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RESEARCH ARTICLE

REVISED Metallothionein expression on oysters (*Crassostrea cuculata and Crassostrea glomerata*) from the southern coastal region of East Java [version 2; peer review: 2 approved]

Asus Maizar Suryanto Hertika, Kusriani Kusriani, Erlinda Indrayani, Defri Yona, Renanda Baghaz Dzulhamdhani Surya Putra

Faculty of Fisheries and Marine Science, University of Brawijaya, Malang, East Java, 65165, Indonesia

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Abstract

Background: This study aimed to analyse levels of heavy metals (Pb, Hg and Cd) in the aquatic body, gills and stomach of the oysters *Crassostrea cuculata* and *Crassostrea glomerata*, the metallothionein (MT) level in the gills and stomach of both oysters, and relationships between heavy metals level (Pb, Hg and Cd) in the gills and stomach to MT level in both species of oysters.

Methods: The research method utilized was a descriptive method. The oyster samples were taken from three stations: Sendang Biru, Popoh and Prigi beaches. MT values were assessed using confocal laser scanning microscopy. The heavy metal levels were assessed using atomic absorption spectrophotometry method.

Results: Both oyster heavy metal content obtained in the southern coastal waters exceeded the safe limit set by the State Minister of Environment No. 51 of 2004. In general, the expression of MT was found to be higher in stomach tissue compared to gill tissue.

Conclusions: The levels of the heavy metals Pb, Hg, and has a strong relationship with MT levels in the gills and stomach in both types of oysters.

Keywords

Heavy Metal, Biomarkers, Metallothionein, CLSM

Open Peer Review

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1 **Ima Yudha Perwira**, Udayana University, Jimbaran, Indonesia

2 **Rossita Shapawi**, Universiti Malaysia Sabah, Kota Kinabalu, Malaysia

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Corresponding author: Asus Maizar Suryanto Hertika (asusmaizar@yahoo.com)

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REVISED **Amendments from Version 1**

Thanks for the reviewer's comments about our paper/article. The latest version of our paper has deeper explaining the characters in each sampling station. Furthermore, we rearrange the data in result and discussion for easier to read follow the suggestion of the reviewer. Overall, the paper more detailed and easier to read.

Any further responses from the reviewers can be found at the end of the article

Introduction

Coastal areas are often under the pressure of ecological pollution originating from human activities. One kind of pollutant is heavy metals, such as cadmium (Cd), Mercury (Hg), and lead (Pb), which originate from household and industrial waste effluent[1,2]. Heavy metals settle on the bottom of the seabed by sedimentation. This can contaminate marine biota with heavy metals and threaten human health as consumers[3–7].

Metallothionein (MT) is a non-enzymatic protein in a low molecular weight which has a high cysteine content, does not have aromatic amino acids and is not heat-stable. The multiple thiol groups (-SH), formed by cysteine residues, allow MT to bind heavy metals[8–10]. MT has a specific metal binding ability. Each MT only binds one type of metal, with Cd M, Hg and Pb each binding a different MT[11,12]. MT has been widely used as a specific biomarker because the expression of MT reflects the presence of heavy metals[13–15]. Previous research has revealed that the induction of MT expression increases after the organism is exposed to heavy metals[16]. Hertika *et al.*[17] found the existence of positive relationships between heavy metals and MT expression in North East coast oysters.

MT possess the ability to bind a certain amount metal in a cell and restore the ability to function of inactive proteins due to metal cadmium[18]. According to Prabowo[19], heavy metals contained in waters can enter the body of aquatic biota. Heavy metals pass through the mouth and digestive organs, such as the surface of the gills. Therefore, organisms that live in waters with higher levels of heavy metal contamination will have higher heavy metal level.

Oysters, including benthic macrofauna species, are one of the best bioindicators of heavy metal contamination in an area[20]. Oysters are potential biota contaminated by heavy metals, as these are filter feeders, and express MT, which is able to bind heavy metals Therefore, oysters can be used as test animals in monitoring the accumulation of heavy metal levels in polluted waters[21]. This study aims to analyze heavy metal level (Pb, Hg, and Cd) in the tissues (gills and stomach) of oysters (*Crassostrea cuculata* and *Crassostrea glomerata*) and the coastal waters of the South coast of East Java to determine their relationship to MT (MT) expression.

Methods

Sample collection

In total 108 oyster were used in this study. Three samples of oyster (*Crassostrea cuculata* and *Crassostrea glomerata*) were collected in three of each of the three sub-stations on the Sendang Biru (Malang) coast, Prigi beach (Trenggalek) and Popoh beach (Tulungagung). Sub-station 1 located in port, sub-station 2 located in fish market, sub-station 3 located in between mangrove and beach. Sub-station 1, 2 and 3 on the Sendang Biru beach are geographically located at 8°26'01.3"S 112°41'01.8"E, 8°26'04.7"S 112°40'55.3"E and 8°25'48.2"S 112°41'17.0"E, respectively. Sub-stations 1, 2 and 3 of the Prigi coast are geographically located at 8°25' 48.2"S 112°41'17.0"E, 8°15'47.9"S 111°48'11.6"E and 8°15'44.4"S 111°48'13.0"E, respectively. Sub-stations 1, 2 and 3 from Popoh beach are geographically located at 8°17'11.9"S 111°43'41.9"E, 8°17'13.2"S 111°43'47.2"E and 8°17'11.8"S 111°43'33.1"E, respectively. Oyster samples were taken three times for gills and stomach tissue taken in each sub-station and each was analyzed separately.

Heavy metal analysis

Heavy metals (Pb, Cd, and Hg) in oysters (gills and stomach tissue) and the seawater at each sub-station were measured by atomic absorption spectrophotometry (AAS) following the measurement procedures in previous studies carried out by Hertika *et al.*, 2018[17] A total of 50 ml seawater samples obtained from each substation were filtered with a 0.45-mm polycarbonate membrane to separate particles which caused contamination in heavy metal measurements. Next, 1 M nitric acid was added to the water sample to obtain a pH value below 2.

The gill tissue and stomach taken from the oyster samples in each substation were prepared according to the method of Trinchella *et al.*[22]. In order to obtain a complete oxidation process in the decomposition of organic substances, to each sample of gill and stomach tissue (0.2 grams), 2 ml of HNO_3 were added. The samples were incubated for 30 minutes at low temperatures (5–8°C) to avoid minerals lost during the evaporation process. The sample is centrifuged for 15 minutes at 12,000 *g*. The supernatant produced from the centrifugation process was taken to measure the heavy metal content. Measurement of heavy metals (Pb, Cd, and Hg) was carried out using the A220 Atomic Absorption Spectrophotometer Variant (Variant, Inc.).

Analysis of MT expressions

To analyse MT expression in this study, confocal laser scanning microscopy (CLSM) was performed (Confocal Olympus FluoView™ FV1000) based on previous research by Ockleford[23] and Mongan *et al.*[24]. This observation system utilized a reverse-light-path fiber-optic signal that transmits Nomarski DIC signals to a second detector to visualize immunofluorescent and refractive index (RI) images. Images were observed using an Olympus U-TBI90 Microscope (Olympus, Japan) and inputted to Olympus Fluoview v4.2a, Japan for calculating MT expression quantities. Briefly, the gills and stomach in oyster samples (*Crassostrea cuculata* and *Crassostrea glomerata*) were preserved into 10% formaldehyde. The sample was cut into 2–3 mm sections using a microtome and dehydrated using the Tissue Tex Processor. The samples were twice soaked with xylol (#CAT 1086612511, Merck, Japan) for 10 minutes each. Then the sample was fixed with absolute ethanol 90%, for 5 minutes. Immediately, the sample was soaked with 10 mM pH 6 buffer citrate for 15 minutes. Samples were blocked with PBST containing BSA 2% (CAT# 15561020, Thermo Fisher, USA) for 1 hour in room temperature. Furthermore, the sample was labelled with the Anti-MT Primary monoclonal mouse Antibody (1:1000, CAT# UC1MT, Gene Tex, USA) which contained 2% BSA for 1 hour at room temperature. Samples were rinsed with PBST for 8 minutes. Furthermore, the sample is labeled with rhodamine-conjugated Mouse IgG Antibody (CAT#610-1002, ROCKLAND Immunochemical Inc,USA) containing 2% BSA for 1 hour at room temperature. The sample was rinsed using PBST and dried. Lastly, glycerol was added into the sample and observed using CLSM.

