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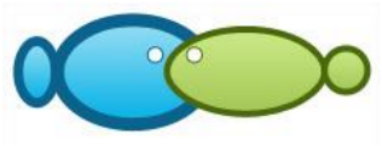

# Antioxidative responses in the skin mucus of *Periophthalmodon schlosseri* as biomarkers for the assessment of heavy metal pollution in the coastal wetlands of Kuala Lupak estuary of the Barito River, Indonesia

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**Abstract**. One of the main factors that concern the well-being of estuarine water ecosystems is heavy metal pollution by anthropogenic activities. Heavy metals can cause oxidative stress in fish by increasing the formation of reactive oxygen species (ROS) and, thus, directly disrupt the antioxidant defense system. This article aimed to determine the responses of antioxidant enzymes in the skin mucus of *Periophthalmodon schlosseri* as a biomarker of oxidative stress. *P. schlosseri* or the giant mudskipper is a typical fauna of the contaminated Barito River estuary. The study showed that the activity of the antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) was higher in the skin mucus of the mudskipper from estuary waters compared to that from coastal waters, while the level of $H_2O_2$ was lower. According to *in silico* studies, heavy metals can act as activators for binding Nrf2-KEAP1, causing antioxidant expression to be disrupted. The activity of antioxidant enzymes such as SOD, CAT, and GSH-Px in *P. schlosseri* skin mucus has the potential to be used as an oxidative stress biomarker in non-invasive biomonitoring assessment of heavy metal pollution in estuaries. The giant mudskipper has the potential to be a sentinel species and a sensitive bioindicator in monitoring/assessing heavy metal pollution in estuaries for ecotoxicological studies in polluted water bodies.

**Key Words**: antioxidative defense system, biomonitoring, heavy metal pollution, *in silico*, oxidative stress biomarkers, skin mucus.

**Introduction**. Estuaries and other coastal environments have been subjected to heavy metal pollutants and their derivatives. However, the toxicity and effects of these pollutants are still unknown (Salgado et al 2019). Heavy metal pollution is a major environmental issue that harms the health of the biota and the aquatic environment. In aquatic organisms, the presence and exposure to non-essential heavy metals (Hg, Pb, Cd) and/or essential heavy metals (Zn, Cu, Fe) produces oxidative stress and adverse reactions. Anthropogenic activities primarily cause heavy metal pollution in the aquatic environment (Adeogun et al 2020). Settlements, industrial activities, fisheries, agriculture, and coal transportation on land along the Barito River have the potential to produce heavy metal waste harmful to the aquatic environment. Therefore, biomonitoring measures are required for early detection of the detrimental effects of pollution.

There is insufficient data on biomonitoring heavy metal pollution at the Barito river estuary. Invasive monitoring of the Barito River estuary quality reveals that heavy metals Cd, Pb, Hg, Fe, Zn, and Cu in the water bodies, sediments, liver, and kidneys of *Periophthalmodon schlosseri* surpasses the prescribed quality standards. Non-invasive biomonitoring using fish mucus skin as a biological matrix produced the same results (Santoso et al 2021a). Heavy metal accumulation in fish tissues might be viewed as an indirect marker of heavy metal exposure in aquatic ecosystems. Monitoring/evaluating fish tissue exposure to heavy metals acts as an early warning indicator of poor water quality and heavy metal pollution of sediment, allowing necessary actions to be carried out to protect public health and the environment (El-Shenawy et al 2021).

Fish are extremely vulnerable to heavy metal-induced oxidative stress. Heavy metal-mediated oxidative damage is a complex process, in which redox-active metals including Fe, Cu, Cr, and Va generate ROS through the redox cycle. Metals with no redox potential, such as Hg, Ni, Pb, and Cd, can interfere with antioxidant defenses (Sevcikova et al 2011). These heavy metals can inhibit antioxidant gene expression through the NRF2 (the nuclear factor erythroid 2–related factor 2), KEAP1 (Kelch-like ECH-associated protein1), and ARE (the antioxidant responsive element) pathways, resulting in decreased antioxidant activity. As a result, ROS levels increase and the balance of cellular antioxidant activity shifts (Adeogun et al 2020).

