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Evaluation of Glutathione S-transferases Expression as Biomarkers by Heavy Metals in *Geloina expansa* from Sepang Besar River, Selangor, Malaysia

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ABSTRACT

Glutathione S-transferases (GSTs) are enzymes involved in phase II of detoxification metabolism and could be used as biomarkers for water pollution. This study aims to determine heavy metal concentrations in the soft tissue of the mangrove clam *Geloina expansa*, as well as the expression of GSTs in the species. The acid digestion method was used to digest the samples, followed by a standard USEPA 6010B procedure using inductively coupled plasma optical emission spectrometry (ICP-OES) to measure the heavy metal contents in the samples. GST enzyme activity was measured using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate. One-way ANOVA was performed to compare the mean values of heavy metal concentration, protein concentration, enzyme activity, and specific activity. There was a significant difference ($p<0.05$) for Zn, total protein, and specific activity in *G. expansa*, but no significant difference in Pb, Cu and enzyme activity. GST enzyme activities were estimated at 0.16 ± 0.01 µmol/min, with a protein content of 1.24 ± 0.04 mg. The specific activity for GST was 0.13 ± 0.01 µmol/min/mg, calculated as the ratio of enzyme activity to the total protein. GST-specific activity positively correlates with Pb concentration in the soft tissue of *G. expansa.* Detailed studies on the effects of pollution on the expression of GST need to be further investigated for the future use of this species as an efficient biomarker model.

Keywords: Biomarker, bivalves, *Geloina expansa*, glutathione S-transferase, heavy metals, SDG 14

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INTRODUCTION

Pollution is often linked to heavy metals in the environment, which must be monitored. Heavy metal pollution in aquatic environments such as mangroves, rivers, estuaries and coastal wetlands has increased significantly due to human activities (Shukor et al., 2023). Due to the increasing industrialisation and urbanisation of most Malaysian cities, significant amounts of pollution are now entering the rivers, leading to concerns about increasing water pollution (Krishnan et al., 2022). Heavy metals in the coastal areas may originate from various sources, including fossil fuel extraction, combustion, agriculture, refining, chemical production, and intentional and unintentional discharges. Heavy metals also enter coastal waters through natural processes such as rock weathering and river discharges (Krishnakumar et al., 2018). These metals are dangerous because of their high toxicity, longevity, and ability to bioaccumulate in the tissues of living organisms (Sharaf & Shehata, 2015). In ecosystems threatened by global environmental change, bivalves have proven to be important bioindicators, providing valuable insights into biodiversity and the state of ecosystems (Lamine, 2023). Bivalves can adapt to changing environmental conditions and store high levels of heavy metals in their tissue (Yap et al., 2016; Yap et al., 2019). Monitoring heavy metals in aquatic ecosystems alligns with Sustainable Development Gold 14 (SDG 14) and Life Below Water (Secretary-General, 2017).

Bivalves are among the first-choice organisms to serve as bioindicator species for chemical and environmental stresses. Bivalves are benthic sentinel species that reside in numerous ecological compartments and live as filter feeders (Helmholz et al., 2016). Because of the widespread distribution of bivalves and their ability to tolerate a wide range of environmental conditions, resulting in organisms directly taking up and accumulating a wide range of pollutants in water, these organisms are a valuable tool for biomonitoring contaminants (Boillot et al., 2015). Bivalves are often used to assess the toxicity and availability of man-made toxins in the aquatic environment (McCarty et al., 2002). Chemical bioaccumulation and alterations in cellular and molecular responses (biomarkers) can be studied on this species with great success (Regoli et al., 2014).

The mangrove clam, *Geloina expansa*, is a bivalve widely distributed in mangrove forests throughout the Indo-West Pacific region (Poutiers, 1998) and is common in tropical countries such as Malaysia (Ong et al., 2017). As a popular shellfish in Malaysian mangroves, it is a commonly harvested resource (Yahya et al., 2018). *Geloina expansa* Mousson, 1849 of the family Cyrenidae is a typical tropical mangrove clam that lives partially submerged in sediment for most of its life. This clam has two equal-sized shells connected by two adductor muscles and a burrowing foot (Morton, 1976). This species was previously known as *Polymesoda expansa* or *Polymesoda erosa* (Yahya et al., 2020; WoRMS Editorial Board, 2022) and was recently reclassified in the World Register of Marine Species. *Geloina expansa* is economically valuable as an edible mollusc and

is directly consumed by humans as a regular food source (Hamli et al., 2012). *Geloina expansa* is highly tolerant to extreme environmental conditions, surviving in a wide range of salinities and inhabiting muddy, brackish, and near-freshwater areas of mangrove swamps (Morton, 1976).