Water quality measurement

Water quality in this study was measured based on standard methods[25]. Dissolved oxygen concentration was measured *in situ* using an oximeter (YSI PRO 20). The pH was also measured *in situ* using a pH pen (PH 2011 ATC) at each sub-station. Temperature and salinity were measured using a mercury thermometer and refractometer (RHS-10ATC, SINOTECH), respectively.

Data analysis

This study used regression correlation analysis with a simple linear regression model in SPSS version 16.0 software. Professional charts are created using the GraphPad Prism 7.00 application. Using the method outlined bn Hertika *et al*.[17], the relationship between heavy metal levels with MT (MT) expression was obtained from multiple regression results with variable Y exhibiting heavy metals in oyster gills or stomach tissue. Variable X exhibited levels of Pb, Cd, and Hg.

Results and discussion

Water quality analysis

The research station was taken from three different locations. The first station is Sendang Biru Coastal area, one of the major ports on the southern coast of East Java and tourist area. The second station is the Popoh Beach area which is a tourist area and settlement. The third station is Prigi Beach which is a tourist beach, settlement and fishing port area. We observed physical and chemical water quality parameters that support the life of *Crassostrea cuculata* and *Crassostrea glomerata*, namely temperature, acidity (pH), dissolved oxygen (DO) and salinity (Figure 1).

Water quality monitoring exhibited that there is no significant difference between each station and exhibited that the water quality is good for the oyster ecology. According to KEPMENLH. 51 of 2004[26], it indicates the temperature suitable for oysters growth is 25–34°C. Furthermore, the pH level suitable for oysters' ecosystem ranging from 6.8 to 8.8. DO levels of more than 5 mg l^{-1} are required to support aquatic organisms' survival[26].

Heavy metal levels in the waters

Analysis of the Pb, Cd and Hg content at the three research stations (Sendang Biru, Popoh, Prigi) is shown in Figure 2. The range of highest Pb concentrations in each substation

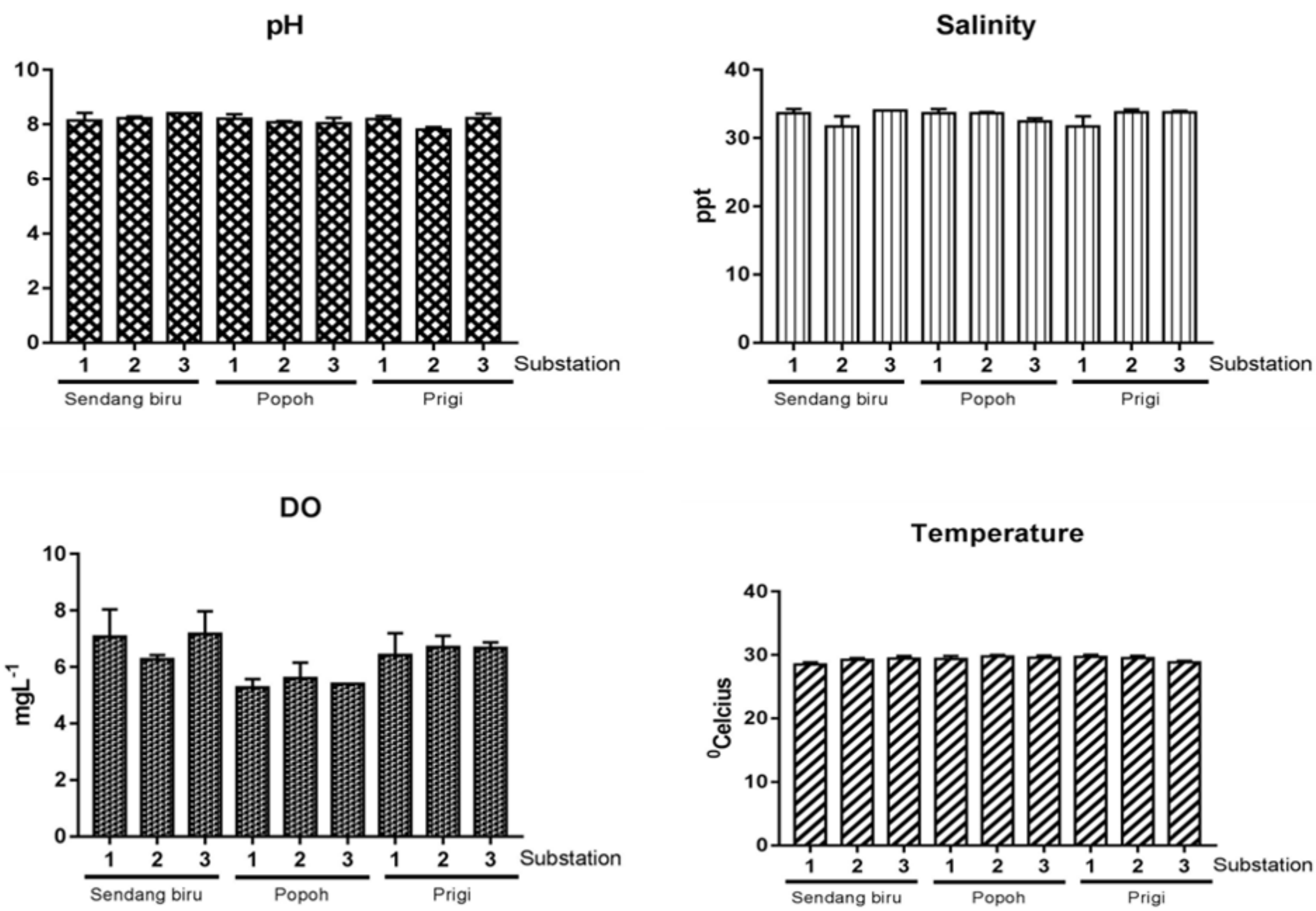

Figure 1. Analysis of water quality at the three research stations.

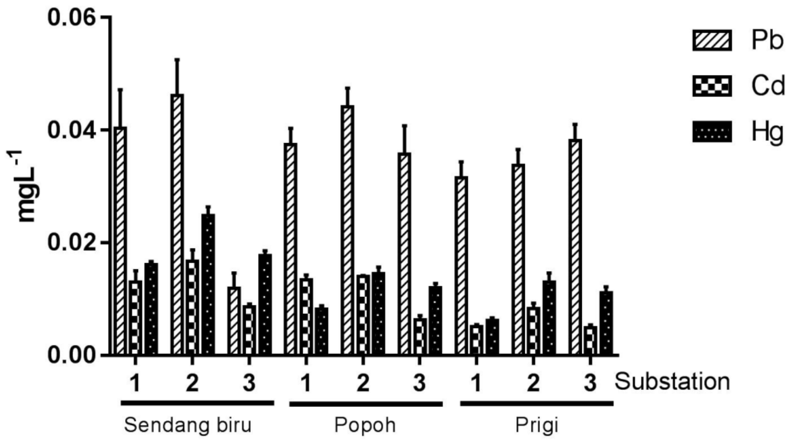

Figure 2. Analysis of Pb, Cd and Hg levels in seawater samples from each substation of Sendang Biru, Popoh, and Prigi stations.

was 0.03–0.054 mg l^{-1}; the highest Hg concentration was 0.01–0.026 mg l^{-1} and that of Cd was 0.009–0.018 mg l^{-1}. The highest concentration of Pb, Cd and Hg heavy metals were found at sub-station 2 in Sendang Biru for 0.054, 0.018, 0.026 mg l^{-1}. In general, some measured heavy metal content has passed the specified quality standard. Based on the Decree of the State Minister of Environment No. 51 of 2004 concerning seawater quality standards for heavy metal content[26], the Hg content appropriates the aquatic environment must not exceed 0.003 mg l^{-1}, Pb 0.05 mg l^{-1} and Cd 0.01 mg l^{-1}.