Fish have complex antioxidant enzyme defense mechanisms that prevent excessive ROS and oxidative damage, including superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), and glutathione peroxidase (GPx) (Soliman et al 2019). This defensive mechanisms assist in mitigating the harmful effects of ROS produced by heavy metal metabolism. Antioxidant enzymes such as SOD, CAT, and GPx are useful as heavy metal pollution biomarkers in water (El-Shenawy et al 2021). Antioxidants of low molecular weight, such as glutathione (GSH) and vitamin A, have also been shown to neutralize ROS. Endogenous enzymatic and non-enzymatic antioxidants are required for the conversion of ROS to innocuous metabolites to restore metabolism and cellular function (Orbea et al 2002; Nnamdi et al 2015; Pastorino et al 2020).

Prior studies on *P. schlosseri* exposed to heavy metals revealed damage to liver and kidney cells (Santoso et al 2021b), indicating a failure of antioxidant enzyme activity to reduce excessive quantities of ROS or free radicals. Excess ROS or free radicals produce oxidative stress, which leads to the oxidation of biological membranes, decreased cell stability, cell damage, disease, and death (Magara et al 2021). The appearance of antioxidant enzymes can show cell damage produced by the oxidation of biological membranes, allowing researchers to establish the mechanism of heavy metals contamination in *P. schlosseri*. Given the information provided above, further studies are needed to establish the potential of *P. schlosseri* as a bioindicator species of heavy metal contamination in the Indonesian Barito river estuary. The concentrations of heavy metals Fe, Zn, Cu, Hg, Cd, and Pb in water bodies, sediments, and skin mucus of mudskipper have been previously studied and published by Santoso et al (2021a), but the interaction description between heavy metals and antioxidant enzymes is not defined. The purpose of this article was to assess the activity of antioxidant enzymes in *P. schlosseri* skin mucus in response to oxidative stress due to exposure to heavy metals and to examine the visualization of interactions between heavy metals and antioxidant enzyme activity *in silico*. Changes in the parameters of antioxidant enzyme activity in fish skin mucus can be used as biomarkers of oxidative stress for non-invasive biomonitoring of heavy metal pollution in estuaries.

## Material and Method

***Description of the study sites***. The longest and largest river in South Kalimantan is the Barito River. The Barito River starts from the Schwaner Mountains and flows approximately 1000 km from the Central Kalimantan region in the northern part of Borneo to the river's estuary in the Java Sea. The river is 650 to 800 m wide and 8 m deep. The width of the river in the funnel-shaped estuary reaches 1000 m, making the Barito River Indonesia's widest river. Starting from the upper parts of the river, the

longest section of the Barito River is in Central Kalimantan, while the remainder, up to the river mouth, is in South Kalimantan. Before reaching the mouth of the Barito River in the Barito Kuala region, this river meets the estuary of the Negara River. This study was carried out in the estuary waters (station 1) and the coastal waters of Kuala Lupak (station 2), which are both located along the west coast of the Barito River estuary (Figure 1). Table 1 shows a description of the research locations for each station.

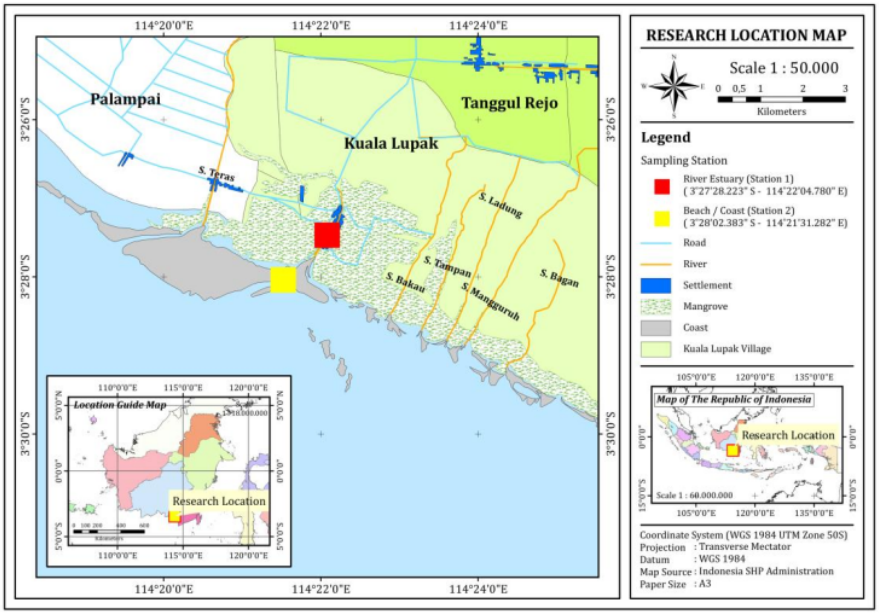


Figure 1. Map of research locations and stations.

