Relationship between Metallothionein and Mercury (Hg) in the Gill Tissue of the *Barbonymus altus* in the Brantas River Jombang, East Java

Aang Setyawan Anjasmara[1], Asus Maizar Suryanto Hertika[2*], Uun Yanuhar[2]

[1]Postgraduate Program, Faculty of Fisheries and Marine Science, University of Brawijaya, Malang, Indonesia
[2]Department of Water Resources Management, Faculty of Fisheries and Marine Science, University of Brawijaya, Malang, Indonesia

Abstract

Pollution of river waters is most often the result of human activities in managing agricultural land, industry, and transportation. The entry of pollutants such as heavy metal mercury (Hg) into the waters can interfere with the survival of fish waters. Red Bader Fish (*Barbonymus altus*) can be used as a biomarker in analyzing aquatic environmental conditions in the Brantas River, Jombang, East Java. This study aimed to analyze the relationship between metallothionein and heavy metal mercury in the gill tissue of *Barbonymus altus*. Sampling was conducted at three stations with three replications from February to March 2022. Data analysis used regression and correlation methods to determine the relationship between metallothionein and metal mercury in gill tissue. The density and intensity of metallothionein in the gill tissue of *Barbonymus altus* were analyzed using the immunohistochemical method. The mercury (Hg) content in the gill tissue of *Barbonymus altus* was analyzed using Atomic Absorption Spectroscopy (AAS). The results showed that the metallothionein density at each station included station 1 of $252.52 \times 10^{-6} – 497.79 \times 10^{-6}$ MT.μm^{-2}, station 2 of $277.78 \times 10^{-6} – 378.78 \times 10^{-6}$ MT.μm^{-2}, and station 3 of $303.03 \times 10^{-6} – 404.04 \times 10^{-6}$ MT.μm^{-2}. The relationship between the metallothionein density value and mercury in the gill tissue has a moderate correlation. In contrast, the metallothionein intensity value with mercury in the gill tissue strongly correlated with a significance value of sig < 0.05. The Hg levels in the gill tissue, density, and intensity have significant values at the all three locations based on the real difference test (sig. 0.00).

Keywords: *Barbonymus altus*, density, intensity, mercury, river.

INTRODUCTION

Rivers are public aquatic ecosystems that act as habitats for air biota and human life, including agriculture, fisheries, industry, and transportation. Increasingly dense community activities will harm rivers, one of which is the accumulation of heavy metals and a decrease in air quality [1]. Jombang is located in the central part of East Java and has a strategic position at the crossroads of Java's northern and southern islands [2]. The community commonly used the Jombang Brantas River for agricultural, plantation, and industrial activities. Due to final waste, the rapid development of industry and agriculture will affect environmental problems, including air and water conditions. Water pollution can affect organisms and air plants in aquatic waters. The effect is not only on species and populations but also on natural biological communities [3].

Water pollution is commonly caused by heavy metal pollution in the environment, such as mercury. Mercury can easily enter the atmosphere through various processes such as natural human activities and mineral breakdown processes in rocks, burning fossil fuels, using agricultural fertilizers, and disposing of industrial wastewater [4]. This pollution comes from anthropogenic contaminants in the aquatic environment that can cause threats to organisms due to toxicity, persistence, and bioaccumulation. Heavy metal mercury in the Jombang Brantas River can come from agriculture using pesticides, hospital waste, and final processing waste from animal feed factories. Metallothionein is a low molecular weight cysteine-rich metal-binding protein that plays a role in transporting and storing essential metals and protects against the toxic effects of heavy metal exposure. Metallothionein induction in fish has the highest value in tissues directly involved in the uptake, storage, and excretion of metals, including gills, liver, kidneys, intestines, and muscles [5].

Fish are sensitive to environmental changes, have relatively long life, and accumulation of heavy metals in the body. The absorption of heavy metals in aquatic organisms will increase with increasing trophic levels in the ecosystem. The fish often found in the Brantas River is the Red Bader Fish (*Barbonymus altus*) which humans often use for consumption [6]. The Red Bader Fish (*B. altus*) is categorized in the

*Correspondence address:
Asus Maizar Suryanto Hertika
Email : asusmaizar@ub.ac.id
Address : University of Brawijaya, Veteran, Malang, Indonesia, 65145.

Cyprinidae species, which can be used as a toxicity test as a biomarker agent in determining river pollution that receives input from the pollutant load from community activities. Biomarkers in fish can be used as an early warning tool in evaluating the pollution burden in the aquatic environment caused by environmental threats [7]. This study aims to determine the relationship between metallothionein and heavy metal mercury (Hg) in the Red Bader Fish (*B. altus*) gill tissue in the Brantas River, Jombang, East Java.

