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Density and Intensity of Metallothionein in Gill and Interior Cavity of Taiwan Mussels (*Anodonta woodiana*) after Exposure to Lead (Pb) at Sub-Chronic Level using Immunohistochemical Technique

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Abstract

Lead (Pb) is a heavy metal which is less useful and even toxic but at low concentration. Organisms respond to heavy metal stress with their distinctive defense system, such as the isolation, the compartmentalization, the complex building and the synthesis of adherent protein such as Metallothionein (MT) and Phytochelatins (PC). The increase of MT synthesis is related to the capacity building to bind the metal and to protect the binding from metal toxicity. The objective of research is to understand the density and intensity of MT expressed in the gill and interior cavity of Taiwan Mussels in various length sizes such 6, 8 and 10 cm after exposure to the lead at sub-chronic level. Research method is an experiment. Preliminary result showed that LC-$50_{96\ hours}$ of $PbNO_3$was obtained on Taiwan Mussels at dose 80.3 ppm. The result was used as the guide for sub-chronic level of exposure at various dosage(0 %, 12.5%, 25 %, 32.5 %, and 50 %) of LC-$50_{96\ hours}$ guidance dose. Research was conducted for 3 months. Sample was taken at 96 hours since the beginning of exposure.The coloring of sample preparation was by immunohistochemical technique, while the measurement of sample's density and intensity was facilitated by Olympus SN 3K19322 Microscope and ImageJ program application. Result of research indicated that density and intensity of MT increase with the increasing of exposure dose. The increase of MT's density and intensity was occurred at exposure of 30 ppm, but it was then decreasing at exposure of 40 ppm. Result of research also showed that the MT's density and intensity averages in interior cavity were higher compared to the gill of Taiwan Mussels.

Keywords: lead, metallothionein,immunohistochemical

1. Introduction

Excessive concentration of some metals in biosphere is a threat for the health. Such metals may be essential metals (Cu, Cr, Zn, Mn, Fe, Ni and Mo) and non-essential metals (Cd, Pb and Hg). These metals cause toxicity at high concentration, and accumulate in the organism body (Murty *et al*, 2011). Lead (Pb) is a toxic heavy metal for certain organism, although at low concentration. Some organisms find this metal less useful (Suhendrayatna, 2001; Yorulmazlar and Gul, 2003). This mental is not essential substance for life creature, but it is toxic for animal and human because it is accumulated in the tissues.

General toxicity mechanism of metal ion is divided into three categories, such as (1) the prevention of the important biology groups such as protein and enzyme, from functioning as usual, (2) the replacement of essential metal ion in the bio-molecule, and (3) the modification of the active structure of bio-molecule which disturbs its specific function (Murthy *et al*, 2011). Organisms respond to heavy metal stress using different defense systems such as the isolation, the compartmentalization, the complex building and the synthesis of adherent protein such as Metallothionein (MT) and Phytochelatins (PC) (Binz and Kagi, 2000., Murthy *et al*, 2011). Metallothionein (MT) is non-enzymatic protein with low molecular weights (Zatta, 2008) such as 10 KDa (Couillard *et al*, 1993), 3-15 KDa (Couillard *et al*, 1995), 6-7 KDa (Fowler *et al*, 1987), and 14.3 KDa (Murthy, 2011). It has high cysteine content but without aromatic amino acid, and it is unstable by heat. Thiol cluster (-SH) in this group represents a cysteine residue which allows MT to bind heavy metals(Zatta, 2008). MT firstly is found in horse kidney cortexbyMargoshes and Valle in 1957(Roesijadi, 1992, Carpene *et.al*,2007). MT is also reported in vertebrate, including fish species (Roesijadi, *et al*, 2009) and in water invertebrate (Roesijadi and Fowler, 1991), such as mollusc (Couillard *et al*, 1993; Isani *et al*, 2000).

The function of Metallothionein (Carpene *et.al*, 2007., Amiard *et a*l, 2006; Couillard *et al*, 1993; Couillard *et al*, 1995) is assumed as involved within the homeostasis process to detoxify the excess of essential and no-essential metals. The long-hour treatment against heavy metal contamination due to the increased MT

concentration is significantly found in bivalvia. It is evident in bivalvia which migrates from clean waters to those contaminated by heavy metals (Couillard *et al*, 1995). Three most influential invertebrate taxa in the ecology cycle of waters are mollusc, crustacean, and annelide. These three taxa are used often as the device to examine waters condition (biomarker) by understanding its MT content. In addition to its usefulness as the biomarker, MT also plays a key role for the adaptation of these taxa to metal exposure, and therefore, it contributes to the large growth of these taxa in the estuary and coast area (Amiard *et al*, 2006). Next, Roesijadi (1994) explains that Metallothionein plays a central role in the intracellular regulation of various metals such as copper, zinc, and cadmium. The improvement of MT synthesis is related to the increase of capacity to bind the metal and to protect the binding from metal toxicity. According to Amiard *et al*, (2006), the increased MT concentration in certain organism will reduce the sensitiveness of organism to heavy metals (becoming less sensitive or becoming resistant). Furthermore, MT concentration in organisms is influenced by metal polluter concentration (Amiard *et al*, 2006; Roesijadi, 1994; Couillard, 1993), organism species and exposed organ.

