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139 EKSAKTA journal.uin.ac.id/eksakta August 2020, Volume 1, Issue 2, 139-146 Research Article Analysis Metallothionein of Carp fish in The Brantas River, Indonesia Putri Ayu Ika Setiyowati^{1,*}, Rofiatun Solekha¹, Sri Bintang Sahara², Febianti Dwi Hapsari¹ 1 Departement of Biology, Faculty of Sains and Technology, Universitas Muhammadiyah Lamongan, East Java, Indonesia 2 Departement of Pharmacy, Faculty of Medicine, Universitas Muhammadiyah Lamongan, East Java, Indonesia * Corresponding author: putriayuika@uiml.ac.id Received: 26 June 2020; Accepted: 28 August 2020; Published: 6 September 2020 Abstract: This study aimed to detection of protein profile, expression of metallothionein (MT) protein, and analyze difference of MT density in liver and gill in one of Carp fish that is *Barbonymus balleroides* in the upstream and downstream of Brantas river.

The method used observasional analytic, Two individual of *Barbonymus balleroides* samples taken three times (with difference of month) from two station there are upstream and downstream of Brantas river. Analysis protein profile used electrophoresis SDS-PAGE (15%), expression and density of MT used western blot method and imageJ software. Difference between MT density in liver and gill of *Barbonymus balleroides* in the upstream and downstream of Brantas river analyzed with Two-way ANOVA.

The results showed, protein profile in liver and gill *Barbonymus balleroides* in the upstream and downstream Brantas river have molecular weight about 8-93 kDa, expression of MT showed band of MT with molecular weight 24 kDa, results of MT density in liver and gill on upstream and downstream, continuously 231.29 MT/ μm^2 and 229.87MT/ μm^2 , 232.41 MT/ μm^2 and 231.56 MT/ μm^2 but there is not significant.

Keywords: Brantas river, metallothionein, liver, gills, Carp fish *Barbonymus balleroides*
Introduction Industrialization that occurs in several developing countries such as

Indonesia is one of the causes of environmental pollution.

Pollution is usually common in water environment such as river. River have important benefits for people's live [1]. In the province of East Java there is the Brantas river. The condition of Brantas river both upstream (Karangkates Reservoir) and downstream (Surabaya River) is said to have been contaminated with heavy metal. The heavy metal elements (Pb, Cu, Cr, and Cd) that found in the upstream and downstream of the Brantas River are fluctuative [2], usually dependent on the seasons.

In the dry season, level of heavy metal is higher than the rainy season due to heavy metals will tend to settle in sediment [3]. Related The effect of this heavy metals are so dangerous, especially for fish that live on the river. Heavy metals elements can be absorbed, and bind the receptors on the cells. *Barbonymus balleroides* is one type of carp fish that can be found in the upstream and downstream of the Brantas river. In the river, fish is one of the water biota that can be used as environmental monitoring (bio-monitoring) [4]. The heavy metal elements in the river will enter the cytoplasm of fish cells and interact with proteins called metallothionein.

MT Protein (0,5 – 36 kDa) is a type of protein that has thiol groups to bind heavy metals [5]. There are several organs that accumulate heavy metals and have high levels of metallothionein, such as liver and gills [6]. Concentration of heavy metal (Pb, Cu, Cr, and Cd) in Brantas river reached re 0,03 It's exceeds maximum allowed the n of Health Minister No. 492/2010 [7]. Based on the description, few studies were conducted on levels and expression of metallothionein, so, the purpose of this study is to analyze the expression of MT protein (observing molecular weight and calculate MT density) in liver and gill of red bader fish (*Barbonymus balleroides*) that live in the upstream and downstream of Brantas river. It is beneficial to acknowledge about the quality of *Barbonymus balleroides* in Brantas river because this fish is highly consumed by local society.

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EKSAKTA journal.uui.ac.id/eksakta August 2020, Volume 1, Issue 2, 139-146 Materials and Methods Sampling Site and Collection of Fish Samples The fish were collected from two locations in the upstream, the point of sampling and downstream of Brantas river. The method for collection of fish was through trawling (fish trawl net). The sampling was done in three times, March (1st month), June (2nd month), September (3rd month) 2016.

In each sampling, two *Barbonymus balleroides* with body weight approximately 80-200 gram were collected, fish were labeled and dissected directly in sampling location. The excised liver and gill samples were stored at -800C until isolation prossessed. Protein

Isolation Sampling tissues (liver and gill) were freshly weighed (50 mg) and placed in a microtube, then gently homogenized with mortar in 500 µL buffer lysis. The homogenization buffer contained 250 mM Tris-HCL (pH= 8), 50 mM EDTA (pH=8), 100 mM NaCl, and 2% SDS The homogenate was vortexed for 10-20 second, and incubated at temperature 55 oC for 10 minute.

