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Alteration of Gills and Liver Histological Structure of *Cyprinus carpio* Exposed to Leachate

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Abstract

One of the main problems in the waste management in Indonesia is the treatment of leachate, which mostly dumped to the river This research is aimed to obtain information of histological alteration in gills and liver of *C. carpio* L. exposed to leachate. Measurements on the water quality parameters comprised water temperature, pH, and dissolved oxygen (DO). This research was conducted by exposing leachate to *C. carpio* for 96 hours. The concentration of leachate were 0 ppm, 80 ppm, and 100 ppm. Histological preparation were made on the gills and liver using 10% fixative Neutral Buffered Formalin and Ehrlich Hematoxylin-Eosin staining with qualitative observation descriptive analyses for discussion. The result showed that increasing water temperature is directly proportional to the leachate concentration in the aquaria, while the value of pH and DO inversely proportional to the leachate concentration. Damages on the gills with 80 ppm leachate concentrasion were identified as follows: fusion of secondary gill filaments and hyperplasia of epithelial cell, along with karyorrhexis and hydropic degeneration on the liver. Damages on the gills of fishes exposed to leachate with 100 ppm concentrasion were identified as follows: fusion of secondary gill filaments, hyperplasia of epithelial cell, congestion, and edema along with karyorrhexis, hydropic degeneration and *melanomacrophage centre* (MMC) found on the liver. The results of this study can be used as an overview of the impact of an environmental pollution by leachate as indicated from histological damage to the gills and liver of *C. carpio*, thus contribute significan information to aquaculture sector and endorse better waste management

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INTRODUCTION

Based on data from the Central Statistics Agency (BPS) since 2000 to 2015, the number of Indonesia's population has increased by 0.8% from ± 205.1 million to 255 million. This increasing number of the population is indirectly proportional to the increasing number of private consumption, which considerably contributes to the high volume of waste products (garbage). In Indonesia, 60% of the total waste being transported to the final disposal (TPA) is processed in the landfill (waste management in an open field), due to the easy and relatively inexpensive operation. This is the reason why TPA method has widely been used. However, this waste management gives a negative impact to the environment, one of which is the contamination of the waters due to the leachate (Susanto et al., 2004).

Leachate can be interpreted as liquid waste arising from the entry of water into the garbage heap and dissolving soluble materials contained in the heap (Hartati, 2007). Generally, leachate contains organic and inorganic substances with high concentration, such as ammonia, heavy metals, pathogenic or non-pathogenic bacteria, and microbial parasites (Susanto et al., 2004; Hartati, 2007). The entry of contaminants in water can result in alteration on community structure, decline on biomass or productivity, changes in behaviour, decreasing growth rate, disruption of reproductive system, and the effects, which can disrupt the balance of the aquatic ecosystem (Riani, 2012).

One place deemed potential for leachate production on a large scale is TPA Sarimukti, District Cipatat, West Bandung regency. TPA Sarimukti has a land area of ± 25.2 hectares with the volume of waste reaching to 1.200 tons per day. Sarimukti landfill exloads leachate into Citarum river that flows to the north (Suganda et al., 2012). The use of water from the river to fulfil the population's needs in daily lives is still dependeing on the surrounding community, one of which is for fishing culture activity. Resulting leachate contamination of the river by the living and farmed fish can accumulate pollutants in their bodies, which surely is not safe for consumption.

Fish can be used as one of the test animals in assessing biological effects of contaminants and environmental quality. This is due to low response to toxic substances (Neelima et al., 2015). According to Price (1979), assessment of toxicological effects of some chemical pollutants in the environment can be tested by using a species that lives in waters with pollution. One species of fish generally used as test animals is the common carp (*Cyprinus carpio* L.).

Histopathological changes have widely been used as a tool of biomonitoring health status of fish exposed to a mixture of chemicals on a laboratory scale research and investigation in the field (Stentiford et al., 2003; Boran et al., 2012). The gills are important organs in terms of respiration, guard acid-base balance, osmoregulation and excretion of residual nitrogen in fish (Evans et al., 2005). The occurrence of direct contact between the gills with outside environment makes the gills have high sensitivity, including in terms of stress or pressure, pollutants, and changes in the environment (Palar, 2004). In addition to the gills, liver can also be used as one of the objects of the pollution indicators. Fish liver plays an important role in vital functions in basic metabolism and serves as the main organ of accumulation, biotransformation, and excretion of contaminants in fish (Authman et al., 2013). Most of the toxic substances that enter the body after being absorbed by the cells will be carried to the liver by the portal vein liver. This is what causes the liver accumulate toxic substances in large quantities (Setyowati et al., 2010).

