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Research Article

Analysis Metallothionein of Carp fish in The Brantas River, Indonesia

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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 has reached more than 0,03 ppm. It's means exceeds the maximum limited allowed by the regulation of Health Minister No. 492/2013 [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.

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 -80°C until isolation processed.

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 °C for 10 minute. Homogenate was added 500 µL PCL (phenol chloroform isoamil alcohol), centrifuged at 1200 rpm for 10 minutes at 25°C. Supernatant were added 500 µL *ethanol absolute*, 50 mL *ammonium acetat* 7,5 M and stored at -20°C 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 reduced 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 4°C 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 4°C, 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

Descriptive analysis was used to analysis protein MT expression. Prior to statistical analyses, Results (MT density) were reported as mean values ± standard deviation (SD), after that, data were tested for normality (Kolmogorov-Smirnov test) and equality of variances (Levene's test), the data was normal distribution and homogen, and then Two-Way ANOVA test was used to determine significant differences among the different organs for MT density in upstream and downstream Brantas river. The significance level adopted throughout this study was 95% ($\alpha=0.05$).

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).

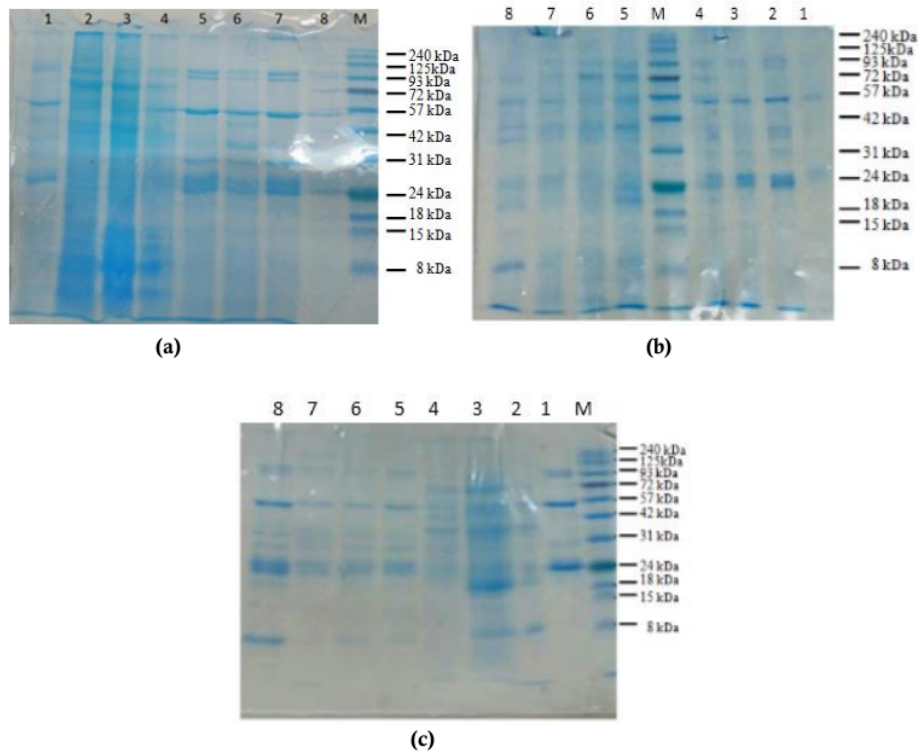


Figure 1. Results of protein profile SDS-PAGE

- (a) Liver and gills of two *Barbonymus ballerooides* that live in the upstream and down stream based on first month (continously) 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 ballerooides* that live in the upstream and down stream based on second month (continously) 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 ballerooides* that live in the upstream and down stream based on third month (continously) 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 ballerooides* 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 ballerooides* MT. MT protein from fish liver and gill samples exhibited band at 24 kDa.