One species belonged to bivalvia group is Taiwan Mussels. This animal is *filter feeder* because the food is obtained by filtrating water into the body. The volume of water filtrated by Taiwan Mussels is 2.5 liters per adult individual per hour (Mamun and Khan, 2011). The food which enters with water will be directed, squeezed and digested by the assistance of cilia (vibrated hair). Cilia can do 2-20 vibrations per second. According to McIvor (2004), Taiwan Mussels eat some foods such as zooplankton, phytoplankton, bacteria, flagellate, protozoa, diatom, detritus, algae and various suspended matters in the waters. Mussels cannot swim like fish. Therefore, mussels' food is greatly relying on the condition of waters where they live.

According to Regoli *et al.* (2002), many reviews of expression or synthesis of metallothionein in organism have been made, and further development of metal toxicity test is also reviewed from metallothionein measurement. Review of MT expression has found that fish and mussels have specific ability to accumulate higher level of heavy metals than other organism in waters. Hanson (2009), Metallothionein expression, mainly associated with exposure to certain metals in the environment of the organism, this is because according to Binz and Kagi (2000), metallothionein is the only biological compounds that interact with metals in the body of organism. Interactions between proteins with metal ions will form metal-thiolate clusters. Amiard *et al*, (2006) the observation of MT is mostly conducted in the gill and interior cavity organs. Bebiano *et al*. (1993) have explained that the expression of MT has been detected in gill, digestive channel, and other important tissues of mollusk *Ruditapes decussatus* when this species is exposed to Cd and Cu.

The current research observes MT expression in the gill and interior cavity organs of Taiwan Mussels by using immunohistochemical technique. Indeed, this technique is identifying specific protein in the tissue or cell using antibody. The binding between antibody and specific protein is identified by the marker which usually adheres to antibody and visualized directly or with reaction to identify the marker. The marker may be colored compound, fluorescence substance, heavy metal, radioactive label, or enzyme (Larasati, 2010). Immunohistochemical is often used in the fundamental research to acknowledge the distribution and location of biomarker or protein which is expressed in various body tissues (Ramos-vara, 2005). The objective of research is to understand the density and intensity of MT expressed in the gill and interior cavity of Taiwan Mussels (*Anodonta woodiana*) which is exposed to PbNO3 at sub-chronic level.

2. Research Method

Research method used was experiment. This research was the continuation of previous research which involves toxicity test which obtained LC-$50_{96\ hours}$ of $PbNO_3$ on Taiwan Mussels at dose 80.03 ppm. Indeed, LC-$50_{96\ hours}$was used as the guide for sub-chronic level of exposure at various doses such as 0 %, 12.5%, 25 %, 32.5 %, and 50 % of LC-$50_{96\ hours}$ guidance dose 80.03 ppm (LPPT-UGM, 2012). Research lasted for 3 months and emphasized on various sizes (S) of Taiwan Mussels such as 6, 8 and 10 cm. Sampling was begun 96 hours from the beginning of exposure. Sample preparation is colored by immunohistochemical technique, while sample's density and intensity were measured by Olympus SN 3K19322 Microscope and ImageJ program application. Research stages included as followed.

2.1 Research Preparation

The sample was taken from column no. 23 in Implementation Unit of Freshwater culture, Punten, Batu, East Java. Sample was cleanly washed and put into acclimatization batch. This batch was controlled for its temperature, pH and dissolved oxygen, and fed by freshwater phytoplankton and fish pellet (pre starter crumble product by central proteinaprima). This stage took place for 1 month.

2.2 Treatment Stage

The stage was subjecting Taiwan Mussels (*Anodonta woodiana*) to treatment. It included preparing the batch and aerator and installing aerator hose. The treatment batch has 20 litre capacity for 5 dosage (D). The solution of PbNO3 at sub-chronic level of exposure was 0 %, 12.5%, 25 %, 32.5 %, and 50 % of the LC-$50_{96\ hours}$ guidance dose 80.3 ppm. The percentage-based determination of solution produces some treatment doses (D) such as 0 ppm, 10 ppm, 20 ppm, 30 ppm and 40 ppm.

2.3 Organ Collection

Gill and interior cavity organs were collected after 96 hours exposure (Geffard *et al*, 2001). The sample was cut by scissor or sectioning set. Gill and interior cavity samples were entered into 10 % formalin solution. Gill and interior cavity organs were shown in Figure 1.

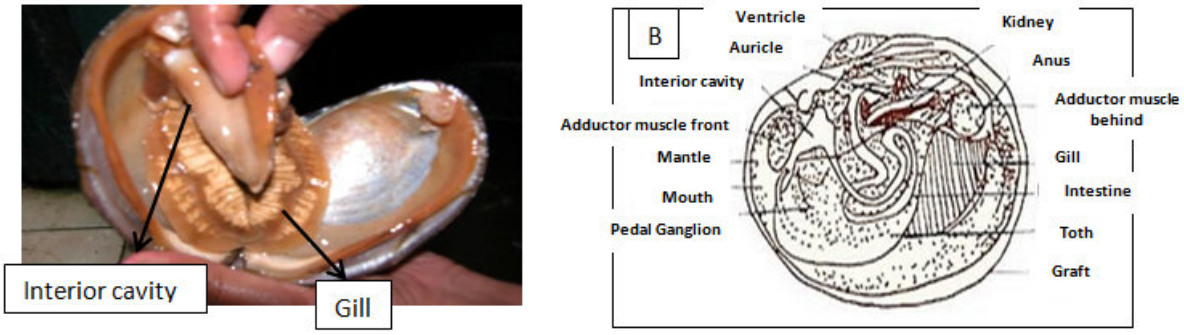

Figure 1. (A) The position of gill and interior cavity organs and (B) The organ structure in Taiwan Mussels.