Table 1

Description of stations 1 and 2

| *No* | *Station* | *Description* |
|---|---|---|
| 1 | Estuary/River estuary | Kuala Lupak is a Wildlife Reserve Area with a mangrove (swamp forest) environment. There are residential areas around the mouth of the Kuala Lupak River. The Kuala Lupak River flows into the Java Sea. Samples were collected in the estuary waters at 3°27′28.223″S; 114°22′04.780″E. |
| 2 | Coastal waters | Kuala Lupak's coastal waters are about 8 km west of the mouth of the Barito River, located on the coast of the Java Sea with a coastline of 30 km. Mangrove swamp forests can be found along the waters of Kuala Lupak's coastline. The samples were collected in coastal waters near the river's mouth at 3°28'02.383″S; 114°21'31.282″E. |

Water, sediment, and *P. schlosseri* were collected between February 2-3, 2021, using the "purposive sampling" method. The stations were selected based on the source of activities suspected of producing pollution loads. The settlements in the area justify sampling estuaries and coastal waters, since it is believed that human activity has contaminated the Barito River's water flow. Landslides, sedimentation, flooding, and other natural occurrences are also believed to play a role in polluting. As a result of

anthropogenic activities in the river's upstream and intermediate watersheds, which will have an impact on the estuary area, the samplings further consider that estuaries and coastal locations are the most vulnerable to biodegradation.

***Sampling***. At each site, 10 *P. schlosseri* were caught. Their average body length was 15.47±0.84 cm, and the average mass was 150.95±3.41 g. Skilled fishermen manually caught the fish by inserting their hands into the fish burrows. To remove contaminants, water was used to clean the fish from each location. The skin mucus of *P. schlosseri* was prepared using the method described by Fernández-Alacid et al (2018). Clean fish were placed on a tray, and the skin mucus was carefully collected using a sterile glass slide by gently rubbing from the region behind the gills down to the caudal fin. A sterile slide was softly slid down on the animal's flanks two or three times, and the skin mucus was then delicately pushed and gathered in a sterile tube (2 mL). A large portion of mucus might be collected by repeatedly touching the skin, but doing so is not recommended because it could lead to epidermal lesions and contaminate the samples with blood or other cells. This method must be carried out precisely, without rewetting the animal and without any contact with the operculum, ventral, anal, or caudal fins, to prevent mucus dilution by the water. For further analysis, the mucus was immediately frozen in liquid nitrogen and preserved at -80°C.

All procedures were carried out following the ethical standards given by the Health Research Ethics Committee No. 549/KPEK-FK ULM/EC/III/2021 Faculty of Medicine, Lambung Mangkurat University, Banjarmasin, Indonesia, which was formed to control and regulate the animal experiments.

***Evaluation of antioxidative stress in the skin mucus of P. schlosseri***. Antioxidant levels were determined by measuring the activities of SOD, CAT, and GSH-Px. The supernatant was prepared from skin mucus samples in 10 volumes of 0.1 M Tris-EDTA buffer (pH 7.4), centrifuged at 1000×g at 4°C for 30 min. The kit's instructions were then followed to determine the various oxidative stress/antioxidant parameters. The supernatant was collected for enzyme activity analysis. SOD activity was measured spectrophotometrically at 560 nm, referencing Misra & Fridovich (1977). One unit of SOD is defined as the amount of enzyme required to prevent 50% of epinephrine autooxidation. SOD activity is measured in units of SOD $mg^{-1}$ protein. CAT activity was determined in the samples using the approach of Aebi (1984), by monitoring the enzyme's decrease in $H_2O_2$ absorbance at 240 nm. For 3 min, the decrease in absorbance was recorded, and the change was calculated in nmol of $H_2O_2$ decomposed/min/mg protein (Lakra et al 2021). $H_2O_2$ levels were measured using ammonium molybdate according to Suhartono et al (2013), who used hydrogen peroxide as a standard. The 100 µL of the supernatant sample was stirred with 100 µL of 32.4 mM ammonium molybdate. After 10 min incubation, the absorbance was measured at 405 nm wavelength with a UV-Visible spectrophotometer. The activities of GSH-Px were detected using the GSH-Px Activity Assay Kit (Elabscience®) according to the manufacturer's instructions. All antioxidant enzyme activities were measured at 37°C with a Shimadzu UV-2100 spectrophotometer.

