Methallothionein expression on the gills and stomach of Chinese pond mussels exposed to lead (Pb)

H Kartikaningsih, A M Suryanto and D Arfiati

Faculty of Fisheries and Marine Science, Brawijaya University, Malang.
E-mail: hartatikartan@ub.ac.id

Abstract. In freshwaters area, Pb originates from rocks (naturally), industries, and pesticides. The ability of Chinese pond mussels as biofilters to absorb heavy metal (Pb) was demonstrated in water circulation system using ten 8 cm mussels. PbNO₃ (0, 10, 20, and 30 ppm) was administered into water containing mussels. Carp culture was done for 30 days, and Pb accumulation in carps was measured every week (week 0, 1, 2, and 3). The results showed that the highest Pb ion accumulation was found in the gills of mussels. The examination using hematoxylin-eosin showed that tissues were damage due to haemorrhage, cell ruptures, and cell deaths. The results of the measurement of metallothionein (MT) showed that MT molecular weight was 12.84 kDa.

1. Introduction
Biofilter is usually used in closed cultivation system (recirculation). The recirculation system is used mainly to reduce water usage. In addition to aquatic plants, biofilters common to aquatic environment are organisms that live at the bottom of waters, e.g. gastropods and bivalves. Mussels have the potential to be biofilters because they are filter feeder [1]. An adult’s capacity of food filtering is very high up to 2.5 litres/hour [2]. Mussels are also resistant to high levels of pollution and able to filter out water rapidly, giving them the potential to be biofilters [3].

Organisms adapt to the heavy metal pollution in the environment through a defence system, including exclusion, compartmentalization, and complex formation and synthesis of binding proteins such as metallothionein (MT) and phytochelatins (PC) [4]. Metallothionein is a protein possessed by all organisms to bind both essential and non-essential metals [5]. Plants, however, also have phytochelatins in addition to metallothionein.

The expression of metal-binding proteins (MT) in each individual in response to the presence of heavy metal pollution varies. MT is a non-enzymatic protein with a low molecular weight (10 kDa [6], 3-15 kDa [5], 6-7 kDa [7], 14.3 kDa [4]), which has high cysteine content and no aromatic amino acids, and is unstable by heat. The thiol group of MT (-SH) is a cysteine residue which allows MT to bind heavy metals. MT was reported to be present in vertebrates, including many species of fish [8], and aquatic invertebrates, particularly molluscs [4, 6]. MT is a protein that has sulphur content and binds metal ions. The sulfur content reaches 20 % of the total molecular weight of MT. In mammals, MT is characterized by a molecular weight of 6-7 kDa, 60-68 amino acid residue content, and binds total 7 equiv. bivalent metal ions [9].

Chinese pond mussels (Anodonta woodiana) live on the bottom of a pond or lake. Chinese pond mussels can “devour” pollutants, including heavy metal Pb that is suspended in the water. Because they are more resistant to pollutants than fish, Chinese pond mussels are used as ‘the cleaner’ of the environment [3]. As a filter feeder, an Anodonta woodiana gets food by filtering the water entering its body. The volume of water that can be filtered by an adult Chinese pond mussels is 2.5 litres per hour. The food that comes with the water is removed, squeezed, and digested with the help of cilia that
move 2—20 times per second. Foods that enter can be zooplankton, phytoplankton, bacteria, flagellata, protozoa, detritus, algae or various other substances suspended in the water where the mussels live [10].

The purpose of this study was to determine the ability of Chinese pond mussels as biofilters based on Pb concentration in the stomachs and gills of Chinese pond mussels, the metallothionein expression as well as its molecular weight.

2. Methodology

Chinese pond mussels (Anodonta woodiana) (8 cm in length) and carps (Cyprinus carpio) fingerlings (5 cm) were obtained from the Fish Seed Bureau, Punten, Batu, East Java. Chinese pond mussels were placed in a 20-litre tub. Mussels and carps were exposed to Pb(NO3)2 for 96 hours [11] at doses of 0 %, 12.5 %, 25 %, and 37.5 %. The PbNO3 doses used in the chronic exposure of this study were 0, 10, 20, and 30 ppm. Plastic tubs measuring 40 cm in height and 100 cm in diameter were filled with ten 8 cm Chinese pond mussels. The water in this tub was channelled into another fibre-sized tub that was filled with ten 5 cm carps (Cyprinus carpio). The water in the tub containing the fish was channelled back into the tub containing gravel, resulting in closed system circulation. During rearing, the fish were fed with Hi-Pro-Fit 781-1 (CP Prima). On the other hand, the Chinese pond mussels were fed with phytoplankton and pellet (Feng Li Code FL0 flour, PT Matalhari Sakti). The rearing tubs were aerated to ensure sufficient oxygen. On day 8th, the Chinese pond mussels were inserted into a recirculation-model tank and were left for a day without being fed (acclimatization process).

