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Sublethal Toxicity Test of Mercury (Hg) in The Flesh and Tissue of Tilapia (*Oreochromis niloticus*)

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ABSTRACT

This study tested the sublethal toxicity of mercury (Hg) in the flesh and tissue of tilapia (*Oreochromis niloticus*). This research used an experimental method completely randomized design (CRD) with 3 treatments and 3 replications. The treatments used different Hg concentrations: Treatment A = 0.08 ppm; B = 0.16 ppm; C = 0.24 ppm; and the control group = without the addition of Hg. The parameters observed were the accumulation of mercury (Hg) in the flesh and tissue damage (histopathology) of the gills, kidneys and liver of tilapia. The results showed that the highest Hg accumulation was at the highest Hg concentration (treatment C = 0.24 ppm). Tissue damage was mainly found in the kidneys, liver and gills of fish exposed to Hg with indications of bleeding, tubular necrosis, vacuolization of epithelial cells, and mononuclear cell infiltration. The results showed that the toxicity of mercury to the organs increased with the increase in the concentration of Hg in water.

Keywords : flesh, mercury, *Oreochromis niloticus*, sublethal, tissue.

ABSTRAK

Penelitian ini menguji toksisitas subletal merkuri (Hg) pada daging dan jaringan ikan nila (*Oreochromis niloticus*). Penelitian ini menggunakan metode eksperimen Rancangan Acak Lengkap (RAL) dengan 3 perlakuan dan 3 ulangan. Perlakuan menggunakan konsentrasi Hg yang berbeda: Perlakuan A = 0,08 ppm; B = 0,16 ppm; C = 0,24 ppm; dan kelompok kontrol = tanpa penambahan Hg. Parameter yang diamati adalah akumulasi merkuri (Hg) pada daging dan kerusakan jaringan (histopatologi) insang, ginjal dan hati ikan nila. Hasil penelitian menunjukkan bahwa akumulasi Hg tertinggi terdapat pada konsentrasi Hg tertinggi (perlakuan C = 0,24 ppm). Kerusakan jaringan terutama ditemukan di ginjal, hati, dan insang ikan yang terpapar Hg dengan indikasi perdarahan, nekrosis tubulus, vakuolisasi sel epitel, dan infiltrasi sel mononuklear. Hasil penelitian menunjukkan bahwa toksisitas merkuri pada organ meningkat sejalan dengan meningkatnya konsentrasi Hg dalam air.

Kata kunci: daging, mercury, *Oreochromis niloticus*, sublethal, jaringan.

1. Introduction

Fish farming has become an increasingly popular activity in the fishery industry executed to fulfill animal protein needs. Fish provide valuable nutrients, inexpensive source of protein and minerals, and are rich in unsaturated fatty acids and omega-3 which help reduce cholesterol in the human blood and prevent damage to the liver (arteriosclerosis) (Connor, 2004; Harris, 2007; Mohebi-Nejad and Bikdeli, 2014; Tilami and Samples, 2017). Unfortunately, large quantities of pollutants and their spread in many places give rise to adverse water quality and disrupt the quality and quantity of fish therein (Karataş and Karataş, 2016; Austin 1998; Fatima et al., 2015; Irianto, et al., 2017).

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Environmental pollution is a problem found in almost all parts of the world. Industrial progress has led to increased pollutant emissions into the ecosystem. Mining and industrialization are one of the main causes of water pollution. Environmental pollution from heavy metals is one of the direst forms of pollution that needs to be kept in consideration as it can cause poisoning, diseases and even death of aquatic creatures (fish). This is compounded by the high absorption or accumulation of pollutants in individual aquatic organisms exposed to heavy metals above their load capacity.

Almost any contaminant at a sufficient concentration has biomagnification and bioaccumulation capability with a wide spectrum of effects not excluding inducing stress responses from aquatic organisms (Censi et al., 2006). This leads, in particular, to a steady decline in aquatic biota. It has now been recognized that fish are more prone to stress due to their intimate reliance on their surroundings (Ogundiran et al., 2007). Accumulation and enrichment of contaminants in aquatic organisms, such as fish, transpire directly from the surrounding contaminated water through respiration and indirectly through their diet (Riba et al., 2004; Ashraf, 2005; Borgå, 2008). Environmental stress can influence the bodies of organism and trigger morphological and physiological disruptions. Some stress reactions can be detected by gross or microscopic examination of various organs or tissues (Harper and Wolf, 2009; Liebel et al., 2013).

