amount of free radicals and antioxidants is known as oxidative stress. In addition to the relatively common contaminants, several studies have shown that environmental factors can influence the formation of Reactive oxygen species (ROS) and free radicals, exceeding the antioxidant defense's protective capability and leading to oxidative stress [12]. Changes in ROS levels in the body are directly related to oxidative stress biomarkers [13]. Oxidative stress can lead to lipid peroxidation, protein carbonyl production, and enzyme inactivation when antioxidant defences are compromised or overcome. Oxidative stress is caused by three factors: increased production of oxidants, decreased antioxidant defenses, and the ability to repair oxidative damage. Oxidative stress can lead to DNA, protein and lipid damage and decreased antioxidant defenses.

Marine bivalves are the most commercially important species in aquaculture. Hypoxia, heat stress, and ocean acidification all pose dangers to this species' survival. *Perna viridis*, a thick-shelled mussel, is found in abundance throughout the subtropical coast. This mussel lives in coastal habitats, adhering to hard surfaces, forming huge subtidal beds, and playing a significant role in coastal systems. This mussel is regarded as an important aquaculture species in the majority of Asian countries. Despite its economic and ecological importance, there is a scarcity of knowledge on the effects of global environmental stressors like acidification. Although OA is recognized as serious threats to marine life, little information exists on oxidative stress due to long term effect of OA on green mussel and hence present study was designed to observe the chronic effect of OA on the oxidative stress and the corresponding antioxidant response in haemolypmh of *P. viridis.*

In the present investigation, different pH were selected to study their effect on oxidative stress of *P. viridis*, namely carbonyl protein (CP), lipid peroridation in terms of MDA (Malondialdehyde), high and low thiol levels in the haemolymph of mussels. To assess the extent of oxidative stress induced by OA, sublethal pH values at environmentally realistic levels were tested and the total antioxidant capacity was determined. The results indicate that *P. viridis* may be a suitable indicator for detecting oxidative stress caused by OA.

2. MATERIALS AND METHODS

2.1 Animal Collection

Mature *Perna viridis* mussels weighing 60-10 g were collected and brought to the laboratory from the sea mouth of the Pulicat Lake in Chennai, Tamil Nadu, India. The mussels were kept in large seawater-filled glass aquaria (60 X 30 X 45 cm) and fed a fresh phytoplankton culture. The mussels were acclimatised to laboratory conditions and a feeding regimen for a week and the water was changed daily. Dissolved oxygen (5–7 mg/L), salinity (30 ppt), temperature (28^oC), and pH (8.1) were the holding conditions in the tank. A 14:10 h light-dark cycle was used to provide illumination.

2.2 Experimental Design

The consequences of ocean acidification were studied by reducing the pH of seawater and subjecting mussels to three distinct pH situations, including a control pH of 8.1 and three pH levels expected under different climate change scenarios: 7.7, 7.4, and 7.1 are the three levels of severity. By bubbling CO_2 gas into the seawater medium, the pH of the seawater was decreased. Totally six mussels were exposed in each glass tank and duplicate chamber was maintained for each group. The impacts of seawater acidification were assessed by exposing the mussels for 30 days. Sampling was done after 15 days and 30 days of exposure three mussels from each tank (six individuals per group) was taken and sacrificed for the analysis. Throughout the experiment, the mussels were fed with chlorella sp. To minimise fluctuation, the temperature and salinity were maintained and measured daily with a probe, and the pH of seawater was recorded twice a day using a carefully calibrated pH metre. Using a 23-G needle connected 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, 200 µl of haemolymph were collected. Haemolymph was quickly mixed with the buffer to prevent haemocyte clumping. For the purposes of the investigation, mussel haemolymph samples were not combined.