Homogenate was added 500 µL PCL (phenol chloroform isoamil alcohol), centrifuged at 1200 rpm for 10 minutes at 250C. Supernatant were added 500 µL ethanol absolute, 50 mL ammonium acetate 7,5 M and stored at -200C until they were required for western blot analysis. SDS-PAGE Analysis Electrophoresis SDS-PAGE was performed according to the method of Laemmli, 1970. SDS-PAGE was used with the discontinuous system in 15% of separating gel. Protein sample added by Tris-HCl with pH 6,8 and reducing sample buffer (1:1), heated the sample in boiling water for 5 minutes.

Running electrophoresis was conducted at constant current of 100 volt for 90 minutes by Bio-Rad electrophoresis power supply unit. Protein marker was also run into every gel. After running, gels were stained with Commasie Brilliant Blue solution for 120 minutes, and then fixed with fixative solution (methanol: acetic acid = 3:1) for 1 hour and in the water for 1 hour, and scan each gel using a scanner. To determine molecular weights of protein, a standard curve was constructed from the relative of mobility (Rf) by each marker protein.

Then the relative of mobility (Rf) by the sample proteins can be plotted and their molecular weights calculated by interpolation with Excel program [8]. Western Blotting Briefly, Protein concentration of the whole homogenates was determined according to Biuret method. Protein (15µg) were separated by SDS-PAGE (15%) without staining with Commasie Brilliant Blue. Electrotransferred to nitrocellulose membranes for 120 minutes at a constant current of 100 V.

The membranes were blocked at 40C for 1 h with 5% BSA, washed twice with 0,2% PBS-Tween for 5 minutes followed incubation with primary antibody (Rabbit polyclonal anti-MT antibody (ab36882), 1:1000 dilutions) overnight at 40C, washed three times with 0,2% PBS-Tween for 5 minutes. After being washed, the membranes were incubated with HRP conjugated secondary antibody (goat anti rabbit 1:500 in PBS- Tween). The height of the bands (molecular weight) and their density was calculated scanning the blots and applying the ImageJ software.

Statistical Analyses Results and Discussions Electrophoresis SDS-PAGE (15%) The results electrophoresis SDS-PAGE (15%) revealed variation of molecular weight in each liver and gill. Profile protein in liver and gill *Barbonymus balleroides* that live on upstream

and downstream of Brantas river with variation of sampling time were expressed on range 8-93 kDa (Figure 1). EIS 2720-2 P-SN 2716- 0459 analysis used analysis MT to analyses, (MT were as values standard (SD), that, were for normality test) equality variances test), data normal distribution homogen, then ANOVA was to significant among different for density upstream downstream river. significance level adopted throughout this study was 95% ($\alpha=0.05$). 141 EKSAKTA journal.uui.ac.id/eksakta August 2020, Volume 1, Issue 2, 139-146 Figure 1.

Results of protein profile SDS-PAGE (a) Liver and gills of two *Barbonymus balleroides* that live in the upstream and down stream based on first month (continuously) were shown on well number 5 and 6; 7 and 8 (upstream); 1 and 2; 3 and 4 (downstream). (b) Liver and gills of two *Barbonymus balleroides* that live in the upstream and down stream based on second month (continuously) were shown on well number 7 and 8; 5 and 6 (upstream); 3 and 4; 1 and 2 (downstream). (c) Liver and gills of two *Barbonymus balleroides* that live in the upstream and down stream based on third month (continuously) were shown on well number 5 and 6; 7 and 8 (upstream); 1 and 2; 3 and 4 (downstream).

Expression and density of MT protein MT protein expression was assessed from liver and gill tissues of *Barbonymus balleroides* by western blot method using Rabbit polyclonal Anti-MT primary antibody and HRP conjugated goat anti-rabbit secondary antibody. Antibody reacts with specific protein after electrophoresis in 15% SDS-PAGE. An antibody bound by the immobilized antigen was detected by a secondary, labeled antibody directed against the primary antibody. Western blot analysis (Figure 2) revealed that the selected rabbit anti-MT antibody cross-reacted specifically with *Barbonymus balleroides* MT. MT protein from fish liver and gill samples exhibited band at 24 kDa.

