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Performance of Metallothionein Biomarker from *Sulcospira testudinaria* to Assess Heavy Metal Pollution in the Brantas River Watershed, Indonesia

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ABSTRACT

Heavy metal pollution in freshwater ecosystems is a critical issue because it threatens the ecosystem as well as public health. Early detection of these pollutants is therefore essential, and biomarker analysis can be an ideal way to achieve this. Metallothionein (MT) protein is a widely-used molecular biomarker related to the physiological and biological changes which suitable bioindicators, like freshwater snails, undergo in stressful environments. The purpose of this research is to assess the relationship between the heavy metals lead (Pb), cadmium (Cd) and mercury (Hg) and MT levels in freshwater snails (*Sulcospira testudinaria)* in the Brantas River watershed. Heavy metals were assayed using an atomic absorption spectrophotometer, while MT levels were analyzed using indirect enzyme-linked immunosorbent assay (ELISA). Water quality parameters including temperature, dissolved oxygen (DO), biological oxygen demand (BOD), ammonia concentration, and phenol concentration were also measured. Samples were obtained from ten sampling sites at Brantas River watershed. The results indicated that Pb concentration ranged from 0.001–0.006 mg/L, Hg from 0.001–0.005 mg/L, and Cd from 0.005–0.03 mg/L, while MT concentration ranged from 0.40–0.80 ng/g. Relationship analysis between heavy metals and MT level in this study revealed the significant effect of Pb concentration on MT values in *Sulcospira testudinaria*. Therefore, MT in this snail is a potential biomarker for Pb contamination.

Keywords: aquatic monitoring; biomonitoring; freshwater ecosystem; water contamination.

INTRODUCTION

Contamination of freshwater ecosystems with heavy metals is a major concern around the world because metals have a tendency to bioaccumulate in aquatic species [van der Oost et al., 2003]. Unlike organic pollutants, heavy metals are non-degradable [Zhou et al., 2008]. Heavy metals including cadmium (Cd), lead (Pb), and mercury (Hg) continue to pose a health threat to organisms, and their widespread distribution is due to anthropogenic activities [M'kandawire et al., 2017]. Heavy metal pollution should be detected early, before it causes deleterious effects to ecosystems and public health. Because chemical water monitoring is both technically and financially challenging, there is a pressing need to measure particular biomarkers [Kovářová and Svobodová, 2009].

Biomarkers are used in environmental assessments as 'early warning' techniques. [McCarthy and Shugart, 1990] and can be used to measure hazardous chemical or environmental stress exposure [Paustenbach and Galbraith, 2006]. Among biomarkers, molecular biomarkers are linked to physiological characteristics found in stressful situations [Livingstone, 1993; Fabrin et al., 2018]. Sentinel species can therefore be regarded as bioindicators in both aquatic and terrestrial ecosystems [Hodkinson and Jackson, 2005]. The importance of molecular biomarker tests is that they reveal early changes in biological processes as a result of pollution exposure [van der Oost et al., 2003]. Furthermore, because pollutants may be conveyed by the river, the identification of these materials by typical chemical studies may be difficult, since the untreated effluent is the main source of pollution in aquatic ecosystems [Fabrin et al., 2018]. Bioindicator species are often connected to a specific contaminant [Livingstone, 1993], which can be indicative of an ecosystem's overall health. Bioindicator species include both vertebrate and invertebrate species [Li et al., 2010].

Metallothioneins (MTs) are widely-used molecular biomarkers associated with metal pollution [Fabrin et al., 2018]. MTs are metal-binding proteins with a low molecular mass and a high level of cysteine [Kaegi and Schaeffer, 1988]. The sequestration function of MT in the presence of toxic metals is linked to cellular defence against metal toxicity [Roesijadi, 1992]. Under experimental conditions, heavy metals such as zinc (Zn), copper (Cu), and cadmium (Cd) elicit the promotion of MT in aquatic organisms [Linde and Garcia-Vazquez, 2006]. These findings have allowed MT to be considered as a promising biomarker for metal exposure. However, in field studies, where environmental circumstances are less well defined than in laboratory research, MT induction in aquatic species caused by environmental exposure to heavy metals is more difficult to assess [Linde et al., 2001]. Therefore, the choice of an appropriate organism as bioindicator is important [Chen et al., 2021].

