Analysis of the density and intensity of metallothionein in

Crassostrea cucullata oyster hulls in the coastal fishing port of

Mayangan Probolinggo, East Java using

immunohistochemical techniques

W Isroni¹⁴, N Maulida², M I Mubaroqi³

¹Department of Fish Health Management and Aquaculture, Faculty of Fisheries and Marine, Universitas Airlangga, Kampus C Jalan Mulyorejo, Surabaya 60115 Jawa Timur, Indonesia.
²Faculty of agriculture enginering, Brawijaya University
³Faculty of Fisheries and Marine Science, Brawijaya University
⁴Author correspondence: wahyu.isroni@fpk.unair.ac.id

Abstract. The purpose of this study was to determine the relationship between heavy metal levels in the stomach of Crassostrea cucullata. Descriptive methods and oyster sampling were carried out at 3 stations and 3 oyster samples were taken from each station. Station I, it is suspected that the pollution came from waste from ship engine oil, or waste from ship repair, Station II is suspected of having contamination originating from TPI or SPN. Station III, it is suspected that the pollution came from domestic waste from the community who used the river as a disposal site. The results of the metallothionein density are in accordance with the response of the oyster hull to heavy metal absorption which indicates that the heavy metal content in the stomach at station 1 is higher when compared to station 2 and station 3. The highest metallothionein intensity is found at station 1 which is the dock area, while the lowest metallothionein intensity is found at station 3, namely the mangrove area. The color intensity of metallothionein varies at each station depending on the absorption rate of heavy metals by the oyster body. Average Metallothionein Intensity Graph.

1.Introduction

The main function of a fishing port is as a shelter, a place for fishing vessels to dock, a marketing place, a fishing industry development place, a place to control fish resources and control the quality of fishery products [1]. In the last few years, Mayangan coastal waters have started to be threatened by heavy metal pollution. Seawater in the coastal waters of Mayangan already contains heavy metal elements, namely Hg, Pb and Cd, although the concentration is still low. According to the preliminary test results, it is known that the Mayangan coastal waters contain heavy metal Hg 0.0092 ppm; Pb 0.0172 ppm and Cd 0.0697 ppm, the maximum recommended content of heavy metals in waters is Hg 0.002 ppm; Cd 0.01 ppm and Pb 0.03 ppm [2].

Shellfish is a biota that has the potential to be contaminated with heavy metals, because of its filter feeder properties, so that this biota is often used as test animals in monitoring the level of heavy metal accumulation in marine organisms. Oysters are also an effective filter feeder organism to reduce heavy metal concentrations [3].

Pollutants in the waters can be in the form of organic or inorganic materials. Organic pollutants, among others, come from agricultural waste, livestock, residential areas and others. While inorganic
pollutants are heavy metals such as Mercury (Hg), Lead (Pb), Arsenic (As), Cadmium (Cd), Chromium (Cr), and Nickel (Ni), heavy metals that often contaminate waters are mercury lead [4].

Immunohistochemistry is a method for detecting proteins in the cells of tissue using the principle of binding between antibodies and antigens to living tissue. Immunohistochemical staining is widely used in examining abnormal cells such as cancer cells. Specific molecules will colour certain cells such as dividing cells or dead cells so that they can be distinguished from normal cells. This examination requires a tissue with a number and thickness that varies depending on the purpose of the examination. Generally, tissue originating from the body will be cut into very thin pieces using a device called a vibrating microtome.

2. Materials and methods

2.1. Place and time of research
This research was conducted in June 2019 in the waters of Pantai Mayangan Fishery Port, Laboratory of Pathology Anatomy, Faculty of Medicine, University of Brawijaya Malang, Laboratory of Basic Chemical Environment, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya Malang, and Laboratory (Human/Animal Physiology) FAAL Faculty of Medicine, Brawijaya University. Malang.

2.2. Tools and materials
The material in this study is the density and intensity of metallothionein in the hull tissue of *Crassostrea cucullata* from the waters of the Mayangan Coast Fishery Port which is suspected of being contaminated by heavy metals.

