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Integrated biomarker response to assess toxic impacts of iron and manganese on deep-sea mussel *Gigantidas platifrons* under a deep-sea mining activity scenario*

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Abstract Deep-sea mining activities can potentially release metals, which pose a toxicological threat to deep-sea ecosystems. Nevertheless, due to the remoteness and inaccessibility of the deep-sea biosphere, there is insufficient knowledge about the impact of metal exposure on its inhabitants. In this study, deep-sea mussel *Gigantidas platifrons*, a commonly used deep-sea toxicology model organism, was exposed to manganese (100, 1 000 μg/L) or iron (500, 5 000 μg/L) for 7 d, respectively. Manganese and iron were chosen for their high levels of occurrence within deep-sea deposits. Metal accumulation and a battery of biochemical biomarkers that related to antioxidative stress in superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA); immune function in alkaline phosphatase (AKP), acid phosphatase (ACP); and energy metabolism in pyruvate kinase (PK) and hexokinase (HK) were assessed in mussel gills. Results showed that deep-sea mussel *G. platifrons* exhibited high capacity to accumulate Mn/Fe. In addition, most tested biochemical parameters were altered by metal exposure, demonstrating that metals could induce oxidative stress, suppress the immune system, and affect energy metabolism of deep-sea mussels. The integrated biomarker response (IBR) approach indicated that the exposure to Mn/Fe had a negative impact on deep-sea mussels, and Mn demonstrated a more harmful impact on deep-sea mussels than Fe. Additionally, SOD and CAT biomarkers had the greatest impact on IBR values in Mn treatments, while ACP and HK were most influential for the low- and high-dose Fe groups, respectively. This study represents the first application of the IBR approach to evaluate the toxicity of metals on deep-sea fauna and serves as a crucial framework for risk assessment of deep-sea mining-associated metal exposure.

Keyword: mussel; metal; deep-sea mining; biomarker; environmental monitoring

1 INTRODUCTION

The deep-sea deposit hosts an abundance of highly coveted minerals including iron, cobalt, copper, zinc, and manganese, alongside rare earth elements that are critical for modern technology and renewable energy sources (Hauton et al., 2017). With terrestrial mineral reserves depleting, deep-sea mining has emerged as a new avenue for humans to acquire these resources. The prospecting of minerals is probable to cause the emergence of new fluid sources and consequent release of metal complexes

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from sedimentary deposits. These metals can be desorbed upon interaction with oxygenated water, rendering them easily available for biological uptake (Roberts, 2012). Such an elevation in metal bioavailability could adversely affect the health and well-being of deep-sea organisms, negatively affect the fragile and largely unexplored deep-sea environment, eventually leading to reduced biodiversity and the depletion of vital habitats (Gollner et al., 2017; Santos et al., 2018). As a result of the challenging remoteness and inaccessibility of the deep-sea biosphere, research into the possible ecotoxicological repercussions of mining operations on deep-sea organisms has been notably scarce. Thus, it is imperative to carry out related work, which is crucial in developing appropriate guidelines and regulations for environmental risk assessments, and for responsible decision-making within the deep-sea mining industries.

The deep-sea mussel within the subfamily Bathymodiolinae is one of the most successful fauna that thrive at both deep-sea cold seeps and hydrothermal vents worldwide (Laming et al., 2018). Its abundant presence among deep-sea macrofauna, coupled with its significant biomass, highlights its crucial role in shaping community structure and ecosystem dynamics of deep-sea chemosynthetic environment. Bathymodiolinae mussels, like their coastal counterparts, have the ability to accumulate metals in their tissues and are extremely susceptible to environmental toxins, making them ideal "sentinels" for monitoring the deep-sea ecosystem (Martins et al., 2017; Zhou et al., 2021, 2023). Among the diverse range of metals deep-sea fauna often encounter, iron (Fe) and manganese (Mn) are two important metals that are found mostly in deep-sea ores. Fe and Mn are transition metals that contribute significantly to the proper growth, development, functioning, and maintenance of living organisms (Valko et al., 2005). Fe, for example, is essential for various physiological processes including respiration, energy production, and hemoglobin synthesis (Valkova et al., 2020). Likewise, Mn is a vital element involved in the maintenance of both the nervous and immune systems, and acting as a critical cofactor in numerous enzymes including superoxide dismutase, arginase, and pyruvate carboxylase (O'Neal and Zheng, 2015). Nevertheless, excessive amounts of Fe and Mn can be detrimental to aquatic organisms, leading to severe consequences or even fatality. The toxic effects of Fe on aquatic organisms are known to cause inhibition or suffocation on gills, eggs, or other body surfaces, which restrict organisms from obtaining fundamental resources like oxygen and food and are considered indirect effects (Valkova et al., 2020). Mn toxicity could trigger oxidative stress, induce cell death pathways, cause immunosuppression, and increase the risk of infections (Hernroth et al., 2020).