***Ligand and protein preparation***. Through *in silico* studies, the interaction between proteins involved in antioxidant activity and ligands in the form of heavy metals can be described. $Fe^{2+}$, $Zn^{2+}$, $Cu^{2+}$, $Hg^{2+}$, and $Cd^{2+}$ ligands were obtained from MIB: Metal Ion-Binding site prediction and server docking (http://bioinmfo.cmu.edu.tw/MIB/) (Pratidina et al 2022; Lahdimawan et al 2022). The previous study of Santoso et al (2021a) provided the basis for using these heavy metals as ligands. Meanwhile, the protein used as a target was KEAP1 obtained from the RCSB Protein Data Bank (https://www.rcsb.org/search) with PDB code: 5cgj (Sari & Bare 2021). The protein was prepared by removing the natural ligand residue present in the protein. The Chimera 1.15 program (https://www.cgl.ucsf.edu/chimera/download.html) was used to prepare the ligands and the protein.

***Analysis methods***. Mean and standard deviation (SD) were used to analyze the distribution and variability of antioxidant enzyme activity and hydrogen peroxide level. The Kruskall Wallis test, with a 5% significance value, was performed to assess whether there is a significant median difference between the activity of antioxidant enzymes and the levels of hydrogen peroxide in two stations (estuary and coastal waters). Docking data are processed and visualized using the Chimera 1.15 program. Visualization is used to explain the interaction between ligands and receptor protein residues, specifically the interacting amino acids and the distance between the receptor protein ligands (Pratidina et al 2022; Lahdimawan et al 2022).

**Results and Discussion**

***Antioxidants biomarkers in skin mucus P. schlosseri***. Antioxidant enzyme activity was recorded in fish skin mucus in response to the accumulation of heavy metals such as Fe, Zn, Cu, Hg, Cd, and Pb in water bodies, sediments, and fish skin mucus. The heavy metal accumulation data has been previously published (Santoso et al 2021a). Fish skin mucus from estuary waters had an average activity of the SOD enzyme (39.3±1.6 unit of SOD $mg^{-1}$ protein) higher than that of fish from coastal waters (20.46±5.4 unit of SOD $mg^{-1}$ protein). The average CAT enzyme activity in fish skin mucus from estuary waters (18.59±4.82 µmol $min^{-1}$ mg $protein^{-1}$) was higher than that from fish from coastal waters (8.8±2.94 µmol $min^{-1}$ mg $protein^{-1}$). The average activity of the GSH-Px enzyme in fish skin mucus from estuary waters (42.7±4.65 nmol $min^{-1}$ mg $protein^{-1}$) was higher than that of fish from coastal waters (28.43±5.53 nmol $min^{-1}$ mg $protein^{-1}$). Meanwhile, the average level of $H_2O_2$ in fish skin mucus from estuary waters (11.95±2.38 mmol $g^{-1}$) was lower than that of fish from coastal waters (17.83±6.21 mmol $g^{-1}$) (Table 2). The findings of nonparametric statistical tests (Kruskall Wallis) revealed that antioxidant enzyme activity and $H_2O_2$ levels in fish skin mucus from the estuary and coastal waters differed significantly (Figure 2). Table 2 reveals that the CAT and $H_2O_2$ enzymes have statistically insignificantly different levels of activity.

Table 2

Antioxidant enzyme activity and $H_2O_2$ level in *Periophthalmodon schlosseri* skin mucus

| *Antioxidant biomarkers* | *Station* | |
|---|---|---|
| | *Estuary (Station 1)* | *Coastal waters (Station 2)* |
| SOD | 39.3±1.6[b] | 20.46±5.4[b] |
| CAT | 18.59±4.82[c] | 8.8±2.94[c] |
| GSH-Px | 42.7±4.65[a] | 28.43±5.53[a] |
| $H_2O_2$ | 11.95±2.38[c] | 17.83±6.21[c] |

Note: data are presented as mean ±SD of three replicates; n=10 samples of each station; different superscripts shpw significant differences at $p<0.05$; SOD (unit of SOD $mg^{-1}$ protein) - superoxide dismutase; CAT (µmol $min^{-1}$ mg $protein^{-1}$) - catalase; GSH-Px (nmol $min^{-1}$ mg $protein^{-1}$) - glutathione peroxidase; $H_2O_2$ level (mmol $g^{-1}$).