This research was conducted as a result of exposure biomonitoring leachate from the landfill Sarimukti to examine any changes of histological structure in gills and liver of *C. carpio*, and also to determine the concentration of exposure that can cause the greatest changes in histological structure. This research is expected to provide an overview and scientific information on the changes in gill and liver histology *C. carpio* exposed leachate so that it can serve as consideration for further study.

METHODS

Leachate samples obtained from landfill Sarimukti West Bandung regency, the reservoirs have already received treatment and are flowing towards the river (outlet). Leachate was first sterilised before being used in the experiments to avoid microbial activity during the experiments.

This study used carp fish (*Cyprinus Capio* L.) healthy male with an average weight of 90-100 grams as test animals. *C. Carpio* obtained from a fish pond Cileunyi, West Java. Before treatment, the fish were acclimated beforehand for 3 days at room temperature (25°C) and fed twice a day.

After acclimatisation, test animals were divided into three groups treatments in separated aquaria. The fish were exposed by water added by leachate with concentration of 0 ppm, 80 ppm and 100 ppm for 96 hours (4 days). At the end of the experimental period, organs like gills and liver were isolated for the preparation of permanent incision histology (Karthigayani et al., 2014). Supporting physical and chemical parameters used in this study is the water temperature, acidity (pH), and dissolved oxygen (DO).

Gills and liver of *C. carpio* were isolated and then fixed in a solution of 10%-NBF (Neutral Buffered Formalin). Samples in NBF were removed from the fixative after 48 hours and then washed with water, then dehydrated by placing samples in rising concentrations of ethanol (70%, 80%, 90%, 95% and 100%), cleared using xylene, embedded using paraffin, sliced using a microtome with a thickness of 4-5 μm, and stained using Hematoxyline-Eosin (H&E) (Humason, 1967). The parameters observed in this study was the structure change of the gills and liver organ histologic of *C. carpio* and analysed both qualitatively and descriptively.

RESULTS AND DISCUSSION

The parameters used for the measurement of water quality in this research were the water temperature, acidity (pH), and dissolved oxygen (DO). These parameters were measured twice, namely at the time of acclimatisation and after 96 hours of administration of the leachate for each different concentration. The results of measurements of water quality parameter are available in Table 1.

The measurement results in each water temperature in this study were included in the category of optimal range of the living *C. carpio* based on the classification of Costa-Pierce et al. (1990), which is in the water temperatures ranging between 25-27 °C. Based on the data in Table 1, it can be seen that there was an increase in the water temperature in greater exposure concentrations of leachate. The higher the pollutant concentrations, the higher the water temperature became. The addition of pollutants to the water column also triggered the fish stress. It can be seen from the increase of the movement in swimming speed and operculum.

One of the causes of temperature change from the normal state becomes hotter or colder in the waters is the entry of pollutants like sewage into the waters. High and low temperature changes are depending on the magnitude of the number of triggers that appear or are contained in the water (Mamangkey, 2011). According to Campbell (2002), changes in water temperature are great or even 1 °C alone and sudden in onset, can be felt and affect the adaptation of fish. The influence of these changes can be seen from the changes in the activity of the body, swimming speed, and nerve stimulation.

Fish acclimatised to a relatively high temperature will increase their respiration rate, which can be observed from the changes in the movement of the fish operculum. High water temperature can lead to reduced DO gas, resulting in fish accelerating the movement of the operculum to get oxygen gas as quickly as they need the respiration process. In addition, the increase in water temperature can also cause increasing solubility of toxic substances such as crude oil pollutants and pesticides, as well as increasing toxicity of heavy metals (Fardiaz, 1992).

The DO was lower than the value of DO during acclimatisation time and keep decreasing during treatment obviously on aquaria with leachate concentration of 100 ppm. Decreased oxygen levels in the water may be due binding of oxygen with the chemical compounds and organic material contained in the leachate. This is aligned with Mamangkey (2011) that some of the factors affect the concentration of DO in water include chemical compounds, organic substances, stirring period of water, temperature, flow, and water depth.