Heavy metal in the gills and stomach tissue

Heavy metals (Pb, Cd, and Hg) in *Crassostrea cuculata* and *Crassostrea glomerata* gills and stomach tissue is exhibited in Figure 3. The highest Pb content in the gills of *Crassostrea cuculata* was obtained at the Prigi station, at sub-station 1 at 0.13 mg l^{-1}, the highest Cd and Hg concentrations were obtained from Sendang Biru station in sub-station 2 at 0.08 mg l^{-1} and 0.09 mg l^{-1}, respectively (Figure 3A). Whereas the highest heavy metal level in *Crassostrea cuculata* stomach tissue Pb, Hg and Cd levels were observed at the Sendang Biru station in sub-station 1, at 0.067, 0.036 and 0.077 mg l^{-1} respectively (Figure 3B).

Furthermore, the heavy metal content in the gills of *Crassostrea glomerata* is exhibited in Figure 3C. The highest Pb, Hg, and Cd values were obtained at Sendang Biru station at sub-station 1, at 0.142, 0.071 and 0.11 mg l^{-1}, respectively. The highest value of stomach Pb and Cd content was observed in Prigi stations in substations 1 and 3: 0.145 and 0.047 mg l^{-1}, respectively (Figure 3D). The highest value of Hg was obtained at Sendang Biru station at sub-station 1, which was 0.078 mg l^{-1}.

The accumulation of heavy metals in this study exhibit the same pattern in the study conducted by Bilgin *et al.*[27], which found that accumulation of heavy metals in the soft tissue of mollusks, Pb was found higher than Cd or Hg. Aquatic organisms are capable of absorbing and accumulating heavy metals in several ways: through the respiratory tract (gills), digestive tract, and skin surface diffusion[28]. In this study, the highest accumulation of heavy metals was found in gill tissue. According to Hutagulung[29], the high accumulation of heavy metals in gills is closely related to the nature of biota. The oyster's food intake is conducted through filtering water (filter feeders). Furthermore, Soto *et al.*[30] revealed that gills are the main target tissue for absorption contamination of dissolved heavy metal ions in aquatic bodies.

Research result exhibited that *Crassostrea cuculata* and *Crassostrea glomerata* have different values of heavy metals as each organism has a different ability to accumulate heavy metals. Based on the results of the study by Fattorini *et al.*[31] *Mytilus galloprovincialis* is able to accumulate Pb, Cd and Hg heavy metals at 0.29–2.95 mg l^{-1}, 0.41–1.60 mg l^{-1}, and 0.02–0.19 mg l^{-1}, respectively. However, Kucuksezgin *et al.*[32] achieved different results; *Thylacodes decussatus* was observed to absorb Pb at levels ranging from 0.38–1.2 mg l^{-1}, Cd at 0.03–0.24 mg l^{-1}, and Hg at 0.04–0.13 mg l^{-1}. This may be related to the tendency of specific bioaccumulation of bivalves, based on different habitats, lifestyles, and abundance of food. Some studies emphasize that metal accumulation has presented different species-specific capacities for bivalves[33,34]. It is claimed that this difference is related to the metabolic rate of bivalve species[35]. The bioaccumulation pattern of metals can generally be attributed to the presence of anthropogenic inputs or lithogenic sources affecting the area. Seasonal variations in bivalves metal concentrations result from many factors, such as large differences in water temperature, particulate metal runoff to coastal waters, food availability. It is caused by transferring metals from water to feeding-filtering organisms, body weight changes during gonadal development, and biomass release associated with sexual reproduction[36–38]. For bivalves, the accumulated changes depend on the metal and the ability of different species or genera to store or/and remove metals from the tissue. In general, metal concentrations in bivalves increase with increasing shell size; however, in some cases, metal concentrations may decrease due

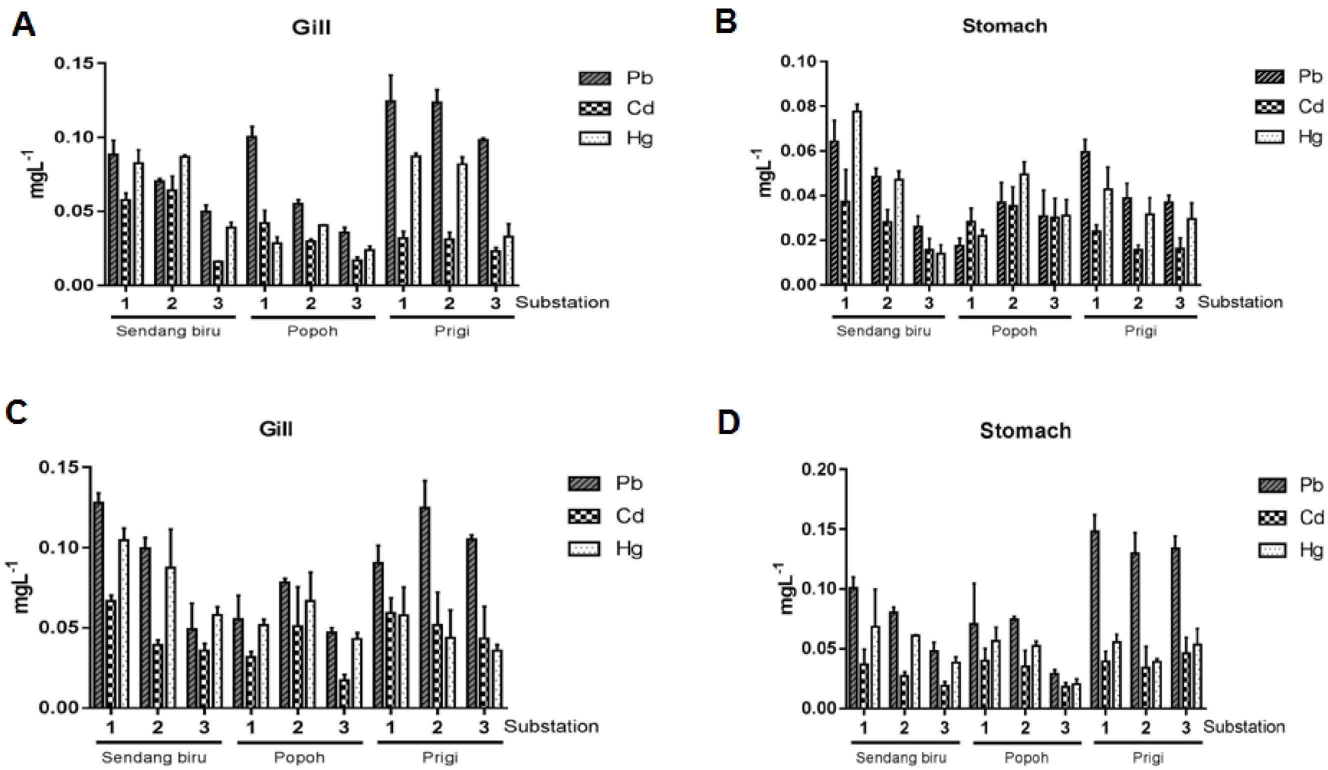

Figure 3. Pb, Cd, and Hg levels in the gills and stomach of (**A**, **B**) *Crassostrea cuculata* and (**C**, **D**) *Crassostrea glomerata* at Sendang Biru, Popoh and Prigi stations.

to the detoxification process in these organisms[39,40]. Raw data are available on OSF[41].

MT levels in the gills and stomach of *Crassostrea cuculata*

MT content analysis is exhibited in Figure 4. Research results exhibited that in MT stomach tissue overall expression was higher than gill tissue (Figure 4A). The highest metallothionine expression in gill tissue was obtained at Prigi station at 810.876–1387.61 arbitrary units. The lowest expression on the stomach tissue was found at Sendang Biru station ranging from 453.246–511.098 arbitrary units. Highest MT expression in gill tissue was also found at Prigi station ranging from 325.976–622.534 arbitrary units. On the other hand, the lowest value was obtained at the Sendang Biru station at 276.254–498.512 arbitrary units. MT expression in the stomach tissue is higher than that in the gill tissue. This is supported by assessment of the morphology of MT expression using rhodamine-labelled MT in the gill and stomach tissue. In Figure 4B, Rhodamine-MT as metallothionine marker is expressed brighter in stomach tissue compared to gill tissue. Figure 4C shows rhodamine-MT absorption, as an MT marker, is recorded to have a higher intensity in stomach tissues compared to gill tissues.