The Pb levels in the gills and stomachs of Chinese pond mussels were observed 96 hours after the exposure, and the Pb levels in carps were observed in weeks 0, 1, 2 and 3. The accumulation of Pb ions in the gills and stomach was observed using SEM/EDX. In this stage, qualitative measurement was performed with SDS PAGE and western blotting to determine the molecular weight of expressed MT in kilodalton (kDa). Observations on gill tissue damage and gastric gravity were also carried out with hematoxylin-eosin staining.

The expression of MT in the gills and stomachs of Chinese pond mussels was observed with the Olympus SN 3K19322 microscope. The quantitative measurement of MT was performed according to Ana and Garcia-Vasquez [12] with the standard curve of glutathione (GSH), assuming that 1 mole of MT contains 20 moles of cysteine.

MT molecular weight measurements were performed with SDS-PAGE according to Murthy et al. [4] method with standard BM from Thermo Scientific. The microanatomy condition of Taiwanese gills exposed to Pb was identified using SEM method, and a test with Energy Dispersive X-ray (SEM/EDX) was conducted to determine the levels of Pb accumulation in the target tissues.

3. Result and discussion

One day after the exposure, there was an increase in the mucus excretion around the Chinese pond mussels. High levels of PbNO3 in the water resulted in the inhibition of the activity of enzyme and cell membrane due to the bonding of toxic materials with enzymes and cell membranes. This metabolism may cause stress which eventually resulted to caused excessive mucus excretion. This is a self-defence instinct of aquatic organisms to adapt to poor environmental quality. According to Palar [13], the effect of lead toxicity (Pb) primarily depends on the length of contact time, which caused intoxication of aquatic organisms as their response to heavy metals by increasing the mucus fraction of the gills, causing the respiratory and metabolic processes to be disrupted. Binelli et al. [14] described that heavy metals enter the body along with water or food. At first, the heavy metals will accumulate in the gills, then in the digestive tract, and eventually in the kidneys. Subsequently, the Chinese pond mussels will die if the heavy metals entering has exceeded the tolerable threshold. Pb is known to cause oxidative effects of the enzyme d-aminolevulinic acid dehydratase in molluscs. It is especially disruptive in the synthesis pathway.

The gills and stomachs of the Chinese pond mussels were collected 4 days (96 hours) after the beginning of the exposure. This was done by referring to the study conducted by Geffard et al. [11], indicating that the induction of metallothionein in Mytilus galloprovincialis larvae exposed to metal gives a noticeable effect at 96 hours after exposure. By that time, the metal will experience dilution and strong correlation with the increase in metal on the cytosolic fraction.
3.1. Molecular Weight (MW) MT

Metallothionein (MT) is a non-enzymatic protein with a molecular weight of 6—7 kDa [9], 10 kDa [6], 3—15 kDa [5] or 14.3 kDa [4]. MT has high cysteine content and no aromatic amino acids and is unstable to heat. The thiol group of MT (-SH) is a cysteine residue which allows MT to bind heavy metals. The first MT was found on horseshoe cortex. In addition, MT has been reported to be found in vertebrates, including many species of fish [8], and invertebrates [9], especially molluscs [6].

The estimated molecular weight of MT is presented in tables 1 and 2, with an estimated MT molecular weight of Chinese mussel gills of 12.18 kDa. MT is a protein rich in cysteine, low molecular weight, and forms complexes with heavy metals such as Cd, Co and Zn which are essential metals for growth. MT also acts as in detoxification process for toxic metals such as Cd and Hg [5]. The results of Vergani et al. [16] showed that MT in mussel and fish had MW of 12 kDa, but MT on mussel was more reactive which was structurally different which was suspected due to life style differences in both organisms. Jenny et al. [17] mentioned that MT is a metal-binding protein with MW 6-7kDa with cysteine repetition (Cys-X-Cys or Cys-Xn-Cys) that plays a role in homeostatic metal, detoxification and stress response.