Geloina expansa meets a number of the listed requirements of an excellent biomonitor. This species is a sedentary filter-feeder widely distributed in mangrove areas, accumulates pollutants, and can adapt to stressful conditions and a long-life span. It feeds by filtering much stirred-up material from the waters. This feeding method traps many elements in its body, including various nutrients and potential toxins such as trace metals from the water column and surface sediments. Because of its ability to filter water, this species has often been used as a bioindicator in monitoring heavy metals (Edward et al., 2009). To this date, metal pollution studies of cadmium, nickel, chromium, iron, copper, lead, zinc, and arsenic using *G. expansa* as a bioindicator of environmental pollution have been conducted in several areas in Malaysia (Mohd Hamdan et al., 2020; Marlin et al., 2019; Harsonon et al., 2017; Ong et al., 2017; Ong & Ibrahim, 2017; Dabwan & Taufiq, 2016; Yap et al., 2014; Yap & Chew, 2011; Edward et al., 2009).

As a result of water pollution by metal contaminants and accumulation in the tissues of aquatic animals, there is an excessive formation of hydroxyl radicals and superoxide radicals as reactive oxygen species (ROS), including hydrogen peroxide. Besides the increase of oxidative damage and the lack of cellular antioxidants, the accumulation of radicals also contributes to the disturbance of physiological and biochemical mechanisms (Çomaklı et al., 2015). Oxidative stress induced by the accumulation of heavy metals in bivalve tissues can lead to protein degradation, lipid peroxidation (LPO) and other cellular changes such as DNA damage and inactive enzymes (Regoli, 2000; Xu et al., 1999). Bivalves typically perform defence mechanisms to combat free radical formation and ROS, which include changes in respiratory and metabolic rates, activation of alternative energy production pathways, and activation of antioxidant defence and repair processes (de Almeida et al., 2007).

An organism's metabolism involves several antioxidant defence mechanisms, whether enzymatic or not. The glutathione defence system is one of the most important of these defence mechanisms. Glutathione S-transferase (GST) is one of the enzymatic antioxidants in glutathione metabolism. The GST enzyme plays an important role in the metabolism of xenobiotics. GSTs belong to the phase II detoxification enzymes, which can decrease the cellular toxicity of various endogenous and environmental substances by providing a nucleophilic attack on reduced glutathione (GSH) (Espinoza et al., 2012). GST is a vital phase II biotransformation enzyme, and it catalyses the conjugation of glutathione (GSH) to electrophilic xenobiotics and oxidised components to boost their hydrophilicity and permit the elimination of toxins (Ketterer et al., 1983). GSTs have been well studied in

ecotoxicology because of their essential role in detoxification pathways and oxidative stress response (Hoarau et al., 2006). Nowadays, investigating potential biomarkers using a molecular biology approach has gained popularity as a robust method for monitoring environmental pollution studies.

The present study aims to determine the heavy metal concentrations of copper (Cu), zinc (Zn), cadmium (Cd) and lead (Pb) in the soft tissue of *G. expansa* from Sepang Besar River. The correlation between GST activity and heavy metals was assessed to evaluate the potential of GST as a molecular biomarker for monitoring metal pollution. Because of the broad range of tasks that GST enzymes perform in cells, such as the effects of oxidative stress, GST enzymes were selected as biomarkers in the present study. GST-related studies have been conducted on different species of bivalves, with the same emphasis on identifying bivalve GST for environmental contamination. The scope of this information may contain critical facts for environmental management and further enhance the potential of *G. expansa* as an efficient biomarker.