Based on the classification of Costa-Pierce et al. (1990), DO value obtained at the time of acclimatisation and leachate concentration of 0 ppm can be categorised as the optimal environmental conditions for the life of *C. carpio*, namely above 6 mg/L. While the aquaria with leachate concentrations of 80 and 100 ppm, based on the grouping Swingle (1969) are included in the category of environmental conditions that are not

Table 1. Measurement Results of Temperature, DO, and pH During Acclimatisation and After 96 hours of Treatments

Parameter	Unit	During acclimatisation	Leachate concentration in the water		
			0 ppm	80 ppm	100 ppm
Water Temperature	°C	25	25	25.5	26.5
DO	mg/L	8.7	8.3	5.5	4.4
pH	-	7	7	6.5	6.5

favourable for *C. carpio* and therefore can cause physiological disorders, namely the DO value ranging from 4 to 5.5 mg/L.

Acidity of water for aquatic animal becomes one important factor in maintaining a state of homeostasis of animals, with the increase or the decrease which can cause acid-base balance, in regulation of ion and ammonia excretion (Wood, 2001). In general, leachate pH are ranging from 5.8 to 8.5 (Renou et al., 2008). A decrease in pH occuring in this study may be due to the chemical contents of the leachate from organic and inorganic materials, especially heavy metals, ammonium, and biological activity of microbial. Decreasing pH to 6.5 will cause stress on the fish, which can be seen from the increase of their swimming speed and body respiration. Fish can experience stress at pH ranging from 4.0 to 6.5 and from 9.0 to 11.0. In general, a pH of 7.0 to 8.5 is the optimum pH for fish life as according to biological productivity. Death of fish can occur at pH of less than 4.0 or more than 11.0 (Bhatnagar & Devi, 2013).

Based on the identification of preparations, gills exposed to leachate with a concentration of 0 ppm showed the result of a normal gill structure that is based on the book Atlas of Fish Histology by Genten et al. (2009). In the book, there was a description of some *C. carpio* gill compiler structures that are branching of the gill filaments (hemibranch), the primary lamella composed by chondrocytes, calcified cartilage, arteriolar blood and epithelium, as well as secondary lamella arranged by arteriolar blood, empty lumen, pillar cells, and epithelial cell layer that surrounds it. This gill histological structure can be seen in the Figure 1.A.

The identification results presented leachate gill preparations with a concentration of 80 ppm, obtained in the form of secondary lamella fusion and hyperplasia epithelial cell. As for the identification preparations, damages were found on the gills exposed to leachate with a concentration of 100 ppm in the form of lamella secondary fusion, hyperplasia epithelial cell, edema and congestion. Gill damage in the form of secondary lamella fusion and hyperplasia epithelial cell can be seen in the Figure 1.B, whereas edema in the