Table 1. Estimation of MT molecular weights with standard MW marker using Western Blotting method

| Band No. | BM | Range (cm) | Rf (x) | Log BM (y) | Equation |
|----------|----|------------|--------|------------|----------|
| 1        | 40 | 0.8        | 0.1495 | 1.60       | Y = -1.1461x + |
| 2        | 25 | 1.75       | 0.3271 | 1.40       | 1.7848   |
| 3        | 15 | 2.9        | 0.5421 | 1.18       | R=0.9438 |
| 4        | 10 | 4.25       | 0.7944 | 1          |          |
| 5        | 4.6 | 4.7       | 0.8785 | 0.66       |          |

Table 2. Molecular weight estimation after the exposure of PbNO3

| Well | Rf     | Log MW | BM   |
|------|--------|--------|------|
| 1    | 0.6075 | 1.0886 | 12.26|
| 2    | 0.6075 | 1.0886 | 12.26|
| 3    | 0.6075 | 1.0886 | 12.26|
| 4    | 0.6168 | 1.0776 | 11.96|

Table 3. Data of MT content in gills and stomach.

| Content of PbNO3 (ppm) | Gill Repetition | Stomach Repetition |
|------------------------|-----------------|--------------------|
|                        | 1     | 2     | 3     | Average | 1     | 2     | 3     | Average |
| 0                      | 2600  | 2600  | 2600  | 2600    | 9250  | 9250  | 9250  | 9250    |
| 10                     | 14150 | 5700  | 5600  | 8483    | 18000 | 14750 | 10100 | 14283   |
| 20                     | 27350 | 26450 | 9050  | 20950   | 17900 | 17150 | 18150 | 17733   |
| 30                     | 31350 | 32800 | 29000 | 31050   | 11600 | 11850 | 11000 | 11483   |

3.2. Metallothionein in the stomachs and gills of Chinese pond mussels

MT induction is a “specific biomarker” in response to a heavily polluted environment. Increased MT synthesis relates to metal binding capability and increased resistance to metallic toxicity [15]. The expression of MT is a metal accumulation [18]. The results of measurements of MT values in each treatment on the gills and stomachs of Chinese pond mussels are presented in table 3.

Metallothionein is a protein class that has a low molecular weight, high cysteine levels, and ability to bind IB and IIB metals [6]. MT has a protein and polypeptide content which is affected by metal.
exposure. MT primarily functions in metabolic processes and detoxification of essential and non-essential metals. The results of previous studies explain that MT has a role in the regulation and control of intracellular availability of Cu, Zn, and Cd as well as other metals that have been studied. MT is able to control the removal of Cu and Zn with the help of appropriate receptor molecules such as metalloenzymes. Thus, metal setting is very specific. Because of MT’s selectivity and role in the process of metabolism of metals in organism bodies, MT is believed by researchers to be a protein that plays a role in protecting organisms from metal toxicity. This protein binds to toxic metals such as Cd, where MT acts as a Cd receptor [8]. Many studies have been conducted on MT in relation to metal pollution, allowing this protein to be used as a biological marker for metal pollution in the water [19]. MT level in the stomach decreased in the exposure of Pb at 30 ppm with an average value of 11.483 ng/mL. It was suspected as a metabolic disorder because the Chinese pond mussels had been poisoned as the dose of PbNO\textsubscript{3} became untolerable. The poisoning in the organism was caused by the decrease in the immune system. According to Dafre et al. [20], poisoning or decrease in the immune system in bivalves results from the oxidative reaction of the enzyme d-aminolevulinic acid dehydratase due to the high levels of heavy metals accumulated in bivalves.

3.3. Pb levels in the gills and stomachs of Chinese pond mussels
The results showed that the Pb content was directly proportional to the doses of PbNO\textsubscript{3} administered. The lead content in the gills tended to be higher at higher doses of PbNO\textsubscript{3} exposure. According to [4], lead (Pb) is a heavy metal that will be continuously accumulated in organisms because it has properties that tend to be difficult to regulate by living things. The lead content in the stomach tends to be higher at higher exposure doses as well. This is due to the fact that the absorption of heavy metals also takes place along the digestive tract. The higher the level of Pb directly given, the higher the heavy metal content mixed with the food and accumulated in the digestive tract. According to [21], the absorption of heavy metals by organisms generally takes place in the gills and digestive tract. Therefore, Pb tends to accumulate in the gills and stomach. According to Indonesian National Standard [22], the safe limit of consumption of heavy metal Pb contained in bivalves is 1.5 ppm. The Pb levels in the stomach and gills are presented in figure 1.

![Figure 1. Pb content in the gills (a) and stomachs (b) of Chinese pond mussels](image)

3.4. Pb content in carp
Carp s are frequently used in toxicity tests because they have properties that are sensitive to environmental changes. The most sensitive, and the first, organ in contact with the environment is the gill. Carps are among the fish species that have low tolerance to water quality as well as to the toxic substances contained. The Pb content of carp fish gill decreased in week 3 and week 4 as the heavy metals were reduced. According to Liu et al. [23], Chinese pond mussels (Anodonta
woodiana) are able to accumulate good heavy metals in freshwater because they serve as bio-accumulators based on high heavy metal content.

