JEE Journal of Ecological Engineering

Journal of Ecological Engineering, 2026, 27(3), 399–415
https://doi.org/10.12911/22998993/214172
ISSN 2299–8993, License CC-BY 4.0

Received: 2025.09.30
Accepted: 2025.01.15
Published: 2026.02.01

Integrating metallothionein expression and heavy metal bioavailability for river biomonitoring with *Cheumatopsyche* sp. larvae

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ABSTRACT

Monitoring heavy metal pollution in riverine ecosystems requires a comprehensive understanding of their distribution, bioavailability, and assimilation into aquatic biota, including potential risks to humans. Macroinvertebrates provide valuable insights into metal contamination; this study investigated metal assimilation in rivers by the caddisfly larva *Cheumatopsyche* sp. It was hypothesized that the heavy metals derived from water and sediments, largely originating from anthropogenic activities, are assimilated and reflected through the expression of metallothionein (MT) in *Cheumatopsyche* sp. Accordingly, this study aimed to evaluate the relationship between metal bioavailability and MT responses as a molecular biomarker. Field sampling was conducted at five ecologically representative locations distributed along the longitudinal gradient of the Bone River watershed, spanning from the upper reach to downstream. Heavy metals were analyzed using the atomic absorption spectrophotometry method, while MT density and intensity were assessed through immunohistochemistry. The research revealed significant spatial variation in both heavy metal concentrations and MT expression across stations. The metal levels in water and sediment were strongly correlated with bioaccumulation in biota. Cadmium (Cd) exhibited the highest bioavailability, whereas generalized additive models (GAMs) identified mercury (Hg) as the primary determinant of MT density and intensity, with lead (Pb) influencing intensity only, and Cd showing no significant effect. Collectively, the obtained findings demonstrate that *Cheumatopsyche* sp. larvae effectively trace the mobilization and distribution of contaminants in aquatic systems, underscoring their potential as bioindicators for ecosystem management in polluted rivers.

Keywords: aquatic bioindicators, biomonitoring, *Cheumatopsyche* sp., heavy metals contamination, metallothionein, riverine pollution.

INTRODUCTION

Rivers are biologically diverse and highly productive ecosystems that provide habitats for insect larvae, fish, and other aquatic organisms, supporting higher trophic levels and fisheries. They also deliver essential ecosystem services to humans, which has led to numerous anthropogenic activities that potentially cause pollution, including the introduction of inorganic and organic contaminants into water and sediments (De Girolamo et al., 2017). Organic contaminants in rivers are often degraded into their constituent elements and rendered harmless through natural processes (Yang et al., 2020). Heavy metals, however, can remain toxic even when broken down into their elemental forms and may persist in riverine environments for hundreds of years (Mohiuddin et al., 2010).

Heavy metals accumulate in aquatic sediments and can become bioavailable within them (Zhuang et al., 2018). River flow facilitates the migration of dissolved heavy metal particles into surrounding sediments, where they can be assimilated by resident aquatic organisms (Yu et al., 2020). The exposure to heavy metals affects growth, development, reproduction, immune responses, neuromuscular function, and behavior in aquatic organisms (Kadim and Arfiati, 2022). Animals may be exposed through direct ingestion, contaminated food or particles, dissolved metals in water, or through metal adsorption onto their bodies (Razak et al., 2021; Tabrez et al., 2021).

Heavy metal pollution is a significant environmental issue affecting freshwater ecosystems, particularly in the river basins impacted by anthropogenic activities such as mining, agriculture, and urban runoff (Griboff et al., 2018). Globally, heavy metal contamination has emerged as a major threat to aquatic biodiversity and the quality of water resources (Sanae et al., 2021). Toxic metals, such as lead (Pb), cadmium (Cd), and mercury (Hg) exhibit high bioaccumulation potential within aquatic ecosystems, especially in sediments and biota (Bertrand et al., 2018; Islam et al., 2015). Long-term exposure to heavy metals can induce physiological stress, behavioral alterations, and impairments in growth and reproduction among aquatic organisms. Furthermore, the toxic effects of heavy metals can lead to biodiversity loss and disruption of aquatic ecosystem balance. The fate and transport of metals in an ecosystem are determined by biological, geological, and chemical factors, as well as by site-specific food web dynamics (Fritsch et al., 2011).

The movement of most metals within biota is primarily diet-driven; thus, macroinvertebrates, dominant in river ecosystems, serve as key vectors transferring contaminants from sediments to higher trophic levels in both aquatic and adjacent terrestrial ecosystems (Bere et al., 2016). Sampling benthic macroinvertebrates and identifying them to species level is challenging and often requires the expertise of multiple taxonomic specialists (Tszydel et al., 2015). Therefore, this study relied on the presence of selected organisms that can represent specific flow types or habitats. The genus Cheumatopsyche (family Hydropsychidae) was selected as the indicator organism for analyzing heavy metal concentrations because of its wide distribution from the upstream to downstream sections of the Bone River (Kadim et al., 2022a).

In bioindicator studies, Trichoptera larvae from the family Hydropsychidae are particularly relevant for assessing freshwater quality. They inhabit rocky riverbed substrates and are highly sensitive to environmental changes, including heavy metal contamination. Hydropsychidae are relatively tolerant to metals (Awrahman et al., 2016; Thamsenanupap et al., 2021), and their long-term exposure and ability to accumulate heavy metals make them suitable indicators of metal pollution (Hornberger, 2024; Let et al., 2022). This family is generally classified as an indicator of mild to moderate pollution (Prommi et al., 2025). Cheumatopsyche species within this family are relatively sensitive to polluted waters (Sudarso and Yoga, 2015), play an important ecological role (Yoga et al., 2014), and are among the dominant aquatic insects in the Bone River under Pb, Cd, and Hg contamination (Kadim et al., 2022b). Therefore, Cheumatopsyche larvae have strong potential as bioindicators of contaminated aquatic ecosystem condition.

One of the primary biological mechanisms by which organisms respond to heavy metal exposure is the production of metallothionein (MT). Metallothioneins are low-molecular-weight proteins that play a key role in binding and detoxifying heavy metals inside cells. MT expression is widely employed as a biological biomarker to detect the level of heavy metal contamination in aquatic organisms (Wong and Stillman, 2020). Numerous studies have demonstrated that elevated MT levels correlate with the heavy metal concentrations in organism tissues (Kadim and Risjani, 2022; Le et al., 2016), making them an effective tool for environmental monitoring.

The effectiveness of bioindicators in assessing river ecosystem health depends not only on the sensitivity of organisms to pollution, but also on the strength of the correlation between biomarker responses and actual environmental conditions. Therefore, an integrated approach combining field data, biomarker responses, and ecological variables is essential for water quality monitoring. Previous studies have shown that the biomarker-based approaches can provide deeper insights into the impacts of heavy metal contamination compared to conventional monitoring methods (Kadim and Risjani, 2022). By combining metallothionein expression analysis, water quality data, and biotic community composition, this study aims to evaluate the effectiveness of *Cheumatopsyche* sp. as a bioindicator of heavy metal pollution in freshwater ecosystems.