MATERIAL AND METHOD

Study Site

This research was conducted on the Brantas River, Jombang, East Java. Sampling was done by using the purposive sampling method. The location of research was carried out at three stations suspected of receiving different waste inputs, including Station 1, adjacent to agricultural land and boat crossings; Station 2, adjacent to land use in agriculture; and Station 3, adjacent to waste disposal from industrial end products. The research locations of the three stations can be seen in Figure 1.

Fish and Water Sampling

Water and fish blood samples were taken every two weeks for three repetitions from February to March 2022. Fish samples were taken using fishing nets. The content of Hg in the gill tissue of Red Bader Fish (*B. altus*) was measured using the Atomic Absorption Spectroscopy (AAS) method conducted at the Chemical Laboratory of Brawijaya University [8] conducted at the Chemistry Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya. Meanwhile, metallothionein testing of the gill tissue of Red Bader fish (*B. altus*) was carried out using an immunohistochemical method, including adding 1-3 drops of Phosphate Buffer Saline (PBS) EDTA (Ethylene Diamine Tetra Acetic Acid) and Na citrate solution added to each sample, putting the sample into an oven at 95°C for 15 minutes, wash the sample with 0.3% H_2O_2 twice with PBS for three times every 5 minutes, incubate the sample with 1% PBS serum for 30 minutes, add the primary antibody of metallothionein (Mouse-METALLOTHIONEIN-IgG1/Thermofisher Scientific) before incubation in the refrigerator overnight, measuring the density and intensity of metallothionein by transferring the sample to a microscope slide, analyzing the density and intensity of metallothionein with ImageJ software [9].

Data Analysis

Statistical analysis was used to correlate the density and intensity of metallothionein with heavy metal mercury (Hg) in the gill tissue of *B. altus* using regression and correlation analysis. Regression analysis was used to determine the relationship between the dependent and independent variables, while correlation coefficient was used to determine the close relationship between the two variables [10]. The dependent variable in this study was the density of metallothionein and intensity of metallothionein, while the independent variable was heavy metal Hg in the gill tissue of *B. altus*.

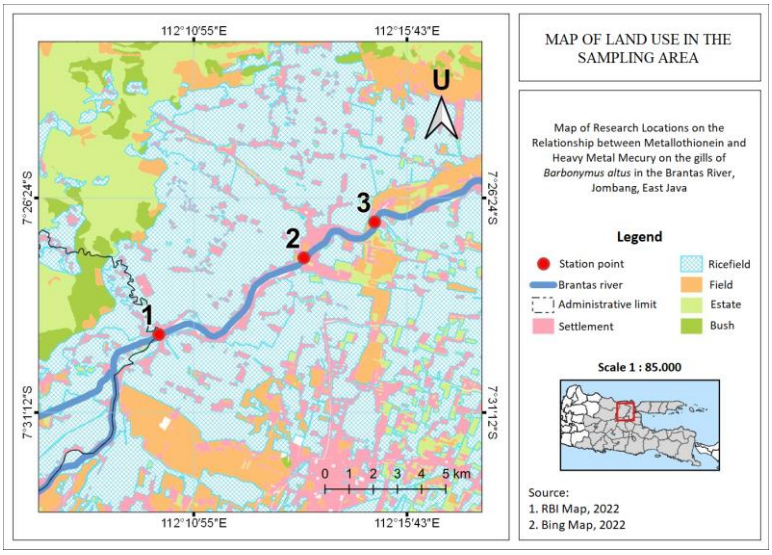

Figure 1. Map of sampling location

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RESULT AND DISCUSSION

In this study, the metallothionein density results at the three stations include station 1 of $252.52 \times 10^{-6} - 497.79 \times 10^{-6}$ MT.µm^{-2}, station 2 of $277.78 \times 10^{-6} - 378.78 \times 10^{-6}$ MT.µm^{-2}, and station 3 of $303.03 \times 10^{-6} - 404.04 \times 10^{-6}$ MT.µm^{-2} is shown in Figure 2. The results showed that stations 1 and 3 were relatively higher than stations 2. It was related to the concentration of gill heavy metals in *B. altus* with an average of 0.047 mg.kg^{-1} (station 1), 0.034 mg.kg^{-1} (station 2), and 0.039 mg.kg^{-1} (station 3). The graph on the measurement of heavy metal mercury in gill tissue is shown in Figure 3. The density of metallothionein and heavy metal mercury in the gill tissue of *B. altus* showed that stations 1 and 3 were relatively higher than station 2. It was due to sampling at station 1, adjacent to agricultural land and boat crossings, while station 3 was adjacent to industrial waste disposal. Hg can come from industrial waste, using mercury compounds in agriculture, rock weathering processes, and volcanic eruptions. Mercury in waters in high concentrations can lead to decreased water quality and the death of aquatic life [11]. Hg emissions have increased along with increasing industrialization by burning fossil fuels, mining, and production processes from industrial products [12].