A strong bioindicator should meet specific requirements, such as a well-characterized biology, potential to be used for early warning of changes in ecosystem health, widespread distribution in the relevant environment, representation of an essential function in the ecosystem, and a connection between the biomarker node and pollutant level [Reguera et al., 2018]. Molluscs have been recognized as ideal bioindicators for decades, as they often fit these requirements [Oehlmann and Schulte-Oehlmann, 2003]. Snails, which are common in freshwater habitats, can account for up to 60% of overall macroinvertebrate biomass in some freshwater systems [Tallarico, 2015]. Some snail species are known to react to external contaminants; for example, there is a relationship between the degree of metal exposure and its impact on *Biomphalaria alexandrina* [Habib et al., 2016] and *Pomacea canaliculate* [Campoy-Diaz et al., 2018].

Sulcospira testudinaria is a snail from the gastropod class which is widely distributed in fresh waters in Indonesia, especially on the island of Java. The habitats of this species are rivers or lakes with calm or swift currents. Previous research found that *Sulcospira testudinaria* was present in the Cikeruh River [Lutfi et al., 2020], Code River [Hellen et al., 2020], Meru Betiri National Park [Suratno et al., 2020], Gunung Kidul [Isnaningsih and Listiawan, 2010], and the Brantas River [Febbyanto et al., 2015]. This species was used as a bioindicator for aquatic environmental health [Hertika et al., 2021], and was used to measure the results of efforts to reduce levels of organic matter in aquaculture wastewater [Lailiyah et al., 2021].

This study aims to assess the relationship between heavy metals (Pb, Cd, Hg) and MT levels in the freshwater snail *Sulcospira testudinaria* in the Brantas River watershed. The river is susceptible to contamination of various kinds due to the substantial human activity around it: it is located in Indonesia's most populous region and industrial center on Java Island [Roosmini et al., 2018; Buwono et al., 2021]. While many studies have utilized *Sulcospira testudinaria* as bioindicator, the use of this organism as a bioindicator for heavy metal contamination by analysing MT levels is limited.

METHODS AND MATERIALS

Study area

This study was conducted at Brantas River. Figure 1 depicts ten sampling locations chosen based on the geomorphological properties of the Brantas river basin. Each sampling location was subdivided into three carefully chosen sub-sites. Monthly sampling was carried out between February and April of 2019.

Heavy metal determination

The body of *Sulcospira testudinaria* was separated from the shell, then mashed. To separate the mud particles which were present in the body of the snail, 0.9% Na-physiological solution was sprayed onto the snail. Up to two grams of *Sulcospira testudinaria* was placed in a 25 ml Erlenmeyer flask, and 10 ml of aquaregia with a ratio of concentrated HCl to HNO_3 of 3:1 was added in