2.3. Procedure of research preparation

2.3.1. Station Determination
Oyster sampling was carried out in 3 locations, namely Location I which is located in the anchoring pond I (Dermaga), Location II is located in the anchoring pond II (TPI) and Location III is located in a mangrove area adjacent to residential areas and agricultural activities. The location I (Labuh Pond I) is a part of the port used as a berth for fishing boats. Location II (Labuh II Pond) is part of the port used as a berth for ships, Fish Auction Place (TPI), SPDN (Solar Package Dealer for Fishermen) and close to food stalls for fishermen. Meanwhile, Location III is a mangrove area.

2.3.2. method of collecting data
In this study, researchers observed the metallothionein content of oyster hulls from several determinations of stations in Mayangan PPP which were suspected to have been contaminated by taking oysters from that location, then dissected the stomach and carried out observations using immunohistochemical techniques to determine the density and intensity of metallothionein. In addition, sea water quality observations were carried out at the time of sampling to determine the relationship with the density and intensity of metallothionein in oyster hulls.

3. Result and Discussion

3.1. Heavy metal content in the oyster stomach *Crassostrea cucullata*
The absorption of heavy metals by oyster hulls is different at each station. The average heavy metal content of Pb, Cd and Hg in the stomach at each research station can be seen in Figure 1.
The diagram in Figure 1 above shows that the oyster stomach absorbs more lead (Pb) than cadmium (Cd) or mercury (Hg). At station 1, the response of oyster hulls to absorption of heavy metals, especially Pb, is greater than that of stations 2 and 3. This shows that the accumulation rate of oysters against heavy metal Pb is quite high. The accumulation factor in each type of marine biota is relatively different, this is due to differences in the biological characteristics (type, age and physiology) of each type of biota, also due to differences in physical and chemical properties and activities of each. Each location is an example of heavy metal Pb [5].

3.2 Expression of Metallothionein in C. cucullata Oyster Stomach by Immunohistochemical Technique

Metallothionein is a protein with a low molecular mass (6-7 kDa) and its main property is that it contains 26-33% "cysteine" and does not have aromatic amino acids or histidine. Immunohistochemical methods on the gastric tissue of Crassostrea cucullata were found in three areas with different levels of contamination, indicating that the metallothionein in the image is a brown block. The brown block can be divided into three levels of colour intensity for positive reactions and one colour intensity for negative reactions [6]. Positive reactions consist of a strong positive which is indicated by dark brown to blackish colour (**), medium positive which is indicated by the colour dark brown (**) and a weak positive which is indicated by the colour brown mixed with blue (*). The darker the brown, the more the amount of metallothionein expressed and the higher the levels of heavy metals. Can be seen in Figure 2.

Figure 1. Graph of Average Heavy Metal Contents of Pb, Cd and Hg in Oyster Stomach *C. cucullata*

Figure 2. Expression of Metallothione on a scale of 20 μm (A) Strong Positive (B) Moderately Positive (C) Weak Positive
Metallothionein appears to appear dark brown in the hull tissue of the oyster to bind heavy metals that enter when the oyster is breathing, mostly found at station 1, which is a dock area, while metallothionein that appears slightly appears at station 3 which is a mangrove area. The image can be seen in Figure 3.

The process of forming a brown colour is explained by the principle of the immunohistochemical staining of the peroxidase method, namely that the antigens in the tissue are bound with specific primary antibodies [7]. Then the primary antibody that is bound to the antigen is then tied to the secondary antibody (primary anti antibody) which has been labelled the peroxidase enzyme. The addition of a substrate containing chromogen and H$_2$O$_2$ will give rise to brown and H$_2$O deposits. The brown precipitate is the result of the decomposition of the substrate (chromogen and H$_2$O) by the peroxidase enzyme. The brown colour that appears indicates a positive reaction (+), which means that there are antigens in the tissue. If there is no antigen in the tissue, it will not appear brown.