This study presents a novel approach to monitoring heavy metal pollution by employing *Cheumatopsyche* sp. larvae as bioindicators through metallothionein (MT) expression. Authors' previous research demonstrated that these larvae can accumulate heavy metals, such as lead (Pb), cadmium (Cd), and mercury (Hg) in aquatic environments (Kadim et al., 2024; Kadim et al., 2022a). MT, as a metal-binding protein, plays a critical role in heavy metal detoxification and homeostasis (Thanomsit, 2016) and serves as a biomarker of metal exposure. Although the use of insects as bioindicators has been recognized in various studies, their application in heavy metal pollution monitoring remains limited compared to other organism groups, such as fish and aquatic plants (Liaqat et al., 2023). Furthermore, while MT is widely acknowledged as a biomarker of metal exposure, its application in *Cheumatopsyche* sp. is relatively new, as most previous studies have focused on other organisms, such as fish (Adam et al., 2022; Dewi et al., 2015) and molluscs (Freitas et al., 2012; Hertika et al., 2020). Despite growing interest, research gaps remain in the application of MT as a bioindicator in aquatic ecosystems, particularly in regions like Indonesia, where industrial and anthropogenic activities pose significant heavy metal pollution threats. The novelty of this study lies in employing MT expression as an early warning system to detect sublethal heavy metal exposure before broader ecological impacts occur. By integrating MT expression with metal concentration data, this research offers a more comprehensive monitoring approach that not only provides insights into the bioavailability of heavy metals but also advances ecological risk assessment in aquatic ecosystems.

METHODS AND MATERIALS

Study area

This study was conducted in the Bone River, Gorontalo Province, Indonesia. Previous research showed that the macrozoobenthic community, water quality parameters particularly biological oxygen demand (BOD), chemical oxygen demand (COD), and ammonia (Kadim and Pasisingi, 2024a) as well as heavy metal concentrations (Kadim and Pasisingi, 2024b) exhibited spatial variation, dividing the river into two pollution categories: slightly polluted (upstream) and moderately polluted (midstream to downstream). In general, land use along the Bone River Basin is dominated by agricultural and residential activities, with gold and sand mining present at several points along the river. On the basis of these conditions, five observation stations were established using purposive sampling to represent the upstream–downstream gradient of the Bone River Basin (Figure 1). Each sampling location was further divided into three carefully selected sub-stations.

In addition to anthropogenic factors that may serve as sources of metal contamination, the selection of sampling stations was also considered river substrate characteristics. The chosen locations featured substrates composed of pebbles, cobbles, and gravel, which are suitable for supporting the presence of Hydropsychidae larvae, the target organisms of this study. One observation station was located in the upstream section of the Bone River, specifically in the Pinogu Village, which served as a control station due to its relatively minimal anthropogenic disturbance. A description of the sampling stations is presented in Table 1.

Sampling was carried out in stages from September 2022 to February 2024, considering the hydrological conditions of the Bone River, specifically during low-flow or rain-free periods to minimize current disturbances and sedimentation. The access to one of the upstream stations, particularly Pinogu (BR-R), was challenging and became even more difficult during the rainy season. Additionally, the extremely small size of *Cheumatopsyche* sp. larvae made it difficult to meet the minimum weight requirement for AAS analysis. The combination of weather conditions, terrain, and organism characteristics contributed to a prolonged sampling process for larvae, sediments, and water.

Sample collection

Larvae samples were collected using the kicking technique with a hand net measuring 200–400

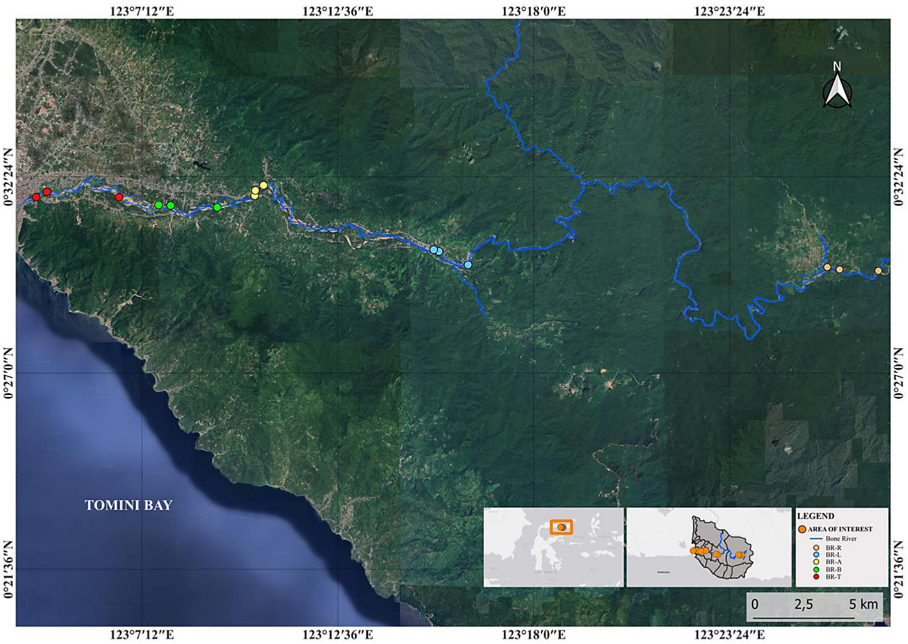

Figure 1. Map of observation stations

Table 1. Description of sampling station

Sampling locations	Code	Coordinate	Land use
Pinogu (Reference station)	BR-R	0°29’48.07”U 123°27’30.57”T	Primary forest
Lombongo	BR-L	0°32’9.18”N 123°10’32.38”E	Agriculture and settlement
Alale	BR-A	0°32’0.99”N 123°10’20.59”E	Dam, agriculture, and settlement
Bubeya	BR-B	0°31’37.63”N 123° 7’38.89”E	Settlement, agriculture and farm
Tanggilingo	BR-T	0°31’48.83”N 123° 6’33.66”E	Settlement, agriculture and sand quarry

mm in width, 2–3 m in height, reinforced with a 100–200 mm shoulder frame, and a mesh size of 500 µm, with a maximum reach of 10 m. Hydropsychidae larvae were also manually collected by searching stones within the sampling area, covering a maximum distance of 100 m. *Cheumatopsyche* sp. larvae were sorted and identified under a binocular microscope at 8–32× magnification. Sampling was considered complete once a sufficient number of individuals had been collected to allow three replicates per station. The sorted larvae were preserved in 10 mL vials containing 4% formaldehyde.

Water and sediment samples were collected from the same stations as the larvae. The water samples were taken using a 1 L dipper and stored in 300 mL bottles. The sediment samples were collected with a soil ring sampler modified from PVC pipes, with approximately 250 g of sediment stored in 300 mL plastic containers. All samples were stored in a cool box and transported to the laboratory for analysis.

Heavy metal determination

The concentrations of Pb, Cd, and Hg in water, sediment, and *Cheumatopsyche* sp. larvae samples were analyzed using an Atomic Absorption Spectrophotometer (AAS) Shimadzu model AA-6200. Heavy metal determination followed the procedure of Hertika et al. (2023), with wavelengths of 217 nm for Pb, 253.7 nm for Hg, and 228.8 nm for Cd.

For larval tissue analysis, a minimum of 1–2 grams of samples from each sampling station were required, and heavy metal concentrations were measured in whole bodies.