MATERIALS AND METHODS

Study Area

This study was conducted in the Sepang Besar River (SBR) mangrove estuary in Selangor, Peninsular Malaysia (2°35'30" N, 101°43'1" E). The mangrove and estuary ecosystem is a dynamic environment with one of the world's highest biodiversity and productive resources (Bianchi, 2013). SBR funnels into the Strait of Malacca, one of the country's main shipping routes and fishing grounds. Within a five-kilometre radius of the SBR confluence, an extensive mangrove area is located near fishing villages, aquaculture facilities, charcoal power plants, and urban development areas (Ya et al., 2014). Several anthropogenic activities, including agricultural activities such as oil palm plantations and upstream aquaculture, have been identified as potential sources of heavy metal pollution to the SBR. River cruising, fishing, fishing boats, and boat repair using anti-fouling agents also contribute to the heavy metal pollution in the river.

Sample Collection

Mangrove clam (*G. expansa*) was collected from the SBR's mangrove area at three sampling sites. Site 1 is located at the upper estuary (2°39' 14.8 N, 101°44' 34.1 E), Site 2 is at the middle estuary (2°38' 48.9 N, 101°43' 53.6 E), and Site 3 is located nearest to the river mouth, i.e., down-estuary (2°38' 36.8 N, 101°43' 39.7 E). The site selection is based on preliminary studies and surveys conducted in several areas, from the estuary's upper to the down section. The choice of location depends on the accessibility of the area where a clam can be found and the level of low water at the time of sampling. Field observations show

that this species prefers sites with softer sediments, such as water channels and puddles. The sampling was carried out during the rainy season in December 2021. During the collection of clam samples, a long metal rod was dipped into the mud, and the clam's presence was indicated by a clicking sound when the metal came in contact with the clam surface. The clams were collected and placed in an icebox before being taken to the laboratory for examination. Samples were sealed in polyethene bags, transported to the research facility, and stored at -20°C for further analysis.

Laboratory Pre-analysis

Glassware and other laboratory equipment were cleaned with 10% nitric acid, rinsed with milli-Q water and dried as a precaution. Clams collected for observations were cleaned with water, and their morphometric measurements (length, height, width) were recorded with a digital calliper. The dimensions of the shells were 63.90 to 108.26 mm in length, 58.87 to 105.79 mm in height, and 35.55 to 64.59 mm in width. The soft tissue was dissected from the shell for each clam sample, weighed, transferred to a glass Petri dish, and oven-dried at 60°C until a constant weight was reached. The wet weight of the tissue samples ranged from 8.51 to 50.7 g, and the dry weight ranged from 0.91 to 7.17 g. The dried samples were then pulverised using a porcelain mortar and pestle.

Determination of Heavy Metal Concentrations in the Soft Tissue of *G. expansa*

Cu, Zn, Cd, and Pb heavy metals were selected because they are bivalves' most common environmental contaminants (Zarykhta et al., 2019; Ong et al., 2017; Ong & Ibrahim, 2017). Acid digestion for clam soft tissue was performed using the standard USEPA 3050B procedure. First, 1 g of the dry weight of the soft tissue sample was digested with nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) additions. Then, the original digestate was treated with hydrochloric acid (HCL), and the sample was kept under reflux. After filtration of the digestate, the filter paper and residues were rinsed twice, once with hot HCL and once with hot reagent water. Before re-filtering, the filter paper and residue were placed back into the digestion flask and refluxed with more HCL. The filter paper and residues were then returned to the digestion flask and refluxed with more HCL before filtering again. The distilled digestion was made up to a final volume of 100 mL with distilled water. Finally, the soft tissue's heavy metal content (Cu, Zn, Cd, Pb) was measured according to the standard procedure US EPA 6010B using the inductively coupled plasma 5100 optical emission spectrometer (ICP-OES). The analytical procedures for the clams were validated using standard reference material (SRM 2976) mussel tissue by the National Institute of Standards and Technology. The recovery percentage is calculated based on the following formula:

$$\text{Recovery (\%)} = (\text{Measured values}) / (\text{Certified values} \times 100\ \%).$$

Homogenisation and Centrifugation of the Samples

The soft tissue of the clam was homogenised (1:5 w/v) in 25 mM phosphate buffer (pH 7.4), which also contained 1.0 mM ethylenediaminetetraacetic acid or tetrasodium salt (EDTA), 0.1 mM dithiothreitol (DTT), 0.1 mM phenylthiourea (PTU), and 10 mM phenylmethylsulfonyl fluoride (PMSF) using IKA Ultra-Turrax T25 homogeniser. The homogenised sample was centrifuged for one hour at 17500 rpm. The pellet was removed, and the supernatant was collected. All procedures were performed in the protein laboratory using a cool box with ice at 4°C to prevent protein degradation.