Biomarkers are advantageous tools in determining the level of a specific pollutant present in an organism and serve as warning signals at an early stage. Excessive amount of metals in the environment can trigger a surge in the production of reactive oxygen species (ROS) in organisms, resulting in oxidative damage to cells. Consequently, biomarkers for oxidative stress including superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) can serve as valuable tools for assessing the level of oxidative damage induced by pollution. Previous toxicological experiments have shown that deep-sea mussels possess the capability to produce antioxidant molecules as a defense mechanism against metal-induced oxidative stress (Company et al., 2004, 2006; Zhou et al., 2023). In addition, it has been reported that metals can also disturb immune and energy homeostasis in deep-sea mussels (Zhou et al., 2021, 2023). Therefore, an array of biomarkers associated with immune responses, such as alkaline phosphatase (AKP) and acid phosphatase (ACP), along with biomarkers linked to energy metabolism, namely pyruvate kinase (PK) and hexokinase (HK), have been widely utilized for the assessment of metal contamination in aquatic fauna. Overall, biomarkers utilized in environmental assessments can offer crucial insights into the ecological health of various ecosystems, the effects of pollution, and the consequences of anthropogenic activities on the environment. These valuable insights can aid in formulating tactics to mitigate the deleterious impact of pollution caused by deep-sea mining.

Integrated biomarker response (IBR) is a comprehensive technique that employs an array of biomarkers to assess multifaceted biological impacts of contaminant exposure on an organism (Beliaeff and Burgeot, 2002). This approach considers multiple biomarkers that respond to different mechanisms of toxicity and is regarded a more sensitive and comprehensive approach than analyzing the individual biomarkers in isolation. This method has widely employed in numerous environmental risk assessment studies conducted

both in laboratory settings and in the field (Damiens et al., 2007; Kim et al., 2010). Nevertheless, to date, there has been no report of studies using IBR approach to evaluate the toxic impacts of metals on deep-sea creatures.

This research examines the impact of metals on *Gigantidas platifrons*, a bivalve species commonly found in the cold seeps and hydrothermal vents of the West Pacific Ocean. Mn and Fe were chosen as the exposure metals based on their established high levels of occurrence within deep-sea deposits. After being exposed to Mn (100 or 1 000 μg/L) or Fe (500 or 5 000 μg/L) for 7 d, respectively, a battery of biochemical biomarkers (SOD, CAT, MDA, ACP, AKP, HK, and PK) were assessed in deep-sea mussel gills. To better understand the overall toxicological effects of metal exposure on *G. platifrons*, IBR approach was used to establish the integrated data for the first time. The findings of this research fill in the gaps of current understanding regarding the possible ecotoxicological consequences imposed on deep-sea organisms by mining activities. Additionally, these results aid in developing methods that could be incorporated into upcoming environmental impact assessment (EIA) guidelines for deep-sea mining operations.

2 MATERIAL AND METHOD

2.1 Experiment design

The specimens of *G. platifrons* were obtained from a cold seep in the South China Sea located at 22°06.919′N and 119°17.140′E at a depth of 1 119 m using a remote operated vehicle (ROV) named Faxian which was deployed from the R/V *Kexue*. An insulated container was used to transport the mussels to the surface of the water, maintaining the natural environmental temperature of 4 °C. Upon arrival, the deep-sea specimens were placed into a regulated aquarium system that was specifically designed for the cultivation and monitoring of deep-sea organisms during the research expedition. To maintain their habitat-like conditions, the deep-sea mussels were cultured using 4 °C-filtered seawater that was renewed daily. In addition, these mussels were supplemented with methane gas as a source of food and energy.

After a 48-h acclimation period, mussels (shell length mean±SD, 55.6±10.91 mm) were arbitrarily allocated to five high-density polypropylene containers (capacity: 15 L), with a rearing density of one mussel per liter of seawater and eight individuals per tank. For a duration of 7 d, the treatment tanks were received dissolved Mn (100- or 1 000-μg/L nominal concentrations) or Fe (500- or 5 000-μg/L nominal concentrations). An experiment control tank was also included. The solutions of iron and manganese were respectively prepared using $Fe_3Cl{\cdot}6H_2O$ and $MnCl_2$ (analytical grades), with concentrations that were environmentally relevant to hydrothermal environments (Zeng, 2011; Zhou et al., 2020). Daily replenishment of filtered seawater and metal solutions was performed throughout the entire exposure experiment, while all other conditions remained consistently in line with those of the acclimation period. No mortality was observed during the experiment.