Heavy metals are one of the main sources of pollution in estuary water ecosystems. Several heavy metals, such as Cu, Fe, Mn, and Zn, are essential to biological processes, they can also be hazardous in excessive doses. Harmful effects from Cd, Cr, Pb, and Hg can occur even at very low amounts (Akinsanya et al 2020). Heavy metals significantly increase ROS like superoxide anions, hydroxyl radicals, singlet oxygen, and hydrogen peroxide in the fish body. This may result in cell degeneration due to damage to the cell membrane and genotoxicity. Fish can accumulate these heavy metals and experience severe oxidative stress as a result of their harmful effects, making them an effective bioindicator (Kumar et al 2021). Oxidative stress occurs due to an imbalance between pro-oxidants such as ROS and defense cellular antioxidant enzyme systems (Pastorino et al 2020). Antioxidant biomarkers are used for early detection of the harmful effects of heavy metals on several aquatic organisms (Hamed et al 2020). The first line of defense against excessive ROS brought on by heavy metals is provided by antioxidant enzymes such as SOD and CAT (Kumar et al 2021). Additionally, fish tissues and blood experience

modifications in their physiological and biochemical processes due to heavy metals. Its harmful effects result in oxidative phosphorylation blockade, glutathione enzyme decrease, inhibition of antioxidant enzymatic activity, excessive ROS production, and inhibition of cell repair mechanisms (El-Shenawy et al 2021).

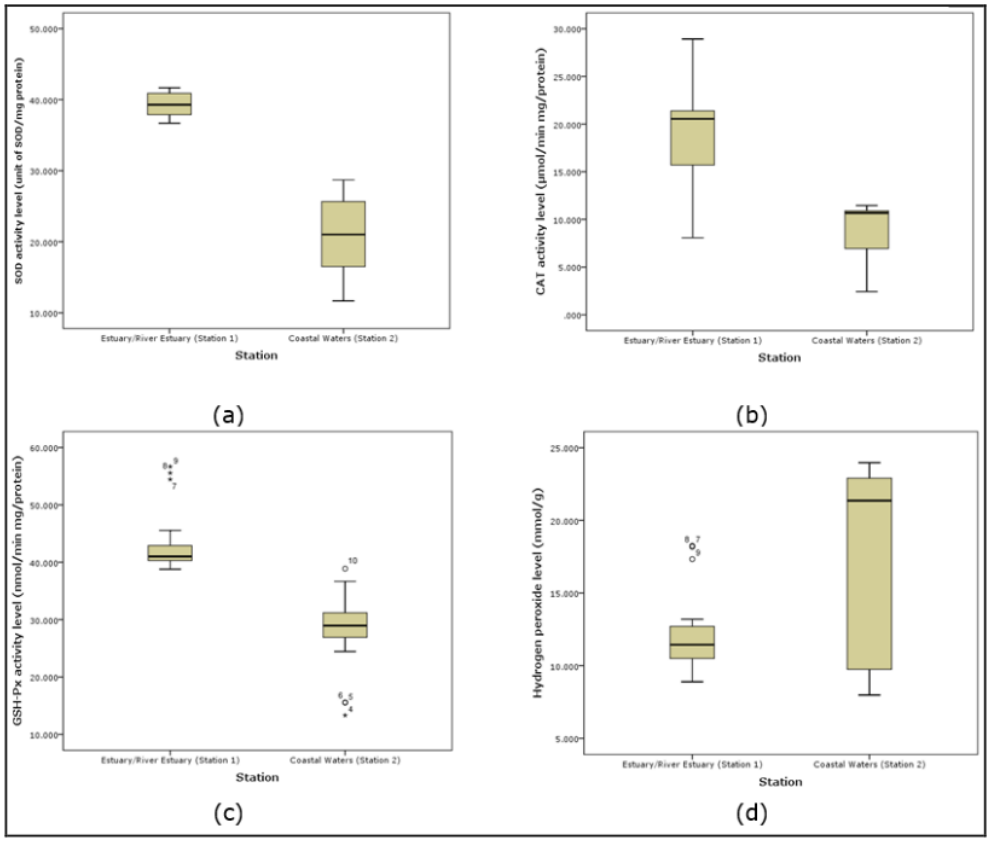


Figure 2. Antioxidant enzyme activity in *Periophthalmodon schlosseri* skin mucus: (a) SOD (unit of SOD $mg^{-1}$ protein); (b) CAT (µmol $min^{-1}$ mg $protein^{-1}$); (c) GSH-Px (nmol $min^{-1}$ mg $protein^{-1}$); (d) hydrogen peroxide (mmol $g^{-1}$).