Immunohistochemistry for analysis of MT expression

According to Tszydel et al. (2016), molting occurs at each developmental stage of Hydropsychidae larvae. This mechanism is thought to be one of the rapid ways to eliminate harmful elements from the larval body; therefore, this study used *Cheumatopsyche* sp. larvae at the fifth instar stage.

The fifth instar in all Hydropsychidae can be easily distinguished from earlier stages by the presence of small, fleshy, pointed projections (bud-like structures) along the lateral midline on both sides of abdominal segments VI and VII (Kumanski et al., 2004). However, if obtaining larvae at this stage was challenging, individuals measuring 1–1.5 cm in length were used, which is the typical size of Cheumatopsyche at the final larval stage (Zhang and Zhou, 2021).

The larvae of *Cheumatopsyche* sp. collected from each observation station were sectioned, with abdominal segments 1–9 processed for histological examination. Immunohistochemical staining was performed using an anti-MT primary antibody following the protocol outlined by Hertika et al. (2021). Metallothionein (MT) expression was subsequently visualized with a Dot Slide Microscope (OLYMPUS SN 3 K19322), and quantitative assessments of MT density as well as intensity were also conducted in accordance with Hertika et al. (2021) using ImageJ software. MT expression was identified by the presence of brown coloration in the tissues, resulting from antigen–antibody interactions during the immunohistochemical process, and expressed in units of MT/mm^2. MT density was calculated based on the proportion of stained tissue area, whereas intensity was determined by variations in color brightness (dark to light) within the gill and stomach tissues. A darker coloration indicated lower intensity but corresponded to higher MT concentrations, while lighter coloration reflected higher intensity yet lower MT concentrations, expressed in pixel units.

Data analysis

The concentrations of Pb, Cd, and Hg in the water, sediment, and biota samples were compared with the quality standards listed in Table 2.

Statistical analyses were performed to evaluate the relationships between heavy metal concentrations in the environment (water and sediment), metal concentrations in biota, and metallothionein (MT) expression as a biomarker of contamination. The Kruskal–Wallis test was applied to detect significant differences among stations, followed by Dunn's post-hoc test to determine station pairs that differed significantly for each variable. Spearman's correlation analysis assessed the relationships between heavy metal concentrations in water and sediment and those in biota, as well as between metal concentrations in biota and MT expression (density and intensity). These non-parametric tests were chosen to identify the strength and direction of associations without assuming data normality. The relationships between heavy metals (Pb, Cd, Hg) and metallothionein responses were modeled using semi-parametric Generalized Additive Models (GAMs) with a Gaussian identity link and smoothness parameter (k = 4), fitted using the 'mgcv' package. All analyses were performed in R version 4.5.0, with a significance level set at $\alpha = 0.05$. Results were summarized in tables and visualized in graphs for ease of interpretation.

Table 2. Quality standards for Pb, Cd, and Hg in water, sediment, and biota

Heavy metals	Sample	Quality standard (Concentration in ppm)*
Pb	Water	0.03
	Sediment	50;31;30.2
	Biota	0.2
Cd	Water	0.01
	Sediment	1.5;0.6;0.7
	Biota	0.1
Hg	Water	0.002
	Sediment	0.15;0.2;0.13
	Biota	0.5

Note: *For water samples, the quality standards followed Indonesian Government Regulation No. 22 of 2021. Sediment samples were evaluated against ANZECC (Australian and New Zealand Environment and Conservation Council) guidelines (Simpson et al., 2013), OSQG LEL (Ontario Sediment Quality Guidelines – Lowest Effect Level), and CCME TEL (Canadian Council of Ministers of the Environment – Threshold Effect Level) (Edward, 2020). For biota samples, the standards were based on the Indonesian Food and Drug Authority or BPOM Regulation No. 5 of 2018.

RESULT AND DISCUSSION

Heavy metal concentrations in water, sediment, and *Cheumatopsyche* sp. from the Bone river

The results of heavy metal analysis across five sampling stations are summarized in Table 3. Pb consistently exhibited higher mean concentrations in both water and sediment compared to Cd and Hg. In water, Pb concentrations ranged from 0.0028–0.0047 ppm for Pb, 0.0013–0.0028 ppm for Cd, and 0.0023–0.0034 ppm for Hg. In sediment, the maximum Pb concentration (44.2 ppm) occurred at BR-A, Cd peaked at BR-T (0.168 ppm), and Hg at BR-L (0.213 ppm). The lowest levels of all three metals were recorded at BR-R (24.1, 0.071, and 0.145 ppm for Pb, Cd, and Hg, respectively). Across sampling stations, Cd consistently showed the lowest concentrations among the three metals.

The results indicate that the Pb and Cd concentrations in water complied with the quality standards for all designated classes as stipulated in Government Regulation No. 22 of 2021, whereas the Hg concentrations at all observation stations exceeded the Class III standard. In sediments, the Pb concentrations at BR-L, BR-A, BR-B, and BR-T were higher than the OSQG LEL and CCME TEL thresholds but remained below the ANZECC guideline values. Hg concentrations exceeded the ANZECC standards at BR-L and BR-A, CCME TEL at all stations, and OSQG LEL at BR-L. In contrast, Cd concentrations remained within the permissible limits across all three standards. In this study, sediment metal concentrations increased from BR-R to BR-A, then decreased at BR-B, and rose again at BR-T. These findings also reveal that the relative proportions of metals were consistent across sampling stations, suggesting that contamination patterns may lead to spatial differences in sediment metal concentrations.

The data in Table 3 shows that heavy metals (Pb, Cd, and Hg) accumulated in the bodies of *Cheumatopsyche* sp. larvae. The highest concentrations in larvae were recorded at BR-A, with Hg at 0.479 ppm, followed by Pb (0.446 ppm) and Cd (0.392 ppm). Larvae accumulated lower metal concentrations in less contaminated areas (BR-R), higher levels at BR-A, then a decline at BR-B, and an increase again at BR-T.

Measurements also revealed that Pb (at all stations) and Cd (except BR-R) concentrations in larvae exceeded the limits set by Indonesian Food and Drug Authority Regulation No. 5 of 2018, while Hg, although detected in larvae, remained below the threshold. Variations in concentrations across water, sediment, and larvae reflect station-specific physicochemical conditions and the differential heavy metal accumulation capacity of *Cheumatopsyche* sp.

Figure 2 illustrates that heavy metal concentrations were generally lowest in water, higher in sediment, and highest in Cheumatopsyche sp. larvae, reflecting bioaccumulation potential across matrices. The stations located in Lombongo Village (BR-L), Alale Village (BR-A), and Tanggilingo Village (BR-T) exhibited elevated heavy metal concentrations in water, sediment, and biota tissues. The detection of Hg in the Bone River indicates contamination, likely associated with the gold mining activities. The spatial gradient pattern demonstrates similar distribution trends for metal concentrations across water, sediment, and larval tissues. Unlike Pb and Cd, the Hg gradient at BR-A, BR-B, and BR-T showed a decreasing trend downstream in both sediment and larvae. The lowest heavy metal concentrations were recorded at the upstream station (BR-R), while the highest concentrations were observed at BR-A. This gradient pattern may reflect bioavailability levels, rather than absolute sediment metal concentrations. Furthermore, statistical analysis revealed significant spatial heterogeneity in heavy metal contamination across the study area. BR-A consistently exhibited higher metal concentrations in biota, whereas BR-R generally showed lower contamination levels. The bioaccumulation patterns of heavy metals in *Cheumatopsyche* sp. differed significantly among sampling stations. Pb bioaccumulation varied significantly across stations, with BR-A showing the highest tissue concentrations, significantly different from BR-R. Cd bioaccumulation also displayed significant spatial variation, with BR-A having higher values than BR-R. Similarly, Hg bioaccumulation was significantly different among stations, with BR-A exhibiting higher levels than BR-R, as confirmed by the Kruskal–Wallis and Dunn's post-hoc tests ($p < 0.05$ in all cases).