Upon complete of the 7-d exposure period, mussels were euthanized, and their gills were dissected with the use of plastic instruments. Mussel gills were segregated into two separate sample sets, with one set being used to measure the metal content and the other set being used for biochemical analysis. The tissue intended for metal content analysis was subjected to meticulous washing using deionized water to eliminate any surface contaminants. All samples were promptly cryopreserved using liquid nitrogen and subsequently stored at a temperature of -80 °C until subsequent analysis.

2.2 Metal determination

The method employed for quantifying metal content in deep-sea mussel gills was carried out following earlier established protocols (Wang et al., 2017; Zhou et al., 2020). Specifically, freeze-dried gill tissue (approximately 0.1 g) per mussel was deposited in a capped polytetrafluoroethylene (PTFE) griffin beaker for metal measurements. The samples were subjected to digestion with 1-mL perchloric acid and 3-mL nitric acid, and then were exposed to heat first at 150 °C for six hours and later at 220 °C for an additional three hours. Subsequently, after the solution became translucent and colorless, the specimens were heat-dried at 220 °C without the cap and allowed to cool down to room temperature. Following this, 0.5 mL of nitric acid (1∶1, v/v) was added to each sample and then it was standardized by adding ultrapure water to reach a fixed volume of 10 mL.

Inductively coupled plasma mass spectrometry (ICP-MS, Thermo-Scientific iCAPQc, Bremen, Germany) was employed to assess the concentrations of Fe and Mn in mussel gills. To

ensure quality control, certified reference material from a scallop (GBW10024/GSB-15) was utilized. To verify accuracy, the ICP-MS measurements were compared with the certified values of the reference control. An accuracy of less than 10% variance between a given measurement and its certified value affirmed the precision. The metal concentration results obtained from the gill tissues were reported in milligrams per kilogram of dry tissue weight.

2.3 Biochemical biomarker determination

The mussel gill samples were assessed for seven biochemical biomarkers, including superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), alkaline phosphatase (AKP), acid phosphatase (ACP), pyruvate kinase (PK) and hexokinase (HK). These biomarkers were quantified through the use of commercial kits from Nanjing Jiancheng Bioengineering Institute (China).

The method of McCord and Fridovich (1969) was employed to assess the activity of SOD, while the activity of CAT was evaluated using the method outlined by Góth (1991). The determination of GSH was accomplished by utilizing Ellman's reagent, 5,5′dithiobis-(2-nitrobenzoic acid) (DTNB) (Sedlak and Lindsay, 1968). The levels of MAD were determined by quantifying the reactivity of thiobarbituric acid (TBA) (Uchiyama and Mihara, 1978). King (1965)'s method was utilized to monitor the activities of AKP and ACP. HK activity was ascertained by utilizing Crabtree and Newsholme (1972)'s method, and the activity of PK was determined using Malcovati and Valentini (1982)'s method.

2.4 Integrated biomarker response

The IBR values were computed utilizing the approach outlined by Beliaeff and Burgeot (2002), with adjustments as detailed in the literature by Sanchez et al. (2013), which provided a comprehensive representation of biomarker responses as a singular value or graphical depiction. The process of calculating the IBR index can be simplified as follows. The individual biomarker data (X_i) were initially compared to the reference data (X_0) and log transformed to obtain a new parameter (Y_i) using the formula $Y_i=\log X_i/X_0$. Next, the average (μ) and standard deviation (s) of all biomarker parameter (Y_i) were determined for each treatment, and then transformed into standardized values using the formula $Z_i=(Y_i-\mu)/s$. The biomarker deviation index (A) was then calculated by taking the average of the standardized biomarker response (Z_i) and the average of the reference biomarker data (Z_0), resulting in $A_i=Z_i-Z_0$. To determine the IBR value, the absolute values of A parameters for each biomarker were summed. The biomarker data for each treatment group were presented in star graphs, and the biomarker deviation index (A) was represented in the star graphs to show the deviation of the studied biomarker for each treatment in comparison to the control group. A positive value indicated biomarker induction, while a negative value suggested biomarker inhibition. Since the IBR value is dependent on the number of biomarkers in the dataset, we calculated the IBR/n value by dividing the IBR value by the number of biomarkers used in each case (n=7), according to the approach proposed by Broeg and Lehtonen (2006).