Heavy metal content was inversely proportional to MT expression in the gill and stomach tissues. The high content of heavy metals in the gills stimulates high MT expression to bind and detoxify heavy metals quickly. Therefore, MT quantity detected on the tissue decreases. Conversely, heavy metal in stomach tissue accumulates less compared to gill tissue. Hence, the detoxification process is slower. It indicates that MT quantity detected in this study is higher. According to Ringwood *et al.*[42], there is a positive relationship between MT and heavy metal pollutants. Heavy metal contaminants can cause systemic damage to an organism and result in increased MT production. Previous research has revealed that MT has a crucial role in various processes of biological activity; it binds heavy metals and conduct recovery process from systemic damage caused by heavy metals through homeostasis process (dynamic balancing of the body's biological processes) to heavy metals[43,44], and heavy metal detoxification[45,46]. The function of MT in heavy metal detoxification mainly depends on the high-affinity bond between heavy metals and MTs, which causes heavy metal absorption to be higher than that of important macromolecules[45,47]. It indicates that MT plays an important role in protecting cells from heavy metal poisoning[48–51]. It is proven that MT could be a biomarker useful for predicting heavy metal toxicity and heavy metal detoxification toxic to organisms[52,53]. Raw data are available on OSF[41].

Analysis of MT levels in *Crassostrea glomerata* gills and stomach

MT content analysis is exhibited in Figure 5. Similar results were obtained in the analysis of *Crassostrea glomerata* gills and

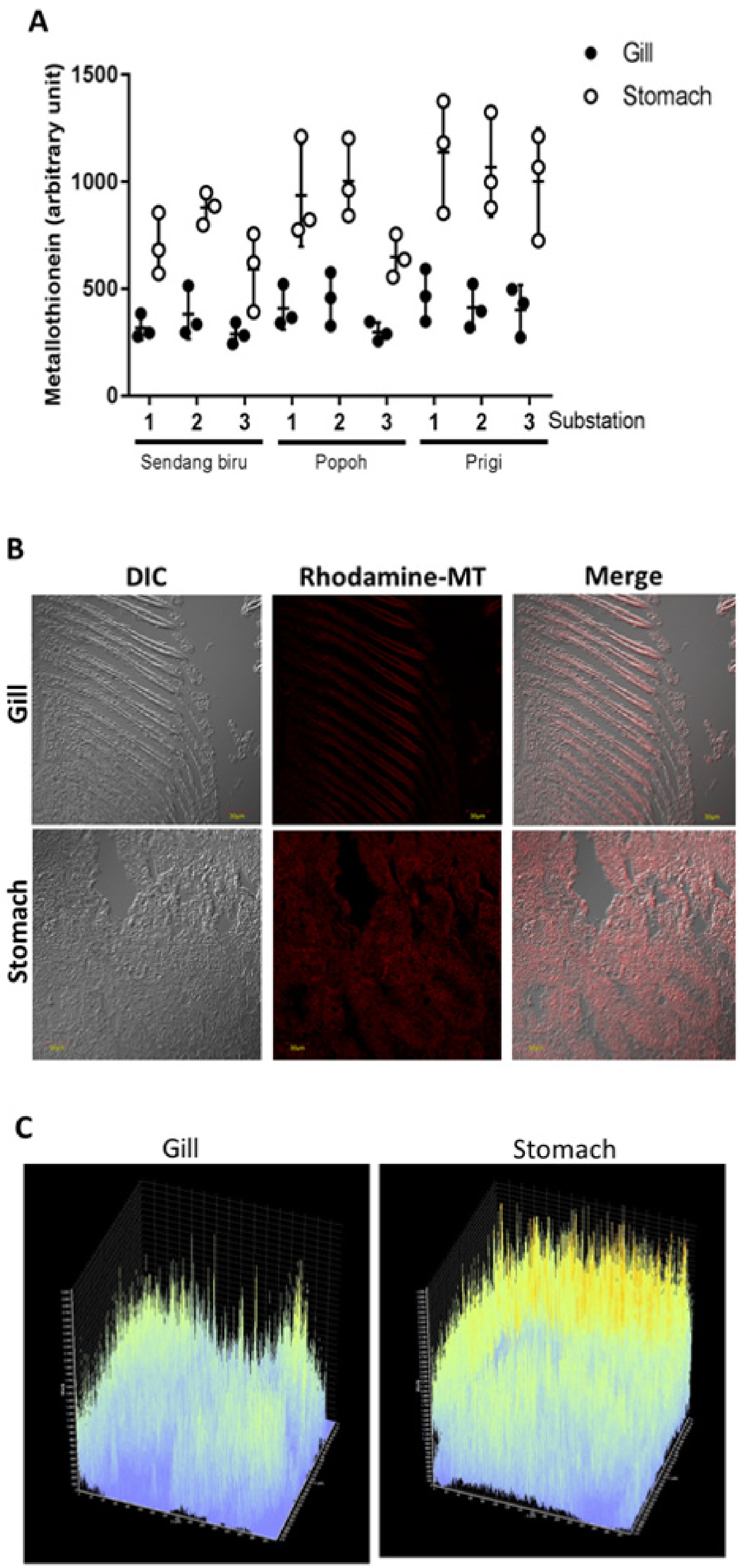

Figure 4. Metallothionein (MT) expression in *Crassostrea cuculata* gills and stomach. (**A**) The quantity of MT expression at each station. (**B**) Morphology of MT expression in gills and stomach, (**C**) Absorbance gating of MT expression from a representative experiment.

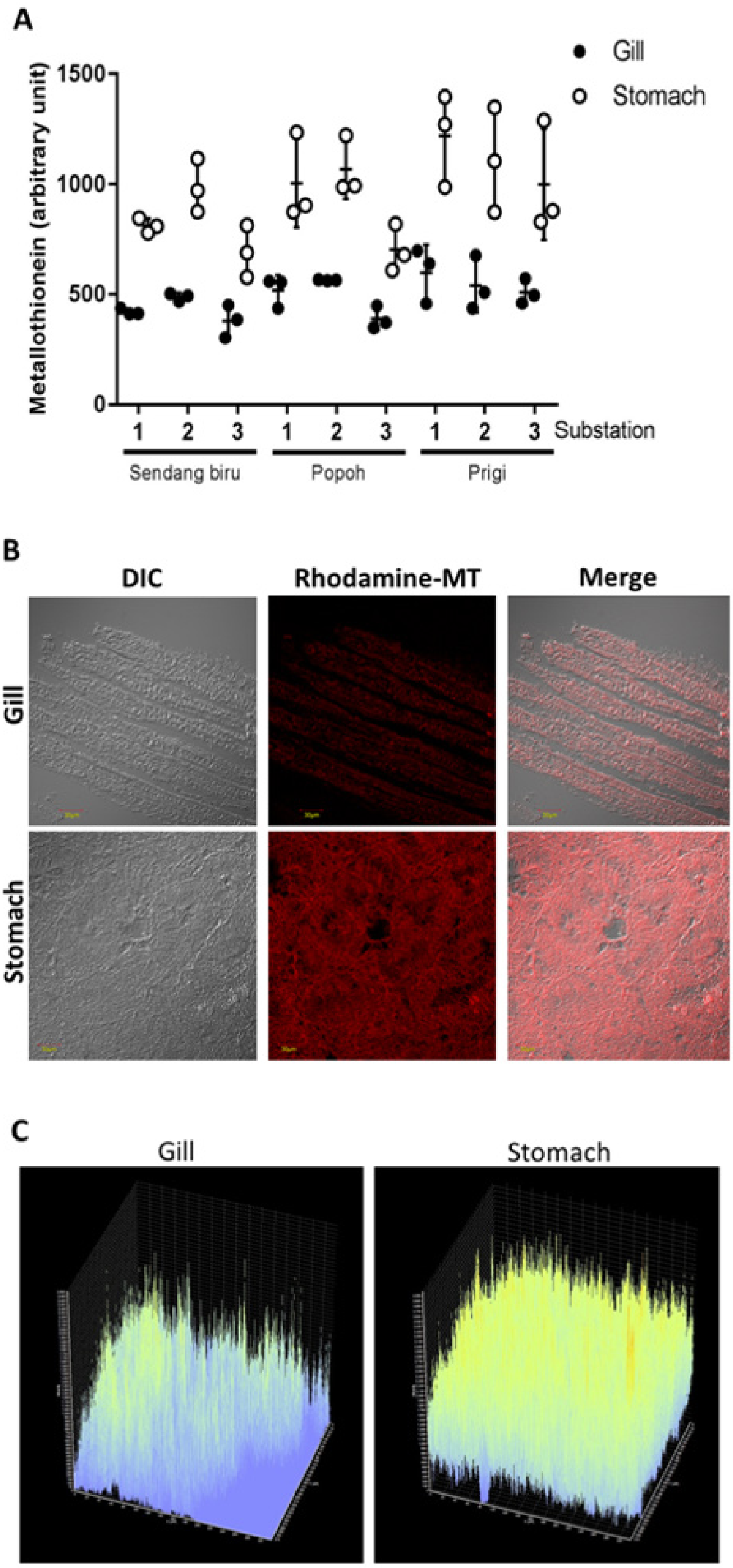

Figure 5. Metallothionein (MT) expression in gills and stomach of *Crassostrea cuculata*. (**A**) The quantity of MT expression at each station. (**B**) Morphology of MT expression in gills and stomach. (**C**) Absorbance gating of MT expression from a representative experiment.