2.3 Analysis of Oxidative Stress in the Haemolymph of Mussels

The carbonyl (CP) content of proteins was measured using the 2,4-dinitrophenylhydrazine (DNPH) process described by Lushchak et al. [14]. 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. Lipid peroxidation (LPO) was determined using Devasagayam and Tarachand's [15] method and expressed in terms of malondialdehyde (MDA) levels as nmol of MDA created/unit protein. As previously stated, free thiols were quantified using the Ellman [16] method with DTNB as described in Lushchak and Bagnyukova [17]. The thiol content was calculated using a molar extinction value of 1.4 X 10^{-3} M^{-1} cm $^{-1}$ and absorption measured at 412 nm. The concentrations of L-SH and H-SH thiols were expressed as micromoles of SH- groups per unit protein.

2.4 Analysis of Total Antioxidant Capacity in the Haemolymph of Mussels

Total antioxidant capacity (TAC) was determined by following the methodology by Kaloyianni et al., [18]. Briefly TAC is determined by the antioxidants in an organic or inorganic liquid decolorizing the 3,3′,5,5′ tetramethylbenzidine (TMB) cation. One TMB tablet was dissolved in 10 mL phosphate citrate buffer (0.05 mM, pH 5) to make the TMB solution (substrate buffer). The TMB solution was combined with 50 µL of newly made strong oxidant, ammonium persulfate (0.4 percent), incubated for 5 minutes at room temperature, and then utilised right away. In each well 10 µL of each sample in duplicate, standard, or blank (distilled water) were mixed with 200 µL of the above working solution, and the plate was incubated in a dark area at room temperature for 3–4 minutes. Each well received 50 µL of 2 N HCl, and the plate was incubated in the dark for 45 minutes. Finally, using an ELISA reader, the plate was measured at 450 nm and the absorbance of the samples and standards were subtracted from the blank absorbance. Serial dilutions of uric acid standard were used to create a standard curve. The results represent the means and standard deviations of six individuals in µM uric acid.

2.5 Statistical Analysis

Two-way analysis of variance (ANOVA) performed using SPSS Version 20.0 software for statistical comparisons. In brief, duplicates of three mussels were collected for each group, and the results were reported as the mean 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 two-way analysis of variance (ANOVA) was used to check whether any differences existed between the groups. Significant was defined as a p-value < 0.05. To establish the statistical difference between different treatment groups, the Tukey's multiple comparison post hoc tests were performed.

3. RESULTS

3.1 Carbonyl Protein Levels in Mussels Exposed to Ocean Acidification

Carbonyl protein (CP) content concentrations vary depending on the pH of the exposure medium (Fig. 1). After both time points, CP was found in greater levels in the haemolymph of mussels subjected to the lowest pH. After 15 days of exposure, the highest levels of CP in haemolymph were found between pH 7.1 and 7.4, whereas the lowest concentrations were found at pH 7.7. When the mussels were exposed for 30 days, the results were significant. All of the CP levels were significantly higher (p<0.01) in the experimental group compared to the respective control group (pH 8.1).

3.2 Lipid peroxidation Levels in Mussels Exposed to Ocean Acidification

Malondialdehyde (MDA) levels in the mussels' haemolymph component were used to determine lipid peroxidation levels. OA exposure caused a statistically significant increase in oxidative stress in mussels, as measured by MDA levels in their haemolymph (Fig. 2). After both exposure periods, the LPO levels of haemolymph were higher in mussels subjected to OA. In mussels exposed to lower pH and for longer exposure period haemolymph magnitudes were higher for MDA content. Although the LPO level in mussels exposed to pH 7.7 increased after 15 days, there was no statistically significant difference when compared to the respective control group. On contrary the LPO level showed a significant increase in the entire exposed group including pH 7.7.

3.3 Low Thiol Levels in Mussels Exposed to Ocean Acidification

Low thiol (L-SH) levels in mussel haemolymph increased with decreasing pH. After 15 and 30 days of exposure, greater level of L-SH was found in mussel haemolymph subjected to 7.1 pH, while the least L-SH was found in pH 7.4 and 7.7 pH (Fig. 3). When compared to the control group L-SH content in haemolymph was significantly higher at the lowest 7.1 pH evaluated, and the increase in L-SH level was significantly higher in the exposure group subjected to lowest pH 7.1 after 30 days of exposure.