Enzyme Activity and Protein Content

GST (EC 2.5.1.18) activity is measured in the presence of reduced gluthathione (GSH) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate (Habig et al., 1974). The conjugation of glutathione with 1-chloro-2,4-dinitrobenzene (CDNB) resulted in thioether glutathione dinitrobenzene (molecular extinction coefficient of 9.6 $mM^{-1}cm^{-1}$). GST enzyme activities were determined at 25°C in a Victor X5 Perkin Elmer microplate reader. The total volume of each assay was 200 µL. The reaction mixture is prepared by mixing 190 µL of 0.1 M sodium phosphate buffer (pH 6.5), 3.34 µL of sample, 3.34 µL of 60 mM GSH, and 3.34 µL of 60 mM CDNB. The change in absorbance at 340 nm was recorded for 10 minutes. The reaction solution without the samples served as a blank. Total enzyme activity was determined as µmol/min at 25°C and specific activity as µmol/min/mg protein. The Bradford protein assay was used with bovine serum albumin as the standard to determine protein concentration (Bradford, 1976).

Statistical Analysis

Six replicates were performed for the clam samples, and the significance ($p < 0.05$) between sites was determined using one-way Analysis of Variance (ANOVA). A post hoc comparison of means using Tukey's honest significant difference (HSD) was done when there was a significant difference between groups ($p < 0.05$). A correlation analysis was later constructed (Pearson's coefficient, $p < 0.05$). All statistical analyses were performed using SPSS ver. 22.0.

RESULTS AND DISCUSSION

Heavy Metals Concentration

Heavy metal concentrations in the soft tissue of *G. expansa* at different sampling sites are shown in Table 1. Cd was not detectable at ICP-OES for all samples. The per cent recovery of SRM 2976 for the heavy metal analyses was acceptable, with a value of 144.28% for Cu, 103.88% for Zn, and 105.045% for Pb. According to Krishnan et al. (2023), the accuracy of

the analytical method was evaluated by a recovery test with the certified reference materials SRM 1566b and SRM 2976, which provided excellent results with values between 92.3% and 141.2%. The results demonstrate the reliability and precision of the analytical method and its ability to accurately determine the heavy metal concentration in the samples. In another study by Ong and Ibrahim, 2017, the heavy metals showed a good percentage recovery of over 90% for the certified reference material used with DOLT-4 dogfish liver as a benchmark, so it can be assumed that the analyses and methods used to determine the metal concentrations are reliable.

Heavy metal analyses of soft tissue samples recorded higher concentrations of Zn and Cu compared to Pb. The highest concentration of Zn was recorded in the soft tissues (mean: 262.86 ± 36.64 mg/kg) at all sites. Cu follows it with 17.03 ± 2.89 mg/kg concentrations, while Pb concentrations were 0.58 ± 0.008 mg/kg (Table 1). Concentrations of individual heavy metals varied significantly at different sites (Table 1). Soft tissues from Site 3 (down-estuary) have significantly greater ($p<0.05$) Zn concentration (328.17 ± 33.61 mg/kg) compared to those collected from other sites. In contrast, Cu and Pb in the tissues were considerably similar ($p>0.05$) between sites.

Table 1
Concentrations of heavy metals in G. expansa *tissue from three sites in Sepang Besar River*

Site	Heavy metal concentration in tissue (mg/kg of dry weight)		
	Cu	Zn	Pb
1	21.42 ± 5.46^{a}	201.42 ± 31.66^{a}	0.75 ± 0.17^{a}
2	11.58 ± 1.32^{a}	259.00 ± 30.77^{ab}	0.50 ± 0.00^{a}
3	18.08 ±2.09^{a}	328.17 ± 33.61^{b}	0.50 ± 0.00^{a}
All Sites	17.03 ± 2.89	262.86 ± 36.64	0.58 ± 0.01

Note. Site 1 = upper estuary; Site 2 = middle estuary; Site 3 = down-estuary. Concentrations of heavy metals are presented in mean ± standard error. Values sharing a similar superscript alphabet (a, b, c) within the same column are not significantly different