2.5 Statistical analysis

For metal concentrations and biochemical biomarkers, means and standard deviations were calculated, and the homogeneity of variance and normality of fitted residuals were examined prior to formal statistical analysis. Parametric variables underwent analysis of variance (ANOVA) while nonparametric variables underwent Kruskal-Wallis tests. When significant differences (P<0.05) were obtained, pairwise comparisons were performed using Tukey's range test or Mann-Whitney U tests to determine which treatment or control groups showed significant variances. The statistical analyses of the dataset were carried out through the utilization of the GraphPad Prism version 9.3.1 for Windows (GraphPad Software, San Diego, CA, USA)

3 RESULT

3.1 Metal accumulation in deep-sea mussel gills

Figure 1 illustrates the concentrations of accumulated Fe and Mn in the gills of deep-sea mussel *G. platifrons*. The findings indicated that *G. platifrons* mussels could accumulate substantial quantities of Mn/Fe from their surroundings, and the higher the concentration of Mn/Fe present in the environment, the greater the accumulation of Mn/Fe found in the gills of *G. platifrons* (Fig.1). Furthermore, it is noteworthy that *G. platifrons* exhibited a greater capacity for accumulating Fe as opposed to Mn, as evidenced by the higher accumulation of Fe in the mussel gills.

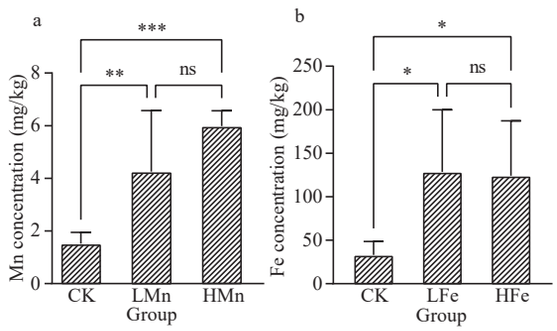

Fig.1 Accumulation of Mn/Fe concentrations in the gills of *Gigantidas platifrons* following a 7-d exposure to Mn/Fe (*n*=8)

The treatments included in the study were CK (control), LMn (100 μg/L Mn), HMn (1 000 μg/L Mn), LFe (500 μg/L Fe), and HFe (5 000 μg/L Fe). *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$. ns: no significance between treatments.

3.2 Biochemical biomarkers in deep-sea mussel gills

The Mn/Fe exposure in this study had a noticeable impact on most biochemical biomarkers, as depicted in Fig.2. SOD activity exhibited a significant raise in mussels exposed to low-dose Mn, but significantly lowered in mussels exposed to high-dose Mn. Exposure to Fe resulted in a decline of SOD activity, regardless of the dose. CAT activity remained unchanged in Mn-treated groups, but notably declined in mussels exposed to high-dose Fe. MDA levels in all treatment groups showed a significant decrease. ACP was only considerably lowered in mussels exposed to high-dose Mn, whereas it remained unaffected in mussels exposed to Fe. AKP exhibited a significant decline only in mussels exposed to low-dose Mn and remained unaltered in Fe-exposed groups. HK showed changes in the high-dose Mn group only, while no changes observed in mussels from the Fe-treated groups. PK was greatly affected in all treatment groups.

3.3 Integrated biomarker response (IBR)

The IBR values were computed for different treatment groups using seven biomarkers, which included SOD, CAT, MDA, AKP, ACP, HK, and PK (Fig.3). Based on the star plots, it was observed that varying degrees of induction or inhibition among multiple biomarkers were exhibited in different treatment groups. Normalized data from all seven biomarker endpoints were utilized to calculate the IBR/*n* index using the star plots demonstrated in Fig.4. A higher IBR/*n* value indicates greater stress and poorer health in deep-sea mussels. Mussels exposed to low-dose Mn had a higher IBR value than those from the low-dose Fe group, indicating that Mn has a more detrimental effect on the health of deep-sea mussels than Fe.

The relative importance of each biomarker in IBR values for each treatment can be determined based on the absolute *A* value. Results showed that oxidative stress-related biomarkers including SOD and CAT had the highest weight in IBR values for both Mn treatments. On the other hand, ACP had the greatest weight in IBR values in mussels exposed to low-dose Fe, and HK had the most significant weight in IBR values in mussels from the high-dose Fe group.