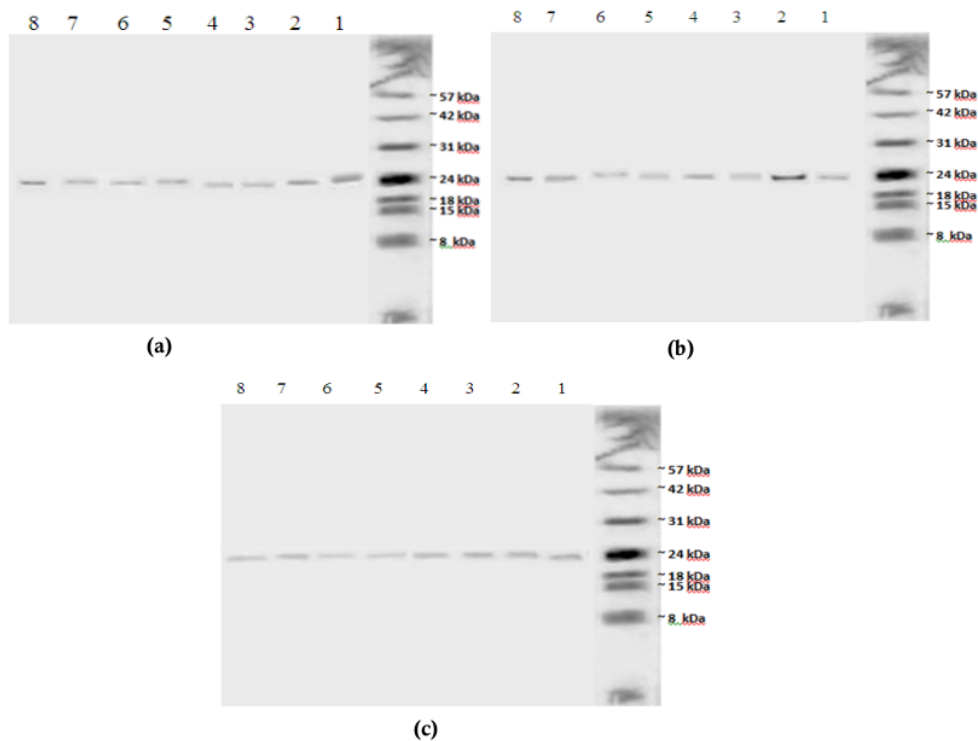


Figure 2. Expression of MT protein with western blot method based on difference of month.

- (a)** Liver and gills of two *Barbonymus ballerooides* that live in the upstream and down stream based on first month (continously) 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 ballerooides* that live in the upstream and down stream based on second month (continously) 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 ballerooides* that live in the upstream and down stream based on third month (continously) 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 ballerooides* 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 ballerooides* in the upstream and downstream based on variaton of sampling time, from the diagram have been known that there were no significant.

Table 1. MT density in liver of *Barbonymus baleroides* that live on upstream and downstream Brantas river based on sampling time.

Loc.	Sampling time/individual replication of <i>B.balleroides</i>	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	(Month1 st / <i>B.balleroides</i> 1)	231.400		231.290	0.729
	(Month1 st / <i>B.balleroides</i> 2)	231.722	231.561		
	(Month2 nd / <i>B.balleroides</i> 1)	229.824			
	(Month2 nd / <i>B.balleroides</i> 2)	231.529	230.677		
	(Month3 rd / <i>B.balleroides</i> 1)	231.729			
	(Month3 rd / <i>B.balleroides</i> 2)	231.538	231.634		
Down stream	(Month1 st / <i>B.balleroides</i> 1)	232.422		231.910	0.611
	(Month1 st / <i>B.balleroides</i> 2)	231.804	232.113		
	(Month2 nd / <i>B.balleroides</i> 1)	231.529			
	(Month2 nd / <i>B.balleroides</i> 2)	231.294	231.412		
	(Month3 rd / <i>B.balleroides</i> 1)	232.875			
	(Month3 rd / <i>B.balleroides</i> 2)	231.538	231.207		

Table 2. MT density in gill of *Barbonymus baleroides* that live on upstream and downstream Brantas river based on sampling time.