In general, metal concentrations in *G. expansa* in the present study are considerably similar to those reported in previous studies. Yap et al. (2014) reported concentrations of Zn at 365 ± 0.00 µg/g and Cu at 21.0 ± 0.00 µg/g in *Polymesoda erosa* collected from different sites in the adjacent Sepang Kecil River. Bivalve species may differ in metal composition due to physicochemical and biotic variables that affect metal bioaccumulation (Rajeshkumar & Li, 2018). The variation in the amount of metal that *G. expansa* accumulated showed its affinity for a particular element. Because of their importance in metabolic processes, Zn and Cu are essential elements, while Pb is non-essential and toxic even in very low concentrations (Hussein & Khaled, 2014). The high metal concentrations in the bivalve indicated high metal bioavailabilities of the metals in the sampling site (Yap & Al-Mutairi, 2022; Yap et al., 2006). Many marine organisms require zinc, which they absorb through

their food and the surrounding aquatic environment, but excessive zinc uptake can have toxic effects (Turkmen et al., 2005). Zn poisoning leads to death, impaired growth and reproductive problems (Sorenson, 1991).

As a filter-feeder, *G. expansa* consumes microscopic algae, bacteria, and detritus. They filter a huge amount of seawater by syphoning it in from the posterior ventral side with the inhalation syphon, filtering it through the gills, and then expelling it through the exhalation syphon (Famme et al., 1986). In this way, their tissues absorb pollutants and food particles from the water. This bivalve absorbs trace elements from the surrounding aquatic environment through the cell membrane and ingests food (Boening, 1999). Compared to the amounts found in the aquatic environment in which they live, bivalves have been reported to accumulate up to 100-100,000 times more trace metals in their tissues (Farrington et al., 2016; Casas et al., 2008). Thus, it is possible to find trace metals and other chemical pollutants in bivalve tissues undetectable in seawater (Farrington et al., 2016).

Cu and Zn are expected due to the intensive aquaculture operations along the SBR. For example, shrimp farming contributes greatly to the Cu content of the river, as this element is required to grow and develop aquaculture species. Cu is added to aquatic feed to increase immunity, antioxidant capacity and growth performance (Yuan et al., 2019; Wang et al., 2009). An earlier study in the SBR found that pig farming was the main cause of heavy metal contamination in the area (Ismail & Ramli, 1997). High concentrations of Zn and Cu are fed to pigs, where they act as growth promoters and are added to pig feed to prevent parasite infestation (Zhang et al., 2017; Zhu et al., 2013; Moral et al*.,* 2008). Pig slurry is likely the cause of Cu and Zn contamination (Marszalek et al., 2019; Jensen et al., 2018; Li et al., 2014). Sediment samples collected in 1996 from the mangroves upstream of the SBR, where pig farm effluents were discharged, revealed high Zn and Cu levels of 210–670 mg/kg and 250–550 mg/kg, respectively (Ismail & Ramli, 1997). After one year, the sediment sample collected in 1997 had a high Cu content, which Hossain et al. (2001) found to be 213–518 mg/kg upstream of the SBR. In 2001, Zn and Cu contents were reported to be 430–602 mg/kg and 400–574 mg/kg, respectively (Saed et al., 2002). Due to the novel Nipah disease (*Japanese encephalitis*) in Bukit Pelanduk, located upstream of the SBR, a ban on pig farming was introduced there in 1998 (Singh, 2014). In 2003, after the ban on pig farming, Zn and Cu concentrations of 375–416 and 5.23–22.73 mg/kg, respectively, were detected in the sediment sample (Yap et al., 2007). Ramsie et al. (2014) also reported Zn and Cu concentrations in sediment samples of 246.38 and 77.17 mg/kg, indicating that anthropogenic pollution sources are decreasing over time and the surrounding mangrove ecosystem is recovering from pollution.

In an exponential decay model, decreasing Zn and Cu levels in surface sediments of the SBR were used to describe the inverse correlation between Zn levels and a ban on pig farming over the years. The exponential regression can simulate a scenario in which

Zn levels in SBR sediment decrease more rapidly before and after a ban on pig farming. According to the model, the ban reduced the "considerable ecological risk" of the SBR estuary to "minimal ecological risk" (Yap & Al-Mutairi, 2022). This model was chosen wisely and is appropriate because background levels of metals in sediments will not be zero in naturally occurring sediments worldwide (Wedepohl, 1995) and monitoring studies using surface sediments from SBR as scientific evidence, especially after the ban on pig farming, as well as a detailed assessment of the reduction of metal elements in the previously polluted river. The importance of monitoring metal elements in sediments is beyond question, as is evident from a number of recent publications on these research monitoring activities (Hossain et al., 2021; Wei et al., 2019). According to model simulations with varying degrees of remediation effectiveness, recovery depends largely on source reduction and how close metal concentrations are to background concentrations in the watershed (Moore & Langner, 2012). The current models for Zn and Cu decay in SBR by Yap and Al-Mutairi (2022) can offer intuition into remediation methods and lower restoration costs.