More than 99% of heavy metals entering aquatic systems can be retained in sediments. River sediments act as carriers of heavy metals and therefore play a crucial role in assessing and tracing contamination sources (Maurya et al., 2019). Sediment concentrations are generally

Table 3. Measured concentrations (ppm) of heavy metals in water, sediment, and biota from the Bone River

Station (Variable)		Descriptive	Heavy metal concentration (ppm)		
			Pb	Cd	Hg
BR-R	Water	Mean ± SD	0.0028 ± 0.0003	0.0013 ± 0.0002	0.0034 ± 0.0002
		min – max	0.0025 – 0.0031	0.0012 – 0.0015	0.0022 – 0.0025
	Sediment	Mean ± SD	24.1 ± 2.6	0.0705 ± 0.0075	0.1445 ± 0.0005
		min – max	21.5 – 26.7	0.063 – 0.078	0.144 – 0.145
	Cheumatopsyche sp.	Mean ± SD	0.209 ± 0.036	0.0817 ± 0.0105	0.2277 ± 0.0155
		min – max	0.173 – 0.245	0.071 – 0.092	0.212 – 0.243
BR-L	Water	Mean ± SD	0.0042 ± 0.0004	0.0017 ± 0.0002	0.0042 ± 0.0006
		min – max	0.0039 – 0.0046	0.0015 – 0.0018	0.0032 – 0.0035
	Sediment	Mean ± SD	34.6 ± 2.1	0.1315 ± 0.0155	0.2125 ± 0.0205
		min – max	32.5 – 36.7	0.116 – 0.147	0.192 – 0.233
	Cheumatopsyche sp.	Mean ± SD	0.3257 ± 0.0085	0.2617 ± 0.0055	0.461 ± 0.012
		min – max	0.317 – 0.334	0.256 – 0.267	0.449 – 0.473
BR-A	Water	Mean ± SD	0.0047 ± 0.0004	0.0028 ± 0.0002	0.0032 ± 0.0004
		min – max	0.0043 – 0.0051	0.0026 – 0.0029	0.0028 – 0.0035
	Sediment	Mean ± SD	44.2 ± 1.5	0.147 ± 0.015	0.1875 ± 0.0095
		min – max	42.7 – 45.7	0.132 – 0.162	0.178 – 0.197
	Cheumatopsyche sp.	Mean ± SD	0.446 ± 0.029	0.3917 ± 0.0045	0.4787 ± 0.0105
		min – max	0.417 – 0.475	0.387 – 0.396	0.468 – 0.489
BR-B	Water	Mean ± SD	0.0037 ± 0.0003	0.0023 ± 0.0002	0.0028 ± 0.0002
		min – max	0.0034 – 0.0039	0.0021 – 0.0025	0.0026 – 0.003
	Sediment	Mean ± SD	31.05 ± 2.65	0.1075 ± 0.0185	0.1535 ± 0.0055
		min – max	28.4 – 33.7	0.089 – 0.126	0.148 – 0.159
	Cheumatopsyche sp.	Mean ± SD	0.352 ± 0.025	0.2547 ± 0.0185	0.4477 ± 0.0095
		min – max	0.327 – 0.377	0.236 – 0.273	0.438 – 0.457
BR-T	Water	Mean ± SD	0.0031 ± 0.0003	0.0025 ± 0.0002	0.0028 ± 0.0002
		min – max	0.0028 – 0.0034	0.0024 – 0.0027	0.0026 – 0.0029
	Sediment	Mean ± SD	34.1 ± 2.4	0.168 ± 0.004	0.151 ± 0.002
		min – max	31.7 – 36.5	0.164 – 0.172	0.149 – 0.153
	Cheumatopsyche sp.	Mean ± SD	0.356 ± 0.006	0.352 ± 0.039	0.3647 ± 0.0405
		min – max	0.35 – 0.362	0.313 – 0.391	0.324 – 0.405

higher than those in water, because metals tend to bind and settle, integrating with the deposited sediments (Widiastuti et al., 2019). This process is driven by their higher specific gravity than water, resulting in greater accumulation in sediments. Additionally, heavy metals exhibit a strong affinity for organic matter, further promoting their deposition and incorporation into sediments, leading to significantly higher concentrations compared to water (Suciyono et al., 2024).

The transfer of heavy metals from aquatic insects to secondary consumers (their predators) is strongly influenced by the species and developmental stage at the time of predation. Inter-species variation in tissue and organ distribution results in differences in the proportion of accumulated metals transferred (Chouvelon et al., 2019). Moreover, the total lead burden in insect bodies can increase due to the metals adsorbed onto the exoskeleton or larval casing. During metamorphosis, larvae and juveniles often shed exoskeletons or leave behind larval cases in sediments, which can then be utilized by detritivores as an additional source of metals. Conversely, the metals stored in adult insect bodies can be mobilized into terrestrial environments through emergence (Iqbal et al., 2025). Consequently, contaminated aquatic habitats, particularly those with high insect emergence rates, pose a significant pathway for contaminant transfer to terrestrial ecosystems, increasing the exposure risks at higher trophic levels.