Due to the presence of antioxidant enzymes like SOD, CAT, and GPx in the skin mucus of the giant mudskipper, it is possible to use this species as a bioindicator of heavy metal pollution. El-Shenawy et al (2021) explained that the function of enzymatic activity and scavengers is to eliminate excessive reactive oxygen species, such as hydrogen peroxide ($H_2O_2$) to prevent oxidative damage to cells. This species is a sentinel organism, able to identify the effects of heavy metals in the water and soil of estuarine ecosystems. The findings of this study suggest that environmental risk assessment studies could use oxidative stress biomarkers in the form of cellular antioxidant enzyme expression in *P. schlosseri* skin mucus. This is supported by Hamed et al (2020), who stated that fish exposed to heavy metal pollution develop antioxidant enzymes, which could serve as a biomarker of oxidative stress. Faheem & Lone (2018) confirmed that the antioxidant enzyme activity indices including SOD, CAT, and GPx are frequently used as screening tools to determine the effects of environmental stress.

In this study, the mucus of *P. schlosseri* from estuary waters had stronger antioxidant enzyme activity than that of fish from coastal waters. This is due to increased concentrations of the heavy metals Fe, Cu, Zn, Hg, Pd, and Cd in the water bodies,

sediments, and skin mucus of the mudskipper fish in the Kuala Lupak estuary waters (Santoso et al 2021a). Additionally, there was more anthropogenic influence on this site. Heavy metal accumulation in fish skin mucus results in increased SOD, CAT, and GPx antioxidant activity. Antioxidant defense, according to Prokić et al (2016), functions as a balanced and coordinated system. Heavy metals can induce oxidative stress and disrupt the balance of the antioxidant defense system. SOD includes metalloenzymes that will catalyze the removal of $O_2\bullet-$. into $O_2$ and $H_2O_2$. Additionally, CAT and/or GSH-Px efficiently convert $H_2O_2$ into $H_2O$ and $O_2$. After oxidative stress, SOD and CAT are the first active enzymes to exhibit alteration (Mahamood et al 2021). GSH-Px is a crucial enzyme in preventing hydrogen peroxide damage to cells (Fırat et al 2021). SOD, CAT, GSH, and GPx are important antioxidant enzymes that help shield fish from the harmful effects of ROS. Superoxide dismutase is regarded as the body's first line of defense against oxidative stress, which causes cells to produce hydrogen peroxide from superoxide radicals. CAT and GPx then eliminate the hydrogen peroxide and lessen its damaging effects (Gao et al 2021). The findings of this study are consistent with Lakra et al (2021), who found that all fish tissues exposed to coal mine effluent, and coal mine waste had higher levels of SOD and CAT. Antioxidant enzymes appear in *Clarias batrachus* as an early reaction to heavy metals in coal mine effluent. Farombi et al (2007) found a significant increase in SOD and CAT activity in *Clarias gariepinus* when exposed to heavy metals Pb, Zn, Cu, Cd, and As, whereas Yogeshwaran et al (2020) found that heavy metal exposure to Zn, Cu, Cd, and Pb increased antioxidant enzyme activity and hydrogen peroxide ($H_2O_2$) in *Scylla serrata*. Chang et al (2021) reported that prolonged Cd exposure increases ROS synthesis, resulting in oxidative stress in fish. These exposures to Cd significantly reduced CAT, GSH-Px, and SOD activities, while increasing MDA and ROS concentrations in *Cyprinus carpio*. Biomarker activity can also be influenced by abiotic factors such as pH, dissolved oxygen content, and water temperature. Aquatic species' heavy metal concentrations and antioxidant enzyme activity may fluctuate seasonally and in response to biological and environmental stressors (Pastorino et al 2020).