2.4Histopathology Preparation

The preparations were made in Anatomic Pathology Laboratory of Medical Faculty, University of Brawijaya, Malang. According to Muntiha (2001), histopathology procedures involve the following activities such as: (A) Fixating organs with 10 % formalin overnight, followed by cutting macro tissues to collect the fixated organs. The tissues are cut in 0.3-0.5 mm thickness by arranging tissues in *tissue cassette* based on code and putting *tissue cassette* into specific basket; (B) Implementing dehydration process such as filling the basket with *tissue tex processor* for automatic process. Tissues are subjected to gradual dehydration process in several time cycles such as alcohol 70 % (2 hours), alcohol 80 % (2 hours), alcohol 90 % (2 hours), absolute alcohol (2 hours), and xylol (2 hours); (C) Operating vacuum process, that is by putting the basket in the vacuum machine to eliminate the air from the tissues at temperature 59-60^{0}C for 30 minutes, taking the basket and removing *tissue cassette*; (D) Blocking, which involves: warming the liquid paraffin, pincer, and mold; pouring liquid paraffin to the mold; and entering the tissues from *Tissue Tex Processor* into liquid paraffin contained mold. Tissues are pressed to adhere it toward mold base. The mold is sealed, pressed and labeled in the edge. The mold is stored in the freezer. Tissues are taken from mold after frozen. Block sides are trimmed. Permanent label is given to tissues which are then restored in the freezer; (E) Cutting with microtome, which involves pinching block with microtome and cutting paraffin block with microtome cutter at ± 30^{0}C and ±2-5 microns thickness. The cutting results (the connected thin ribbon/slice) are put into water bath which contains water warmed at 50^{0}C. Object glass box are assembled in the water bath (not installed in reverse). The box is stored in incubator at 60^{0}C.

2.5 Immunohistochemical Procedure

Gill and interior cavity slice preparations are subjected to immunohistochemical coloring procedure. The coloring was carried out at Physiology Laboratory of Physiological Science, Medical Faculty, University of Brawijaya. This procedure is amodification from Larasati (2010), Antibody used were Policlonal antibody anti – MT and secunder anti IGG Rat-MT . The procedure is as follows: (a) de-paraffinating the preparations (paraffin block) with xylol for 3 times for 3 minutes, (b) rehydrating the preparations with ethanol of 100%, 95 % and 70 % each for 2 minutes, 2 minutes and 1 minute, (c) dripping each sample with PBS for 3 times for 5 minutes because PBS stabilizes pH of tissues, (d) dripping with 3CDTA and allowing it for 10 minutes to fixate the tissues, (e) dripping with Na-citrate and allowing it for 10 minutes, (f) rinsing with PBS for 3 times every 5 minutes, (g) dripping with 0.3 % H_2O_2 and allowing it for 30 minutes, (h) rinsing with PBS for 3 times every 5 minutes, (i) incubating 1 % serum in PBS and allowing it for 30 minutes, (j) dripping with Polyclonal antibody Metallothionein and incubating it in the refrigerator overnight, (k) rinsing with PBS for 3 times every 5 minutes, (l) dripping 2nd AB into PBS by comparison 1:200 and allowing it for 60 minutes, (m) rinsing with PBS for 3 times every 5 minutes, (n) dripping with *detection antibody* of MT and allowing it for 60 minutes, (o) rinsing with PBS for 2 times every 5 minutes, (p) washing with aquades and allowing it for 5 minutes, (q) dripping with DAB (*Male Fresh*) by comparison 1:46 until the brown color is obtained, (r) rinsing with D_2H_2O, (s) dripping the sample with haematoxylin for 10 minutes, (t) rinsing with D_2H_2O, and (u) rinsing with tapping water.

2.6 The Observation of Tissues and the Calculation of Density and Intensity of MT

Tissues were observed using Olympus SN 3K19322 Dot Slide Microscope while the density and intensity of MT were calculated using ImageJ program application. Density is the number of individual per width unit, or the volume or mass per volume unit. The measuring unit was usually gram/cm^3 or the number of cell/ml. The intensity of MT at Taiwan Mussels was understood by ImageJ program. Intensity rate was taken from 10 block

points with the densest brown which showed the highest intensity of MT. The intensity rate of the area was average (mean) obtained from the analysis with ImageJ Program (Schmid *et al*, 1993). The measuring unit of intensity was pixel (Melissa *et a*l, 2006).

2.7 Data analysis

The influence of interaction between exposure dose (D) and size (S) on the density and intensity of MT was acknowledged by analyzing data with multi-variance analysis supported by SPSS 17.

3. Result And Discussion

Immunohistochemical is a method to detect protein in the tissue cells using the principle of the binding between antibody and antigen in the living tissues. Immunohistochemical dyeing (coloring) is often used in examining abnormal cell in the case of cancer cells. Specific molecules will dye certain cells, such as the dividing cell or the dead cell to distinguish them from normal cells (Larasati, 2010). Immunohistochemical technique is used to understand the distribution and location of biomarker or the expression of certain protein observed in various tissues of living body (Ramos-Vara, 2005).

Two main components are involved in this dyeing process. One component is primary antibody, or the detection system to identify the result of antigen-antibody reaction. The coloring system (chromogen) has a bridging/connecting component called secondary antibody.Two steps of immunohistochemical are engaged, which are sampling and labeling. Sample preparation is aimed to have tissue preparation from fresh tissue. Sample is prepared by collecting fresh tissues, fixating the tissues using formaldehyde, embedding the tissues into paraffin and freezing it with liquid nitrogen, cutting the tissues using microtome, deparaffinating the retrieval antigen to free the tissue epitope, and blocking the tissues from less specific protein. Sample labeling is using certain materials to color the preparation. Sample labeling involves immuno-detection using primary and secondary antibodies, substrate provisioning, and counter-staining to color other tissues around antibodies (Larasati, 2010). Examples of coloring result with immunohistochemical method over Taiwan Mussels is shown in Figure 2.