3.4 High Thiol Levels in Mussels Exposed to Ocean Acidification

Similar to low thiol, high thiol (H-SH) level in the mussels' haemolymph increased across the pH tested. After 15 and 30 days of exposure, H-SH content was predominantly found high in haemolymph of mussels subjected to the lowest pH (7.1), in comparison to the corresponding control groups. However the H-SH content of mussel haemolymph does not showed any difference when the mussels exposed at pH 7.7 for both 15 and 30 days of exposure (Fig. 4).

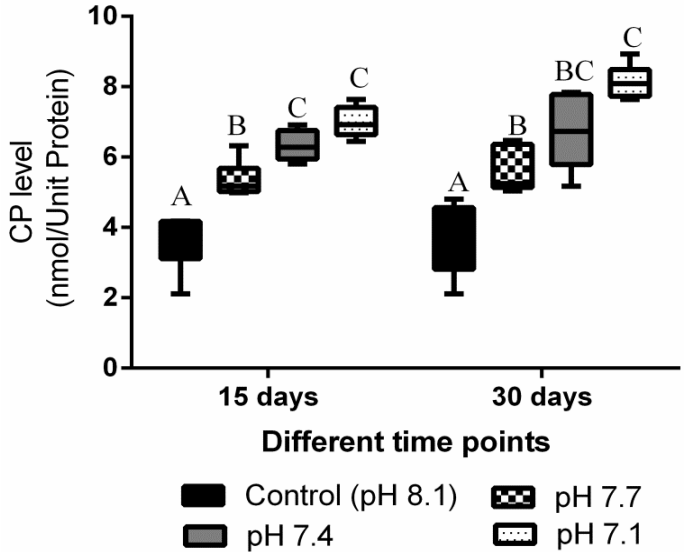

Fig. 1. Carbonyl protein content in mussels exposed to Ocean acidification. The line in each box represent median and whiskers represents the upper and lower 95% confidence intervals of the mean. Two-way analysis of variance followed by Tukey's post hoc test was used. The same letters (A, B, C) indicate no significant difference between the exposure groups whereas different letters indicate statistically significant differences (p < 0.05) between different groups

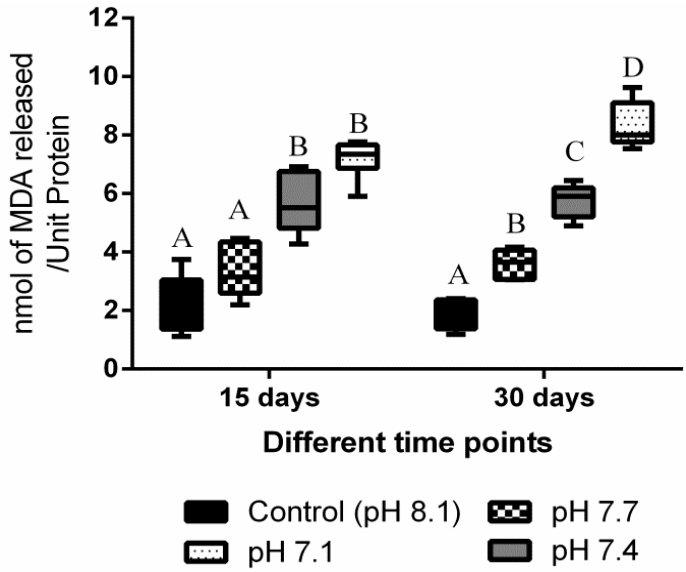

Fig. 2. Lipid peroxidation level in mussels exposed to Ocean acidification. The line in each box represent median and whiskers represents the upper and lower 95% confidence intervals of the mean. Two-way analysis of variance followed by Tukey's post hoc test was used. The same letters (A, B, C,D) indicate no significant difference between the exposure groups whereas different letters indicate statistically significant differences ($p < 0.05$) between different groups