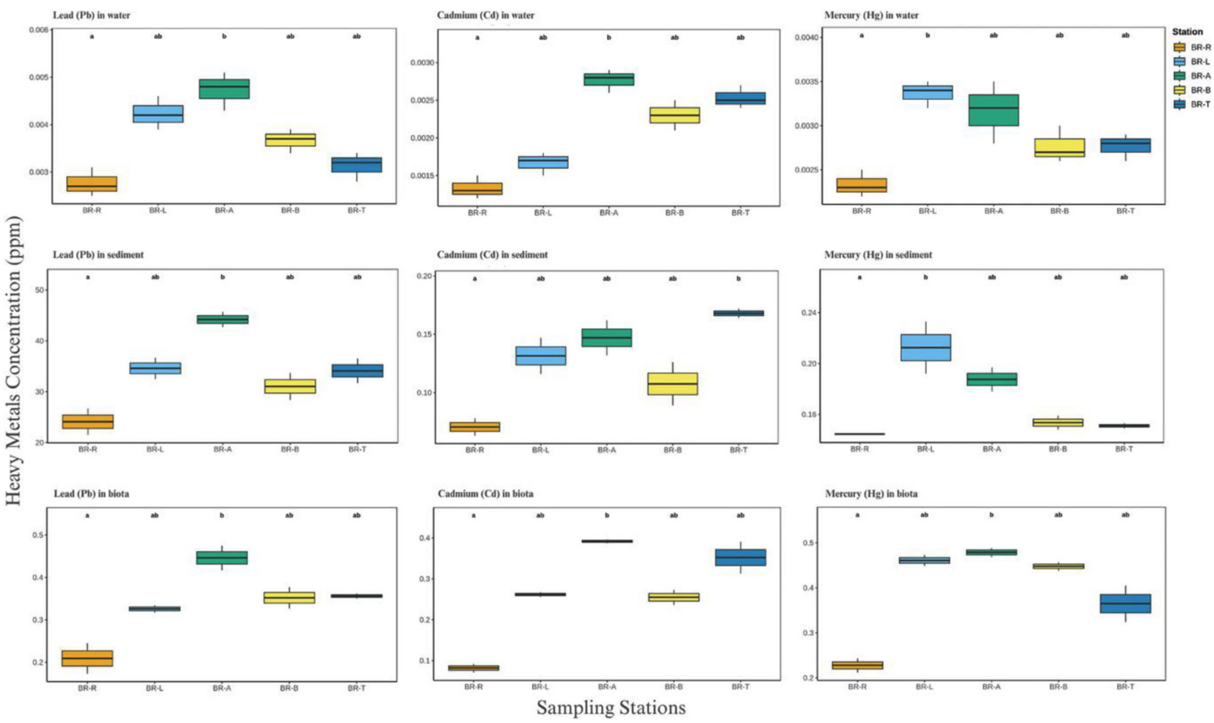

Figure 2. Box plot heavy metal concentration in water, sediment, and larvae of *Cheumatopsyche* sp. Statistical differences among stations are expressed in letters since there were significant differences ($P < 0.05$)

Hydropsychidae are generally sedentary filter-feeding collectors, which likely explains the high accumulation of heavy metals in larval tissues compared to the relatively low concentrations of Pb, Cd, and Hg in water. This accumulation is attributed to the ingestion of contaminated materials suspended in the water column or deposited on the riverbed (Kadim et al., 2024; Tszydel et al., 2016). Continuous exposure, even at low concentrations, may limit the larvae's self-purification capacity and reduce the elimination of heavy metals from their tissues (Chiba et al., 2011). Previous studies have reported a proportional relationship between heavy metal concentrations in water or sediment and those in invertebrate larvae. However, discrepancies often occur between metal concentrations in water and sediment and those in larval tissues. Similar observations were reported by Freitas et al. (2018) in the *Diopatra neapolitana* and *Cerastoderma edule* macroinvertebrates.

The presence of heavy metals in Hydropsychidae larvae can also be influenced by the waste containing nitrates, phosphates, and organic matter from synthetic fertilizers or pesticides, which often contain Cd, Zn, Cu, Ni, and Pb (Mebane et al., 2020; Pola, 2018). Agricultural pollutants, such as nitrate and ammonia can alter water and sediment chemistry by triggering changes in dissolved oxygen and pH, which in turn affect heavy metal speciation and bioavailability (Banerjee et al., 2023; Edwards et al., 2024). The authors' previous research reported that the pollution level in the Bone River is dominated by organic waste originating from agricultural and domestic household activities (Kadim and Pasisingi, 2024), which likely contributes to the presence of Pb and Cd in *Cheumatopsyche* sp. larvae in this study. In addition to anthropogenic impacts, the long life cycle, sedentary behavior, and omnivorous feeding habits of larvae allow them to accumulate heavy metals from water, bottom sediments, and surrounding seston (Haas and Pánik, 2025; Malematja et al., 2023).

Metallothionein expression in *Cheumatopsyche* sp. larvae

Metallothioneins are low-molecular-weight proteins recognized as a defense mechanism, composed of cysteine-rich polypeptides encoded by specific genes. Their primary function is as metal-binding peptides, containing a high number of thiol (sulfhydryl, –SH) groups and lacking histidine residues; these thiol groups bind heavy metals with high affinity and efficiency

(Juárez-Rebollar et al., 2017). On the basis of the immunohistochemical analysis of the abdominal tissues of *Cheumatopsyche* sp., metallothionein was detected as brown-stained blocks in tissue sections from stations with varying contamination levels (see Figure 3).

The formation of the brown coloration is explained by the principle of the peroxidase-based immunohistochemical staining method, in which antigens in the tissue bind to specific primary antibodies, producing a brown precipitate as a result of substrate (chromogen and H_2O_2) breakdown by the peroxidase enzyme (Ramos-Vara, 2005). The brown color indicates a positive reaction (+), meaning that the antigen is present in the tissue. In the absence of an antigen, no brown staining appears. The brown-stained areas can be classified into three intensity levels for positive reactions and one for negative reactions. Positive reactions include strong positive (+++), indicated by dark brown to black staining; moderate positive (++), indicated by dark brown; and weak positive (+), indicated by light brown mixed with blue (Hertika et al., 2014). Darker staining corresponds to lower intensity, but higher MT concentration, whereas lighter staining indicates higher intensity, but lower MT concentration, measured in pixel units (Hertika et al., 2021).

Metallothionein appeared as dark brown deposits in the abdominal tissues of larvae, binding the heavy metals that entered the digestive organs. As it is shown in Figure 4, station BR-R predominantly exhibited weak positive reactions, while BR-T and BR-B were dominated by dark brown staining, indicating strong and moderate positive reactions. BR-L showed a mix of moderate and weak positives, whereas BR-A was dominated by strong positive reactions.

Metallothionein (MT) is a metal-binding protein that plays a crucial role in the defense mechanisms of organisms against heavy metal exposure and in detoxification processes. According to Hertika et al. (2023), when heavy metals enter the body, thionein proteins immediately bind them, forming the MT complexes that prevent interactions with vital metabolic proteins. If the amount of heavy metals exceeds the organism's capacity to synthesize MT, detoxification efficiency decreases, allowing metals to circulate in the bloodstream, bind to hemoglobin, and remain strongly attached to sulfhydryl groups, which hinders elimination. These interactions can induce structural changes in proteins and enzymes, displace essential metal ions, and inhibit enzymatic activity. Such disruptions impair cellular metabolism, ultimately leading to cell damage or lysis.

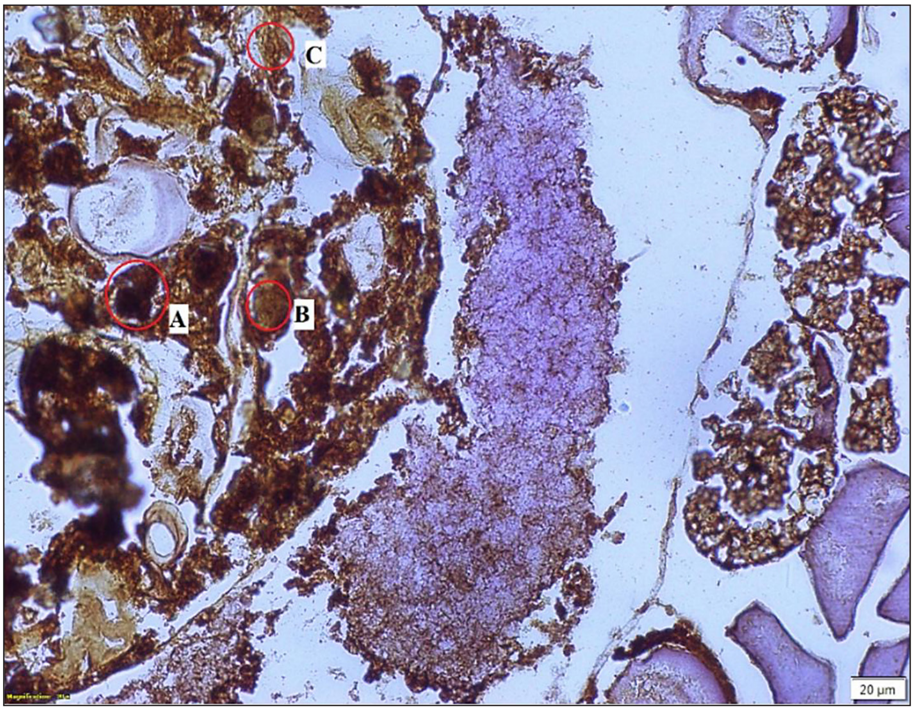

Figure 3. MT expression in *Cheumatopsyche* sp. larval tissues showing positive immunohistochemical reactions (red circles): (A) strong positive; (B) moderate positive; (C) weak positive. Magnification: 400×, scale bar: 20 μm