Since fish tissues are very vulnerable and sensitive, they can be used as a tool to monitor the quality of an aquatic environment. Fish histopathology can be used as an indicator of environmental stress (Ruiz-Picos et al., 2015; Irianto, et al., 2017; Nur and Yusnaini, 2018). Pollution of organic and inorganic materials in waters can cause bodily failures when the toxicants enter the fish body. There is a clear correlation between exposure to pollutants and damage to fish liver. For example, lesions in fish liver are a symptom of damage caused by toxic substances and this phenomenon is used as biomarkers of chemical contamination in waters (Stehr et al., 2004).

Mercury is one of the water pollutants of the non-essential heavy metal group often found in various bodies of waters, both in river and sea. Accordingly, the accumulation of mercury in an aquatic organism can cause eco-physiological disruption of the organism and thus can be used as a biomarker for mercury toxicity. However, the concentrations of these compounds in aquatic organisms vary depending on the metabolic activities and the average life span of the species of the organism concerned as the body of the fish reaches 60-90% due to its high absorption capability during said growth. In addition, absorption and accumulation in different tissue can give different effect.

Mercury is one of the water pollutants of the non-essential heavy metal group often found in various waters, both in rivers and in the sea. Pursuant to this, the accumulation of mercury in aquatic organisms can cause ecophysiological response from organisms which thus can be used as a biomarker of mercury toxicity. However, the concentrations of these compounds in aquatic organisms is diverse depending on the metabolic activities and the average life span of the species of the organism in question, as the fish body grow to 60–90%, due to its high absorption aptitude. Additionally, absorption and accumulation in different tissues can have different effects.

According to Jezierska and Witeska (2006), various factors affect the accumulation of heavy metal in fish, including heavy metal concentration, duration of exposure, path of uptake, environmental condition, and the internal factors of the fish such as fish age and feeding habit. Not many studies have examined the physiological mechanism of fish exposed to heavy metal and the exposure effect on the Tilapia fish health condition in detail. Based on the explanation above, the purpose of this study is to obtain data on the absorption of Hg in the nile fish body with sublethal exposure to different concentrations as well as the description of the damages caused to the vital organs of the fish.

2. Materials and Methods

The material used in this research were nile Tilapia (*Oreochromis niloticus*) with an average body weight of 30 g/fish, pure mercury (Hg) container with size 150x100x30 cm^3, aquariums with size 25x35x40 cm^3, a set of tools to draw and count the blood component of the fish, a set tool for the preparation of histological observation, a set tool for measuring Hg concentration and analyzing water quality. Fish acclimatization done before used for sublethal toxicity test. Bioaccumulation and histopathological observation was carried out after 120 hours exposure.

2.1 Acclimatization procedure

Acclimatization of the test fish was carried out for one week in a fiberglass fish pond. During the acclimatization, aeration was

carried out and pelleted feeding twice a day as much as 3% of the body weight of the fish. Only fish showing health signs were included in the test treatment.

2.2 Hg accumulation procedure

After acclimatization to the media with normal water condition, the test fish were put in their respective treatment aquarium containing water with a volume of 20 L with 8 fish/aquarium. The fish were kept for 120 hours and given fish pellet containing 30% protein. To maintain the water quality of the treatment media, 20% of the total water volume was changed every day with a stock containing mercury concentration according to each treatment. The design used in this study was a Completely Randomized Design (CRD), with 4 treatments and 3 replications, namely: Treatment A = 0.08 ppm; B = 0.16 ppm; C = 0.24 ppm; and control = without addition of Hg. The determination of this sublethal concentrations was based on previous research carried out by Siregar et al. (2018) who found LC50-96 hours in bonylip barb fish (*Osteochilus hasselti*) through toxicity test of Hg at 0.3938 ppm. Fish were dissected, muscle was removed and washed in distilled water, then dried at 80°C for 72 hours. Samples were weighed and digested at 95°C for 3 hours in high purity nitric acid (HNO3) before Hg analyzing. Samples were diluted in ultra-pure water and Hg analyzed on the basis of the AOAC (2012) procedure by Atomic Absorption Spectrophotometry (AAS) using air-acetylene flame (Varian). The outcome value was represented as total Hg mg/kg dry mass, while limit of detection (LOD) was 0.03 mg/kg.