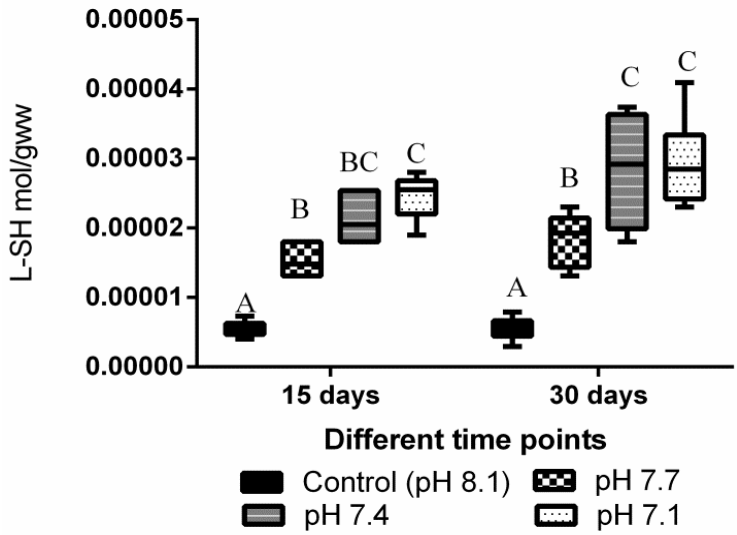

Fig. 3. Low thiol group levels in mussels exposed to Ocean acidification. The line in each box represent median and whiskers represents the upper and lower 95% confidence intervals of the mean. Two-way analysis of variance followed by Tukey's post hoc test was used. The same letters (A, B, C) indicate no significant difference between the exposure groups whereas different letters indicate statistically significant differences ($p < 0.05$) between different groups

3.5 Total Antioxidant Capacity in Mussels Exposed to Ocean Acidification

Total antioxidant capacity (TAC) was determined in mussels' haemolymph exposed to various pH levels and shown to be considerably reduced during the exposure period (Fig. 5), with a rapid decline in the TAC of haemolymph of mussels exposed for 30 days. After 15 and 30 days of exposure, the lowest pH showed a severe decline, and the longest duration indicated a larger reduction in TAC content and moreover the observed TAC variations were concentration dependent.

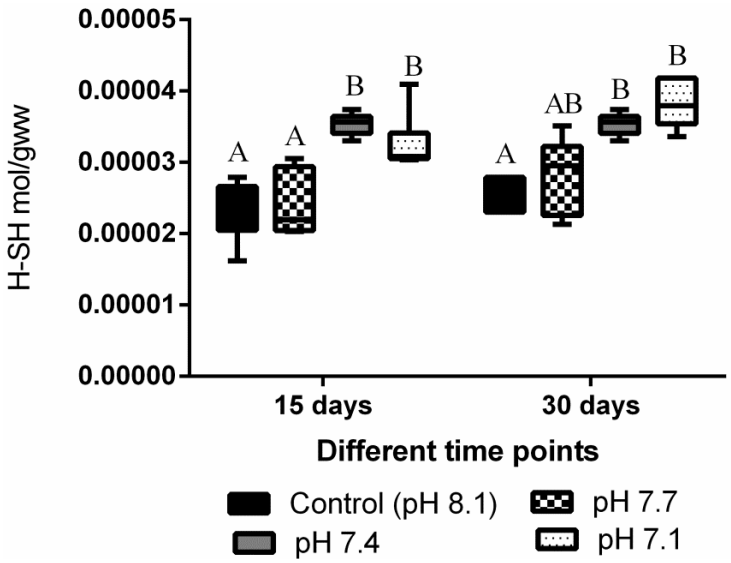

Fig. 4. High thiol group levels in mussels exposed to Ocean acidification. The line in each box represent median and whiskers represents the upper and lower 95% confidence intervals of the mean. Two-way analysis of variance followed by Tukey's post hoc test was used. The same letters (A, B, C) indicate no significant difference between the exposure groups whereas different letters indicate statistically significant differences ($p < 0.05$) between different groups