(a) (b) (c) EIS 2720-2 P-SN 2716- 0459 142 EKSAKTA journal.uui.ac.id/eksakta August 2020, Volume 1, Issue 2, 139-146 Figure 2. Expression of MT protein with western blot method based on difference of month. (a) Liver and gills of two *Barbonymus balleroides* that live in the upstream and down stream based on first month (continuously) were shown on well number 5 and 6; 7 and 8 (upstream); 1 and 2; 3 and 4 (downstream). (b) Liver and gills of two *Barbonymus balleroides* that live in the upstream and down stream based on second month (continuously) were shown on well number 7 and 8; 5 and 6 (upstream); 3 and 4; 1 and 2 (downstream).

(c) Liver and gills of two *Barbonymus balleroides* that live in the upstream and down stream based on third month (continuously) were shown on well number 5 and 6; 7 and 8 (upstream); 1 and 2; 3 and 4 (downstream). Mean of MT density in each organ (liver and

gill) of *Barbonymus balleroides* that live on upstream and downstream Brantas river based on sampling time that analysis use imageJ software provided in Table 1-2, from the data, MT density was higher in downstream Brantas river both of liver and gill with variation of sampling time, and the Figure 3 and Figure 4 showed the difference of MT density in liver and gill *Barbonymus balleroides* in the upstream and downstream based on variation of sampling time, from the diagram have been known that there were no significant. (a) (b) (c) 2720-2 P-SN 2716- 0459 143 EKSAKTA journal.uui.ac.id/eksakta August 2020, Volume 1, Issue 2, 139-146 Table 1.

MT density in liver of *Barbonymus balleroides* that live on upstream and downstream Brantas river based on sampling time. Loc. Sampling time/individual replication of *B.balleroides* MT density (MT/ μm^2) Mean in each month of sampling time (MT/ μm^2) Mean in three month of sampling time (MT/ μm^2) Standard of deviation (MT/ μm^2) Up stream (Month1st/*B.balleroides*1) (Month1st/*B.balleroides*2) (Month2nd/*B.balleroides*1) (Month2nd/*B.balleroides*2) (Month3rd/*B.balleroides*1) (Month3rd/*B.balleroides*2) 231.400 231.722 229.824 231.529 231.729 231.538 231.561 230.677 231.634 231.290 0.729 Down stream (Month1st/*B.balleroides*1) (Month1st/*B.balleroides*2) (Month2nd/*B.balleroides*1) (Month2nd/*B.balleroides*2) (Month3rd/*B.balleroides*1) (Month3rd/*B.balleroides*2) 232.422 231.804 231.529 231.294 232.875 231.538 232.113 231.412 231.207 231.910 0.611 Table 2.

MT density in gill of *Barbonymus balleroides* that live on upstream and downstream Brantas river based on sampling time. Loc. Sampling time/individual replication of *B.balleroides* MT density (MT/ μm^2) Mean in each month (MT/ μm^2) Mean density in three month of sampling time (MT/ μm^2) Standar of Deviation (MT/ μm^2) Up stream (Month1st/*B.balleroides*1) (Month1st/*B.balleroides*2) (Month2nd/*B.balleroides*1) (Month2nd/*B.balleroides*2) (Month3rd/*B.balleroides*1) (Month3rd/*B.balleroides*2) 229.294 230.422 230.353 228.059 228.825 232.294 229.858 229.206 230.560 229.874 1.491 Down stream (Month1st/*B.balleroides*1) (Month1st/*B.balleroides*2) (Month2nd/*B.balleroides*1) (Month2nd/*B.balleroides*2) (Month3rd/*B.balleroides*1) (Month3rd/*B.balleroides*2) 229.743 231.513 230.412 233.529 231.723 232.412 230.628 231.971 232.067 231.5551 1.360 2720-2 P-SN 2716- 0459 144 EKSAKTA journal.uui.ac.id/eksakta August 2020, Volume 1, Issue 2, 139-146 Figure 3.

Difference between liver *Barbonymus balleroides* MT density in the upstream and downstream Brantas river based on sampling time Figure 4. Difference between gill *Barbonymus balleroides* MT density in the upstream and downstream Brantas river based on sampling time. The electrophoresis SDS-PAGE results, appears a band of protein with low molecular weight which is 24-8 kDa. Protein is thought to be a metallothionein protein, the metallothionein protein is one of the groups of intracellular

stress protein that responds to the stressor of heavy metals that enter the body of the organism [9] then molecular weight of 93 kDa, 72 kDa, 57 kDa, and 40 kDa. These proteins are HSP90, HSP70, and HSP40 proteins.