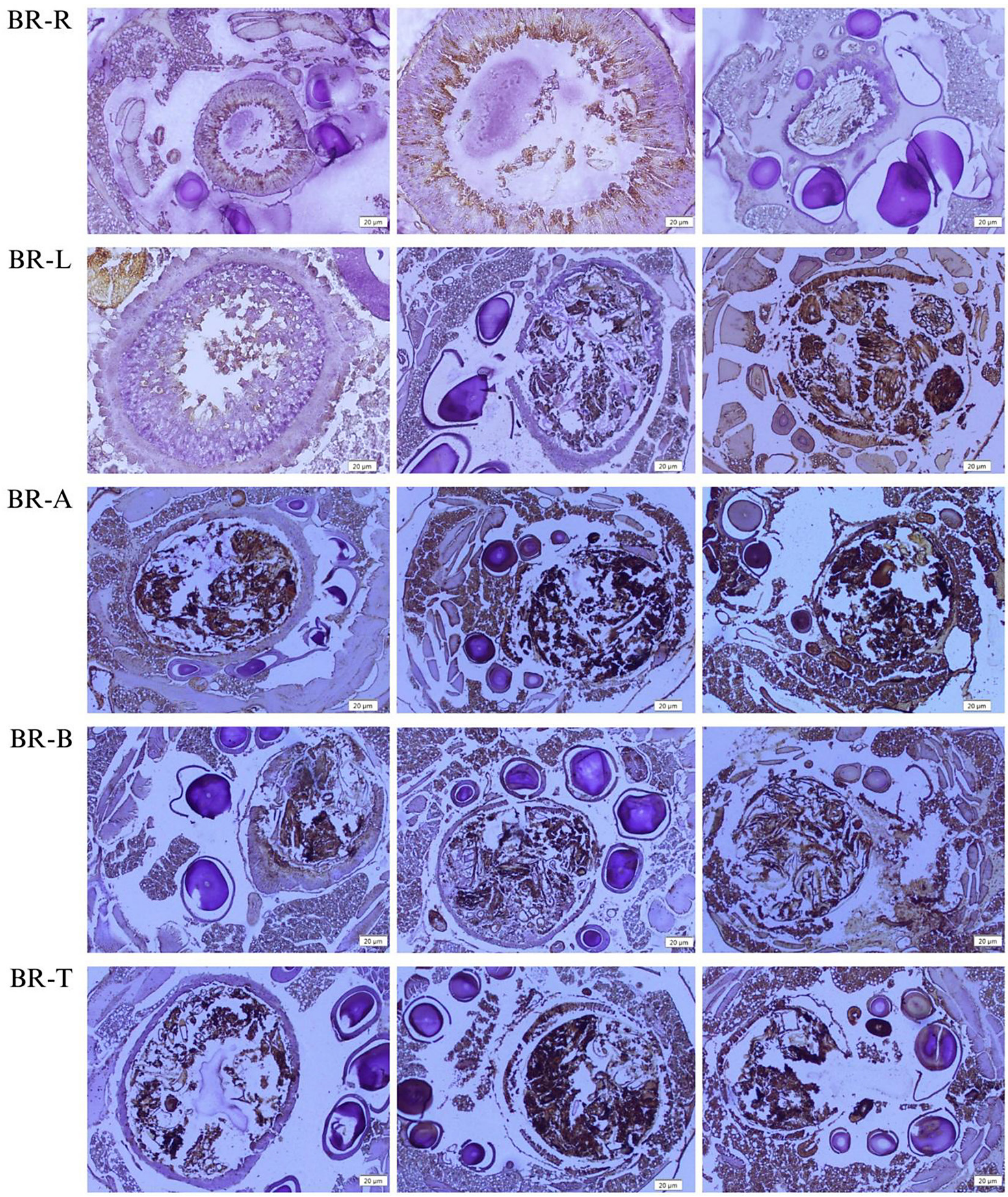

Figure 4. MT expression in *Cheumatopsyche sp*. larval tissues across sampling stations (Magnification: 400×, scale bar: 20 µm.)

Relationship of heavy metal concentration and MT expression in *Cheumatopsyche* sp.

The calculated MT density and intensity revealed that the highest MT density was observed at station BR-A (16.51×10^{-4} MT/µm²), while the lowest was recorded at BR-R (5.44×10^{-4} MT/µm²). Conversely, MT intensity was highest at BR-R (169.3 pixels) and lowest at BR-T (142.44 pixels). These findings reflect the larval response to heavy metal uptake, as the concentrations of Pb, Cd, and Hg in the larvae from BR-A were higher compared to other stations (see Figure 5). This indicates that the concentration of heavy metals accumulated within the organism tends to be positively correlated with the MT density in its tissues.

Figure 6 shows that the samples from BR-R generally exhibited expression dominated by light brown coloration, indicating weak positive staining. Similarly, MT intensity at BR-R was higher compared to other observation stations. According to Isroni et al. (2021), the abundance of MT is reflected by the increasing darkness of the brown coloration, which indicates higher heavy metal concentrations. Greater heavy metal

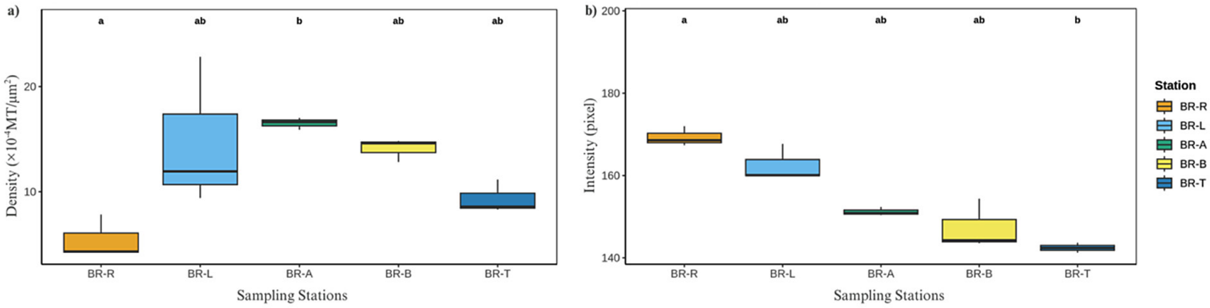

Figure 5. Result of Metallothioneins expression calculation in abdomen larvae of *Cheumatopsyche* sp. from five stations of Bone river: (a) MT density (×10^{-4}MT/µm^2); (b) MT intensity (pixel). Different letters indicate significant differences ($p < 0.05$) among monitored stations

uptake corresponds to higher density or activity of the system within the cells.