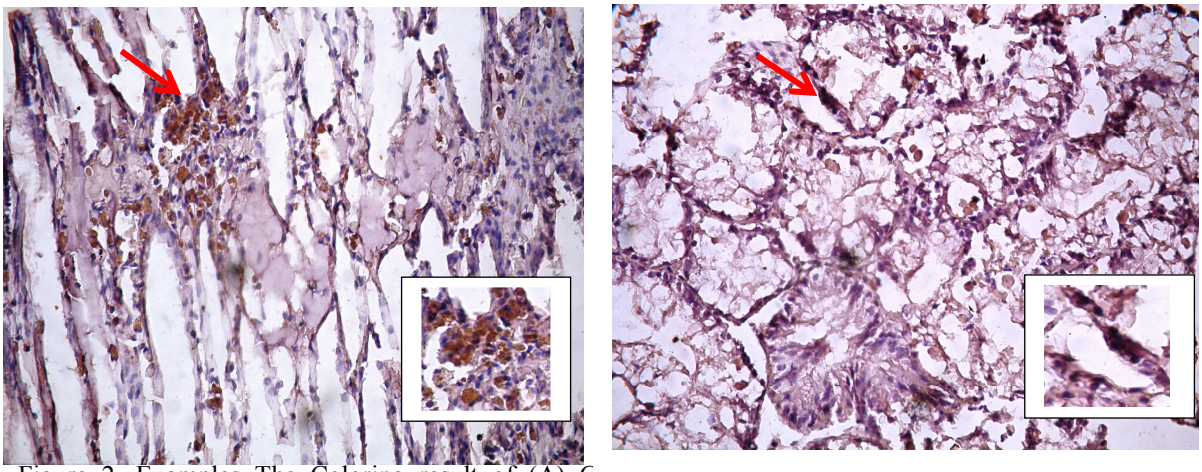

Figure 2. Examples The Coloring result of (A) C
(*Anodonta woodiana*) at size 8 cm with 30 ppm $PbNO_3$ exposure using immunohistochemical method. Brown block (designated arrows)is expressed MT. The observation is by Olympus SN 3K19322, at scale 20 µm.

The establishment of brown block, according to Ramos and Vara (2005), is apparent because it uses peroxidase method principle in which primary antibody will bind the antigen in the tissue. After the bonding complex is made, secondary antibody follows (anti-primary antibody), and this antibody is called as peroxidase enzyme. Brown deposition can be shown up by adding chromogen and H2O2 substrates. Brown deposition is appearing because of substrate decomposition (chromogen and H2O) by peroxidase enzyme. The expression of brown block signifies the positive presence of certain antigen, while the absence of brown block means the presence of negative result with the absence of certain antigen.According to Lehr *et al* (1999), immunohistochemical is useful to learn the distribution of specific enzymes in the intact cell structure (normal/complete), to detect bio-macromolecullar components such as protein and carbohydrate. Schimid *et al* (1993) assert that this method is also useful to see the expression of MT in kidney organ of mice. Result of current research so far indicates that MT in the gill of Taiwan Mussels is greatly expressed in the gill capillary vessel. In the interior cavity, MT is mostly expressed in the soft part of tissues around the interior cavity, and also expressed in part around digestive divercula. Supported by Galtsoff (1964), the digestion and absorption of the food in mollusc is the intracellular process occurring within *digestive divercula*. Moreover, Bebiano and Serafirm (1998) add that the increase of MT is found in gill, digestion gland and other soft tissues. This increase

is so evident during the exposure to heavy metal of CD. Therefore, Galtsoff (1964) underscores that bivalvia's gill represents an organ to sort the food or to be one of food digestion processes.

3.1Metallothionein Density

Density is a unit of how close is a matter with others and measured with the number of matter (mass) per volume unit. The measuring unit, therefore, is mass per volume unit or usually stated in gram/cm^3 or the number of cell/ml. Metallothionein density is a biophysic quantity which has direct relation with the determination of MT number per coverage width.

Result of research shows that MT density in Taiwan Mussels gill is ranging from 7.17 x 10^{-4} MT/µm^2 (treatment at 40 ppm) to 13.33 x 10^{-4} MT/µm^2 (treatment at 30 ppm). MT density in interior cavity ranges between 9.84 x 10^{-4} MT/µm^2 (treatment at 0 ppm) and 15.38 x 10^{-4} MT/µm^2 (treatment at 30 ppm). The graphic of MT density is shown in Figure 3.