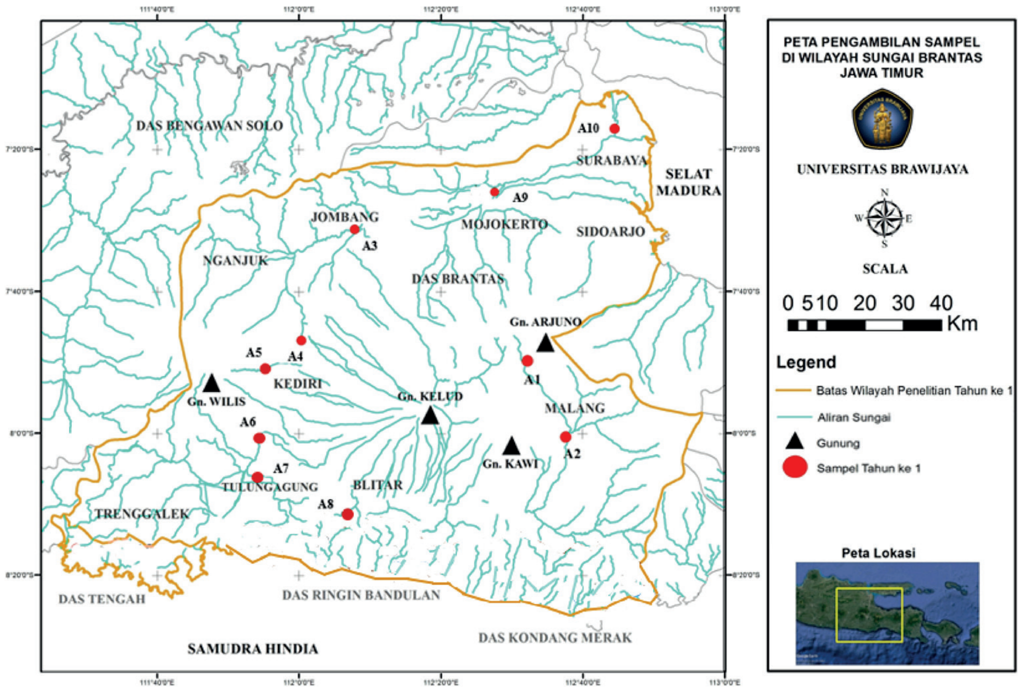

Figure 1. Location of sample sites in the Brantas River watershed

order to extract heavy metals. The samples thus obtained were filtered and homogenized with additional distilled water. Finally, the samples were analyzed by Atomic Absorption Spectrophotometer (AAS) Shimadzu type AA-6200. The wavelengths used for analysis were 217 nm for Pb, 253.7 nm for Hg and 228.8 nm for Cd.

Metallothionein (MT) expression analysis

Indirect enzyme-linked immunosorbent assay (ELISA) was used to determine the MT content. The antigen-to-coating buffer ratio was set to 1:40. The solution was incubated at 4 °C overnight. The ELISA plate was then rinsed six times with a 100 µl PBS/0.2 percent Tween solution. Next, the assay buffer was placed with 100 µl of IgG1 rabbit anti-MT primary antibody (1:400) (Santa Cruz Biotechnology, Cat# J0410). The ELISA plate was then incubated for two hours at room temperature before being rinsed six times with 200 µl 0.2 percent PBS. 100 µl of polyclonal secondary antibody of IgG biotin anti-rabbit (1:800) (Santa Cruz Biotechnology, Cat# L061) was added to the assay buffer. The mixture was incubated at room temperature for one hour before being rinsed six times with 0.2% PBS. 100 µl streptavidin horseradish peroxidase (1:800) was then poured onto the assay buffer to detect the reagent for primary antibodies conjugated to biotin. The solution was incubated at 37 °C in a shaker incubator for one hour before being washed six times with 200 µl of 0.2% PBS Tween. 100 µl of blue 3,3'–5,5'-tetramethylbenzidine was then applied to each well as a substrate for horseradish peroxidase, and the plate was incubated for 20–30 minutes in a dark room. A change in colour to blue suggested that MT was present and that a reaction had occurred. The process was halted by the addition of 100 µl 1 M HCl. At this point, the blue solution turned yellow. Absorbance was measured at 450 nm using an ELISA reader. The MT value was then calculated by converting the values using a standard curve.

Physicochemical water quality measurement

In this study, numerous physicochemical water quality parameters were assessed to determine the water quality condition of the studied area. Temperature, pH, dissolved oxygen (DO), biological oxygen demand (BOD), ammonia (NH3), and phenol were the variables used. Table 1 shows the methods and instruments used for the measurement.

Data analysis

We employed several statistical analyses in this research including one-way analysis of variance (ANOVA), the Tukey test, the Pearson correlation analysis, and multiple linear regression

Table 1. Methods and instruments for assessing water quality

Parameter	Unit	Method/Instrument
Temperature	°C	Lutron PDO-520
pH	-	pH meter
Dissolved oxygen (DO)	mg/L	Lutron PDO-520
Biological oxygen demand (BOD)	mg/L	Winkler method
Ammonia (NH_3)	mg/L	Spectrophotometer
Phenol	mg/L	4-Amino antipyrine method

analysis [Lusiana and Mahmudi, 2021]. The statistical software R (version 3.6.1) was used to support the data analysis.