2.3 Histopathological observation procedure

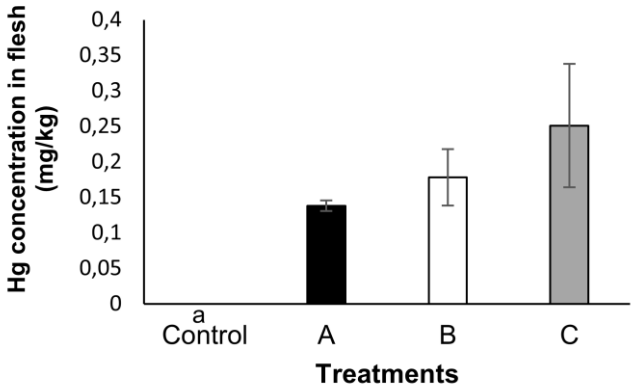

Figure 1. Hg levels in flesh of artificially exposed fish (A = 0.08 ppm; B = 0.16 ppm; C = 0.24 ppm; Control = without the addition of Hg).

Histopathological observation was carried out by taking one fish in each unit of treatment and dissected the target organs (gill, liver, and kidney). Each tissue sample was fixed with 70% formalin, then processed including dehydration, clearing, embedding, section and stained with hematoxylin and eosin (H&E). Histopathology specimens on object glass were observed by microscope stereo (Olympus) for taking documentations.

2.4 Data analysis

Data of Hg accumulation from different concentration treatments obtained were then tabulated and analyzed by variance analysis (ANOVA) at 95% confidence interval with SPSS 16.0 program to determine whether the treatment significantly affected Hg accumulation in fish flesh. If significant effect come to pass, the differences between treatments were analyzed with the Tukey test, while organ damages were analyzed descriptively.

3. Results and Discussion

From the statistical test results of ANOVA diversity test, treatments exhibit differences ($p < 0.05$) relative to each other. Furthermore, from the Tukey test, it is known that A and B treatments as well as the controls did not differ. Though treatments A, B, and C did not differ from one another, treatment C was different from the control. From the data, it can be seen that the higher the Hg concentration in the water media, the higher the Hg level contained in the exposed fish flesh. The highest absorption of Hg in fish flesh was in treatment C with an average of 0.63 mg/kg, followed by treatments B and A respectively at 0.26 mg/kg and 0.11 mg/kg, while the control group presented concentration below the detection limit (< 0.03 mg/kg) or nil. (Figure 1). Mercury can enter the body of fish and accumulate in flesh can be explained as follows. There are two major possible approaches in which mercury go through the aquatic food chain; by direct intake of water and food through the digestive tract and non-dietary routes over permeable membranes such as the flesh, gills and skin (Ribeiro et al., 2005). Mercury accumulates in fish tissues by absorption through the gill surface and the kidney, liver and intestinal walls at higher levels than the ambient concentration. The entry of heavy metals into the organs of the fish occurs primarily by adsorption and absorption; the rate of accumulation depends on the rate of uptake and ablution (Annabi et al., 2013).

Tissue Damage (histopathology)

Histology is an approach that enables the sensitive and selective analysis of the sublethal effect of heavy metals in the environment and aquatic biota (Arellano, et al., 1999). Toxic impacts on organs arise when pathways for excretory, metabolic, storage and detoxification are no longer capable of counteracting uptake (Obasohan et al., 2008) and ultimately lead to physiological and histopathological damages. Histopathological analysis of vital organs comprising of the gill, liver, and kidney of the fish then showed various severe damages in the fish exposed to Hg. The visible damages include hemorrhage, edema, tubular necrosis, vacuolization, epithelial cell release, and mononuclear cell infiltration. The following are some microscopic images of organs alongside organs from fish in