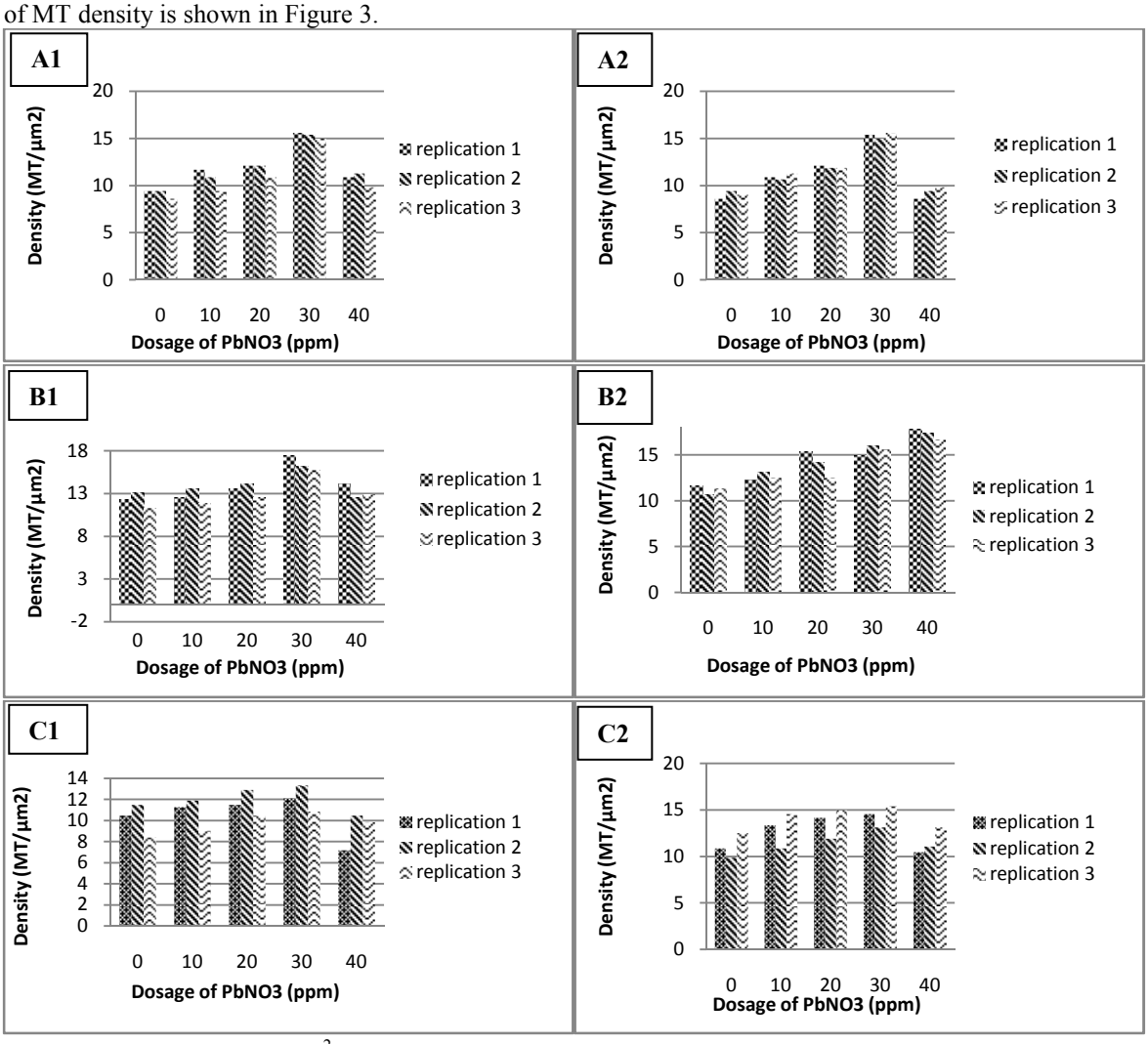

Figure 3. MT density (MT/µm^2) in the (A1) Gill and (A2) Interior Cavity of the 6 cm sized Taiwan Mussels; (B1) Gill and (B2) Interior Cavity of the 8 cm sized Taiwan Mussels; and (C1) Gill and (C2) Interior Cavity of the 10 cm sized Taiwan Mussels.

Figure 3 shows that the rate of MT density in the gill and interior cavity of Taiwan Mussels is always increasing in average with the addition of exposure dose. This increase, however, is decreasing at exposure dose 40 ppm PbNO3. The increase of the density of expressed MT means a presence of reaction toward the increase of MT rate in the gill and interior cavity tissues due to lead exposure. According to Roesijadi (1994), MT also increases with the increased exposure of Cadmium (CD). Couillard *et al* (1993) have researched fresh water mussels and found a strong correlation between MT and CD in the gill, soft tissues, and other certain organs. The highest MT rate is found in the gill than hepatopancreas and other organs. The later research is conducted by Nikpour *et al* (2008), which *Scatophargus argus* is exposed to mercury in several doses (10, 20 and 30 µg/L) and at different timings (24, 48 and 72 hours), and they find an increased MT in the gill from dose 10 to 30 µg/L. Research by Kriskova *et al* (2007) indicates a strong relationship between lead content and MT rate in muscle, gonad, and spleen of fish. The highest MT rate is observed in spleen and liver tissues ranging from 100 to 350 ng

(nano gram) MT per gram of fresh weight. Research by Bebiano and Serafirm (1998) about the exposure to cadmium at sub-lethal concentration, 100 µg/lt, shows that the increased MT in the gill of *Mytilus galloprofincialis* is increasing four times compared to control and also to *Ruditape decussatus* mollusc which the concentration of Metallothionein is increasing only twice. These results so far underline the increasing response of MT rate to the different tissues of any individuals.

However, MT intensity decreases at exposure dose 40 ppm. It is because the response of Taiwan Mussels decreases due to too high exposure of dose. The reduction of MT density, said Roy *et al* (2011), is evident because the absorption rate of heavy metal is exceeding the synthesis rate of MT, thus causing pathology effect over the exposed organisms.Yong *et al.* (1992) Heavy metals in organism body may overwhelm the threshold of the adaptive ability of this organism. The intake of heavy metals may be through water into organism body, or through the consumer food channel. According to Gagnon et al (2006) admit that MT concentration has decreased in freshwater mussel exposed to urban waste. However, there is bioaccumulation for other metal in the tissues possibly because the waste also brings other metals or the waste input has exceeded the threshold of freshwater mussel in tolerating the waste.