stomach. MT expression in the stomach tissue of *Crassostrea glomerata* is expressed higher than gill tissue (Figure 5A). The highest MT expression in stomach tissue was obtained at Prigi Station sub-station 1 with a value of 1412,112 arbitrary units. The lowest MT expression in stomach tissue was obtained from Sendang Biru sub-station 3 with a value of 576,243 arbitrary units. Furthermore, the highest MT expression in gill tissue was obtained from Prigi substation 1 with a value of 756,381 arbitrary units. The lowest MT tissue gill expression was obtained at Sendang Biru substation 3, with a value of 366,125 arbitrary units. Higher MT expression was observed in stomach tissue compared to gill tissue morphologically (Figure 5B). The morphological results exhibited that MT labeled Rhodamine-B in gastric tissue appears brighter than gill tissue. Rhodamine-MT is a MT marker used in this study. Figure 5C exhibited that the Rhodamine-MT absorption as an MT marker possesses a higher intensity in gill tissue compared to stomach tissue.

Rumahlatu, *et al*.[18] stated that MT protein which acts as a metal binding protein can be used as an indicator of pollution, as the presence of MT in oysters serves as a binder of heavy metals that accumulate in the body. Based on the research result, MT expression in *Crassostrea glomerata* and *Crassostrea cuculata* has different results. According to Tapiero and Tew[54], MT expression levels vary between species, these levels are determined by the identity of metal atoms bound to proteins, and the difference in metal distribution between MT isoforms. This may affect MT expression levels, therefore indicating that MT is involved in cellular homeostatic control and element regulation. MT expression in *Crassostrea cuculata* highest value was found in stomach tissue and the lowest value in gill tissue. This is inversely proportional to the heavy metal content, which wsa highest in the gills. In this case, the high content of heavy metals in the gills causes high MT production, which in turn is rapidly used for homeostasis and detoxification from damage caused by these heavy metal toxins. Therefore, MT expression in the gill tissue was detected as lower than that in stomach tissue. In previous studies, MT participated in metal ion homeostasis and detoxification, and anti-oxidative damage[55–57]. Furthermore, MT expression is governed by the rate of accumulation of heavy metals, and MT plays an important role in metal detoxification and homeostasis[58,59]. Some species develop physiological adaptations to tolerate metal pollutants[60] which use two major detoxification mechanisms. The oyster uses metal binding compounds in the cytosol, such as MT (or similar proteins), or mineralization of minerals[61]. The relativity of these two detoxification mechanisms varies greatly depending on the species and habitat. According to Amiard *et al*.[61], a decrease in MT concentration in organisms accumulated by heavy metals is influenced by cytotoxic effects in the detoxification process. Should an organism accumulate high heavy metals, a significant reduction in MT is caused as it is used in the process of suppressing the reactive production of ROS species oxygen responsible for oxygen metabolism[62].

The relationship between Pb, Cd, and Hg levels and MT expression in *Crassostrea cuculata* gills and stomach

The relationship between heavy metal level Pb, Hg, and Cd with MT levels in *Crassostrea cuculata* gills exhibited a very strong value with the coefficient of determination (R^2) of 0.908. Based on the results of multiple linear regression equations of heavy metal level in the aquatic body against MT levels in *Crassostrea cuculata* gill tissue, the following formula was used $Y = 242.337 + 2{,}128.234\ X_1 + 88.354\ X_2 + 2{,}182.218\ X_3$. These results indicate that a 1 ppm Pb (X_1) increase will increase MT expression 2,128,234 arbitrary units. Should Cd increased by 1 ppm (X_2), it will increase MT expression by 88,354 arbitrary units. On the other hand, a 1 ppm Hg (X_3) increase would increase the MT expression of 2,182,218 arbitrary units.

Furthermore, a similar result was found in the relationship between the heavy metal level of Pb, Hg, and Cd with the stomach tissue MT expression. It indicates a strong relationship with the value of the coefficient of determination (R^2) of 0.92. Multiple linear regression equations of heavy metal level in the aquatic body against MT levels *Crassostrea cuculata* stomach assessment obtained the equation $Y = 494.528 + 4{,}075.811\ X_1 + 2{,}852.821\ X_2 + 5{,}990.359\ X_3$. The equation exhibited that Pb (X1) 1 ppm increase would, in turn, increase MT expression 4,075,811 at arbitrary units. Cd (X2) 1 ppm increase would increase MT expression 2,852,821 at arbitrary units. Hg (X3) when rising 1 ppm increase will increase MT expression of 5,990,359 arbitrary units.

Hasan *et al*.[63] stated that when the accumulation of heavy metals in the body of shellfish increases the synthesis of MT will probably reach the maximum level. The research conducted by Li *et al*.[64], exhibited a positive correlation between Cd heavy metal and MT levels in the gills and mantle of the bivalve group, which means that MT can be used as a biomarker for Cd heavy metal pollution. Furthermore, Sakulsak *et al*.[65] stated that the occurrence of exposure to heavy metals and the accumulation of heavy metals in cells can increase MT levels in tissues. Hence, MT can be used as a biomarker in environmental toxicology.

The relationship between Pb, Cd, and Hg levels and MT expression in *Crassostrea glomerata* gills and stomach

A very strong relationship was obtained in Pb, Hg, Cd heavy metal level and gill MT expression of *Crassostrea glomerata* with a coefficient of determination (R2) of 0.943. Multiple linear regression equations of heavy metal level in aquatic body against MT expression of *Crassostrea glomerata* gills is $Y = 320.254 + 2{,}311.778\ X_1 + 910.719\ X_2 + 2{,}173.765\ X_3$. This equation exhibited that a 1 ppm increase in Pb (X_1) will increase MT expression 2,311,778 arbitrary units. Furthermore, a 1 ppm increase in Cd (X_2) will increase MT expression 910,719 arbitrary units. A 1 ppm increase in Hg (X_3) will cause an increase in MT expression at 2,173,765 arbitrary units.

The relationship between the heavy metal level of Pb, Hg, Cd in the aquatic body and MT expression *Crassostrea glomerata* stomach tissue exhibited a strong relationship with the value of the coefficient of determination (R^2) of 0.918. Multiple linear regression equations of heavy metal level in the aquatic body against MT expression in *Crassostrea glomerata* stomach tissue was found to be similar. $Y = 570.492 + 4{,}603.743\ X_1 + 3{,}455.676\ X_2 + 4{,}333.870\ X_3$. The equation exhibited Pb (X_1) up 1 ppm increase will cause an increase in MT expression of 4,603,743 arbitrary units. Furthermore, a 1 ppm increase in Cd (X_2) will cause an increase in MT expression of 3,455,676 arbitrary units. A 1 ppm increase in Hg (X_3) will result in an increase in MT expression of 4,333,870 arbitrary units.

According to Rumahlatu, *et al.*,[18], MT acts as a metal-binding protein. It can be used as an indicator of pollution, as the presence of MT in oysters serves as a binder of heavy metals that accumulate in the body. Although many species can produce MT, oysters have exhibited a higher accumulation rate for metals compared to other species because they are filter feeders and tend to settle in one place[66]. MT can bind metals very strongly, but exchanging bonds with other proteins may take place easily. MT bonds to metals possess high thermodynamic stability but low kinetic stability[67].