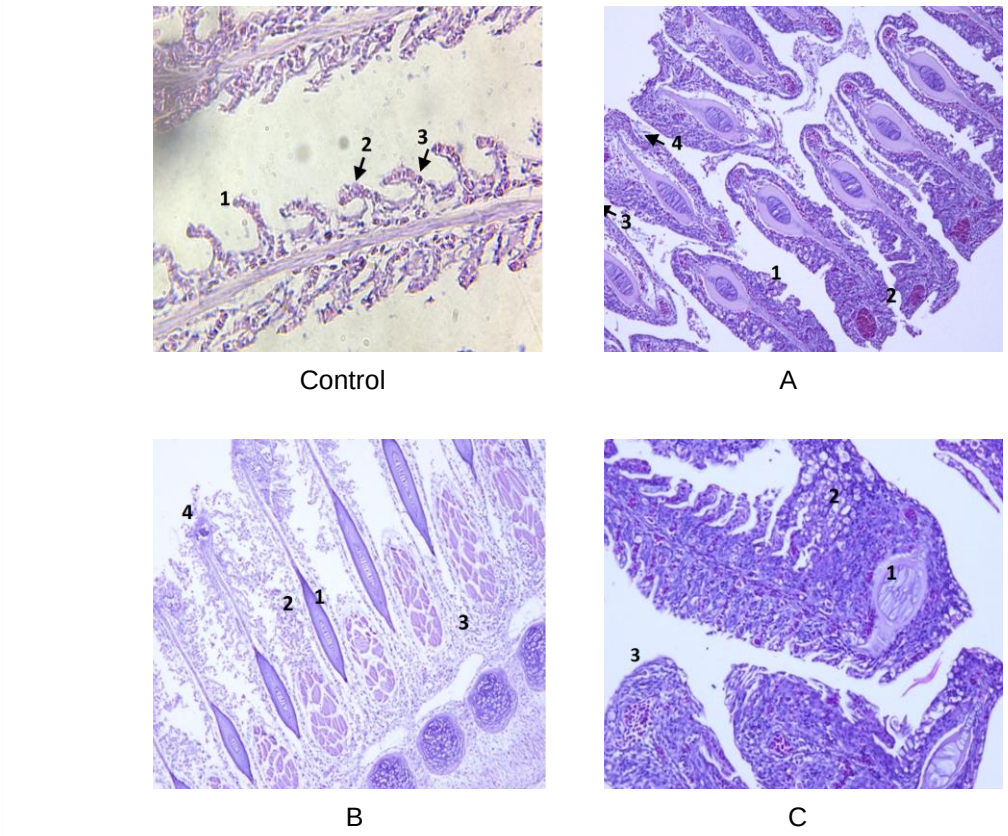

Figure 2. Tissue damages in fish gills exposed to mercury at different concentrations (CL, Control = without the addition of Hg; A = 0.08 ppm; B = 0.16 ppm; C = 0.24 ppm). CL. No significant changes in gill. Secondary gill lamellae (1) with a meniscus shape lining both sides of the gill filaments. The surface of the lamellae is covered by a number of single layers of epithelial cells (2). The capillaries are separated by pillar cells (3) which spread out in the secondary cell lamellae. Mucous cells and chloride cells are present in secondary gill lamellae. Epithelial cells and pillar cells are easy to undergo changes that occur in a short time due to physical, chemical, biological damage. The cells undergo degenerative changes (or necrosis) that eventually rupture and bleeding in the area. A. Hemorrhagic necrosis of the gill. 1. Lamella, 2. Congestion, 3. Hemorrhage and 4. Lamella necrosis. HE 100x. B. Necrosis and edema in the gill tissues. 1. Lamella, 2. Gill epithelial cell necrosis, 3. Edema and 4. Epithelial detachment. C. Necrosis in the gill tissues. 1. Lamella, 2. Lamellae vacuolization and 3. Hemorrhage.

the control group.

From various previous researchers, there are affinity differences of various fish organs toward heavy metal. In general, the vital organs that showed the greatest accumulation are the liver, kidney, and gill. However, previous researchers have also shown that the digestive tract is a part of the body of the fish with accumulation it provides pathway for the uptake and entry into the fish body (Giguere et al., 2004; Hastuti et al., 2019). While Yamazaki et al. (1996) found that swimming bubbles and kidney accumulated various types of heavy metals. Other organs such as the gonad, bone, and brain also showed high levels of metal accumulation, while the muscle, compared to other tissues, usually showed low metal concentration though is still often examined because this particular body parts of the fish is consumed by human (Jezierska and Witeska, 2006; Nur et al., 2020).