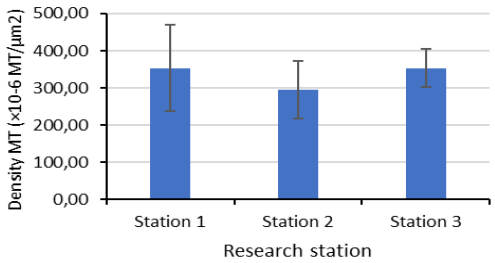

Figure 2. Calculation of metallothionein density; **Notes:** Each station with three replications, one-way ANOVA results is significantly different (sig. > 0.05).

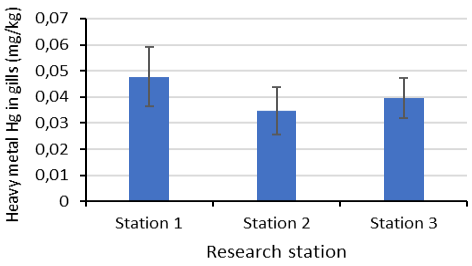

Figure 3. Calculation of heavy metal Hg in gill tissue **Notes:** Each station with three replications, one-way ANOVA results is significantly different (sig. > 0.05)

The entry of heavy metal mercury into the body of aquatic organisms can be done in three ways, including the food chain, skin surface diffusion, and gills. The process of entering large amounts of mercury into the body is likely through the food chain because almost 90% of toxic substances enter the body. Phytoplankton has an essential role in absorbing organic mercury during photosynthesis. The characteristics of mercury as a lipophilic substance indicate that it easily diffuses through the skin membrane and then enters the body's tissues. So large fish that have eaten small fish contaminated by methyl mercury is suspected of having mercury content in the aquatic biota network by the biomagnification process in the aquatic environment [13].

Metallothionein is a non-enzymatic protein with a high cysteine content, does not have aromatic amino acids, and is unstable by heat. The thiol group (-SH) contained in MT is a cysteine residue that allows MT to bind heavy metals [14]. The accumulation of heavy metals in fish begins with entering through the gills and then being absorbed into all body tissues. The factors affecting mercury accumulation include metabolic rate, size, type, alkalinity, and pH. The effect of mercury accumulation can come from the organism's level, time, source, and level of life, which is significantly 70% of the food processor and absorption in the fish body. It will impact fish mortality caused by pollutants that damage the gills and gill-related organs due to the thin epithelium and direct contact with polluted and suspended water as a living medium.

The function of the gills is as a regulator of osmosis so that any damage will result in respiratory problems. It will disrupt the lamellae's circulatory system, resulting in edema or swelling of cells around the blood vessels. This condition can reduce the efficiency of gas diffusion because the absorption of the secondary gill lamella surface area will be narrowed [15]. The gills and skin accumulate appreciable toxins through direct contact with contaminated water and respiratory, osmotic, and ion regulatory functions. Exposure to mercury from food causes only sublethal toxic effects despite prolonged exposure to contaminated food. In contrast to the bioaccumulation process, eliminating heavy metal Hg in gill tissue will be slow, so it can lead to biomagnification of mercury throughout the food chain, which poses a considerable risk if consumed by humans [16].

The immunohistochemical method detects enzyme content by observing the color intensity. The intensity obtained was divided into three categories: strong positive, characterized by dark brown to blackish brown; medium positive, characterized by dark brown to light brown; and weak positive, indicated by reddish brown color [17]. Observation of metallothionein intensity at the three stations included station 1 of 317807 - 639330 pixels, station 2 of 214051 - 418366 pixels, and station 3 of 412767 pixels - 624239 pixels. The results showed that the highest value was stations 1 and 3, while the lowest was stations 2. Measurement of the color intensity of metallothionein can be seen in Figure 4. The results of immunohistochemical staining from the three research stations are shown in Figure 5.

The intensity unit used in the form of pixels is a quantitative analysis unit of protein expression for using immunofluorescent as a dark color distinguishing agent in metallothionein testing. The algorithm can be used to measure the expression of biomarkers from the cellular and subcellular levels. Information from Aquascore can be obtained from the pixel intensity of an area with the value in the analyzed image so that the average intensity of the number of pixels can be known. The study's results by observing the intensity of metallothionein showed that the highest values were at stations 1 and 3, while the lowest values were at stations 2.