Conclusion

Based on the results of the study it can be concluded that the heavy metal levels in the three locations assessed (Sendang Biru, Popoh, and Prigi) have exceeded the specified quality threshold. Furthermore, the relationship between Pb, Hg, and Cd heavy metal level in the aquatic body has a strong relationship with the expression of MT in oysters' stomach and gills (*Crassostrea cuculata* and *Crassostrea glomerata)*.

Data availability

Raw data from the present study, including heavy metal levels in all oyster samples and all raw immunofluorescent images, are available on OSF. DOI: https://doi.org/10.17605/OSF.IO/37BVQ[41].

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References

1. Emersida I, Sukendi S, Amin B: **Kandungan Logam Berat Pada Air Dan Tiram (*Crassostrea cucullata born*) di Muara Sungai Loskala Kota Lhokseumawe Provinsi Aceh.** *Berkala Perikanan Terubuk.* 2014; **42**(1): 69–79.
Reference Source
2. Vélez D, Montoro R: **Arsenic speciation in manufactured seafood products.** *J Food Prot.* 1998; **61**(9): 1240–1245.
PubMed Abstract | Publisher Full Text
3. Setiawan H: **Akumulasi dan Distribusi Logam Berat pada Vegetasi Mangrove di Perairan Pesisir Sulawesi Selatan.** *Jurnal Ilmu Kehutanan.* 2013; **7**(1): 12–24.
Reference Source
4. Doney SC: **The growing human footprint on coastal and open-ocean biogeochemistry.** *Science.* 2010; **328**(5985): 1512–1516.
PubMed Abstract | Publisher Full Text
5. Valdés J: **Heavy metal distribution and enrichment in sediments of Mejillones Bay (23° S): Chile: a spatial and temporal approach.** *Environ Monit Assess.* 2012; **184**(9): 5283–5294.
PubMed Abstract | Publisher Full Text
6. Järup L: **Hazards of heavy metal contamination.** *Br Med Bull.* 2003; **68**(1): 167–182.
PubMed Abstract | Publisher Full Text
7. Caussy D, Gochfeld M, Gurzau E, *et al.*: **Lessons from case studies of metals: investigating exposure, bioavailability, and risk.** *Ecotoxicol Environ Saf.* 2003; **56**(1): 45–51.
PubMed Abstract | Publisher Full Text
8. Couillard Y, Campbell PG, Tessier A: **Response of metallothionein concentrations in a freshwater bivalve (Anodonta grandis) along an environmental cadmium gradient.** *Limnol Oceanogr.* 1993; **38**(2): 299–313.
Publisher Full Text
9. Lasut MT: **'Metallothionein': Suatu Parameter Kunci Yang Penting Dalam Penetapan Baku Mutu Air Laut (BMAL) Indonesia.** *Ekoton.* 2002; **2**(1): 61–68.
Reference Source
10. Macovei A, Ventura L, Donà M, *et al.*: **Effects of heavy metal treatments on metallothionein expression profiles in white poplar (Populus alba L.) cell suspension cultures.** *An Univ Oradea Fasc Biol.* 2010; **1**: 194–198.
11. Astutik LW, Zulaika E: **Viabilitas *Azotobacter* A1a, A5 dan A9 pada Medium yang Mengandung Logam Pb.** *Jurnal Sains Dan Seni Its.* 2015; **4**(1): 2337–3520.
Reference Source
12. Isani G, Andreani G, Kindt M, *et al.*: **Metallothioneins (MTs) in marine molluscs.** *Cell Mol Biol (Noisy-le-grand).* 2000; **46**(2): 311–330.
PubMed Abstract
13. Dewi NK, Purwanto, Sunoko HR: **Metallothionein at Fish Liver As Pollution Biomarker of Cadmium (Cd) In Kaligarang river of Semarang.** *J Human and Environment.* 2014; **21**(3): 304–309.
Reference Source
14. Desouky MMA: **Metallothionein is Up-Regulated in Molluscan Responses to Cadmium, but not Aluminum, Exposure.** *The Journal of Basic and Applied Zoology.* 2012; **65**(2): 139–143.
Publisher Full Text
15. Carpenè E, Andreani G, Isani G: **Metallothionein functions and structural characteristics.** *J Trace Elem Med Biol.* 2007; **21 Suppl 1**: 35–39.
PubMed Abstract | Publisher Full Text
16. Wu JP, Chen HC: **Metallothionein induction and heavy metal accumulation in white shrimp *Litopenaeus vannamei* exposed to cadmium and zinc.** *Comp Biochem Physiol C Toxicol Pharmacol.* 2005; **140**(3–4): 383–394.
PubMed Abstract | Publisher Full Text
17. Hertika AMS, Kusriani K, Indrayani E, *et al.*: **Relationship between levels of the heavy metals lead, cadmium and mercury, and metallothionein in the gills and stomach of *Crassostrea iredalei* and *Crassostrea glomerata* [version 1; referees: 2 approved].** *F1000Res.* 2018; **7**: 1239.
PubMed Abstract | Publisher Full Text | Free Full Text
18. Rumahlatu D, Corebima AD, Amin M, *et al.*: **Kadmium dan Efeknya terhadap Ekspresi Protein Metallothionein pada *Deadema setosum* (Echinoidea; Echinodermata).** *Jurnal Penelitian Perikanan.* 2012; **1**(1): 26–35.
Reference Source
19. Prabowo R: **Akumulasi Kadmium Pada Daging Ikan Bandeng Accumulation Cadmium Of Bandeng Fish Flesh.** *Mediagro.* 2005; **1**(2): 58–74.
Reference Source
20. Irnidayanti Y, Septiana S, Layli FA: **Metallothionein gene expression in green mussels (Perna viridis) as a bioindicator for heavy metal on the North Coast of Java and its impact on the development of reproductive organs.** *In World Congress of Reproductive Biology.* BioScientifica. 2014; **1**: 312.
Publisher Full Text
21. Wulandari E, Herawati EY, Arfiati D: **Kandungan logam berat Pb pada air laut dan tiram *Saccostrea glomerata* sebagai bioindikator kualitas perairan Prigi, Trenggalek, Jawa Timur.** *Jurnal Penelitian Perikanan.* 2012; **1**(1): 10–14.
Reference Source
22. Trinchella F, Esposito MG, Simoniello P, *et al.*: **Cadmium, lead and metallothionein contents in cultivated mussels (*Mytilus galloprovincialis*) from the Gulf of Naples (Southern Italy).** *Aquaculture Res.* 2013; **44**(7): 1076–1084.
Publisher Full Text
23. Ockleford C, Editorial.: **The confocal laser scanning microscope (CLSM).** *J Pathol.* 1995; **176**(1): 1–2.
PubMed Abstract | Publisher Full Text