The accumulation of heavy metal in fish exposed to sublethal concentrations depends on the stage or time of exposure. Usually, at the beginning of exposure, the rate of accumulation and absorption of metal is very high, but, ultimately, stability between the accumulation and the rate of excretion to eliminate metal in the body will be achieved. Fish would efficiently absorb methyl mercury, but would at the same time slowly release it. In this study, sublethal concentration was used, in reference to the concentration below the LC50-96 hour of Hg toxicity in silver sharkminnow at 0.396 ppm (Siregar et al., 2018). Sublethal influence occurs in the fish organs, causing damages to the liver, decreasing the amount of blood, reducing the potential for breeding, growth and so on. The lethal concentration

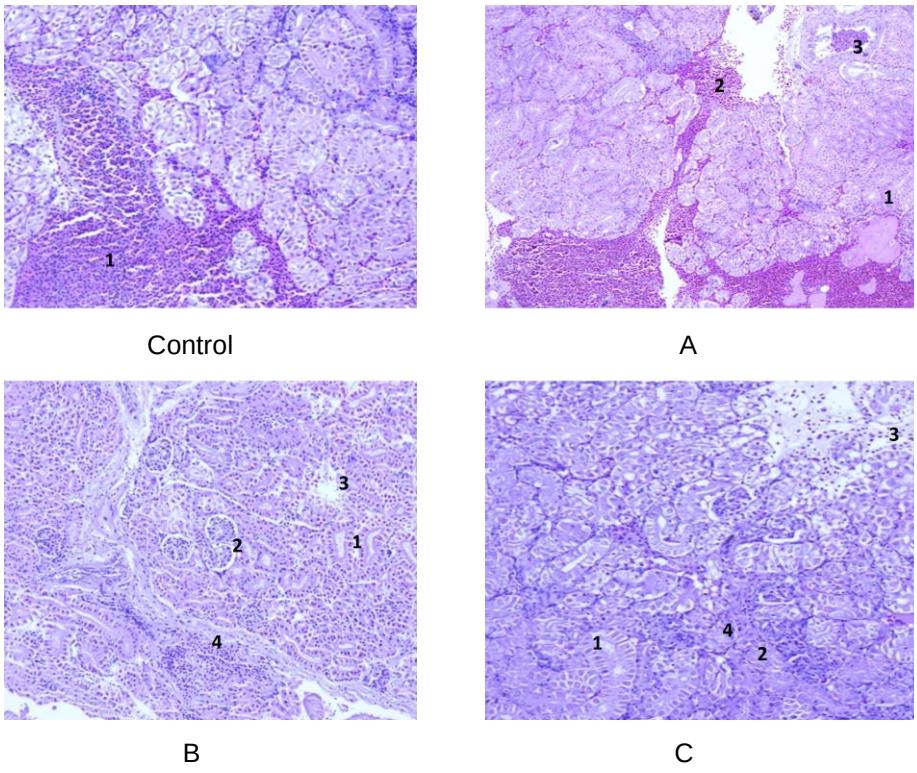

Figure 3. Tissue damage in fish kidneys exposed to mercury at different concentrations (CL= Control = without the addition of Hg; A = 0.08 ppm; B = 0.16 ppm; C = 0.24 ppm). CL. Kidney tissue in the control treatment, there are not many changes in the tissues. 1. Hemorrhage at the edges. A. The kidneys experience hemorrhagic nephritis. 1. Tubules, 2. Haemorrhage, and 3. Mononuclear-lear cell (lymphocyte) infiltration. B. The kidneys experience nephritis. 1. Tubules, 2. Glomerulus, 3. Tubular necrosis and 4. Infiltration of mononuclear cells. C. Kidney has nephritis. 1. Tubules, 2. Glomerulus, 3. Tubular necrosis, and 4. Infiltration of mononuclear cells (lymphocytes)

given give rise to a high toxicity level that caused interference with the central nerve of the organism, resulting in death. Methyl mercury is insoluble and cannot be completely removed or excreted. Accumulation generally happens in the internal organs of the body, though it is also found in the muscle tissue. Mercury bioaccumulation is found in a build up at the adipose tissue, a type of fish tissue that stores fat.

The histology of the normal group fish and fish organs exposed to mercury during the research can be seen in Figure 2-4. The vital organs discussed in this study are the gill, kidney, and liver. The organs are the main organs that accumulated heavy metal pollutant materials (Jezierska and Witeska, 2006). The control group organ displayed a very usual architecture while the fish exposed to mercury showed various alterations for three different sublethal concentrations.