RESULTS AND DISCUSSION

Heavy metal concentration of *Sulcospira testudinaria*

Figure 2 presents the heavy metal (Pb, Hg, Cd) concentration detected in *Sulcospira testudinaria* collected from the Brantas River. Pb concentration ranged from 0.001 – 0.006 mg/L, Hg from 0.001 – 0.005 mg/L, and Cd from 0.005 to 0.03 mg/L, the highest concentration level of the three heavy metals tested. All of these metals showed similar spatial distribution. The lowest heavy metal concentrations were found samples from Batu, while the highest were found in samples from Mojokerto and Surabaya. The concentration of heavy metals in the Batu samples was significantly different from those of samples from all other sites ($p<0.05$, Tukey test for unequal sample). Samples from the other sites (Blitar, Jombang, Kediri, Malang, Mojokerto, Surabaya, and Tulungagung) had nearly identical heavy metal concentrations: differences between samples from these sites were not significant, as measured by the Tukey test ($p>0.05$ in all cases).

Freshwater snails have frequently been employed as bioindicators of heavy metal pollution [Chen et al., 2021]. Using snails has huge benefits for biomonitoring research due to their limited movement and large size in comparison to other freshwater invertebrates [Vukašinović-Pešić et al., 2017]. Furthermore, they are abundant in a wide range of freshwater habitats and are fairly easy to capture and identify. They are able to accumulate large quantities of metals in their tissues. The snail's intoxication is caused not only by sediment ingesting, but also by epithelial absorption of the pollutants [Mostafa et al., 2014].

Heavy metal concentrations in the freshwater snail *Biomphalasia alexandria* in Egypt has been reported as around 0.0366 mg/L for Pb and 0.00229 mg/L for Cd [Mostafa et al., 2014]. Meanwhile, *Eobania vermiculata* was said to accumulate Cd at concentrations as high as 0.00327–0.0044 mg/L and Pb at 0.00515–0.006 mg/L [Mohammadein et al., 2013]. A study conducted in Italy found Cd concentration in *Helix aspera* ranging from 0.00561 to 0.00893 mg/L and Pb concentration ranging from 0.00162 to 0.0805 mg/L [Regoli et al., 2006]. Concentrations of heavy metals found in *Viviparus mamillatus* in another study ranged from 0.16–2.47 mg/L for Pb and 0.06–1.14 mg/L for Cd [Vukašinović-Pešić et al., 2017]. In *Pomacea canaliculata,* one the other hand, Cd concentration was measured as 0.400 mg/L, Pb was not detected, and Hg concentration was measured as 17.3 mg/L; in the same study, concentrations in *Filopaludina martensi* were measured as 7 mg/L for Cd, 31.3 mg/L for Pb, and 22.4 mg/L for Hg [Aroonsrimorakot et al., 2017].

Levels of heavy metal accumulation found in *Sulcospira testudinaria* in this study were relatively low compared to those in the freshwater snail species analyzed in the studies just mentioned. The discrepancies in heavy metal concentrations between the current study and previous research [Regoli et al., 2006] could be attributed to differences in snail species, organism body size, proximity of sampling sites to major roads, and the type of pollution in the ecosystems of the areas studied [Mohammadein et al., 2013].

Accumulation of metals by snails may contaminated their predators. Snails are preyed upon by a variety of predators, including vertebrates such as birds, small mammals, reptiles, along with various invertebrate species [Baroudi et al., 2020]. Snails' role in the transmission of material and energy from primary producers to higher trophic levels suggests that they may be involved in the transport of metallic contaminants along trophic systems [Staikou and Lazaridou-Dimitriadou, 1989].