Prior to this, Spearman correlation analysis was performed to evaluate the relationships between heavy metal concentrations in water and sediment with those in biota, as well as the relationship between the metal concentrations in biota and MT expression (density and intensity) (see Figure 6). The results revealed strong positive associations between environmental heavy metal concentrations (water and sediment) and metal accumulation in biota, with all correlations being significant and ranging from moderate to very strong (r = 0.594–0.869). Cadmium (Cd) exhibited the highest correlation, both between water and biota as well as between sediment and biota, confirming its high bioavailability and greater tendency to accumulate in organism tissues.

Furthermore, the relationship between metals in biota and MT expression displayed an interesting pattern. MT density showed a positive correlation with all metals (r = 0.536–0.793), indicating that higher metal concentrations correspond to increased MT protein density in tissues. Conversely, MT intensity exhibited a significant negative correlation with Pb and Cd, while no significant relationship was observed with Hg. These findings suggest that although biota respond to increased metal exposure by elevating MT density, the intensity of expression may vary depending on the metal type. Hg appeared to elicit the most consistent biological response in terms

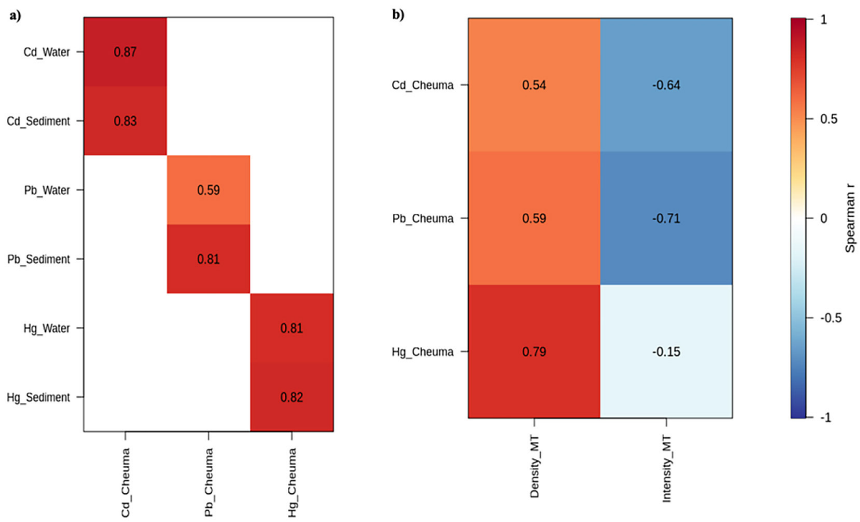

Figure 6. Spearman correlation heatmaps for (a) environmental metals (water and sediment) versus bioaccumulated metals in biota (Cheuma) and (b) biota metals versus MT metrics (density and intensity). Red/orange colors indicate a stronger positive Spearman correlation, while blue indicates a negative correlation. Almost all variable pairs in the matrix exhibited significant correlations ($p < 0.05$). However, Hg Biota was not significant under Spearman correlation ($p \approx 0.585$), whereas distance correlation remained significant, suggesting the presence of a non-linear relationship

of MT density. A subsequent generalized additive model (GAM) analysis was conducted to assess the significance of each heavy metal factor in larval tissues on MT expression in *Cheumatopsyche* sp. The results are presented in Figure 7.

The GAM analysis indicated that the MT expression in biota was differentially influenced by Pb, Cd, and Hg, depending on the response indicator considered (intensity or density). For MT intensity, Pb and Hg exhibited significant effects, but with non-linear patterns, meaning that the changes in Pb and Hg concentrations were not consistently proportional to the changes in MT intensity; instead, the relationship tended to form a curved (non-linear) pattern, suggesting a relatively complex interaction. In contrast, Cd showed no significant effect, indicating the absence of a consistent relationship between Cd concentration and MT intensity. Overall, the model for MT intensity demonstrated excellent explanatory power (adjusted $R^2 \approx 0.94$; deviance explained ≈ 96.5%), suggesting that variations in MT intensity were largely explained by metal concentrations, particularly Pb and Hg.

Meanwhile, for the Density response, only Hg exhibited a significant effect on changes in MT density, whereas neither Pb nor Cd showed any meaningful influence. The relationship pattern observed was relatively simple, approaching linearity. The explanatory power of the model for MT density was moderate (adjusted $R^2 \approx 0.51$; deviance explained ≈ 61.7%), indicating that variations in MT density could only be partially explained by variations in metal concentrations, particularly Hg. Overall, these findings suggest that Hg is the most consistently influential factor affecting both MT expression indicators—intensity and density. Pb also plays an important role, but only in relation to MT intensity, while Cd does not exhibit any significant effect within the context of this dataset. These results align with the role of MT as a biomarker sensitive to specific heavy metals, where biotic responses may vary depending on the type of metal and the biomarker indicator used. Therefore, the observed relationship between MT and the metals in the tissues of *Cheumatopsyche* sp. larvae is biologically meaningful. The presence of heavy metals in water and sediment influences their concentrations in tissues, which ultimately affects the MT expression in *Cheumatopsyche* sp. larvae.

The application of biomarkers for assessing aquatic pollution has been increasingly developed in aquatic organisms. Among these, the use of specific biomarkers such as MT has gained attention for evaluating heavy metal contamination. MT is considered a potential biomarker and has been identified and quantified in the tissues of various aquatic organisms. The accumulation of this metal-binding protein in tissues at the time of measurement can serve as an indicator of heavy metal pollution. According to Wardani

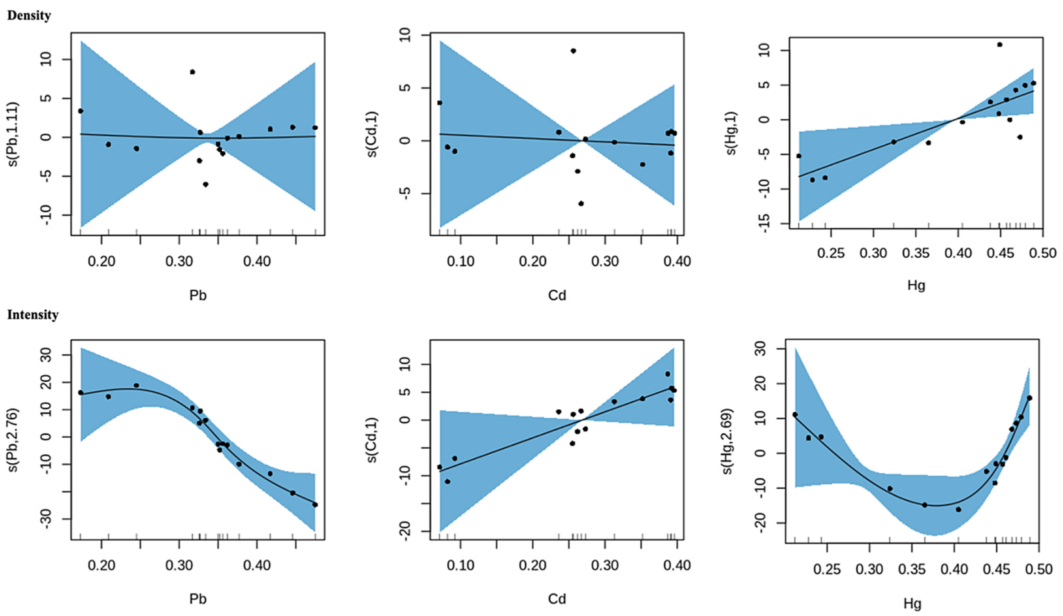

Figure 7. GAMs revealed variations in the effects of Pb, Cd, and Hg concentrations in *Cheumatopsyche* sp. larvae on MT expression, depending on whether the indicator was intensity or density