Gill was one of the organs in the fish's body that show direct implication from the toxic substances contained in water and thus is chosen as a target organ to be an indicator in assessing water quality. The liver is an organ in the body where all kinds of toxic materials are accumulated. This organ is the center of detoxification where toxins are neutralized and improved so that they can be eliminated from the body, whereas the kidney is also very vulnerable because functions as a blood filter.
In general, all organs after experienced necrosis. Cells that experienced necrosis can be identified from their nucleus that change in shape to become smaller or larger. The

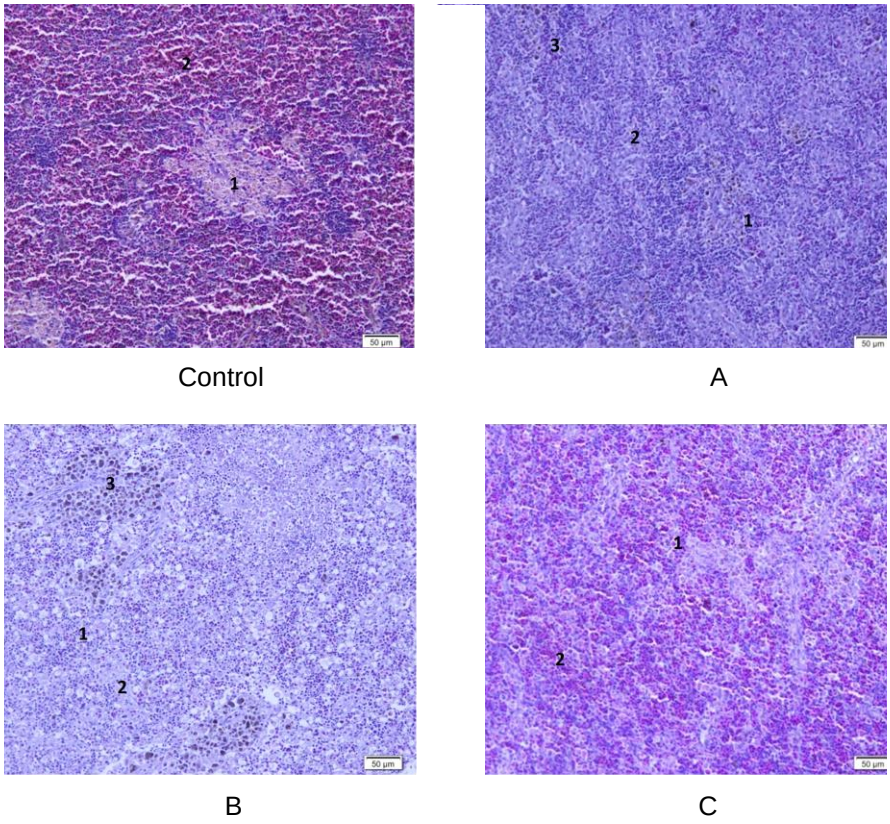

Figure 4. Tissue damages in fish livers exposed to mercury at different concentrations (CL= Control = without the addition of Hg; A = 0.08 ppm; B = 0.16 ppm; C = 0.24 ppm). CL. Liver tissue in control treatment, there was minimal damage to the tissues. 1. Hepatic epithelial cell necrosis, 2. Hemorrhage. A. The liver has hepatitis. 1. Hepatic necrosis, 2. Accumulation of hemosiderin and 3. Infiltration of mononuclear cells (lymphocytes). B. The liver experiences hepatic necrosis. 1. Vacuolization of liver epithelial cells, 2. Coagulative hepatic necrosis and 3. Accumulation of hemosiderin. C. The liver experiences necrosis. 1. Hepatic epithelial cell necrosis and 2. Hemorrhage.

identification is a challenge in and of itself as cytoplasm is missing and thus the absorption of pigment is nil, making it hard to clearly see or recognize the sample.

Fish gill tissue damages due to exposure to Hg during the study can be seen in Figure 2. Hg exposure resulted in a very severe gill epithelial necrosis. Likewise, the lamella disorganization appeared in the result of this study for treatments A and C. The compositions of the primary filament and secondary lamella have changed.