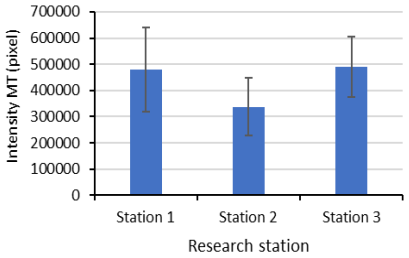

Figure 3. Calculation of metallothionein intensity **Notes:** Each station with three replications, one-way ANOVA results is significantly different (sig. > 0.05)

The concentration of heavy metals Pb, Cd, and Hg significantly affected the metallothionein density value in the gills [9]. Biota that lives in waters with a polluted environment can increase the concentration of metallothionein. The metallothionein concentration accumulates in tissues can change due to hormones, growth factors, and other heavy metals. Heavy metals in water (Zn, CU, Ni, Cr, Pb, Cd, Hg) are classified as substances that can be dangerous due to their toxicity, persistence, and bioaccumulation. Heavy metals accumulating in the gills, liver, kidneys, and muscles of fish can bind strongly to metallothionein or metal-binding proteins. Cations in heavy metals will accumulate in cells, triggering metalloprotein biosynthesis synthesis through the metallothionein gene transcription process [18]. Correlation coefficients can be categorized as 0.00 (no correlation), 0.01-0.20 (very weak correlation), 0.21-0.40 (weak correlation), 0.41-0.70 (medium correlation), 0.71-0.99 (high correlation), and 1.00 (perfect correlation) [19].

The relationship between the density of metallothionein and heavy metal mercury in the gill tissue of *B. altus* showed that the heavy metal mercury in the gill tissue had a significant correlation with the density of metallothionein. It was shown as the equation $\mathbf{Y = 110.67 + 5490.43x.}$ The correlation coefficient (R) value between heavy metal Hg in the gills of 0.693 has a moderate correlation with the density of metallothionein.

Measurement of the intensity associated with heavy metal mercury in the gill tissue of *B. altus* showed that the heavy metal mercury in the gill tissue was significantly correlated with the intensity of metallothionein as indicated by the equation $\mathbf{Y = -41054.452 + 11732045.02x.}$ The value of the correlation coefficient (R) between heavy metal Hg in the gills is 0.873, which correlates highly with the intensity of metallothionein.

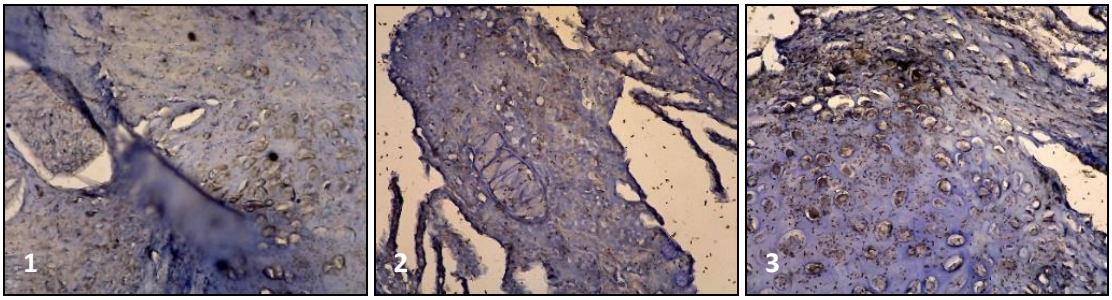

Figure 4. Immunohistochemical staining at the research station

Metallothionein assays on *Crassostrea iredalei* and *Crassostrea glomerata* species resulted in higher MT values in gills than gastric tissue, namely 160,250 $ng.g^{-1}$ in Kenjeran, 123,500 $ng.g^{-1}$ in Mayangan, and 111,500 $ng.g^{-1}$ in Gresik. The results of the regression test stated that the heavy metal content had a significant value ($P<0.0001$) with the MT content in the gills [20]. The gills and skin accumulate appreciable toxins through direct contact with contaminated water and respiratory, osmotic, and ion regulatory functions. Exposure to mercury from food causes only sublethal toxic effects despite prolonged exposure to contaminated food. In contrast to the bioaccumulation process, the elimination of heavy metal Hg in gill tissue will be slow so that it can lead to biomagnification of mercury throughout the food chain, which poses a considerable risk if consumed by humans [16].

CONCLUSION

The relationship between metallothionein and heavy metal mercury (Hg) in the Red Bader Fish (*Barbonymus altus*) gill tissue in the Brantas River, Jombang, at stations 1 and 3 was relatively higher than in station 2. This condition was due to sampling at station 1, adjacent to agricultural land and boat crossings, while station 3 is adjacent to industrial waste disposal. Statistical testing showed that the heavy metal mercury in the gill tissue of *B. altus* positively affected the density and intensity of metallothionein with $sig<0.05$.

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