et al. (2022), heavy metal concentrations exhibit a significant relationship with MT expression. Previous studies have reported that Pb influences MT expression in the gills of the freshwater mussel *Anodonta woodiana* (Hertika et al., 2014) and significantly affects the MT levels in *Sulcospira testudinaria* (Hertika et al., 2023). Similarly, Hg has been shown to affect both MT density and intensity in the gills of *Barbonymus altus* (Anjasmara et al., 2023). Furthermore, Pb, Cd, and Hg in the tissues of aquatic invertebrates, such as *Crassostrea iredalei*, *Crassostrea glomerata* (Hertika et al., 2021), and *Crassostrea cucullata* (Isroni et al., 2021) have demonstrated significant effects on MT density and intensity in both gill as well as stomach tissues. Freitas et al. (2012) also reported that MT can be induced by essential metals (Cu and Zn) as well as non-essential metals (Cd, Ag, and Hg) in both vertebrates and invertebrates, although the degree of induction varies. This variation is both intra- and interspecific and is influenced by environmental as well as physiological factors. Biologically, MT binds dissolved metals in tissues, suggesting that the correlation between MT and metal concentrations should be stronger for dissolved metals to prevent excessive accumulation.

MT is a metal-binding protein crucial for intracellular regulation and detoxification of excess non-essential metals, such as Cd and Hg. Its expression varies across individuals and species, influenced by factors including taxonomic identity, organ type, age, and physiological status (Nordberg and Nordberg, 2022). In aquatic organisms, tissue MT levels are further modulated by metallothionein mRNA upregulation in response to metal influx (Han et al., 2015). As a homeostatic defense mechanism, organisms synthesize MT to sequester and detoxify heavy metals (Kemp et al., 2017). Positive correlations between MT and metals, such as Pb, Cd, and Hg have been reported (Hertika et al., 2018). Heavy metal pollutants induce systemic damage, trigger MT production, and, upon tissue accumulation, drive MT synthesis to its maximum capacity, reflecting the sequestration of metals by MT as part of the organism's defense strategy. Accordingly, MT represents a reliable biomarker when applied judiciously within rigorously designed environmental monitoring programs.

Evaluating heavy metal contamination using water or sediment samples alone is often unreliable, because pollutant quality and quantity in rivers can fluctuate rapidly due to weather conditions, surface runoff, and interactions with other water components (Tszydel et al., 2015). This study confirms that the *Cheumatopsyche* sp. larvae are capable of accumulating heavy metals to a measurable extent, even when their concentrations in water or sediment are relatively low, as previously observed in other Hydropsychidae species. Therefore, *Cheumatopsyche* sp. larvae can serve as effective bioindicators for aquatic pollution, including heavy metal contamination.

Heavy metal contamination in benthic macroinvertebrate communities of the Bone River has been scarcely investigated, particularly in relation to the *Cheumatopsyche* sp. larvae. The number of aquatic bioindicators currently used to assess metal pollution is also very limited. Given that *Cheumatopsyche* sp. is a sessile filter feeder, widely distributed in the Bone River, and characterized by a relatively long larval life cycle, its ability to accumulate heavy metals, confirmed by the expression of MT biomarkers, positions this species as a promising bioindicator of heavy metal contamination. This constitutes a novel aspect of the present study.

This study demonstrated that *Cheumatopsyche* sp. larvae are reliable and sensitive bioindicators of heavy metal contamination in riverine ecosystems. The strong correlation between environmental metal concentrations and bioaccumulation in larvae underscores their effectiveness in tracking contaminant dynamics. Notably, the differential induction of MT across metal types, with Hg emerging as the primary inducer, provides mechanistic validation at the molecular level, extending beyond mere accumulation measurements to reveal specific physiological responses to metal stress. Such MT responses offer a more nuanced tool for distinguishing the toxicological impacts of different metals. From a practical perspective, the use of *Cheumatopsyche* sp. larvae enables targeted and cost-effective river monitoring programs, facilitating the identification of pollution hotspots and the assessment of ecosystem health. These findings support the integration of this species into river management frameworks, providing a scientific basis for regulatory decision-making and remediation strategies. Future research should focus on establishing standardized MT expression thresholds for specific metals to enhance the predictive capacity of this biomarker and explore the long-term effects of sublethal metal exposure on indicator species populations.

Despite establishing the *Cheumatopsyche* sp. larvae as a promising bioindicator, this study has several limitations that warrant consideration. First, the analysis was restricted to a limited set of metals (Pb, Cd, and Hg); responses of MT to other common contaminants, such as arsenic, chromium, or cyanide, remain unexplored, which may influence biomarker interpretation in complex multi-pollutant environments. Second, while immunohistochemistry effectively detects MT, it provides only semi-quantitative estimates of protein density and intensity. Incorporating quantitative techniques such as Enzyme-Linked Immunosorbent Assay (ELISA) could complement these findings and offer more robust validation of metal-induced stress responses. Finally, the results are contextualized within a specific river system. Metal bioavailability and toxicokinetics are strongly influenced by abiotic factors, such as pH, dissolved organic carbon, and water hardness. Therefore, applying MT response thresholds derived from this study to rivers in different geographic regions with varying hydrochemical conditions will require further calibration and validation. Future research should integrate these factors to enhance the predictive power and generalizability of MT-based biomonitoring models.

CONCLUSIONS

Overall, the Pb, Cd, and Hg levels in water, sediment, and *Cheumatopsyche* sp. larvae were relatively low, but sufficient to induce metallothionein (MT) synthesis, enabling MT detection in larval tissues. This finding highlights the high sensitivity of MT as a biomarker, capable of signaling metal exposure even at low environmental concentrations. In more contaminated stations, larvae showed increased MT density, indicating a dose-dependent response to metal stress. Among the metals examined, Hg was the most consistent driver of MT expression, influencing both intensity and density; Pb affected only intensity, while Cd showed no significant effect. These findings confirm the utility of MT as a sensitive biomarker that differentiates physiological responses to specific metals. Collectively, the MT expression in *Cheumatopsyche* sp. larvae reliably reflects metal accumulation and underscores its strong potential as an indicator of aquatic environmental quality.

Acknowledgements

This research was funded by the Ministry of Education, Culture, Research, and Technology of Indonesia through Penelitian Fundamental Reguler 2024 via Decree of Rector of Universitas Negeri Gorontalo No: 0459/E5/PG.02.00/ 2024 under contract no: 063/E5/PG.02.00.PL/2024. We also thanked for everyone who has helped the completion of this research.

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