3.5. Chinese Pond Mussels Histology

*Anodonta woodiana* breathes with two gills and the mantle section. These gills are sheets of structures (lamellae) that contain a lot of gill stems. Meanwhile, between the body and the mantle is a mantle cavity. This cavity is the air exit path [24]. Therefore, the gills of *Anodonta woodiana* are very sensitive to the effects of heavy metal toxicity. The normal gill histology of Chinese pond mussels is presented in figure 3a. In normal gills, the gill lamellae arrangement is still well-constructed. At a PbNO<sub>3</sub> exposure dose of 10 ppm, the Chinese pond mussels suffered from edema and hyperplasia. Edema is the swelling of cells or accumulation of fluid in the body [25]. At a PbNO<sub>3</sub> exposure dose of 20 ppm, the gills showed pathology of hyperplasia, edema, atrophy, and lysis. In abnormal growth, epithelial cells slowly erode the epidermal layer of gill lamellae and push out the lamellae, supported by a liquid dam on the basal membrane, causing erosion of the basement membrane and lysis, or outbreak, of the lamellae. Severe damages occurred in the gills of the Chinese pond mussels at a dose of 30 ppm as the gills could no longer repair cell damages that occurred. Cells that are necrotic will escape their supporting tissues (basement membranes), and nearby tissues become susceptible to irritants [26].

![Figure 2](image)

**Figure 2.** Chinese pond mussel gill histology after PbNO<sub>3</sub> exposure.

3.6. Stomach Histology of Chinese Pond Mussels

Food enters the Chinese pond mussel mouth and continues its way down the esophagus, which is shaped like a dorsoventral flat sponge and seems as if it is the front of the stomach, then into the stomach. The protective outside of the gastric wall is called digestive diverticulum, which is a thin but strong tissue. The thickness of the wall is low to high, ranging between 44.4 μ and 93.3 μ.
Before being exposed to Pb, the outer portion of the Chinese pond mussels is oval, and the inside is squiggly. Silia line up neatly over the surface of the stomach wall.

Digestive diverticula that tend to be round or oval spread out on the outside of the abdomen. According to the results of this observation, the stomachs of the Chinese pond mussels were seen to be in a good condition and had not been damaged yet. At a PbNO₃ exposure dose of 10 ppm, the stomachs of the Chinese pond mussels had edema and hyperplasia in the digestive diverticula. At an exposure dose of above 10 ppm, thickening occurred on the stomach wall, which was accompanied by fluid cavity located at the bottom of cilia. The irregular formation of cilia looked like a pile of cilia leading to the inside of the stomach. Slowly, the pile of cilia merged into one. The histology of the stomachs of the Chinese pond mussels is shown in figure 3.

Figure 3. The histology of the stomachs of the Chinese pond mussels

3.7. Observation of Pb content with SEM/EDX
The Pb in the gills of the Chinese pond mussels observed using an electron microscope is shown in figure 5. The exposure of 30 % PbNO₃ provided more Pb binding effects in the gills of the Chinese pond mussels (table 4). Similar results were obtained by Bebiano and Serafim [27], who induced Mytilus galloprovicialis with Cd at 100 µg/L. The concentration of metal in the organ due to heavy metal induction (table 1, table 4) shows the role of MT as a detoxifier. Similarly, Ghedira [28] also showed that metal concentration and MT induction strengthen the role of MT as in the metal homeostasis and detoxification.

Figure 4. The Pb in the gill of a Chinese pond mussel observed using an electron microscope being marked with red spots.
Table 4. The Accumulation of Pb in the gills of Chinese pond mussels observed using SEM/EDX.

| Treatment | Weight (%) | Atomic (%) |
|-----------|------------|------------|
| PbNO₃ 0 % | 0.179      | 0.001      |
| PbNO₃ 10 %| 0.281      | 0.029      |
| PbNO₃ 20 %| 0.718      | 0.072      |
| PbNO₃ 30 %| 0.683      | 0.072      |

4. Conclusion
Chinese pond mussels can be used as biofilters for carp cultivation in the closed system. The highest Pb accumulation in gills of carp was detected in the first week of treatment, and the accumulation continuously decreased over 30 days. Accumulations of Pb ions in the gills and stomach were detected at a PbNO₃ exposure dose of 30 ppm. The MT molecular weight of Chinese pond mussels was 12.14 kDa.

5. Reference
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