3.2 Metallothionein Intensity

Immunohistochemical method is also used to detect or to measure the enzyme content that is measured by examining the resultant color intensity. The resultant intensity due to this reaction is divided into three classes, which are strong positive shown by range from dark brown to blackish brown (+ + +), moderate positive shown by range from dark brown to light brown (+ +), and weak positive shown by blush brown (+) (Irvan, 2007). MT intensity in the gill of Taiwan Mussels remains, in average, in the range between 26,717 pixels (treatment at 0 ppm) and 47,494 pixels (treatment at 30 ppm), while that in the interior cavity ranges from 22,755 pixels (treatment at 40 ppm) to 51,999 pixels (treatment at 10 ppm). The graphic of MT intensity (pixel) is displayed in Figure 4.

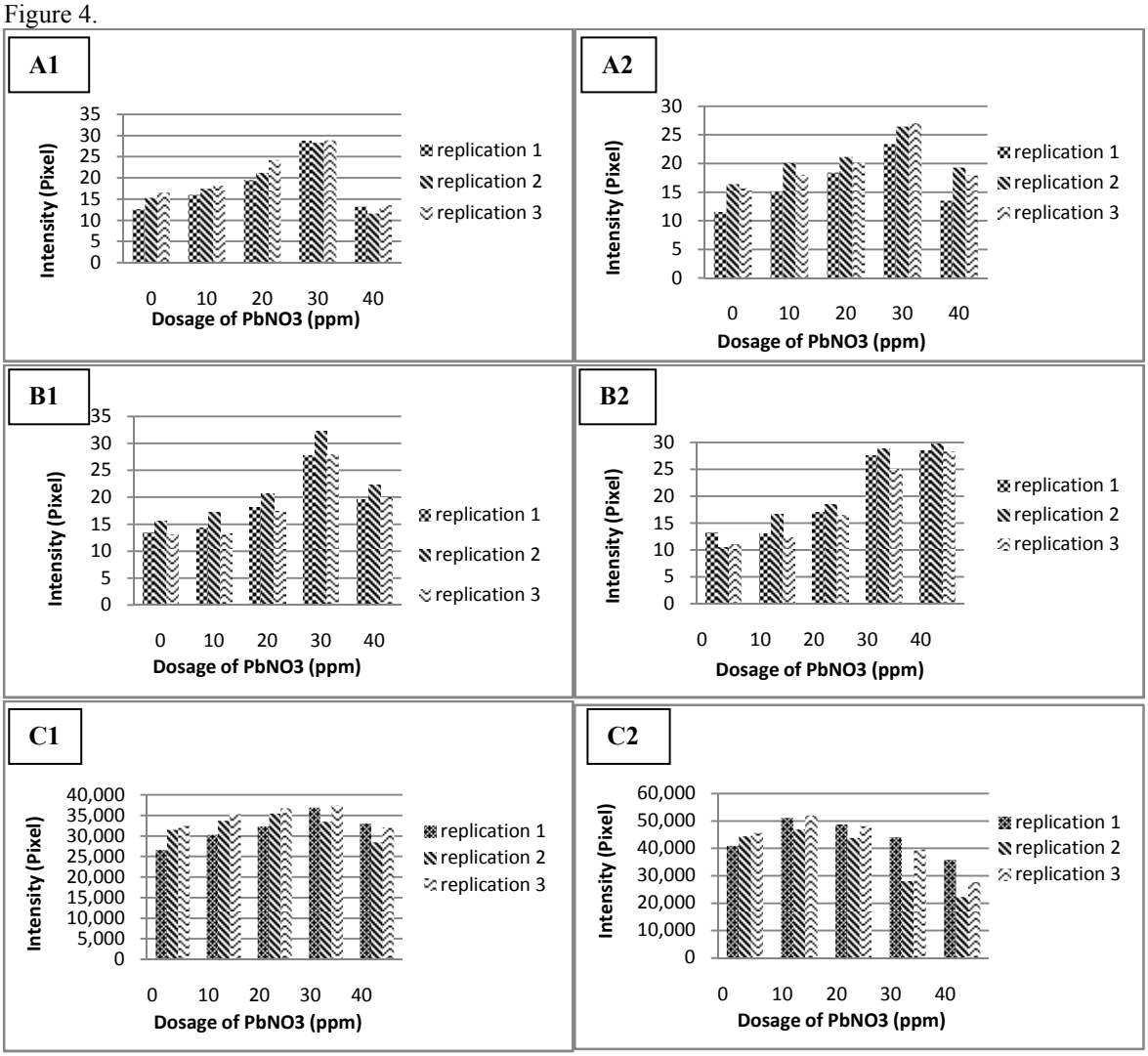

Figure 4. MT intensity (pixel) in the (A1) Gill and (A2) Interior Cavity of the 6 cm sized Taiwan Mussels; (B1) Gill and (B2) Interior Cavity of the 8 cm sized Taiwan Mussels; and (C1) Gill and (C2) Interior Cavity of the 10

cm sized Taiwan Mussels.