24. Mongan LC, Gormally J, Hubbard ARD, *et al.*: **Confocal microscopy: theory and applications.** In: Lambert, editor. *Calcium signalling protocols.* Totowa, NJ: Humana Press. 1998; 1–39.
25. APHA: **Standard methods for the examination of water and wastewater.** New York: American Public Health Association. 2005.
26. KEPMENLH: **Ministerial Decree of Living Environment. Number 51 Year 2004 about Sea water quality standard.** 2004.
27. Bilgin M, Uluturhan-Suzer E: **Assessment of trace metal concentrations and human health risk in clam (*Tapes decussatus*) and mussel (*Mytilus galloprovincialis*) from the Homa Lagoon (Eastern Aegean Sea).** *Environ Sci Pollut Res Int.* 2017; **24**(4): 4174–4184.
PubMed Abstract | Publisher Full Text
28. Darmono S: **Logam Dalam Sistem Biologi Makhluk Hidup.** *UI Press.* Jakarta. 1995.
Reference Source
29. Hutagalung: **Heavy metal In Marine Environment.** *Pewarta Oceana.* 1984; **9**(1): 12–19.
30. Soto M, Cajaraville MP, Marig´omez I: **Tissue and cell distribution of copper, zinc and cadmium in the mussel, Mytilus galloprovincialis, determined by autometallography.** *Tissue Cell.* 1996; **28**(5): 557–568.
PubMed Abstract | Publisher Full Text
31. Fattorini D, Notti A, Di Mento R, *et al.*: **Seasonal, spatial and inter-annual variations of trace metals in mussels from the Adriatic sea: a regional gradient for arsenic and implications for monitoring the impact of off-shore activities.** *Chemosphere.* 2008; **72**(10): 1524–1533.
PubMed Abstract | Publisher Full Text
32. Kucuksezgin F, Kacar A, Kucuksezgin G, *et al.*: **Monitoring metal contamination levels and fecal pollution in clam (*Tapes decussatus*) collected from Izmir Bay (Turkey).** *Environ Monit Asses.* 2010; **162**(1–4): 407–415.
PubMed Abstract | Publisher Full Text
33. Usero J, Gonzalez-Regalado E, Gracia I: **Trace metals in the bivalve molluscs *Ruditapes decussatus* and *Ruditapes philippinarum* from the Atlantic coast of southern Spain.** *Environ Int.* 1997; **23**(3): 291–298.
Publisher Full Text
34. Irato P, Santovito G, Cassini A, *et al.*: **Metal accumulation and binding protein induction in *Mytilus galloprovincialis*, *Scapharca inaequivalvis*, and *Tapes philippinarum* from the Lagoon of Venice.** *Arch Environ Contam Toxicol.* 2003; **44**(4): 476–484.
PubMed Abstract | Publisher Full Text
35. Najdek M, Sapunar J: **Total and methyl-mercury content in bivalves, *Mytilus galloprovincialis* Lamarck and *Ostrea edulis* Linnaeus: relationship of biochemical composition and body size.** *Bull Environ Contam Toxicol.* 1987; **39**(1): 56–62.
PubMed Abstract | Publisher Full Text
36. Szefer P, Kim BS, Kim CK, *et al.*: **Distribution and coassociations of trace elements in soft tissue and byssus of *Mytilus galloprovincialis* relative to the surrounding seawater and suspended matter of the southern part of the Korean Peninsula.** *Environ Pollut.* 2004; **129**(2): 209–28.
PubMed Abstract | Publisher Full Text
37. Giarratano E, Amin OA: **Heavy metals monitoring in the southernmost mussel farm of the world (Beagle Channel, Argentina).** *Ecotoxicol Environ Saf.* 2010; **73**(6): 1378–1384.
PubMed Abstract | Publisher Full Text
38. Uluturhan E, Kontas A, Can E: **Sediment concentrations of heavy metals in the Homa Lagoon (Eastern Aegean Sea): assessment of contamination and ecological risks.** *Mar Pollut Bull.* 2011; **62**(9): 1989–1997.
PubMed Abstract | Publisher Full Text
39. Rainbow PS: **Trace metal concentrations in aquatic invertebrates:why and so what?** *Environ Pollut.* 2002; **120**(3): 497–507.
Publisher Full Text
40. Marsden JD, Smith BD, Rainbow PS: **Effects of environmental and physiological variables on the accumulated concentrations of trace metals in the New Zealand cockle *Austrovenus stutchburyi*.** *Sci Total Environ.* 2014; **470–471**: 324–339.
PubMed Abstract | Publisher Full Text
41. Hertika AMS: **Metallothionein Expression on Oysters (Crassostrea cuculata and Crassostrea glomerata) from the southern coastal of East Java.** 2018.
http://www.doi.org/10.17605/OSF.IO/37BVQ
42. Ringwood AH, Hoguet J, Keppler C, *et al.*: **Linkages between cellular biomarker responses and reproductive success in oysters--*Crassostrea virginica*.** *Mar Environ Res.* 2004; **58**(2–5): 151–155.
PubMed Abstract | Publisher Full Text
43. De Lisle RC, Sarras MP Jr, Hidalgo J, *et al.*: **Metallothionein is a component of exocrine pancreas secretion: implications for zinc homeostasis.** *Am J Physiol.* 1996; **271**(4 Pt 1): C1103–C1110.
PubMed Abstract | Publisher Full Text
44. Pearce LL, Wasserloos K, St Croix CM, *et al.*: **Metallothionein, nitric oxide and zinc homeostasis in vascular endothelial cells.** *J Nutr.* 2000; **130**(5S Suppl): 1467S–1470S.
PubMed Abstract | Publisher Full Text
45. Huang PC, Morris S, Dinman J, *et al.*: **Role of metallothionein in detoxification and tolerance to transition metals.** *Experientia Suppl.* 1987; **52**: 439–446.
PubMed Abstract | Publisher Full Text
46. Ecker DJ, Butt TR, Sternberg EJ, *et al.*: **Yeast metallothionein function in metal ion detoxification.** *J Biol Chem.* 1986; **261**(36): 16895–16900.
PubMed Abstract
47. Klaassen CD, Liu J, Choudhuri S: **Metallothionein: an intracellular protein to protect against cadmium toxicity.** *Annu Rev Pharmacol Toxicol.* 1999; **39**: 267–94.
PubMed Abstract | Publisher Full Text
48. Goering PL, Klaassen CD: **Altered subcellular distribution of cadmium following cadmium pretreatment: possible mechanism of tolerance to cadmium-induced lethality.** *Toxicol Appl Pharmacol.* 1983; **70**(2): 195–203.
PubMed Abstract | Publisher Full Text
49. Park JD, Liu Y, Klaassen CD: **Protective effect of metallothionein against the toxicity of cadmium and other metals(1).** *Toxicology.* 2001; **163**(2–3): 93–100.
PubMed Abstract | Publisher Full Text
50. Conrad CC, Walter CA, Richardson A, *et al.*: **Cadmium toxicity and distribution in metallothionein-I and -II deficient transgenic mice.** *J Toxicol Environ Health.* 1997; **52**(6): 527–543.
PubMed Abstract | Publisher Full Text
51. Brandão R, Santos FW, Farina M, *et al.*: **Antioxidants and metallothionein levels in mercury-treated mice.** *Cell Biol Toxicol.* 2006; **22**(6): 429–438.
PubMed Abstract | Publisher Full Text
52. Shariati F, Esaili Sari A, Mashinchian A, *et al.*: **Metallothionein as potential biomarker of cadmium exposure in Persian sturgeon (*Acipenser persicus*).** *Biol Trace Elem Res.* 2011; **143**(1): 281–291.
PubMed Abstract | Publisher Full Text
53. Knapen D, Reynders H, Bervoets L, *et al.*: **Metallothionein gene and protein expression as a biomarker for metal pollution in natural gudgeon populations.** *Aquat Toxicol.* 2007; **82**(3): 163–172.
PubMed Abstract | Publisher Full Text
54. Tapiero H, Tew KD: **Trace elements in human physiology and pathology: zinc and metallothioneins.** *Biomed Pharmacother.* 2003; **57**(9): 399–411.
PubMed Abstract | Publisher Full Text
55. Peroza EA, Schmucki R, Güntert P, *et al.*: **The beta(E)-domain of wheat E(c)-1 metallothionein: a metal-binding domain with a distinctive structure.** *J Mol Biol.* 2009; **387**(1): 207–218.
PubMed Abstract | Publisher Full Text
56. Coyle P, Philcox JC, Carey LC, *et al.*: **Metallothionein: the multipurpose protein.** *Cell Mol Life Sci.* 2002; **59**(4): 627–47.
PubMed Abstract | Publisher Full Text
57. Palmiter RD: **The elusive function of metallothioneins.** *Proc Natl Acad Sci U S A.* 1998; **95**(15): 8428–8430.
PubMed Abstract | Publisher Full Text | Free Full Text
58. Singh RK, Anandhan S, Singh S, *et al.*: **Metallothionein-like gene from *Cicer microphyllum* is regulated by multiple abiotic stresses.** *Protoplasma.* 2011; **248**(4): 839–847.
PubMed Abstract | Publisher Full Text
59. Huang GY, Wang YS: **Expression analysis of type 2 metallothionein gene in mangrove species (*Bruguiera gymnorrhiza*) under heavy metal stress.** *Chemosphere.* 2009; **77**(7): 1026–9.
PubMed Abstract | Publisher Full Text
60. Aliakrinskaia IO: **Some adaptations of *Monodonta turbinata* (Born, 1780) (Gastropoda, Prosobranchia, Trochidae) to feeding and habitation in the littoral zone.** *Izv Akad Nauk Ser Biol.* 2010; **37**(1): 63–68.
Publisher Full Text
61. Amiard JC, Amiard-Triquet C, Barka S, *et al.*: **Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use as biomarkers.** *Aquat Toxicol.* 2006; **76**(2): 160–202.
PubMed Abstract | Publisher Full Text
62. Roesijadi G: **Metallothionein and its role in toxic metal regulation.** *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol.* 1996; **113**(2): 117–123.
Publisher Full Text
63. Hasan MK, Cheng Y, Kanwar MK, *et al.*: **Responses of plant proteins to heavy metal stress-a review.** *Front Plant Sci.* 2017; **8**: 1492.
PubMed Abstract | Publisher Full Text | Free Full Text
64. Li Y, Yang H, Liu N, *et al.*: **Cadmium accumulation and metallothionein biosynthesis in cadmium-treated freshwater mussel *Anodonta woodiana*.** *PLoS One.* 2015; **10**(2): e0117037.
PubMed Abstract | Publisher Full Text | Free Full Text
65. Sakulsak N, Talek K, Sukjai K, *et al.*: **Metallothionein and epidermal growth factor expressions in wild rodent submandibular gland living in cadmium-contaminated area, Mae Sot, Tak by immunohistochemistry staining**. The 32nd AAT Annual Conference, Thailand. 2009; (suppl): 53–55.
66. Kavun VY, Shulkin VM, Khristoforova NK, *et al.*: **Metal accumulation in mussels of the Kuril Islands, north-west Pacific Ocean.** *Mar Environ Res.* 2002; **53**(3): 219–226.
PubMed Abstract | Publisher Full Text
67. Zangger K, Shen G, Oz G, *et al.*: **Oxidative dimerization in metallothionein is a result of intermolecular disulphide bonds between cysteines in the alpha-domain.** *Biochem J.* 2001; **359**(Pt 2): 353–360.
PubMed Abstract | Publisher Full Text | Free Full Text