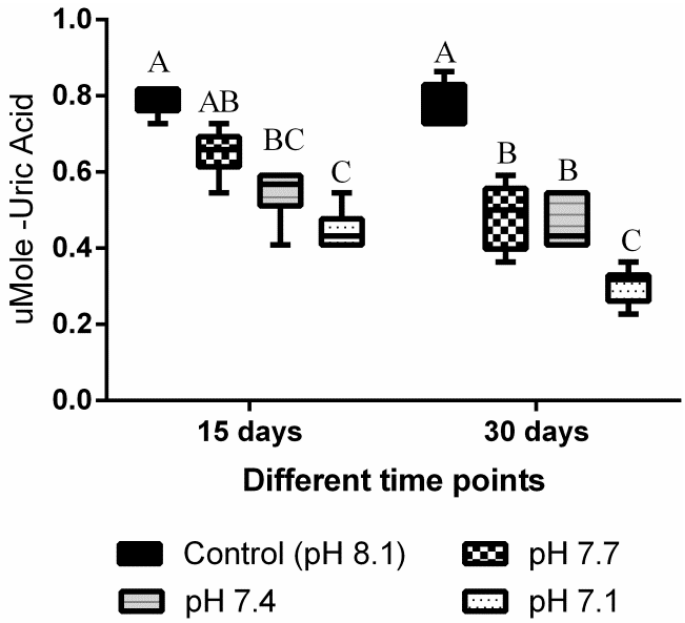

Fig. 5. Total antioxidant capacity (TAC) in mussels exposed to Ocean acidification. The line in each box represent median and whiskers represents the upper and lower 95% confidence intervals of the mean. Two-way analysis of variance followed by Tukey's post hoc test was used. The same letters (A, B, C) indicate no significant difference between the exposure groups whereas different letters indicate statistically significant differences ($p < 0.05$) between different groups

Spearman's Correlation coefficient analysis between antioxidant and oxidative stress measures revealed a strong negative relation between TAC and oxidative stress associated parameters in the haemolymph of mussels exposed for 15 days (Table 1). There is high relationship between the oxidative stresses and scavenging total antioxidant, these relationships revealed the stress developed in the organism due to OA and the total antioxidant response against the stress.

Table 1. Spearman correlations analysis between the oxidative stress and total antioxidant response of the mussels exposed to OA for 15 days

Spearman's rho			TAC15d	CP15d	LPO15d	HSH15d	LSH15d
	TAC15d	Correlation Coefficient	1.000	-.904**	-.912**	-.670**	-.813**
		Sig. (2-tailed)	.000	.000	.000	.000	.000
		N	24	24	24	24	24
	CP15d	Correlation Coefficient		1.000	.892**	.688**	.865**
		Sig. (2-tailed)		.	.000	.000	.000
		N		24	24	24	24
	LPO15d	Correlation Coefficient			1.000	.704**	.806**
		Sig. (2-tailed)			.	.000	.000
		N			24	24	24
	HSH15d	Correlation Coefficient				1.000	.638**
		Sig. (2-tailed)				.	.001
		N				24	24
	LSH15d	Correlation Coefficient					1.000
		Sig. (2-tailed)					.000
		N					24

***. Correlation is significant at the 0.01 level (2-tailed)*

TAC- Total antioxidant capacity, CP- Carbonyl Protein, LPO- lipid peroxidation, HSH-High thiol, LSH-Low thiol

Table 2. Spearman correlations analysis between the oxidative stress and total antioxidant response of the mussels exposed to OA for 30 days