4 DISCUSSION

Deep-sea mussels hold potential as a valuable bio-sentinel for deep-sea metal pollution monitoring due to their ability to accumulate heavy metals, which was further evidenced by the significantly accumulated Mn and Fe in gills of *B. platifrons* from the four treatment groups. Prior studies on bivalves have shown that metal toxicity can trigger reactive oxygen species (ROS) production, causing disruptions in immune and energy homeostasis (Zhou et al., 2023). To unveil the comprehensive adverse impact of metals on deep-sea mussels, this study has focused on biomarkers linked to oxidative stress, immune function, and energy metabolism.

Measuring biomarkers associated with oxidative stress, a condition that arises from the excessive generation of ROS, is a conventional approach utilized to evaluate environmental contaminant exposure (Ruiz et al., 2015). To defend against oxidative stress, bivalves have developed antioxidant defense mechanisms that involve in the activation of primary antioxidant enzymes including superoxide dismutase (SOD) and catalase (CAT), which help mitigate adverse effects of increased ROS levels (Kim et al., 2014). SOD facilitates the transformation of highly reactive superoxide radicals (O_2^-) into a less toxic molecule hydrogen peroxide (H_2O_2) in aquatic organisms (Amorim et al., 2020). Meanwhile, CAT, enables the transformation of H_2O_2 into innocuous byproducts, namely water (H_2O) and molecular oxygen (O_2) (Amorim et al., 2020). The current investigation showed SOD activity elevated in deep-sea mussels exposed to low-dose Mn but decreased in the high-dose Mn group. The increased SOD activity suggested a requirement to metabolize