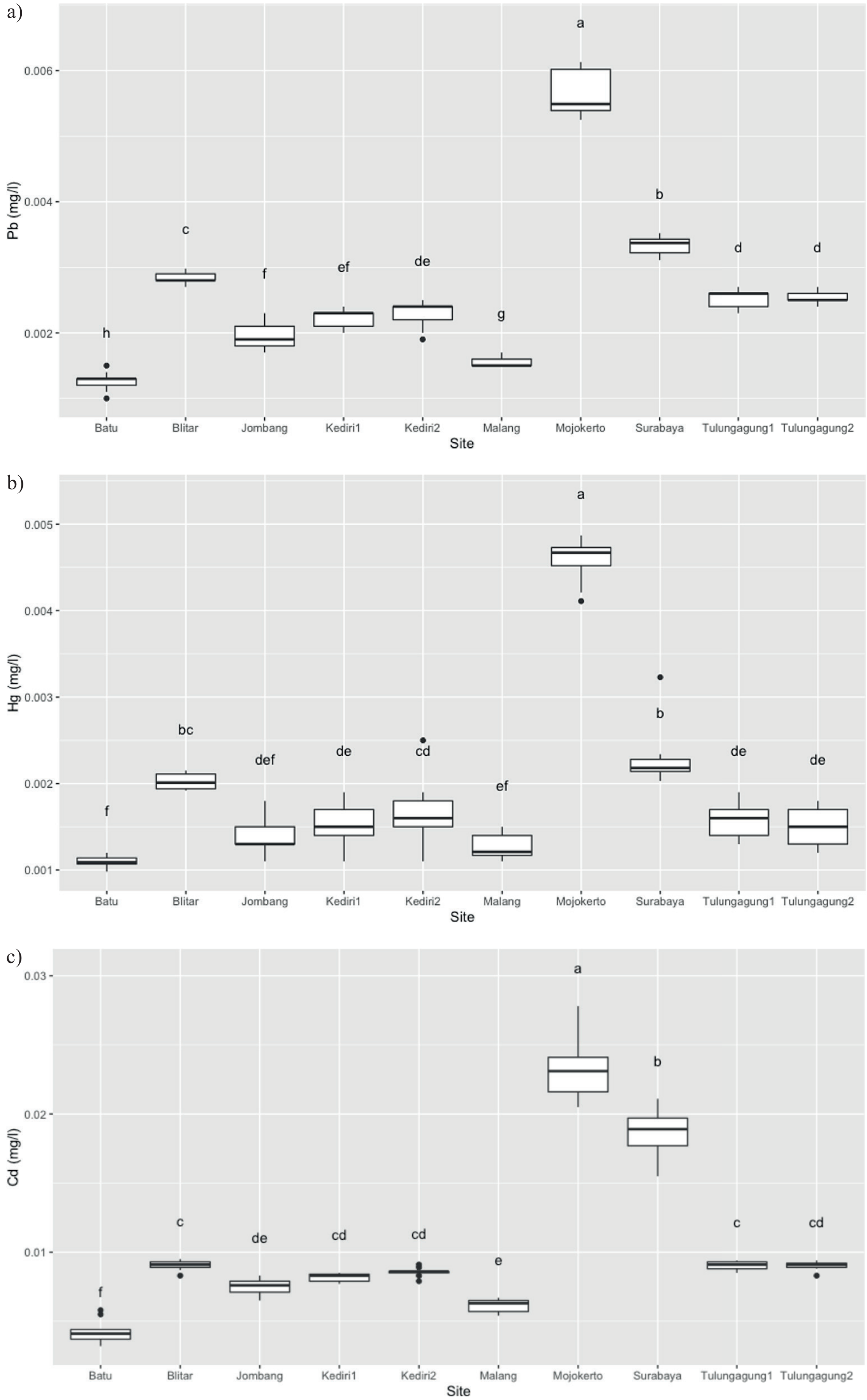

Figure 2. Boxplot of heavy metal concentration in *Sulcospira testudinaria* (a) lead (Pb), (b) mercury (Hg), (c) cadmium (Cd)

MT levels in *Sulcospira testudinaria*

The results of measurement of MT levels in *Sulcospira testudinaria* samples, depicted in Figure 3, ranged from 0.40 to more than 0.80 ng/g. The highest level was found at Mojokerto. Meanwhile, Batu, Malang, and Jombang shared the same letter notation of the Tukey test, and were the three regions with the lowest MT levels.

Each organism has different physiological mechanisms for controlling the body's resistance to heavy metals and avoiding their harmful effects. One such mechanism is regulated by MT, which is a group of metal-binding proteins [Hemmadi, 2016]. MT is activated to control the body's immune function, and plays a role in the metal detoxification process [Wang et al., 2014]. As soon as heavy metals are absorbed by the body, these metals are directly bound by proteins (thionein) and form a protein-metal complex group called metallothionein (MT). The binding of heavy metals by thionein is a defence and protection mechanism to prevent these metals from affecting important proteins in the body's metabolic processes [Suratno et al., 2017].