Figure 4 shows MT intensity rate in the gill and interior cavity of Taiwan Mussels, which are, in average, increasing with the addition of exposure dose. The increase rate is decreasing, however, at exposure dose 40 ppm PbNO3. The increased intensity of the expressed MT means a presence of reaction toward the increase of MT rate in the gill and interior cavity tissues due to lead exposure. The reduction of MT intensity at exposure 40 ppm is obvious because the response of Taiwan Mussels decreases due to too high exposure of dose. According to Schmid *et al* (1993), MT in mice kidney contaminated with a heavy metal, the lead, is increasing based on its color after 6 weeks exposure. However, the intensity is decreasing at Week-16 or smaller than MT intensity in Week-6.

Result of research indicates that the density and intensity of MT expression is higher in interior cavity than gill. Cullaj (2007) with a research on mollusc has explained that the highest MT is found in digestion gland. The different MT rates are observed because heavy metals which induce where the mollusc sample locates are different. The increase of heavy metal rates will increase MT rate in tissues. Moreover, research by Amiard *et al* (2006) on some mollusk species, *Mytilus edulis* and *Mytilus galloprovincialis*, indicates that Metallothionein content in interior cavity is higher than gill. Research by Roesijadi, *et al* (2009) about the induction of CD and the response of MT content has found that the highest MT rate is found in fish intestine than in the gill and liver which are not significantly different. Gagne *et al.* (2003) examines gonad, gill and interior cavity of freshwater mussel sampled from upstream and downstream parts of St. Lawrence River which has been contaminated with Zn waste. It is found that in orderly manner, the highest MT is observed within gonad, then digestive channel and the lowest in the gill

3.3 The Analysis of Interaction of Different Dosage (d) and Size (s) Factors against Metallothionein Density

The interaction of the influence of different PbNO3 exposure dose and mussel size against the density of Metallothionein expression through immunohistochemical coloring procedure is shown in Figure 5.

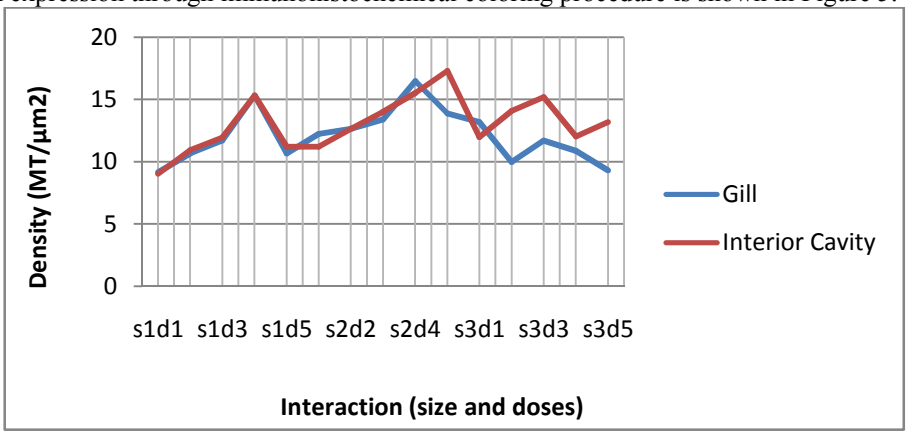

Figure 5. The Interaction of Size (s) and Dose (d) Factors against MT Density in the Gill and Interior Cavity of Taiwan Mussels

The graphic of the interaction of size and dose factors against MT density of the gill and interior cavity is exhibited in Figure 5. The highest density is expressed in the interior cavity of Taiwan Mussels at interaction "s2d5" between 8 cm sized Taiwan Mussels and PbNO3 exposure dose 40 ppm. The lowest density is found also in interior cavity at interaction "s1d1" between 6 cm sized Taiwan Mussels and dose 0 ppm. Data are analyzed by multi-variance analysis type which is ANOVA and followed by smallest obvious differential test. Result of ANOVA, supported by statistic program SPSS 17, is shown in Table 1.

Table 1.Result of ANOVA Test against The Interaction of Size (s) and Dose (d) Factors on MT Density in the Gill and Interior Cavity of Taiwan Mussels

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Size	density_gill	63.044	2	31.522	10.469	.000
	density_interior cavity	46.369	2	23.185	10.046	.000
Dose	density_gill	58.630	4	14.658	4.868	.004
	density_interior cavity	74.833	4	18.708	8.106	.000
Size * Dose	density_gill	66.697	8	8.337	2.769	.020
	density_interior cavity	79.140	8	9.892	4.286	.002
Error	density_gill	90.331	30	3.011		
	density_interior cavity	69.236	30	2.308		
Total	density_gill	6840.076	45			
	density_interior cavity	7918.059	45			

a. R-squared = .676 (Adjusted R-Squared = .525)
b. R-squared = .743 (Adjusted R-Squared = .623)
Data are analyzed in statistic program of SPSS 17. F-table value is $F_{(0.05, 14, 30)} = 2.307$

Table 1 has shown that each F-count is higher than F-table. The significant value of each factor is smaller than obvious degree at α of 5 %. The resultant decision is a significant influence of each factor on MT density in the gill and interior cavity. Smallest obvious differential test is carried out to indicate the factor which gives different influence.Result of smallest obvious differential test shows that the highest average of MT density in the gill of Taiwan Mussels is found in the interaction between 8 cm sized Taiwan Mussels and dose 30 ppm, by the rate of 16.4733MT/μm^2. The lowest average of MT density is obtained in the interaction between 6 cm sized Taiwan Mussels and dose 0 ppm, by the rate of 9.1567MT/μm^2. Furthermore, for MT density in the interior cavity of Taiwan Mussels, the result of smallest obvious differential test over the interaction between dose and size factors indicates that the highest average of MT density in interior cavity is found in the interaction between 8 cm sized Taiwan Mussels and dose 40 ppm by the rate of 17.2933MT/μm^2, while the lowest average is estimated in the interaction between 6 cm sized Taiwan Mussels and dose 0 ppm by the rate of 9.0200MT/μm^2.