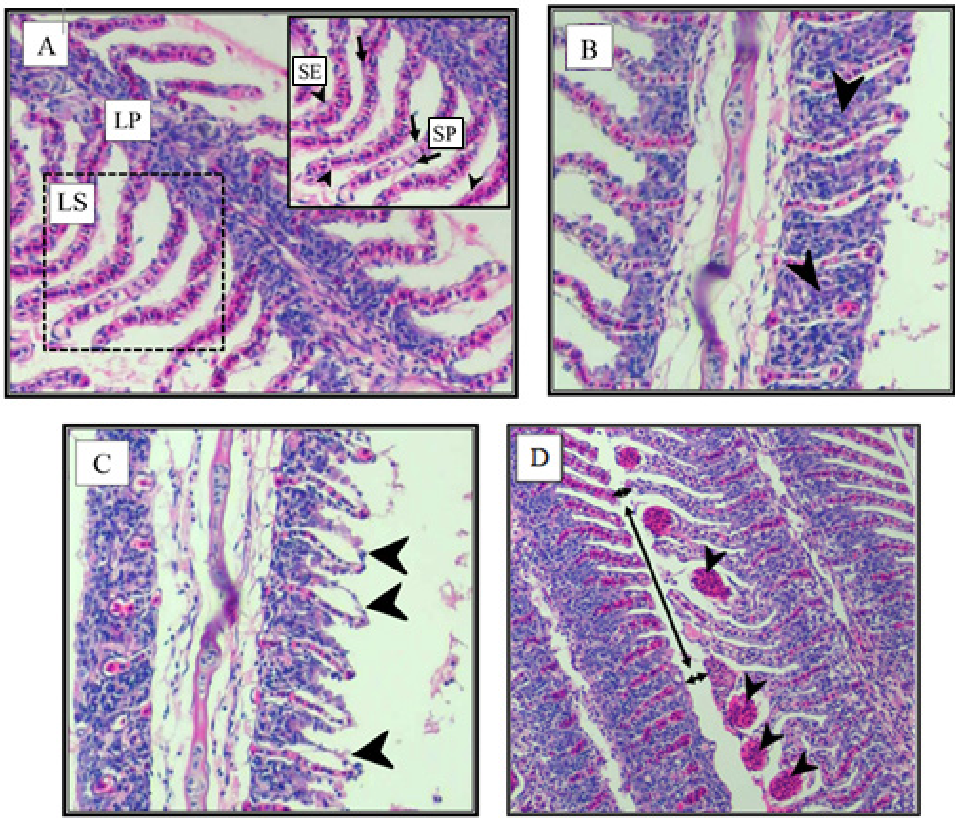

Figure 1. Histological Alteration in gills of *C. carpio* after leachate treatment. **[A]** Gill histology with no leachate exposure (control), showed primary lamella (LP), secondary lamella (LS), epithelial cell (SE), and pillar cell (SP) (Scale: 1,56 cm = 5,524 μm); **[B]** Hyperplasia epithelial cell (arrow) that causes fusion of secondary lamella (Scale: 1,56 cm = 5,524 μm); **[C]** Edema that causes epithelial lifting (arrow) (Scale: 1,56 cm = 5,524 μm); **[D]** Constriction between lamella (two arrows) and Congestion (arrow) (Scale: 1,56 cm = 5,524 μm).

Figure 1.C and congestion in 1.D.

Based on the comparison of damages of the gills histology preparation, it is known that the greatest damages happened in the leachate exposured to a greater concentration, i.e., 100 ppm. This is in line with the statement of Siregar et al. (2012), Setiawan et al. (2013), as well as Kavidha & Muthulingam (2014), that the magnitude of accumulated toxicant found in fish organs is related to the concentration and length of exposure to the toxicated fish. It will be directly proportional to the level of organ damage.

In this study, fusion lamella observed from the primary or secondary lamella was fused with another lamella. Chezhian et al. (2012) and Devaraj et al. (2014) reported the closer move of gill epithelium (via cell hypertrophy) is sometimes considered as one indicator of cell degeneration and eventually signs of early necrosis. The pooling caused the gill surface area reduce the respiration process, so that the respired oxygen decreased. Fusion and hyperplasia of the gill lamella can be triggered by the effects of the toxins that alter cell glycoproteins including mucus, thereby affecting the negative charge of the epithelium and thus supporting the adhesion to the adjacent lamella (Ferguson, 1989). Camargo & Martinez (2007) stated that change such as fusion secondary lamella is one example of a self-defense mechanism. Basically, the increase in the distance between external environment and blood, would be a barrier to the entry of excessive contaminants, but the distance change between the lamella can lead to reduced capability in capturing oxygen that can be transmitted by blood.

Hyperplasia is a state of increasing number of cells in primary lamella due to the division of cells' excessive chloride. In this study, the alleged contents of organic and inorganic materials in leachate, especially for heavy metals and ammonium, are considered as the cause of the hyperplasia of epithelial cell. The same histological changes have also been reported in some studies of *C. carpio* describing ammonium (Devaraj et al., 2014; Chezhian et al., 2012) and the exposure of lead (Pb) (Natalia, 2007). Chemical pollutants and heavy metals that mostly cause hyperplasia are Cadmium (Cd), Cuprum (Cu) and Zinc (Zn) (Saputra et al., 2013). Due to the lamella hyperplasia in primary cause of secondary lamella space between the drains and the enclosed space, mucus production leading to the intake of oxygen into the blood is reduced and creates impaired immune regulation.

Edema was observed in the individuals exposed to leachate concentration of 100 ppm. Based on the results, change of gills with edema is characterised by a white membrane, which does not contain any fluid (former swelling), while visible basement membrane began to stretch off. Edema is swelling of the cell that occurs due to excessive accumulation of fluid in the tissues and it cause the separation between epithelial layer and the underlying systems mast cells that could lead to the destruction of its secondary lamella structure (epithelial lifting). It is a form of physiological adaptation of the fish when experiencing interference from the environment. This damage causes function deficiencies and breathing difficulty in the gills, so that the metabolism began to fail (Robert, 2001; Fitriawan, 2010). In this study, the occurrence of edema could be predicted due to the toxicants of the leachate that have penetrated the gills resulting in cell irritation. Ploeksic et al. (2010) reported that edema often occurs as a result of chemical pollutants exposure, such as heavy metals, metalloids, pesticides, and the use of therapeutic substances (formaldehyde and H_2O_2). Similar observations were reported by Susanah et al. (2013) with *Chanos chanos* (Milkfish) exposed to factory waste in the village pond site Tapak Area Tugu- Tugurejo district of Semarang. Toxic substances in this pond water entered to the gills and made the cells become irritative so that they will cause swelling.