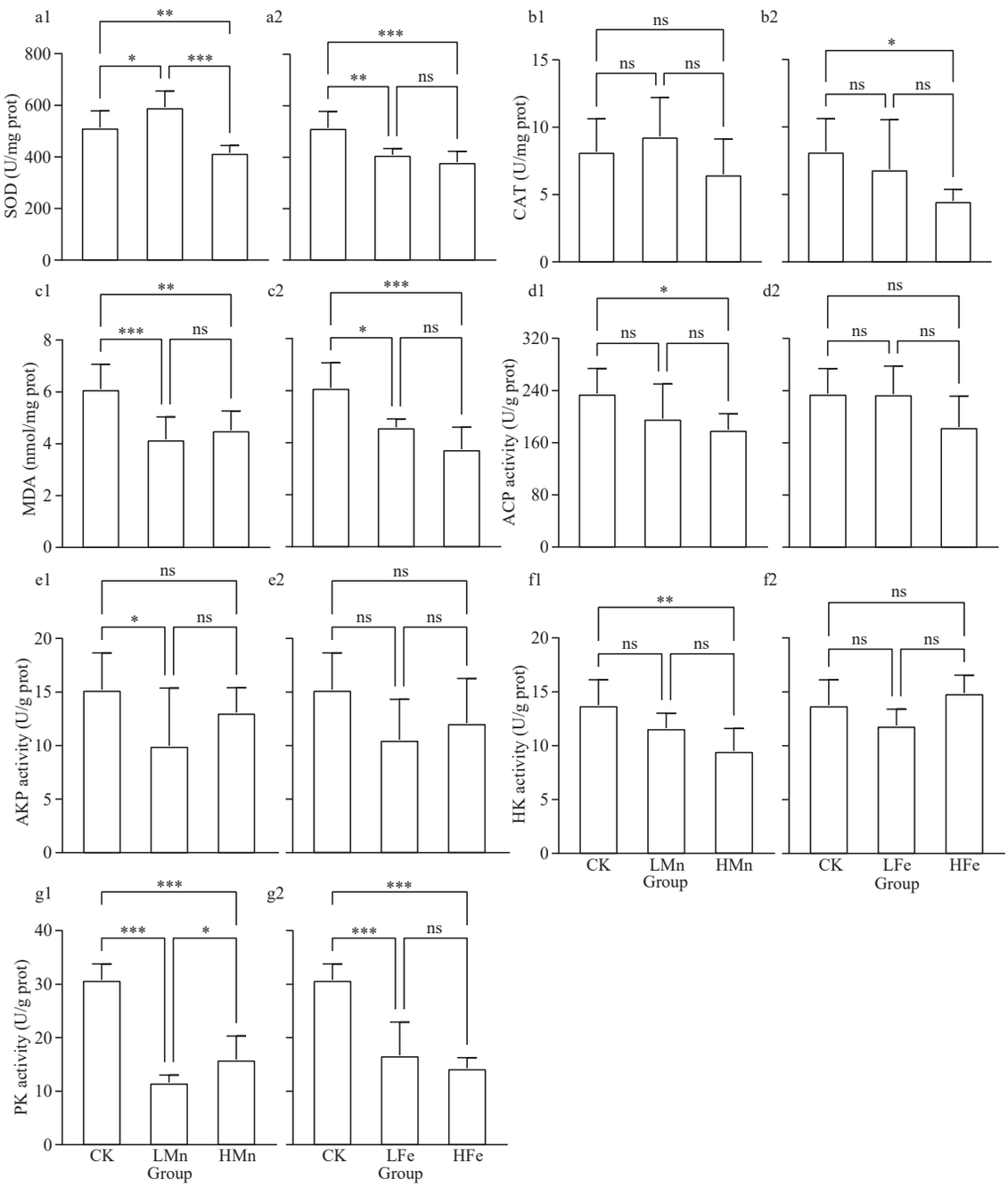

Fig.2 Biochemical biomarkers in the gills of *Gigantidas platifrons* following a 7-d exposure to Mn/Fe (*n*=8)

The treatments included in the study were CK (control), LMn (100 μg/L Mn), HMn (1 000 μg/L Mn), LFe (500 μg/L Fe), and HFe (5 000 μg/L Fe). SOD: superoxide dismutase; CAT: catalase; GSH: glutathione; MDA: malondialdehyde; AKP: alkaline phosphatase; ACP: acid phosphatase; PK: pyruvate kinase; HK: hexokinase. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$. ns: no significance between treatments.

excess O_2^- into H_2O_2 to prevent oxidative damage. Conversely, the drop in SOD activity might have resulted from an overwhelming generation of ROS that exceeded the natural capabilities of the antioxidant defense system in *G. platifrons* (Li et al., 2020), rendering SOD functions ineffective. Additionally, it was observed that the change in CAT activity mirrored that of SOD for each Mn treatment group, indicating a coordinated response of SOD and CAT to Mn-induced oxidative stress. This coordinated response may enable the deep-sea mussels to uphold the redox equilibrium and minimize the damage caused by ROS. Meanwhile, SOD was suppressed in both low- and high-dose Fe

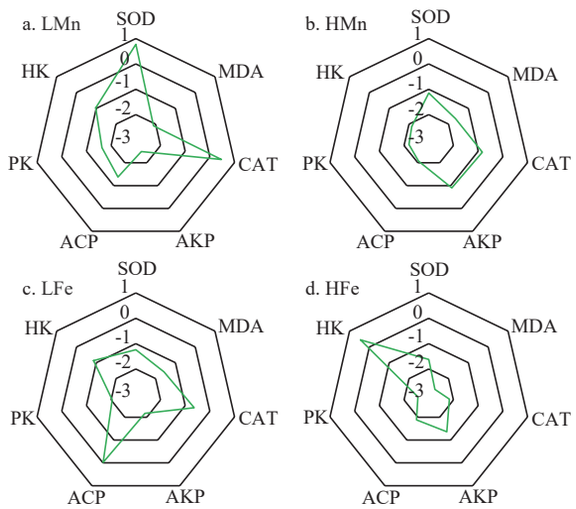

Fig.3 Star graphs showing the integrated biomarker response (IBR) for each treatment group

SOD: superoxide dismutase; MDA: malondialdehyde; CAT: catalase; AKP: alkaline phosphatase; ACP: acid phosphatase; PK: pyruvate kinase; HK: hexokinase.

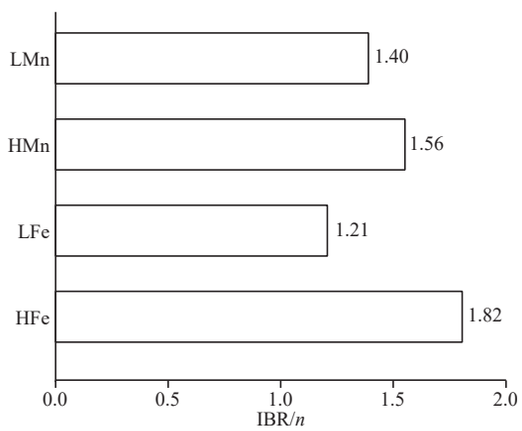

Fig.4 Calculated IBR/*n* index using the biochemical biomarkers measured in *Gigantidas platifrons* following a 7-d exposure to Mn/Fe

The treatments included in the study were CK (control), LMn (100 μg/L), HMn (1 000 μg/L), LFe (500 μg/L), and HFe (5 000 μg/L).

groups and displayed a decreasing trend with rising Fe dosage. This was likely due to the binding of excess iron to the enzyme, causing changes in its structure and interfering with its catalytic activity (Abreu and Cabelli, 2010).