3.4 The Analysis of Interaction of Different Dose (d) and Size (s) Factors against Metallothionein Intensity

The interaction of the influence of different PbNO3 exposure dose and mussel size against the intensity of Metallothionein expression through immunohistochemical coloring procedure is shown in Figure 6.

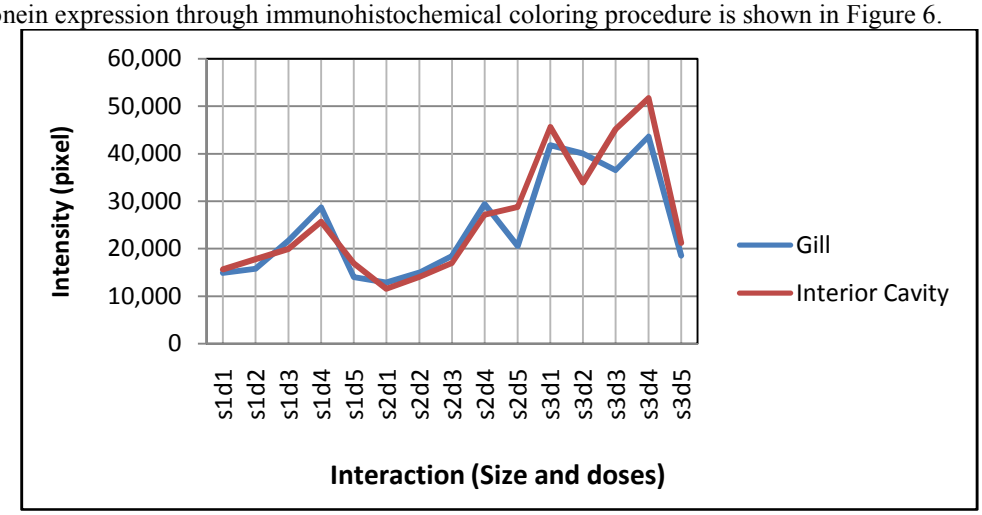

Figure 6. The Interaction of Size (s) and Dose (d) Factors against MT Intensity in the Gill and Interior Cavity of Taiwan Mussels

The graphic of the interaction of size and dose factors against MT intensity of the gill and interior cavity is exhibited in Figure 6. The highest intensity is expressed in interior cavity of Taiwan Mussels at interaction "s3d4" between 10 cm sized Taiwan Mussels and dose 30 ppm. The lowest intensity is found in interior cavity at interaction "s2d1" between 8 cm sized Taiwan Mussels and dose 0 ppm. Data are analyzed by multi-variance analysis type, ANOVA and supported by statistic program SPSS 17, as shown in Table 2.

Table 2.Result of ANOVA Test against The Interaction of Size (s) and Dose (d) Factors on MT Intensity in the Gill and Interior Cavity of Taiwan Mussels

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Size	intensity_gill	2.882E10	2	1.441E10	188.194	.000
	intensity_interior cavity	4.032E10	2	2.016E10	199.937	.000
Dose	intensity_gill	1.081E10	4	2.702E9	35.288	.000
	intensity_interior cavity	2.712E9	4	6.779E8	6.723	.000
Size * Dose	intensity_gill	1.102E10	8	1.378E9	17.996	.000
	intensity_interior cavity	2.400E10	8	3.000E9	29.755	.000
Error	intensity_gill	3.331E10	435	7.658E7		
	intensity_interior cavity	4.386E10	435	1.008E8		
Total	intensity_gill	3.602E10	450			
	intensity_interior cavity	4.183E10	450			

a. R-squared = .603 (Adjusted R-Squared = .591)
b. R-squared = .604 (Adjusted R-Squared = .592)
Data are analyzed in statistic program of SPSS 17. F-table value is $F_{(0.05, 14, 30)} = 1.715$

Table 2 shows that each F-count is higher than F-table. The significant value of each factor (0.000) is smaller than obvious degree at α of 5 %. The resultant decision is a significant influence of each factor on MT intensity in the gill and interior cavity. Smallest obvious differential test is conducted to determine the factor which gives different influence.

Result of smallest obvious differential test shows that the highest average of MT intensity in the gill of Taiwan Mussels is found in the interaction between 10 cm sized Taiwan Mussels and PbNO3 exposure dose 30 ppm, by the rate of 43890.6333 pixel. The lowest average of MT intensity is obtained in the interaction between 8 cm sized Taiwan Mussels and dose 0 ppm, by the rate of 12864.0333 pixel.

4. Conclusion

Result of research indicates that MT is expressed with the emergence of brown block at gill and interior cavity tissues of Taiwan mussels. Brightness or darkness of brown block shows the viscosity rate of MT. This rate is useful to measure density and intensity of brown block. Research concludes that MT density and intensity of Taiwan Mussels expressed in interior cavity are higher than those in the gill. The average of MT density and intensity is increasing with the increased exposure dose. The highest increase is found at PbNO3 exposure dose 30 ppm, but it decreases at dose 40 ppm. Result of interaction test between dose factor of PbNO3 exposure and MT density in interior cavity Taiwan Mussels shows a significant influence. The interaction between size and dose on MT intensity in Taiwan Mussels also produces significant influence in the gill and interior cavity.

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