Loc.	Sampling time/individual replication of <i>B.balleroides</i>	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	(Month1 st / <i>B.balleroides</i> 1)	229.294		229.858	1.491
	(Month1 st / <i>B.balleroides</i> 2)	230.422	229.874		
	(Month2 nd / <i>B.balleroides</i> 1)	230.353			
)	228.059	229.206		
	(Month2 nd / <i>B.balleroides</i> 2)	228.825	230.560		
)	232.294			
Down stream	(Month3 rd / <i>B.balleroides</i> 1)			231.5551	1.360
	(Month3 rd / <i>B.balleroides</i> 2)				
	(Month1 st / <i>B.balleroides</i> 1)	229.743			
	(Month1 st / <i>B.balleroides</i> 2)	231.513	230.628		
	(Month2 nd / <i>B.balleroides</i> 1)	230.412			
	(Month2 nd / <i>B.balleroides</i> 2)	233.529	231.971		
(Month3 rd / <i>B.balleroides</i> 1)	231.723				
(Month3 rd / <i>B.balleroides</i> 2)	232.412				
			232.067		

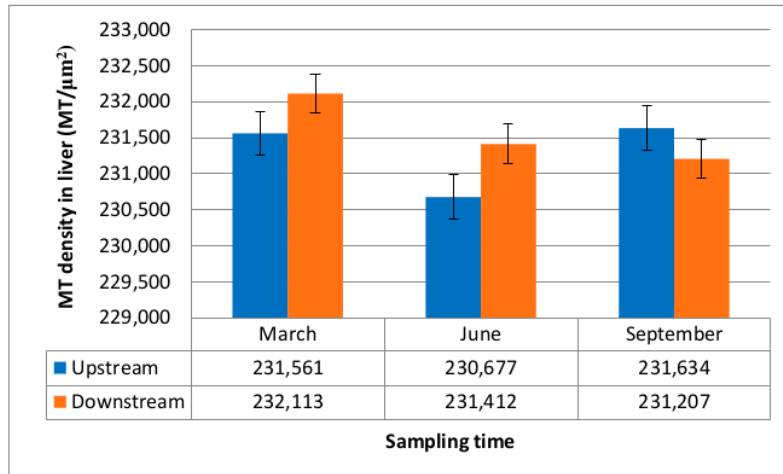


Figure 3. Difference between liver *Barbonymus balleroides* MT density in the upstream and downstream Brantas river based on sampling time

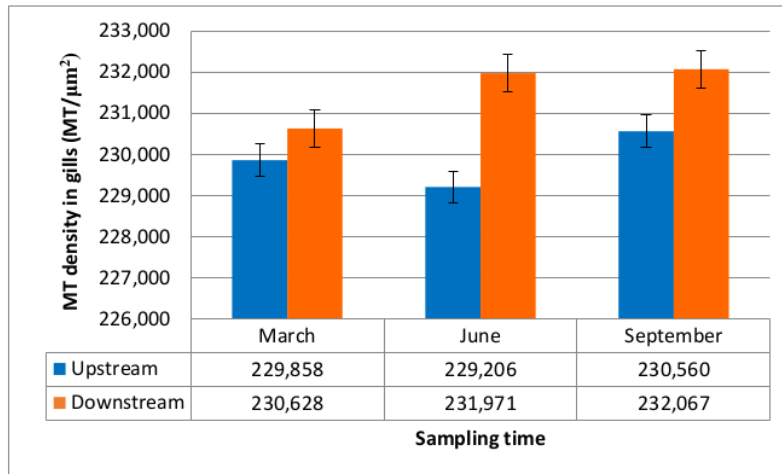


Figure 4. Difference between gill *Barbonymus balleroides* MT density in the upstream and downstream Brantas river based on sampling time.

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

[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 by protein called *metallothionein* [14]. First, ion of heavy metals interact with *Metal-regulatory Factor-1* (MTF-1) and *Metal-synthesis Inhibitor* (MTI), ion binds by 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 have 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 organ 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 balerooides* 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 balerooides* 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 balerooides* that live on upstream and downstream Brantas river based on variation of sampling time.

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