Excessive accumulation of ROS can trigger the onset of lipid peroxidation (LPO) and malondialdehyde (MDA), which can cause various damages to cells and membrane structures. MDA content is often positively correlated with the LPO content, and is commonly employed as a reliable biomarker for assessing the degree of oxidative damage within an organism (Roméo et al., 2000; Livingstone, 2001). The present study demonstrated reduction in MDA levels across all treatment groups, suggesting that the antioxidant defense system effectively combated ROS and/or repaired cellular membrane damage, leading to a decrease in LPO and MDA formation (Géret et al., 2002). This outcome is consistent with previous studies on mussel *Mytilus coruscus* co-exposed to ocean acidification, hypoxia, and high temperature for 30 d (Khan et al., 2021) as well as oyster *Crassostrea gigas* exposed to either cadmium, silver or mercury for 21 d (Géret et al., 2002). In general, metals exposure leads to the disturbance of ROS levels. Low levels of exposure may heighten the activity of antioxidant enzymes in deep-sea mussels, aiding in the removal of excess ROS and maintaining redox balance, while high levels of metal exposure may result in severe oxidative damage, leading to reduced enzyme activity and impaired oxidative defense mechanisms.

The enzymes acid phosphatase (ACP) and alkaline phosphatase (AKP) are widely distributed among organisms and exhibit the ability to hydrolyze phosphate-containing molecules. They are crucial for the non-specific immunity of aquatic organisms and thus can be taken as reliable biomarkers for assessing immune responses triggered by environmental contaminants (Mazorra et al., 2002). ACP is primarily found in lysosomes and is used as a key biomarker for lysosomal membrane injury in bivalves (Mazorra et al., 2002). Moreover, ACP has a significant function in the process of biomineralization by facilitating the deposition of calcium and carbonate ions in the shell matrix (Rajalakshmi and Mohandas, 2005). Therefore, the ACP activity decreased by cadmium exposure in deep-sea mussel *G. platifrons* reported by Zhou et al. (2021) may be attributed to the dysfunction of lysosomal membrane stability. Similarly, the observed decline in ACP activity in pacific oyster *Crassostrea gigas* exposed to tralopyril, as observed by Wang et al. (2022), may indicate reduced shell deposition. In this study, the noteworthy decrease in ACP activity in deep-sea mussel from high-dose Mn exposure may reveal disturbance in lysosomal membrane stability or shell deposition. The role of AKP is multifaceted in bivalves, as evidenced by its involvement in diverse physiological processes such as calcification process, regulation of osmotic balance, and facilitation of oxygen transport (Viarengo and Nott,

1993; Lovett et al., 1994; Lan et al., 1995). It has been demonstrated that environmental stressors can affect the levels of AKP activity (Zhou et al., 2021, 2023). The significant decreased in ACP activity in deep-sea mussels exposed to low-dose Mn indicated suppressed immune function triggered by metals. Significantly, Fe exposure did not impede the activities of ACP and AKP enzymes, indicating that Fe did not interfere with their essential functions.

Exposure to metals disrupts energy allocation in aquatic organisms as the ingested energy, primarily used for maintenance, is redirected towards detoxification and repair mechanisms. This shift may lead to functional deficiencies, ultimately affecting organisms' fitness (Sokolova et al., 2012). Glycolytic pathways are among those affected by metals (Shanmuganathan et al., 2004; Zhou et al., 2021). dos Santos Carvalho and Fernandes (2008) have noted that both hexokinase (HK) and pyruvate kinase (PK) are crucial enzymes in the glycolysis pathway. Specifically, HK functions as the starting point of glycolysis, whereas PK serves as the concluding stage in the glycolytic process for glucose metabolism. Mollusks' PK and HK activities are subject to various factors like temperature, anaerobic conditions, tidal cycle, seasonal changes, and contaminant exposure (Canesi et al., 1998; Katsikatsou et al., 2011).

HK functions by transferring a phosphate group from ATP to glucose. In the present study, only the low-dose Mn group showed a decrease in HK activity. The decline in HK activity is likely to reduce the supply of ATP for both metabolism and metal detoxification. Consequently, this may have a detrimental effect on the metabolic activity of the deep-sea mussels, potentially impacting their overall health and survival. Comparable results have been reported for other organisms, such as *Mytilus galloprovincialis* Lam exposed to Cu, zebrafish exposed to microplastics, and *Macrobrachium nipponense* exposed to nanoplastics (Canesi et al., 1998; Chen et al., 2020; Li et al., 2021a).