If the rate of heavy metals entering the cell exceeds the rate of metallothionein synthesis, the liver's ability to detoxify will decrease, so that excessive heavy metals in the organism's body will be distributed throughout the body through the blood vessels [Jaishankar et al., 2014]. Transport of heavy metals in body tissues is carried out by the blood; the heavy metals are bound to the haemoglobin protein in red blood cells. Once bound to the network, heavy metals are difficult to release again because they have bonded with sulfhydryl groups. Heavy metals that have bonded with sulfhydryl groups will cause disturbances in the structure of proteins and enzymes. This happens because heavy metal ions replace essential metal ions, causing disruption of enzyme activity. Disruption of enzyme activity due to heavy metals disrupts metabolism at the cellular level, and causes cells to become lysed and damaged [Suratno et al., 2017].

Relationship of heavy metal concentration and MT level in *Sulcospira testudinaria*

Relationship analysis between heavy metal and MT levels in *Sulcospira testudinaria* samples collected from the Brantas River watershed is presented in Figure 4 and Table 2. Figure 4 clearly shows that the MT level in *Sulcospira testudinaria* is highly associated with Pb, compared with Hg and Cd. This is because the trend line of Pb vs MT has a greater gradient than those of Hg vs MG or Cd vs MG. These findings are also supported by the multiple linear regression analysis result shown in Table 2. The estimated coefficient for the Pb variable was the highest among the three predictor variables (Pb, Hg, and Cd). Furthermore, the causal effect of Pb concentration on MT levels in *Sulcospira testudinaria* was found to be statistically significant ($p<0.001$). In addition, the regression analysis also produced an R-squared value of 0.656, which indicates that the total MT level variability in *Sulcospira testudinaria* which can be explained by the heavy metals studied was 65.6%, with the remaining 34.4% explained by other factors excluded from the regression analysis.

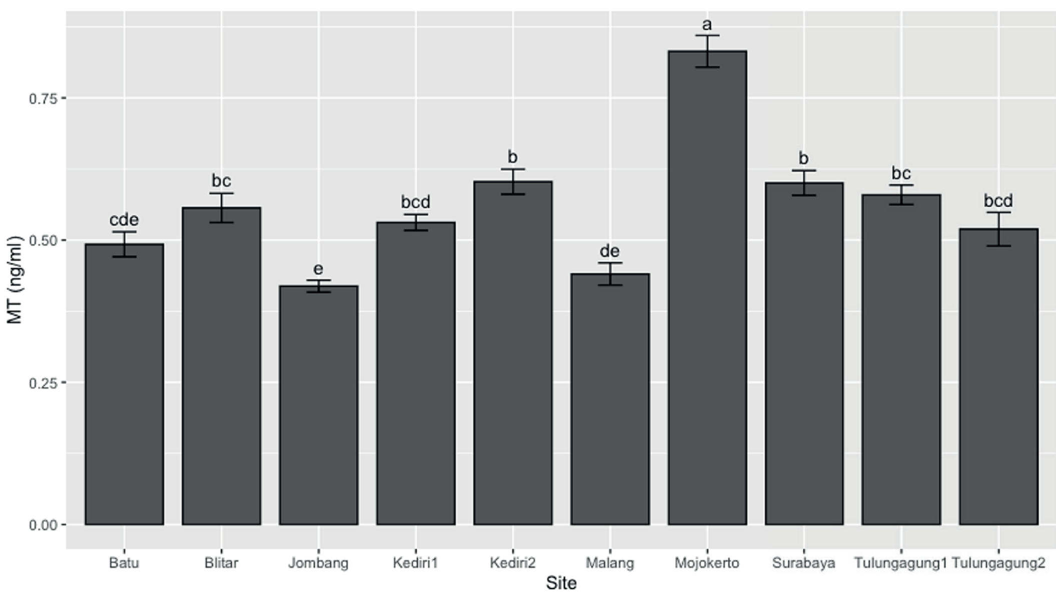

Figure 3. Metallothionein (MT) level in *Sulcospira testudinaria* collected from Brantas River

Table 2. Multiple linear regression results of heavy metals (Pb, Hg, Cd) vs MT level in *Sulcospira testudinaria*

