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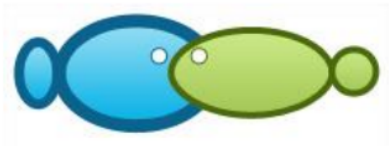

The effect of mercury chloride in different salinities on the histopathology of juvenile *Oreochromis niloticus*

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Abstract. The increased concentration of heavy metals, especially mercury chloride, in the aquatic environment results in toxicity for the life of aquatic organisms, including Nile tilapia (*Oreochromis niloticus* L.). This study aimed to determine the histopathological damage of heavy metal mercury (Hg) exposure in different salinities to juvenile Nile tilapia. The research method was an experimental study with a Completely Randomized Design (CRD), using five different treatments with three replications. The histopathological damage of the gill and skin of Nile tilapia were determined. Data analysis conducted in this study consisted in the Kruskal-Wallis test with the reflection scoring method and continued with the Mann-Whitney U test. The results of histopathological examination of the gills and skin showed damage in the gills, including edema, hyperplasia, hemorrhage and necrosis. The highest damaging effect of mercury chloride exposure on gills, especially hyperplasia, was in treatment A, with 0 ppt salinity and 0.016 mg L^{-1} of chloride. Meanwhile, the histopathological observations in the skin did not show significant differences between treatment groups. Mercury chloride exposure in different salinities had an effect on the histopathological damage of gill organs and the skin of Nile tilapia. The results also showed the potential of salinity to suppress the toxicity of mercury chloride and prevent damage to the gills and skin of Nile tilapia.

Key Words: gills, histopathology, Nile tilapia, $HgCl_2$, skin.

Introduction. Industrial, agricultural, and mining developments facilitate the increased metal pollution in the environment, even in upstream waters and tributaries, among others (Sericano et al 1995). The high concentration of heavy metals in the aquatic environment can be toxic to the life of aquatic organisms. Mercury, as one of the heavy metals found in the environment, is neurotoxic and enters the aquatic ecosystem through industrial waste (Verma & Dwivedi 2013). Mercury levels in freshwater naturally range from 10 to 100 µL, whereas in marine waters it ranges from <10 to 30 µL (Moore 1990). Mercury that accumulates in the body of aquatic animals will damage or stimulate the enzymatic system. As a result, it causes a decrease in the adaptability of these animals to polluted environments. In fish, the organs that accumulate the highest mercury content are the kidneys, liver and gills. The toxicity of heavy metals that injure gills and other external tissue structures can cause the death to the fish. Increasing temperature, decreasing pH, and decreasing water salinity can lead to greater levels of heavy metal bioaccumulation (Wang & Wong 2003). Other studies also suggest that the toxic effects of mercury are influenced by salinity (Modassir 2000), and that metal concentrations will increase with decreasing salinity (Connell & Miller 1984).

Accumulation of heavy metals in the gills can result in changes in the metabolism and function of some enzymes, thus, disrupting the work function of the gills. In addition, the gill tissue damage is characterized by cells experiencing necrosis, hypertrophy and edema (Önning et al 1996). The microscopic image of an organ that experiences necrosis shows changes in tissue color (the color becomes paler) and in tissue consistency (the consistency becomes softer). High levels of mercury in waters are found in developing countries, including Indonesia.

The mercury content in the Surabaya river, Indonesia, increased from 0.0011 to 0.0049 mg L^{-1} in 2001 (Yuniar 2009), and from 0.018 to 0.062 mg L^{-1} in 2011 (Fauziyah 2012). The high level of mercury is not only polluting the waters, but it can also accumulate in the body of fish and other aquatic biota. This can be seen in cases of mercury pollution in Minamata Bay, Japan, where the mercury content in coastal shells in non-polluted areas ranged between 1.7 and 6 mg L^{-1}, while in Minamata Bay it ranged from 11 to 39 mgL^{-1} (Yuniar 2009). Mercury that enters the waters can easily be tied to the chlorine in seawater. Subsequently, mercury enters the body tissues of living organisms through several paths, including the respiratory tract, digestive organs, and skin. Mercury that enters the body cannot be digested, and will be dissolved in fat. Fat-soluble metals are capable of penetrating cell membranes, so eventually mercury ions will accumulate in cells and other organs. The highest accumulation is usually in detoxifying organs (liver) and excretory organs (kidneys) (Dallinger et al 1987). The interaction of fish with its environment affects the digestion, osmoregulation, respiration, reproduction and metabolism. If the condition of the water experiences changes because of pollutants (in this case heavy metals) that enter continuously for long periods of time, it will cause changes in the immune system, blood, organs and other tissue structures in the fish (Hardi 3 Handayani 2015).