Spearman's rho			TAC30d	CP30d	LPO30d	HSH30d	LSH30d
	TAC 30d	Correlation Coefficient	1.000	-.851**	-.881**	-.762**	-.740**
		Sig. (2-tailed)	.000	.000	.000	.000	.000
		N	24	24	24	24	24
	CP 30d	Correlation Coefficient		1.000	.894**	.697**	.791**
		Sig. (2-tailed)		.000	.000	.000	.000
		N		24	24	24	24
	LPO 30d	Correlation Coefficient			1.000	.792**	.820**
		Sig. (2-tailed)			.000	.000	.000
		N			24	24	24
	HSH30d	Correlation Coefficient				1.000	.761**
		Sig. (2-tailed)				.000	.000
		N				24	24
	LSH 30d	Correlation Coefficient					1.000
		Sig. (2-tailed)					.000
		N					24

***. Correlation is significant at the 0.01 level (2-tailed)*

TAC- Total antioxidant capacity, CP- Carbonyl Protein, LPO- lipid peroxidation, HSH-High thiol, LSH-Low thiol

Similar significant relation was observed for 30 days exposure of mussels to varying pH levels (Table 2). However the coefficient value exhibited between the total antioxidant and oxidative stress was relatively high compared with 15 days of exposure. A positive relationship observed between the oxidative stress parameters revealing that all the stress parameters were interdependent with each other.

4. DISCUSSION

Studies on oxidative stress caused by OA have been conducted, with the majority focusing on short-term exposure or specific mussel tissues. The current study looked at the effects of chronic OA exposure on oxidative stress and total antioxidant responses in mussel haemolymph for the first time. In contrast to the previous study, this one looks at the effects of changing pH on oxidative stress in mussel haemolymph in response to total antioxidant response with periodic variations. Environmental stressors that generate ROS via the mitochondrial electron transport chain are known to produce oxidative stress [19]. In the current study, the formation of free radicals in response to OA increased as seawater acidification increased, and these free radicals should be scavenged by various antioxidant systems as a protective reaction to detoxify the ROS formed by these free radicals.

ROS are small, short-lived molecules that are created naturally as by-products of aerobic respiration and metabolism. Antioxidants are necessary for aerobic living because they control the production of ROS. Environmental factors, such as phase I enzymes that can oxidise xenobiotics and other stressors to make them more polar, enabling them to be conjugated and excreted, can cause adaptive responses in aquatic organisms; however, this process involves redox reactions that can generate free radicals, particularly ROS, which influence physiological functions [20]. After 15 and 30 days of OA exposure, oxyradicals may involved in the production hydroxyl radicals through the Haber–Weiss reaction, resulting in more LPO. Higher levels of oxidative stress indicators have been connected to a compensatory response from antioxidant levels in the mussel haemolymph to lower oxidative stress. However the decrease in these productions of total antioxidant capacity could happen in response to oxidative stress induced by OA, on the other hand, has to be examined further.

Lipid peroxidation causes changes in membrane phospholipids, which is one of the most critical mechanisms in oxidative damage [21]. In this investigation, MDA levels were shown to be greater in the haemolymph of mussels subjected to OA. MDA levels in the haemolymph did not decrease as the number of exposure days rose. Other studies that looked at the induction of peroxidative processes in mussels exposed to pollutants found similar results to the present study [22].

In comparison with previous research, the current work demonstrates a novel technique for determining total oxyradical scavenging capability in the mussel *P. viridis* haemolymph as a new biomarker of biological resistance to oxidative stress caused by OA [23]. The diverse processes involved in radicale antioxidant responses may explain each mussel haemolymph's relative radical scavenging capacity against different testing radicals. Mussels that were exposed to OA showed a greater intracellular oxyradical flow. Some of the methods involved in antioxidants' beneficial effects in biological systems include direct quenching of free radicals to terminate the radical chain reaction and operate as reducing agents, or activating antioxidative defence enzyme activities.