Coefficient	Estimate	Standart error	t-value	p-value
Intercept	0.328	0.020	16.198	<0.001
Pb	96.730	24.184	4.000	<0.001
Cd	-5.404	23.746	-0.228	0.821
Hg	-1.254	3.647	-0.344	0.732
F-value = 54.67; p-value < 0.001 R-squared = 0.656				

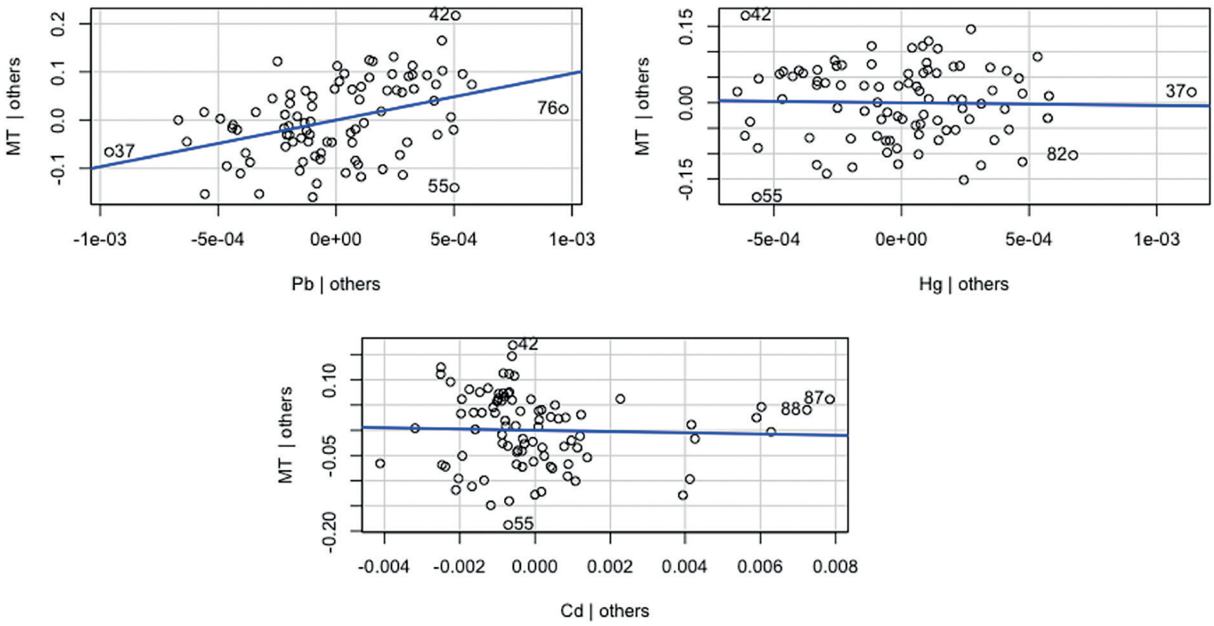

Figure 4. Added variable plots of heavy metals (Pb, Hg, Cd) vs MT level of *Sulcospira testudinaria*

MT is specific – it is only able to bind one type of metal [Dewi et al., 2015]. It is therefore an ideal biomarker for monitoring metal contamination in aquatic organisms [Hertika et al., 2018]. For example, metallothionein-Cd (MT-Cd) will be active only when the exposed organisms live in water contaminated with Cd [Dewi et al., 2015]. Previous research has shown that MT in the digestive gland of *Mytilus galloprovincialis* increased significantly as a result of Cd exposure, but decreased when the organism was exposed to Cu [Bebianno and Langston, 1992].Therefore, MT is an ideal biomarker for heavy metal pollution, and can be used to monitor polluted waters and as an early warning of heavy metal exposure [Fabrin et al., 2018].