***Heavy metal interaction with KEAP1 protein***. The mechanism of heavy metals in inducing ROS formation is thought to be through the Nrf2-KEAP1 binding pathway. Heavy metals work as in the Fenton reaction or the Harber-Weiss reaction, which oxidizes $H_2O_2$ to •OH causing oxidative stress. Oxidative stress can also activate various transcription factors including nuclear factor kappa-B (NF κB), p53, HIF-hypoxia-inducible factor 1α, peroxisome proliferator-activated receptor γ (PPAR-γ), β-catenin/Wnt, and Nrf2. Protein nuclear factor erythroid 2-related (Nrf2) is a key protein in the antioxidant response through the Nrf2/KEAP1 pathway. In inactive conditions, Nrf2 binds to the Kelch protein ECH Associating Protein 1 (KEAP1). In contrast, free Nrf2 will translocate into the cell nucleus and activate the expression of antioxidant genes such as genes encoding SOD, CAT, and peroxidase (Canning et al 2015; Baird & Yamamoto 2020).

The involvement of heavy metals in reducing the expression of antioxidant enzymes is still an interesting study and the mechanism is still unclear. In this study, the interaction of heavy metals with KEAP1 protein is presented in Table 3. The interaction visualization of heavy metals with KEAP1 is presented in Figure 3.

KEAP1 is a protein that has a protein molecular weight of 69-kDa. This protein has a physiological function with the Kelch protein as an actin binder and acts as a negative regulator of Nrf2. Nrf2 further binds to KEAP1 in the cytoplasm. KEAP1 acts as a complex substrate adapter protein and targets E-ligase containing 3 cullin Nrf2. Furthermore, Nrf2 leads to degradation mediated by the proteasome (Sari & Bare 2021).

Electrophilic and oxidative stressors of Nrf2 signaling (CDDO-Im and sulforaphane) interact with residues from KEAP1 to disrupt Nrf2 and proteolytic sequestration (Sari & Bare 2021; Oktaviyanti et al 2022). Heavy metals are predicted to have the potential to be activators of the KEAP1 protein. The five heavy metals, namely $Fe^{3+}$, $Zn^{2+}$, $Cu^{2+}$, $Hg^{2+}$ and $Cd^{2+}$, that interact with KEAP1 protein have the potential to activate the Nrf2 protein bound to its active side. The Nrf2 binding of the Nrf2-KEAP1

complex stimulates an increase in ROS in the cells, so the expression of antioxidant formation can be disrupted.

Table 3

Heavy metals interaction with KEAP1 residues

| *Metals* | *Residues* | *Lengths (A)* |
|---|---|---|
| $Fe^{3+}$ | Ser431 | 2.01 |
| $Zn^{2+}$ | Asp538 | 2.60 |
| $Cu^{2+}$ | His553 | 2.25 |
| $Hg^{2+}$ | Cis368; | 2.712 |
| | Ile425 | 2.682 |
| | Val370 | 2.371 |
| | Val420 | 2.566 |
| $Cd^{2+}$ | ASP585 | 2.18 |