Geloina expansa is capable of accumulating trace elements. The reported Pb concentration in soft tissue at Site 1 is 0.75 ± 0.17 mg/kg, and at Sites 2 and 3, it is 0.5 ± 0.00 mg/kg. Pb levels in SBR reported by Ismail and Ramli (1997) were 3.4–46.5 mg/kg. The research was continued in 1998 by Saed et al. (2002), and the Pb levels were reported from 14.90 to 51.24 mg/kg. No significant difference in Pb levels was observed between 1996 and 2010, possibly because pig effluents did not introduce Pb (Yap & Al-Mutairi, 2022). According to Ramsie et al. (2014), the Pb concentration in SBR sediment was 86.89 mg/kg. The considerable accumulation of Pb in the tissues of bivalves is due to the slow excretion of the amorphous granules and their immobilisation in the shell (Viarengo, 1989). Previous studies have shown that lead accumulation in the bivalve species *Mactra corralina* increases oxidative stress in the gills due to lead exposure (Chetoui et al., 2019). Lead is a heavy metal (non-essential) produced in natural and industrial processes such as mining, refining, petroleum combustion, and cement production. According to (Livingstone, 2001), the effects of accumulated metals cannot be determined by quantification alone because this method cannot demonstrate the toxic effects of pollution on aquatic organisms. Instead, biochemical biomarkers are often used in ecotoxicological studies to assess how contaminants affect species (Livingstone, 1993).

GST Enzyme Activity and Protein Content

The results of total activity and protein in *G. expansa* are shown in Table 2. The mean value of total protein content was estimated at 1.24 ± 0.04 mg, whereas the mean value of total activity was 0.16 ± 0.01 µmol/min. Specific activity for *G. expansa* at Site 1 was slightly higher (0.15 ± 0.01 µmol/min/mg) compared to their counterpart at Site 2 (0.12 ± 0.01 µmol/min/mg) and 3 (0.12 ± 0.01 µmol/min/mg). There was a significant difference

($p<0.05$) in Zn in soft tissue and specific activity of GST at all sites. However, no significant differences were observed between Cu and Pb in soft tissue and total GST activity.

Specific activity positively correlates with Pb concentration in the soft tissue of *G. expansa* ($p<0.05$; $R=0.509$) (Table 3). The accumulation of metal ions promotes the production of reactive radicals in cells, which leads to cell damage (Valko et al., 2005; Leonard et al., 2004). Exposure to Pb increases the formation of ROS, resulting in DNA damage, protein oxidation, and cell membrane damage (Flora et al., 2012; Hsu & Guo, 2002; Ercal et al., 2001; Gurer & Ercal, 2000; Halliwell & Gutteridge, 1989). Organisms must have important antioxidant defence systems such as GST, superoxide dismutase (SOD), and catalase (CAT), the three major enzymatic antioxidants, to protect themselves from oxidative stress. An important metabolic enzyme of phase II, glutathione S-transferase, helps detoxify both xenobiotics and oxidative metabolic end products (Jozefczak et al., 2012; Van der Oost et al., 2003).

The present study shows that Pb of *G. expansa* positively correlated with the specific activity of GST specific activity. GST activity increased significantly, supporting the role of GST in protecting against reactive oxygen radicals and reducing the toxicity of Pb, according to similar research by (Shenai-Tirodkar et al., 2017). Pearson correlation showing a positive relationship between GST and Pb concentration ($p<0.001$) supports this. In the early stages, Pb causes oxidative stress and activates other antioxidant enzymes such as SOD and CAT. At the same time, GST intervenes in the later stages as compensation for the defence system. Pb is one of the most common metals entering water and sediment and can be taken up by most aquatic organisms, particularly bivalves. Filter-feeder bivalves are considered effective toxin carriers because of their relative insensitivity to toxins compared to other aquatic organisms. Bivalve species have different behavioural and physiological defences against various negative stressors. This fact draws attention to the possible function of GST as a detoxification enzyme. GST significantly supports cellular defence against chemically induced toxicity. However, the GST system in bivalves has yet to be comprehensively described (Martins et al., 2014).