In this study, protein-bound carbonyl groups in the mussels' haemolymph were evaluated as a stress indicator. Carbonyl protein levels in OA-exposed haemolymph may be higher in relation to oxidative damage due to their ability to directly increase protein carbonyl formation in response to OA-induced stress. Furthermore, the current study's findings are similar with previous research; however, previous research has focused on the potential for contaminants to cause ROS formation. When a protein is damaged by ROS, carbonyl is generated, which is irreversible and results in a conformational shift [24]. As a result of the modification, enzymatic activity will be lowered, resulting in protein degradation [25]. In the current study, carbonyl protein levels were shown to be increased over both exposure periods. Aggregation, cross-linking, and reduced solubility can all help to stabilise severely oxidised proteins [26]. An increase is expected to be the most severe of the probable consequences. However, it must be assessed to what extent such levels are unlikely to have a negative impact on mussel survival.

Free thiols can protect cells from ROS by changing redox-sensitive activities. Because they are involved in the detoxification of H_2O_2, various peroxides, and free radicals, thiol groups are thought to be the most important defence against ROS. When thiol compounds are oxidised, they lose some of their biological function. In the current investigation, the levels of thiol groups in the haemolymph of mussels exposed to OA followed a similar trend to other oxidative stress indicators. Greater levels of the thiol group in mussels exposed to varied pH values indicate the existence of OA-induced stress. Lagadic et al. [27] stressed the importance of detecting many biomarkers in the same organism at the same time, as this allows for a more accurate assessment of the effects of stress induced by environmental variables or toxins on individuals. Despite the need for more research and validation of findings, as well as a better understanding of the true physiological importance of some of these indications, other biomarkers are being used. This multiparametric technique will examine the

effects of multiple stress-related components present in the aquatic environment by using different and/or complementary biomarkers.

Enzymatic antioxidants are more effective at protecting against active and massive oxidative stress because of their ability to break down ROS. As a result, these antioxidants are critical in the fight against ROS, and most studies that use antioxidant response for oxidative stress use single components like SOD, CAT, GPx, and GST, as well as smaller molecules like vitamin E and -carotene, ascorbic acid, and uric acid to estimate the antioxidant system's efficiency. Because it is impossible to measure individual antioxidant molecules and their antioxidant effects are cumulative, new approaches were needed to provide a more holistic perspective of oxidative stress susceptibility in exposed species. In this line, an unique technique (total antioxidant scavenging capacity, TOSC) has been utilised in a number of field and laboratory investigations to predict species' vulnerability to oxidative stress [28,29]. Similarly, Alamdari et al. [30] used this technique to investigate the antioxidant response of mussels to various pollutants and were the first to measure total antioxidant capacity in mussels.

The TAC test evaluates antioxidants' ability to protect against the oxidative effects of ROS as prooxidants [31]. The significant decline in TAC value in OA-exposed mussels demonstrated in this study indicates that antioxidant defences were weakened, most likely as a result of oxidative stress induced by OA exposure. In mussels exposed to diverse contaminants with decreased antioxidant activity have been reported in other studies [29,32]. The decline in antioxidant capacity, however, was not linear with time or pH concentrations, as no significant differences in haemolymph of mussels exposed to different pH for 15 days were seen. It is possible that the antioxidant capacity of the haemolymph of mussels is destroyed only when the degenerative process causes specific levels of oxidative stress. In the case of mussels exposed to different doses of AO used in this study, antioxidant capacity was time dependent.

5. CONCLUSION

Since invertebrates have representative cells (haemocytes) that play an important role in internal defenses, the components and properties of haemolymph are important indicators of stress and health. Assessment of haemolymph function can provide information about the health of the host. From the results of this study, we can conclude that the effect of OA on *P. viridis* increases the amount of ROS in mussels, which may have an indirect effect on antioxidant activity, which is significant for all markers of oxidative stress, which has been confirmed by significant increase. In conclusion, this study highlighted the link between oxidative stress and potent antioxidant responses and the possible effects of oxidative stress and its components on animal scavenging system

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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