Congestion or blockage of blood vessels can be triggered by the breakdown of cell structure pillars resulting in increasing blood flow in the lamella. Congestion obtained in this study can be caused by metal contents in leachate. This is consistent with the results from Hadi & Alwan (2012) using *Tilapia zillii* exposed with aluminium, a metal that affects the permeability of cell membranes and lead to the occurrence of resistance in the ion exchange system, which in turn lead to the disruptions in the fluid transport into and out of cells. Similar observations were reported by Authman et al. (2013) with *Clarias gariepinus* in heavy metal exposure and also by Salim (2014) with *C. carpio* exposed to heavy metals, pesticides, fertiliser waste, and debris from the river in Kharatrad Gramat Ali river, Basra province.

In the observation of liver histology *C. carpio* some structures making up the liver, the hepatocytes, central venous, and sinusiod were found and did not reveal any changes in the cells. On *C. carpio* liver histology images, sinusiod coated with endothelial cells formed a thin sheet. Cell nuclei elongated and protruded into lumen sinusoidal. The endothelium has small pores (Genten et al., 2009). Incision results of liver histology *C. carpio*

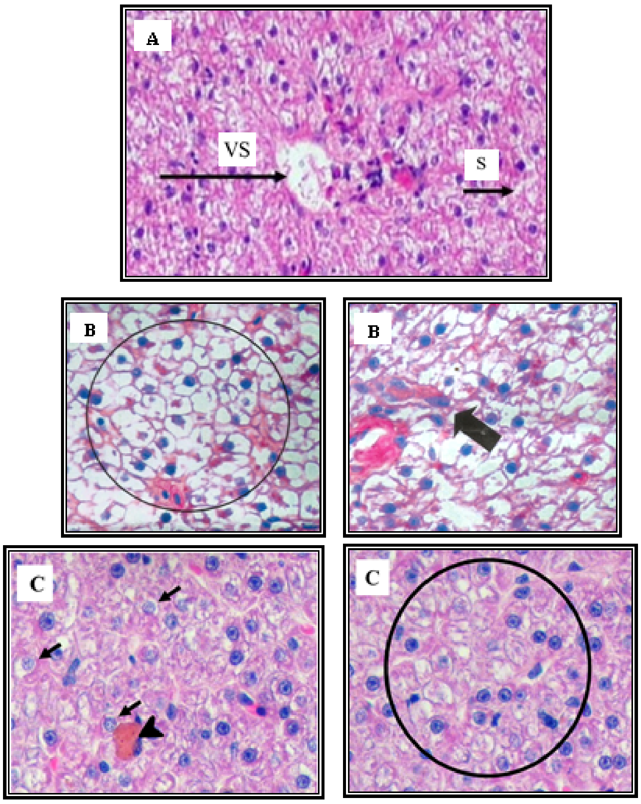

Figure 2. Histological alteration of liver of *C. carpio* after leachate treatment. **[A]** Histologic of liver with no leachate exposure (control), showed vena centralis (VS) and sinusoid (S) (scale: 1,56 cm = 5,524 μm); **[B]** Histologic of liver after leachate exposure of 80 ppm, showed hydropic degeneration (circle) and karyorrhexis (arrow) (scale: 0,66 cm = 1,385 μm); **[C]** Histologic of liver after leachate exposure of 100 ppm, showed karyorrhexis (arrow), melanomacrophage centre (big arrow), and hydropic degeneration (circle) (Scale: 0,84 cm = 1,385) μm).

exposed to leachate with concentrations of 0 ppm showed normal liver histology with characteristics as described previously and it can be seen in the Figure 2.A.

The observation of liver exposed to leachate concentration of 80 ppm showed that there was a change in the form of karyorrhexis cells and hydropic degeneration, while at a concentration of 100 ppm, there was a change in the form of karyorrhexis cells, melanomacrophage center (MMC), and hydropic degeneration. Histology liver with leachate exposure to 80 ppm can be seen in Figure 2.B, whereas changes in fish liver cells with exposure to 100 ppm leachate can be seen in Figure 2.C.