The results of this study indicate that MT in *Sulcospira testudinaria* samples collected from the Brantas River watershed bound Pb specifically, since statistical analysis showed that only this metal had a significant effect on MT levels. Pb is known to cause detrimental effects in aquatic organisms. It enters the aquatic environment as a result of natural events as well as various anthropogenic activities [Nordberg et al., 2007]. The main source of Pb heavy metal pollution in water is industrial waste as result of mining, burning of leaded gasoline, municipal sewage disposal, and paint production and disposal [Yousif et al., 2021]. One study found that Pb also enters the ecosystem through agricultural activities such as the use of pesticides and fertilizers by farmers [Hashmi et al., 2013]. Benthic species consume a diverse range of foods found in wetlands ecosystems, including detritus, soil debris, rotting plant matter, and other organisms [Reichmuth et al., 2009], which can cause higher ranges of Pb accumulation in their bodies than are found in other organisms. A higher level of Pb in aquatic biota can also indicate that they are acting as a secondary predator, and providing a rich magnification of an aquatic ecosystem's trophic structure [Kumar et al., 2012].

Physicochemical water quality parameters

Table 3 shows water quality measurement results at the study area. It can be seen that water

Table 3. Water quality parameters in sampling sites at Brantas River watershed

Site	Temperature (°C)	pH	DO (mg/L)	BOD (mg/L)	Ammonia (mg/L)	Phenol (mg/L)
Batu	23.52	7.48	7.91	8.17	0.45	0.65
Malang	27.10	7.38	6.46	10.34	0.61	0.77
Jombang	29.37	7.29	6.38	11.94	0.66	0.71
Kediri1	28.44	6.87	6.59	12.41	0.73	0.80
Kediri 2	30.22	7.42	7.46	13.33	0.78	0.77
Tulungagung 1	30.16	7.49	7.32	13.96	0.79	0.76
Tulungagung 2	27.87	7.26	6.50	14.22	0.68	0.72
Blitar	28.19	7.42	7.10	14.23	0.49	0.70
Mojokerto	29.08	7.17	6.12	18.40	1.11	1.14
Surabaya	29.73	6.98	6.68	14.77	0.66	0.77
Standard value*	Deviation of 3	6.5 – 8.5	> 6	< 14	< 0.5	<0.001

* Note: based on Indonesia Ministry of Environment Regulation No. 82, 2001.

temperature at all sampling sites was within the normal range (around 30 °C), with the exception of Batu. Furthermore, both pH and DO values were also at normal levels: pH 6.5-8.5 and >6.0 mg/L, respectively. On the other hand, BOD, ammonia, and phenol levels were all above the standard value set by the Indonesia Ministry of Environment Regulation [Ministry of Environment, 2001]. These parameters were particularly high at the Mojokerto and Surabaya sites.

A rise in BOD value is accompanied by a reduction in DO, and therefore might disturb the biota. This is a result of the action of microorganisms which degrade waste or external substances in the water [Susilowati et al., 2018]. The greater the BOD level, the more contaminated the rivers are [Rizki et al., 2021]. If the concentration of ammonia is high enough, it can permeate into the body tissues of fish, and potentially cause harm [Ip and Chew, 2010]. Phenol is derived from waste produced by many industrial sectors, including coal, phenol manufacturing, pharmaceuticals, resin manufacturing, paint, textiles, leather, and oil refining [Naguib and Badawy, 2020]. In animals exposed to phenol by inhalation, reactions include liver damage, kidney failure, neurological damage, developmental problems, skin symptoms, and even death. [Saha et al., 1999]. The presence of phenol in excess of the water threshold can be a chemical stressor for aquatic organisms [Capolupo et al., 2020].

CONCLUSIONS

Brantas River, located on Java Island, is very prone to heavy metal contamination (especially by Pb, Hg, and Cd) as it runs through areas of intensive agricultural and industrial activity. The results of this study on heavy metals in *Sulcospira testudinaria* collected from the river indicate that Pb concentrations ranged from 0.001 – 0.006 mg/L, Hg from 0.001 – 0.005 mg/L, and Cd from 0.005 - 0.03 mg/L. MT concentration ranged from 0.40 - 0.80 ng/g. The highest concentrations of heavy metals and MT in the *Sulcospira testudinaria* samples were found at Mojokerto and Surabaya, which suggests that these regions are the most contaminated sites of those studied. This finding is also supported by the results of measurements made of water quality parameters. Relationship analysis between heavy metals and MT levels in this study revealed a significant effect of Pb concentration on MT levels in *Sulcospira testudinaria*. As a result, it can be concluded that the MT biomarker in this organism is appropriate for use as early warning of Pb contamination.

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