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Current Peer Review Status: ✔ ✔

Version 2

Reviewer Report 11 May 2020

https://doi.org/10.5256/f1000research.26095.r62936

✔

Rossita Shapawi
Borneo Marine Research Institute, Universiti Malaysia Sabah, Kota Kinabalu, Malaysia

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 29 January 2020

https://doi.org/10.5256/f1000research.19007.r57619

?

Rossita Shapawi
Borneo Marine Research Institute, Universiti Malaysia Sabah, Kota Kinabalu, Malaysia

Title

- I would suggest a title that contain "heavy metal" term in it. Not everyone is familiar with the word metallothionein. e.g. "Determination of heavy metal contents of oysters (*Crassostrea cuculata* and *Crassostrea glomerata*) from the .....using metallothionein expression method"

Abstract

- Background - ...MT level in both types of oyster (replace types with species).

- Conclusions - The sentence needs to be improved - Eg. The level of heavy metals ...has a strong relationship with MT...

Introduction

- This section requires language editing. You also need to mention the purpose of comparing two different species of oysters in this study.

Methods

- How many samples (n) altogether were used in the study? I cant find any information on this. The first line stated that three samples of oysters were used? Were these three groups of samples?

- Several typo/formatting issues here to be fixed.

Results and Discussion

- Similarly, this section also requires language editing.

- You mentioned that your findings in soft tissue exhibit the same pattern with Bilgin et al. - Pb>Cd>Hg??? Figure 2 shows Pb>Hg>Cd ????

- There is no discussion on the background of the sampling areas. Any particular differences among the three areas (Eg. industrial activities) that may influence the results?

- I suggest the "Water quality analysis" to be presented before other results and discussion.

General

- The findings of the present study is considered very significant to the local community. I recommend this manuscript to be accepted for publication with minor corrections.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Aquaculture, Aquaculture feed development, fish nutrition, applied phycology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 24 Apr 2020

Asus Hertika, University of Brawijaya, Malang, Indonesia

1. Inquiry
I would suggest a title that contain "heavy metal" term in it. Not everyone is familiar with the word metallothionein. e.g. "Determination of heavy metal contents of oysters (Crassostrea cuculata and Crassostrea glomerata) from the .....using metallothionein expression method"

Response: Thanks for the reviewer's suggestion. We prefer to use this title to reviewer title suggestion. The word “determination of heavy metal….using metallothionein methods” in reviewer suggestion is not proper. Because we can’t determine heavy metal content using metallothionein. However, our title more proper to describe the article..

2. Inquiry

Background - ...MT level in both types of oyster (replace types with species).

Conclusions - The sentence needs to be improved - Eg. The level of heavy metals ...has strong relationship with MT...

Response: Thank you very much for your concern and suggestion. In the background of abstract, we already replace types with species. Furthermore, in the conclusion of abstract we have changed the sentence.

3. Inquiry
This section requires language editing. You also need to mention the purpose of comparing two different species of oysters in this study.

Response: The reviewer raises an interesting concern. The purpose of comparing two different species of oysters in this study detail already put.

4. Inquiry
How many samples (n) altogether were used in the study? I cant find any information on this. The first line stated that three samples of oysters were used? Were these three groups of samples?

Several typo/formatting issues here to be fixed.

Response: Thank you very much for your valuable suggestion. We have written the detailed total sample we have fixed several issues or typo. There are three groups sample from different location that the oyster was taken. They are sendang biru (group 1), Prigi Beach (Group 2), Popo beach (group 3). In each group have 3 substation sampling. In this study there are 108 oyster in total was used.

5. Inquiry
Results and Discussion
Similarly, this section also requires language editing.

You mentioned that your findings in soft tissue exhibit the same pattern with Bilgin et al. - Pb>Cd>Hg??? Figure 2 shows Pb>Hg>Cd ????

Response: the reviewer raises an interesting concern and valuable suggestion. Thanks for the question. In this case, the result showed that the content of Pb was higher than Cd and Hg, however, It still has same result with bilgin et al that Pb has higher content than Cd and Hg. Further, we have made the new sentence of this already.

6. Inquiry There is no discussion on the background of the sampling areas. Any particular differences among the three areas (Eg. industrial activities) that may influence the results?

Response: The reviewer raises an interesting concern and valuable suggestion. Each sampling area has difference characters. We have put the detailed the differences among the three areas.

7. Inquiry

I suggest the "Water quality analysis" to be presented before other results and discussion.

Response: The reviewer raises an interesting concern. We have put the water quality analysis at the before other results and discussion follow reviewer suggestion.

Competing Interests: No competing interests were disclosed.

Reviewer Report 04 February 2019

https://doi.org/10.5256/f1000research.19007.r43030

Ima Yudha Perwira
Aquatic Resource Management, Faculty of Marine Science and Fisheries, Udayana University, Jimbaran, Indonesia

Review:

This paper explains about the usage of Metallothionein (MT) expression as a specific biomarker for the presence of cadmium (Cd), mercury (Hg), and lead (Pb) in the tissues (gills and stomach) of oysters (*Crassostrea cuculata* and *Crassostrea glomerata*) and the coastal waters of the South coast of East Java. This study has used the appropriate methodologies to measure several parameters. The heavy metals (Pb, Cd, and Hg) are measured using atomic absorption spectrophotometry (AAS). Heavy metals in the water samples are directly measured by using AAS, while that in the gill tissue and stomach are extracted (using nitric acid and incubated at 5-8°C for 30 minutes) before the measurement. The expression of MT is observed by using confocal laser scanning microscopy (CLSM). The authors also mentioned the detailed measurement of water quality for supporting the data of this study. The result of this study showed that the heavy metal content in the water is generally past the specific quality standard (based on the Decree of the State Minister of Environment No. 51 of 2004 concerning seawater quality standards for heavy metal content) (Jelaskan kenapa bisa tinggi). The analysis of MT expression on both gill tissue and stomach showed the highest value in the Prigi station. This study also showed a strong relationship between heavy metals (Pb, Cd, and Hg) with gill tissue and stomach in both *Crassostrea cuculata* and *Crassostrea glomerata.*

Comments:

This study is interesting, but some points need to be considered. It would be better for the authors to mention some previous study about the using of MT expression as biomarker in the marine species. This study is also interesting because it was carried out in the south coastal area of Java Sea. There is industrial activity located in this area. It would be nice if the authors could mention the importance of doing this study in that area. The highest heavy metal content in the gill tissue and stomach of oysters is observed in the Prigi and Sendang Biru stations. The author has compared the heavy metal content in the oyster with Mytilus and Thylacodes. It would be better if there is another comparative result on the oyster.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Water polution and ecotoxicology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.