Protein HSP (Heat Shock Protein) is a group of intracellular proteins that are usually induced by a stressor in the form of high temperature (heat), this protein has the main function of improving defense by maintaining cell function [10]. Another protein is protein with a molecular weight of 57 kDa, presumably this protein is Na⁺ / K⁺ + ATPase with sub-unit β. Na⁺ / K⁺ + ATPase is a protein that plays a role in the process of osmoregulation in the body of aquatic animals, especially fish.

Na⁺ / K⁺ + ATPase has a molecular weight of about 100 kDa from the α subunit catalyst and a smaller 50-60 kDa molecular weight of glycosylation of the β subunit [11]. Fish organisms that living in waters polluted by heavy metals will absorb heavy metals passively along with the aeration process. In general, the highest levels of heavy metals in fish are gills. [12] stated that heavy metal ions will accumulate mainly in gills and hepatopancreas. Heavy metals from the water entrance to the cell organisms through the digestive tract, respiratory tract, and skin penetration.

It is related to the study by March June September Upstream 231,561 230,677 231,634 Downstream 232,113 231,412 231,207 229,000 229,500 230,000 230,500 231,000 231,500 232,000 232,500 233,000 MT density in liver (MT/ μm²) Sampling time March June September Upstream 229,858 229,206 230,560 Downstream 230,628 231,971 232,067 226,000 227,000 228,000 229,000 230,000 231,000 232,000 233,000 MT density in gills (MT/ μm²) Sampling time EIS 2720-2 P-SN 2716- 0459 145 EKSAKTA journal.uin.ac.id/eksakta August 2020, Volume 1, Issue 2, 139-146 [13], heavy metals entrance to the cells with the active-passive transport mechanisms and endocytosis.

The ion of heavy metals entrance to the gills (chloride cells) through sodium channel protein facilitated by H⁺ATPase enzyme, then Na⁺/K⁺ ATPase in the basolateral membrane of gills will respond to toxic material (Pb²⁺). In the cells, Pb²⁺ binds to a protein called metallothionein [14]. First, ion of heavy metals interact with Metal- regulatory Factor-1 (MTF-1) and Metal-synthesis Inhibitor (MTI), ion binds to MTI therefore, MTF can be free and give signal to the MREs (Metal-responsibility Elements) to produce Metallothionein (MT) protein [15]. MT is a protein that rich of amino acid, MT has a molecule weight about 0,5-36 kDa localized on Golgi apparatus [16].

In this results, MT protein was expressed on 24 kDa and the density is higher in the liver with total 231,29 MT/μm² (in upstream) and 231,91 MT/μm² (in downstream) than gills with total 229,87 MT/μm² (in upstream) and 231,56 (in downstream). This results,

related to Rose (2014) that revealed in the liver and kidney of *Clarias* sp. after exposure by 20 mg/L cadmium (Cd), showed MT protein was expressed on 6-14 kDa and the density of protein higher in the liver than kidney, it is because liver is an organs that has a function to detoxification.

Toxic conditions encourage the fish to modify the pattern of gene transcription by reducing normal protein synthesis, and synthesize a set of protein stresses, including metallothionein (MT) proteins, and heat shock protein (Hsp) proteins [10]. According to the above explanation, one of the factors that affect the high accumulation of heavy metals is the accumulation of heavy metals in the water, however, these factors are fluctuating depending on the environment and the seasons [17]. Total density of MT protein is higher in the downstream of Brantas river, caused the condition of water quality in the downstream has been polluted. The pollutant source from human activity and industrial waste [18].

The other reason, the accumulation of heavy metals in water affected by season or climate change. In the dry season the accumulation of heavy metals in waters is higher than in the rainy season due to slower water movement resulting in accumulation in sediments [19]. In this study, different sampling time variations did not significantly affect the MT density in the liver and gill because in March, June, and September 2016 it was categorized as rainy season.

In accordance with the results of, Antai (2017) that examined the effects of seasonal variation on heavy metal concentrations in some of the organ (liver, gill, and liver) of catfish (*Clarias gariepinus*) in QUA IBOE River, Southern Nigeria, and the results showed a concentration of heavy metals such as Cu, Cr, Zn, Fe, and Cd in each of the organs, in the dry season the concentration value of heavy metals is higher than the rainy season. Conclusions Total protein results in liver and gill of *Barbonymus baleroides* that live on upstream and downstream Brantas river based on variation sampling time by electrophoresis SDS-PAGE were found protein band with the range 8-93 kDa.

MT protein were expressed in the liver and gill of *Barbonymus balleroides* that live on upstream and downstream Brantas river with molecular weight 24 kDa and no significant data of MT density in liver and gill of *Barbonymus baleroides* that live on upstream and downstream Brantas river based on variation of sampling time.

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