The bioaccumulation of Pb in the bivalves and its possible impact on the bioavailability of other metals could still pose significant environmental risks (Fukunaga & Anderson, 2011). Pb is a non-essential metal, and its environmental presence indicates anthropogenic pollution. Pb is hazardous to aquatic life because it can cause membrane damage and stop vital enzymes from working. GST, as an antioxidant enzyme related to Pb toxicity, has been used to evaluate the toxic effects of Pb in manila clam, *Ruditapes philippinarum* (Aouini et al., 2018). The effectiveness of using biomarkers to evaluate Pb toxicity in marine organisms has been demonstrated (Aouini et al., 2018; Wadige et al., 2014). Pb has the capacity to cause damage to membranes and prevent necessary enzymes from functioning (Krause-Nehring et al., 2012). Pb has a high affinity for the sulfhydryl groups of biologically

significant enzymes such as δ-aminolevulinic acid dehydratase (ALAD) involved in heme biosynthesis. Pb toxicity will result in higher production of ROS, including glutathione peroxidase (GPx), SOD, and CAT, which could indirectly lead to LPO of cell membranes and the creation of malondialdehyde (Wadige et al., 2014).

Bivalves that live as filter feeders in the sediment of mangrove areas can accumulate large quantities of heavy metal pollutants in their tissue (Choi et al., 2011). Antioxidant enzymes are activated in aquatic organisms to protect cells from oxidative stress caused by pollutants (Livingstone, 2001). GST, along with other antioxidant molecules such as glutathione reductase (GR), CAT, GPx, and SOD, is one of the most commonly studied biomarkers to assess oxidative damage caused by Zhang et al. (2014) suggested that GST is the first enzyme to be activated to cope with oxidative stress and that the increase in antioxidant enzymes may be due to a compensatory mechanism of ROS increase in cells. Elevated concentrations of contaminants can impair the function of biomarkers, or hazardous intermediates formed during xenobiotic metabolism can cause enzyme inactivation. GST activities in the clam *Ruditapes philippinarum*, as well as other biomarkers of oxidative stress such as CAT, GPx, GR and SOD, show that the effects of the depuration period differ from Pb concentrations. The decrease in some antioxidant activities could be due to the decrease of Pb in tissues, while the increase in antioxidant activities could be due to a delay in defence mechanisms. The study shows that Pb is not eliminated from the tissues, and the clam could increase its antioxidant activities to overcome toxicity as a preventive mechanism (Aouini et al., 2018).

GST detoxification enzymes have been used as biomarkers in biomonitoring aquatic pollution because they are an important component of adaptation mechanisms to chemical stress. There has been prior evidence of a relationship between the expression and activity of specific GST isoforms in bivalves and the presence of xenobiotics (Hoarau et al., 2006; Hoarau et al., 2002). The toxicity and environmental quality assessment fields will benefit from a better understanding of bivalve GSTs. Bivalve species such as *Mytilus trossulus* (Istomina et al., 2020), *M. edulis* (Yang et al., 2004; Fitzpatrick et al., 1995), *M. galloprovincialis* (Azevedo et al., 2015; Martins et al., 2014; Vasconcelos et al., 2007; Kaaya et al., 1999; Fitzpatrick et al., 1995), *Donux trunculus* (Amira et al., 2018), *Perna perna* (Sáenz et al., 2010; Kaaya et al., 1999), *Corbicula fluminea* (Martins et al., 2014; Puerto et al., 2011; Martins et al., 2009; Vidal et al., 2002), *Asaphis dichotoma* (Yang et al., 2002), *Atactodea striata* (Yang et al., 2003), *Mercenaria mercenaria* (Blanchette et al., 2002), *Anodonta cygnea* (Martins et al., 2014), *Ruditapes decussatus* (Hoarau et al., 2002), *Ruditapes philippinarum* (Revathy et al., 2012; Umasuthan et al., 2012) have been studied for their ability to isolate and characterise GSTs.