### 3.3 Results of Metallothionein Density Analysis in Oyster Stomach

The results showed that the highest metallothionein density was found at station 1 which is the dock area, while the lowest metallothionein density was at station 3, namely the mangrove area. The results of the metallothionein density are in accordance with the response of the oyster hull to heavy metal absorption which shows that the heavy metal content in the stomach at station 1 is higher when compared to station 2 and station 3. This is clearly seen in the Metallothionein density graph which is presented in Figure 4.

| Field Of View | Research Station |
|---------------|------------------|
|               | 1                | 2               | 3               |
| 1             | 27.69            | 31.79           | 29.74           |
| 2             | 30.35            | 36.92           | 29.12           |
| 3             | 28.10            | 35.49           | 26.87           |

The results of the calculation of metallothionein density can be presented in table 1 below.

| Field Of View | Metallothionein Density ( N X 10^-4 Mt/µm²) |
|---------------|---------------------------------------------|
|               | Research Station | 1 | 2 | 3               |
| 1             | 27.69 | 31.79 | 29.74 | 22.15 | 24.82 | 22.56 | 7.79 | 7.38 | 5.12 |
| 2             | 30.35 | 36.92 | 29.12 | 15.79 | 17.64 | 20.51 | 5.33 | 5.94 | 6.36 |
| 3             | 28.10 | 35.49 | 26.87 | 14.15 | 14.76 | 17.02 | 5.74 | 6.35 | 8.00 |

Figure 3. Expression of metallothionein (A) Station 1 (B) Station 2 (C) Station 3
Figure 4. Graph of Metallothionein density in Oyster Stomach

From this graph, it can be seen that the higher the concentration of heavy metals exposed in the organism's body, the greater the metallothionein density in the organism's body and vice versa. Explained that a higher absorption rate of heavy metals would reflect a higher density and/or activity of the system in cells in the hepatopancreas of shellfish [8]. the intestine is located after the stomach, functions to receive large food particles that are not digested by the stomach.

3.4 Results of Metallothionein Intensity Analysis in Oyster Stomach

The results of observations of metallothionein intensity in oyster gills are shown in Figure 5 below.

Figure 5. Metothionein intensity (A) Station 1 (B) Station 2 (C) Station 3

The results of the average metallothionein intensity in each field of view are shown in Table 2 below.

| Field Of View | Metallothionein Density (N X 10^-4 Mt/µm²) |
|---------------|------------------------------------------|
|               | Research Station |
|               | 1        | 2        | 3        |
| 1             | 28.387   | 27.830   | 25.169   | 21.857   | 20.578   | 14.102   | 14.056   | 14.335   |
| 2             | 25.380   | 23.693   | 23.250   | 17.266   | 16.788   | 15.567   | 13.361   | 12.808   | 12.449   |
| 3             | 22.630   | 22.268   | 22.118   | 15.287   | 15.785   | 14.649   | 11.996   | 11.164   | 6.357    |
The highest metallothionein intensity was found at station 1 which is the dock area, while the lowest metallothionein intensity was at station 3, namely the mangrove area. Metallothionein intensity at station 1 ranges from 22,118 pixels to 28,387 pixels, station 2 ranges from 14,649 pixels to 21,857 pixels and at station 3 ranges from 6,357 pixels to 14,102 pixels. Metallothionein colour intensity varies at each station depending on the absorption rate of heavy metals by the oyster body.

4. Conclusion
The results of the Metallothionein density at station 1 ranged from \(26.87 \times 10^{-4}\) MT / \(\mu\text{m}^2\) - \(36.92 \times 10^{-4}\) MT / \(\mu\text{m}^2\), station 2 ranged from \(14.15 \times 10^{-4}\) MT / \(\mu\text{m}^2\) - \(24.82 \times 10^{-4}\) MT / \(\mu\text{m}^2\) and at station 3 ranges from \(5.33 \times 10^{-4}\) MT / \(\mu\text{m}^2\) - \(8.00 \times 10^{-4}\) MT / \(\mu\text{m}^2\). Metallothionein intensity at station 1 ranges between 22,118 pixels - 28,387 pixels, station 2 ranges from 14,649 pixels - 21,857 pixels and at station 3 ranges from 6,357 pixels - 14,102 pixels. The relationship between Density with Metallothionein and Intensity with Metallothionein is that the higher the Metallothionein the higher the heavy metal content.

5. References
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