Some of the gill tissues showed symptoms of telangiectasia or dilation of blood vessels, especially at the end of the secondary lamella which was enlarged and rounded so it looked like a balloon bubble attributed to blockage or blood clotting at the end of the secondary lamella. This damage can cause disruption to the function of respiration, and this could be very problematic if the fish were located at a high temperature area where oxygen solubility is low, whilst their metabolic rate is getting higher, and the retrieval and binding of Hg is also high. Treatments A, B, and C not only show hemorrhage and hyperplasia but also gill bending. This bending took place as cells were enlarged and become abnormal.

At the 120^{th} hour exposure, the gill exhibits hypertrophy, hemorrhage, and hyperplasia. Hemorrhage is seen from spreading of the blood to the gill tissue. However, the control also showed that the gill of the fish experienced hemorrhage in certain sides. Hyperplasia made the gill lamella appear larger than in control fish, and as consequence, the secondary lamella became thick and cannot function properly. In addition, the gill no longer showed clear distinction between the primary and secondary lamellae. Gill in the treatment of Hg exposure experienced blood containment and edema found in gill lamella. The containment is characterized by a very dense build-up of the red blood cells/erythrocytes (Treatment B) in the blood vessels, wherein the red blood cells are thick in color. The accumulation of blood cells can continue and cause the clogging of blood vessels (congestion). If the congestion is very severe, then the blood vessels can burst and it can displace the blood into undue places. The containment is accompanied by edema appearing as white empty space. Edema occurred due to swelling or too much body fluid outside the cell (extracellular) and outside the blood vessel (extra-spectacular), and thus the fluid is stored in the interstitial space or a gap in the body's cell.

In the fish of treatments B and C, some of the pillar cells experienced cell shrinkage. Pilaster cell is primary lamella compiler arranged in a row and wrapped by a thin, semipermeable epidermal membrane. In the other lamella, the clogging occurred is marked by the accumulation of very dense red blood cells/erythrocytes in the blood vessel, and the gill lamella form was destroyed and a very severe flaking of the outer layer of tissue (desquamation) appeared. Most of the gill epitheliums are detached from the organ (A and C Treatments). The existence of gill damages causes fish to be disturbed, and the gill functions in the process of excretion, osmoregulation, and respiration are not optimal (Velmurugan et al., 2009). This is characterized by difficulties in oxygen uptake and ultimately experience of hypoxia. It seemed that the gill is the primary route of the entry of Hg into the fish body.

The differences of the histopathological profile of the kidney organ from each treatment can be seen in Fig 3. Histologically, the kidney is composed of renal tubules or nephron. The positive control histopathology has neat tubular and glomerular structure, although hemorrhage is still found, while the B-D histopathological profiles have destroyed tubular structure and some glomerular were dilate. Moreover, the recognition of glomerular or small blood vessel knot was hard to do as the tissues were damaged by Hg exposure. The Bowman's capsule was no longer visible. Additionally, the fish kidney experienced inflammation or nephritis. In some exposed fish, hypertrophy appeared in the nucleus of the tubular cell.

Liver damage can be used as a marker of the aquatic environment stress. Microscopically, necrosis, hemorrhage, and vacuolization in liver tissue are found in general sample of fish exposed to Hg. Some also display hemosiderin accumulation, an accumulation of pigment from iron (Fe) that occurred due to damage of erythrocyte (Fig 4). This can be ascribed to the disruption of the synthesis of transferrin, a protein that is responsible for binding iron for catalysis. Transferrin is mainly synthesized in the liver and secreted into the plasma (Asmamaw, 2016).

Histopathology has been shown to be consistent with Hg accumulation in the sublethal concentrations exposure to fish, wherein if exposure persists, would result in slow growth and can even cause the death of the fish.

4. Conclusion

The highest Hg accumulation in flesh was found in the treatment with the highest Hg concentration (treatment C = 0.24 ppm). Increasing the concentration of Hg in water media causes an increase in Hg in fish flesh. The effect of Hg in the body of fish also causes tissue changes, especially in the gills in the form of bleeding, edema, fusion, vacuolization of lamellae, and epithelial necrosis. Damage to the kidneys in the form of bleeding, nephritis, mononuclear cell infiltration, and tubular necrosis, as well as liver damage in the form of hemosiderin accumulation and necrosis. If exposure to these sublethal concentrations continues in the fish it can result in fish mortality. The conclusions based on this study indicate that the bioaccumulation and histopathology observations of gills, liver, and kidneys can be used as excellent biomarkers for fish exposed to Hg pollutants.

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