As with other proteins, a specific circumstance leads to the expression of GSTs. Since the expression of GSTs correlates with the amount of substrate exposed, the accumulation of substrate increases the expression of GSTs to the ideal level. Due to their remarkable

ability to detoxify, bivalves are most likely involved in this process, as shown by the fact that they may survive for an extended period after exposure to pollutants. Heavy metals as environmental pollutants can be detoxified by GSTs found in marine animals (Hoarau et al., 2006; Pérez et al., 2004; Fitzpatrick et al., 1997). GST promotes the conjugation of glutathione with foreign substances and helps eliminate reactive oxygen species from the body. Organic electrophiles can be conjugated by GST to the thiol group of glutathione, forming a hydrophobic molecule that is easily eliminated. (Winterbourn, 2008). Increased enzyme activity indicates that the species resists the toxic environment. In contrast, decreased enzyme activity may indicate a less harmful presence in the immediate environment or possibly due to increasing chronic exposure that overcomes the cells' protective system (Cossu et al., 2000).

Table 2
Total protein, total activity and specific activity of GST obtained from soft tissue of G.expansa *(mean ± standard error)*

Site	Total protein (mg)	Total activity (µmol/min)	Specific activity (µmol/min/mg)
1	1.17 ± 0.03^{a}	0.17 ± 0.01^{a}	0.15 ± 0.01^{b}
2	1.32 ± 0.04^{b}	0.16 ± 0.01^{a}	0.12 ± 0.01^{a}
3	1.23 ± 0.03^{ab}	0.15 ± 0.01^{a}	0.12 ± 0.01^{a}
All Sites	1.24 ±0.04	0.16 ± 0.01	0.13 ± 0.01

Note. Values sharing a similar superscript alphabet (a, b, c) within the same column are not significantly different.

Table 3
Pearson correlation matrix among heavy metals and GST biomarker in G. expansa

	Cu	Zn	Pb	GST
Cu	1.00			
Zn	0.275	1.00		
Pb	0.327	0.254	1.00	
GST	0.302	-0.227	**0.509***	1.00

Note.*correlation is significant at the 0.05 level (2-tailed)

Bioaccumulation of pollutants in different organisms is widely used in ecotoxicology because it reflects the bioavailability of pollutants in the ecosystem (Phillips & Rainbow, 1994). *G. expansa*, a sedentary bivalve found in the mangroves of the Sepang Besar River, can serve as a model organism for biomonitoring potentially toxic metals. GST as a biomarker from this bivalve has allowed the detection of specific biological responses to certain pollutants, such as heavy metals, in laboratory studies or mixed anthropogenic pollutants in field studies.

Proteomics has been used in environmental toxicology to assess changes in the proteome of organisms exposed to environmental factors such as pollution and climate

change, which needs to be better understood for continued and sustainable growth. Researchers are concerned about the potential adverse effects of anthropogenic, persistent, industrial, and emerging environmental contaminants on humans and wildlife. Proteomics and other OMICS technologies, such as genomics, metabolomics, and transcriptomics, have provided additional tools for developing biomarkers that are indicators of both chemical exposure and subsequent biological impact. Using omics technologies to preferentially identify biomarkers would be repeatable and allow for the fast, sensitive, and quantitative assessment of massive biomonitoring programmes. Proteomics research in bivalves may represent one of the most promising research areas for biomonitoring marine pollution (Campos et al., 2012). Developing proteomics technology for studying protein biomarkers to detect various pollutants in bivalves is one of the most challenging tasks. Pollutants in the environment affect the health of bivalves and cause economic losses in the aquaculture industry. Further research is needed to investigate the underlying mechanisms, biological pathways, and interrelationships of putative biomarkers and to evaluate the robustness of GST as a biomarker using accurate methods and new populations of *G. expansa* under different pollution situations.

CONCLUSION

G. expansa, as a sedentary organism, can accumulate and tolerate high concentrations of metals. GST, as a biochemical biomarker, correlates positively with Pb concentrations in the soft tissues of *G. expansa*. Therefore, the oxidative stress generated in this bivalve is associated with the heavy metal concentration. Future studies should be conducted to quantitatively evaluate the expression of GST by Pb exposure in *G. expansa* and to investigate the effects of acute Pb toxicity on oxidative stress in the species.

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