In fish liver cells exposed to leachate concentration of 80 ppm and 100 ppm, there was karyorrhexis found, which is considered as a marker of cell death. Cell death can be defined as a permanent loss of plasma membrane integrity. One type of cell death is apoptosis, which can be determined by the changes in the morphological characteristics of nuclei, one of which is fragmentation (karyorrhexis). These changes occur prior to the disappear of plasma membrane integrity (Golstein & Kroemer, 2006). Karyorrhexis is one of the characteristics of cell death characterised by condensation and rupture of the nucleus into particles or nucleus fragmentation (Lescher, 2011). Karyorrhexis can easily be recognised when the nucleus fragmentation process has been completed along with all parts of the destroyed cell nucleus, as fragments will usually appear with irregular shapes (Obe, 1994).

Karyorrhexis is expected to happen to fish due to low oxygen level in the water, called hypoxia. Hypoxia or lack of oxygen is a phenomenon that occurs in aquatic environments where dissolved oxygen (DO; molecular oxygen dissolved in the water) level reduces, affecting aquatic organisms (Mallya, 2007). This is supported by Geng's statement (2003), which stated that decreasing oxygen level in tissue can cause lesions in the form of necrosis and apoptosis in the organ.

Fish liver cells exposed to leachate 80 ppm and 100 ppm were also shown hydropic degeneration. Hydropic degeneration occured due to a disturbance of active transport that has caused inability of the cell to pump out Na^+ ion, resulting the Na^+ ion concentration in the cell to increase. It affects the process of osmosis that causes an influx of water into the cells, causing the cell to swell as the vacuole and nucleus were getting enlarged, granulars in the nucleus can also be seen quite clearly (Robbins et al., 2007).

Hydropic degeneration was also found in liver cells due to the content of ammonia in the leachate. This is supported by the research conducted by Benli et al. (2008), which indicated that the Nile Tilapia (*Oreochromis niloticus* L.) exposed to ammonia has a form of cell changes as hydropic degeneration happens in liver cells. The liver becomes the main organ of various metabolic pathways. Toxic influence of chemicals in general occurs primarily in the liver. Ammonia can be carried away by bloodstream into the liver as nutrient and follow the metabolic pathways (Kucuk, 1999, in Benli et al., 2008). Ammonia and alkali are a strong indicator of toxicity in aquatic environments. Although ion ammonia (NH_4^+) cannot penetrate the cell wall of organism, it can be potentially toxic to fish with the form of molecules (NH_3) which can easily penetrate the tissue, especially when the concentration is quite high (Svobodova et al., 1993 in Emenike et al., 2012).

Melano-Macrophage Centre (MMC) was only found in fish liver cells exposed to 100-ppm leachate concentration. Melano-Macrophage is a type of immune cell that is typical Teleostei and generally found in the spleen. This is phagocytes containing a number of pigments, including melanin (dark brown), hemosiderin, ceroid or lipofuscin (pink yellow to golden brown) located in the vacuole. MM and MMC were also found in the kidney and liver. The form of MMC is considered as a cleaner structure but its role in the immune system is still ambiguous (Genten et al., 2009).

C. carpio exposed to leachate concentration of 100 ppm indicated the presence of MMC in liver cells, which was related to stress and unhealthy condition of the fish due to the leachate exposure. This was in line with Gentens statement et al. (2009) that fish with chronic stress and in unsanitary conditions tend to have a lot more MMC. Leachate contains a number of organic contaminants, so it surely affects water quality and the organisms around. Goyer & Clarkson (2001) also stated that the low oxygen level combined with toxic substances can cause stress response in aquatic ecosystems.

The results of this study are expected to be used as an overview of the impact of an environmental pollution by leachate as indicated from histological damage to the gills and liver of *C. carpio*. So it can give information about the health risk of fish consumption for consideration in the future in better waste management, especially in terms of leachate treatment before being discharged into the water body. It also remains that carp supposed to regional ecological and economic importance in Indonesian.

CONCLUSIONS

The result of this study showed that the greatest damages happened in the leachate exposured to a greater concentration, i.e., 100 ppm. Leachate with concentrations of 80 ppm causes damage in the form of lamella secondary fusion and hyperplasia of epithelial cell in the gills, along with karyorrhexis and hydropic degeneration on the liver. Leachate concentration of 100 ppm causes damage in the form of lamella secondary fusion, hyperplasia of epithelial cell, congestion, and edema in the gills, along with karyorrhexis, hydropic degeneration, and melanomacrophage centre on the liver.

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