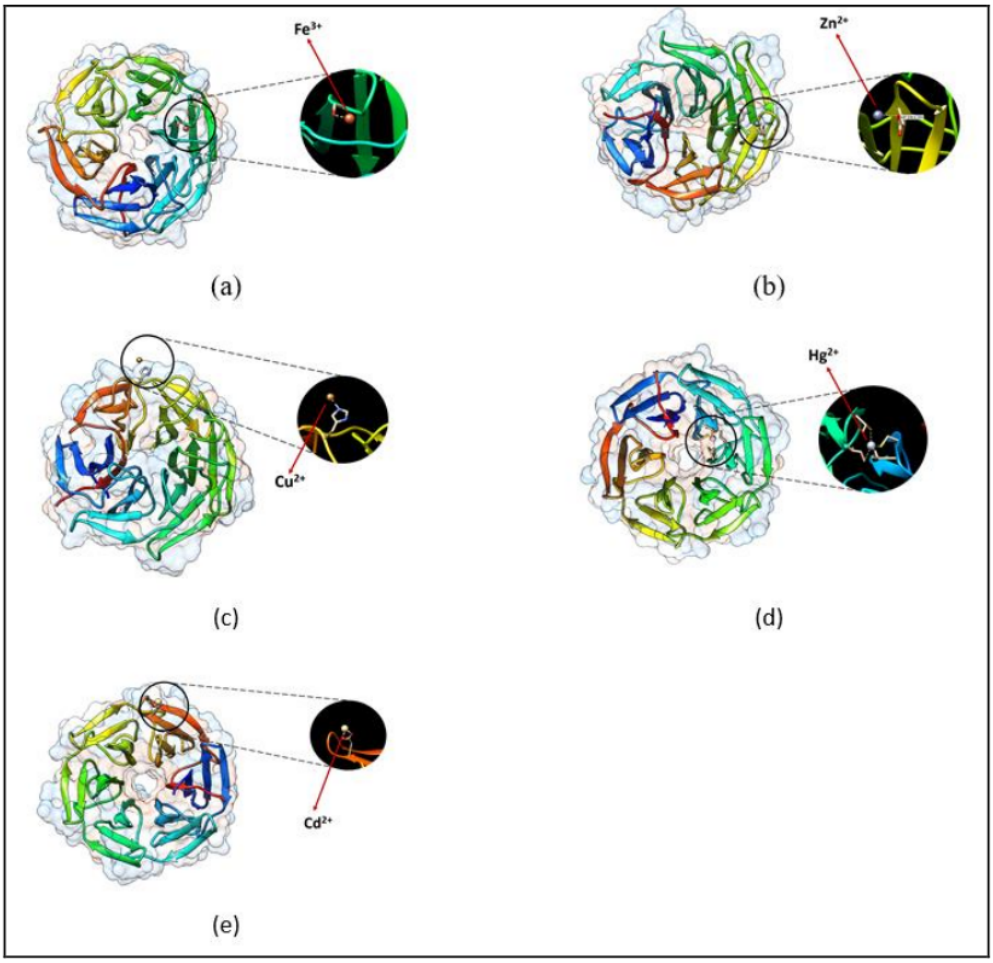


Figure 3. Interaction of KEAP1 to heavy metals; (a) $Fe^{3+}$; (b) $Zn^{2+}$; (c) $Cu^{2+}$; (d) $Hg^{2+}$; (e) $Cd^{2+}$.

In Table 3, it can be seen that $Hg^{2+}$ is chelated with 4 residues, namely Cis368, Ile425, Val370, and Val420. This binding causes $Hg^{2+}$ to accumulate in the KEAP1 protein, thus strengthening the Nrf2-KEAP1 complex. In addition, in Table 3, it can also be seen that $Fe^{3+}$ has the shortest bonding length. A shorter bond will increase the strength of the bond energy between $Fe^{3+}$-Ser431. This also allows $Fe^{3+}$ to accumulate in KEAP1, so that antioxidant expression in the cells will be disrupted. Similarly, $Zn^{2+}$, $Cu^{2+}$, and $Cd^{2+}$ all have the same mechanism. The results of this study are consistent with the research Komari & Suhartono (2020), who note that $Cd^{2+}$ can interact with antioxidants SOD, CAT, and glutathione reductase, disrupting its activity.

**Conclusions**. Heavy metal exposure has a significant negative impact on estuarine ecosystems. Mudskippers are endangered due to heavy metal bioaccumulation. Reactive oxygen species (ROS) are produced when heavy metal accumulation is present, which can lead to an imbalance in the prooxidant or antioxidant homeostasis of the fish. This is referred to as oxidative stress, and it can be measured using a variety of biomarkers, such as alterations in the activity of antioxidant enzymes. The study's findings showed that oxidative stress caused by the bioaccumulation of heavy metals in the skin mucus of *P. schlosseri* is responded to by the appearance of cellular antioxidant enzyme activity, notably SOD, CAT, and GSH-Px as oxidative stress biomarkers. Heavy metals significantly increase the production of ROS and modify the activity of antioxidant enzymes in the mudskipper skin mucus. According to *in silico* studies, heavy metals can activate Nrf2-KEAP1 binding, which disrupts antioxidant expression. The responses of mudskipper fish skin mucus to changes in antioxidant activity are valuable for assessing the quality of estuary waters. The use of antioxidant activity biomarkers in the biomonitoring program serves as a measure to monitor the health of aquatic fauna and assess heavy metal pollution in estuary waters. Therefore, the concentration of heavy metals in water bodies, sediments, and tissues of mudskipper fish should be monitored continuously to control pollution. To maintain the biodiversity of coastal wetland ecosystems, all types of wastewater must be treated before being released into aquatic environments. The findings of this study can be used as a starting point for stakeholders to discuss and explore the appropriate method for evaluating heavy metal concentrations and developing an integrated biomonitoring program.

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**Conflict of Interest**. The authors declare that there is no conflict of interest.

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