PK plays a crucial role in glycolysis by facilitating the transfer of a phosphate group from phosphoenolpyruvate to ADP, thereby yielding ATP and pyruvate. In anaerobic conditions, the produced pyruvate is transformed into lactate via lactate dehydrogenase, which in turn promotes the conversion of NADH to NAD^+ to sustain the glycolytic cycle. Conversely, under aerobic conditions, pyruvate is further converted into acetyl-CoA, which fuels the Krebs cycle leading to the generation of reduced co-enzymes such as NADH and $FADH_2$ (Satyanarayana, 2021). The present study revealed a significant decrease in PK activity across all the experimental groups, which may have resulted from excessive metal accumulation leading to reduction in glycolysis ability (Li et al., 2021b). Additionally, the reduction of PK activity may result from the direct competition of essential bivalent cations, such as Mn/Fe, for binding sites of related proteins, causing changes in enzyme conformation (Abdel-Tawwab and Wafeek, 2017). This phenomenon has been observed in fish Nile tilapia *Oreochromis niloticus* (L.) and grass carp *Ctenopharyngodon Idella* when exposed to cadmium and mercury, respectively (Abdel-Tawwab and Wafeek, 2017; Li et al., 2021b).

The IBR method serves as a valuable tool for evaluating ecological risk by combining various biomarkers. A greater IBR value typically suggests a more serious or challenging situation (Kim et al., 2010; Li et al., 2017). Yu et al. (2021) employed the IBR approach to analyze the overall stress response of turbot fish to nitrate exposure. The findings demonstrated that as the concentration of nitrate increased, the IBR value rose in a dose-dependent manner, indicating a provoked stress response in turbot caused by nitrate exposure. Our study also revealed that *G*. *platifrons* exposed to high doses of Mn/Fe had elevated IBR values compared to low-dose groups. Furthermore, the low-dose Mn group had a higher IBR value than the low-dose Fe group, indicating that Mn has a more detrimental effect on the health of deep-sea mussels than Fe. IBR approach can also discriminate toxicity effects between Mn and Fe on *G*. *platifrons*. In this study, it was observed that different biomarkers play varying roles in the IBR values of deep-sea mussels from different treatments. Our findings reveal that in the Mn treatment, the IBR values were predominantly attributed to biomarkers associated with oxidative stress, namely SOD and CAT. In mussels exposed to low-dose Fe, ACP emerged as the most crucial contributor to IBR values. However, in mussels exposed to high-dose Fe, the weight of HK was found to be of utmost significance.

5 CONCLUSION

This research investigated the biochemical changes in deep-sea mussels *G*. *platifrons*, to the exposure of the most common metals found in deep-sea ores, namely Mn and Fe. The experiment

involved exposing *G. platifrons* to Mn at concentrations of 100 or 1 000 μg/L and Fe at concentrations of 500 or 5 000 μg/L for 7 d. Seven biochemical biomarkers (SOD, CAT, MDA, ACP, AKP, HK, PK) as well as metal accumulation were evaluated to determine the metals' impacts on deep-sea mussels. The results indicated that *G. platifrons* accumulated high levels of metals in its gills. Results also showed that exposure to Mn and Fe caused metal oxidative stress, disrupted immune function, and resulted in an energy imbalance in deep-sea mussels. The IBR analysis revealed that Mn has a more harmful impact on the health of deep-sea mussels than Fe. Additionally, the biomarkers associated with oxidative stress, namely SOD and CAT, had the greatest impact on IBR values for both Mn treatments. Nevertheless, ACP was identified as the most influential biomarker in IBR values for the low-dose Fe group, while HK was found to be the most significant biomarker for the high-dose Fe group. Given the persistence of metals in aquatic environments, the release of these chemicals from deep-sea mining activities is anticipated to have detrimental impacts on the general health of deep-sea mussels. This study highlights the significance of integrated biomarker assessments for evaluating the consequences of pollutants on deep-sea organisms. Future studies should address the chronic effects of metal exposure on deep-sea mussels or other ecologically significant deep-sea organisms, such as holothurians and nematodes. Ultimately, the outcomes of this study provide valuable insights into the possible effects of deep-sea mining activities on deep-sea mussels, emphasizing the importance of comprehensive environmental impact assessments for deep-sea mining activities.

6 DATA AVAILABILITY STATEMENT

The authors declare that all data supporting the findings of this study are available within the article.

7 ACKNOWLEDGMENT

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