Nile tilapia (*Oreochromis niloticus*) is a freshwater fish species farmed in aquaculture, being classified as a fish with a high tolerance to the environmental parameters (Mahasri et al 2018; Mukti et al 2019; Soegianto et al 2017; Zainuddin et al 2017). Furthermore, Environmental Protection Agencies (EPA) suggested the use of Nile tilapia as test animals (Utami et al 2018) in different experiments. This species meets the requirements of extensive distribution, wide cultivation, a high ability to tolerate bad environmental parameters and is easily maintained in the laboratory (Rahim & Tuiyo 2015). In general, fish species have the ability to avoid the effects of water pollution. However, fish that live in limitative habitats (such as rivers, lakes and bays) cannot escape the effects of pollution. Based on these problems, a study was conducted to determine the effect of exposure to mercury (Hg) on Nile tilapia in different salinity concentrations on some organs, especially gills and skin.

Material and Method

Preparation of Nile tilapia and rearing conditions. This research was conducted in January - March 2017. The experiment was carried out for 30 days, with an acclimatization period of three weeks beforehand. Histopathological preparations and observations were carried out at the Laboratory of Anatomy and Microbiology, Faculty of Fisheries and Marine, Airlangga University, Surabaya, Indonesia. The tilapia were obtained from the Pandaan Freshwater Cultivation Service Unit, Pasuruan, Indonesia. The experiment used a total of 90 fish, with a length between 7-8 cm. The fish were transported to an aquarium from the Practical Laboratory. Before the fish were placed in the test media, selection was carried out to separate defective fish from healthy fish. Acclimatization was carried out for one week after the fish was placed in fresh water, then one week in 10 ppt salinity and another week in a salinity of 20 ppt. This was conducted so that fish do not experience stress due to sudden changes in salinity levels. In the process of acclimatization, the fish were fed a standard diet. Acclimatized fish were transferred to the test aquarium according to the salinity prepared beforehand, after a fasting period of 24 hours.

Determination of mercury chloride dosage. The concentration of mercury chloride used was obtained from the results of research conducted by Yuniar (2009). The study states that the safe concentration for Nile tilapia juveniles is 0.016 mg L^{-1} (Yuniar 2009). Dissolution of powdered mercury chloride is carried out in water with a concentration of 10 ppm to produce a stock solution. Afterwards, the stock solution of mercury chloride was dissolved by using Aquadest with a dose of 0.016 mg L^{-1}.

The calculation of heavy metal mercury chloride with standard manufacturing solutions was carried out using molecular weight calculation, molarity, and dissolution.

The weighing process was conducted with analytical scales before mercury was added to the media. Then, the mercury powder was dissolved in distilled water, according to the calculations.

Determination of salinity. The salinity concentrations used were 0 ppt, 10 ppt and 20 ppt. The concentration selection was based on the concentrations that Nile tilapia find in the aquatic environment. The process of making saline water implied diluting seawater in fresh water.

Histopathological analysis. The histopathological preparation was conducted after exposure to mercury. Before being dissected, the fish were monitored. The gills and skin were surgically removed. Subsequently, the organs were inserted into sterile containers and soaked with 10% neutral buffered formalin (BNF) for at least 24 hours.

The results were analyzed using a scoring method, the Pantung method (Table 1), which aimed to determine the organ damage with scores from 0 to 3, depending on the level and extent of changes that occur due to mercury exposure. The histopathological symptoms observed were edema, hyperplasia, hemorrhage and necrosis. The percentage of the scoring values used are presented in Table 1.

Table 1

The scoring value of histopathological changes in juvenile Nile tilapia (*Orechromis niloticus*)

Observed parameter	*Score 0 (normal)*	*Score 1 (mild)*	*Score 2 (moderate)*	*Score 3 (severe)*
Edema	Absent	Less than 30% of the observed area	30% -70% of the observed area	More than 70% of the observed area
Hyperplasia	Absent	Less than 30% of the observed area	30% -70% of the observed area	More than 70% of the observed area
Necrosis	Absent	Less than 30% of the observed area	30% -70% of the observed area	More than 70% of the observed area
Hemorrhage	Absent	Less than 30% of the observed area	30% -70% of the observed area	More than 70% of the observed area

Note: source - Pantung et al (2008).

Experimental design. This study used a completely randomized design with three treatments and a control and five replications, with different salinities and the same mercury chloride ($HgCl_2$) exposure. The treatments in this study were: control (0 ppt salinity without mercury chloride); A (0 ppt salinity with mercury chloride); B (10 ppt salinity with mercury chloride); and C (20 ppt salinity with mercury chloride).

Water quality. The juveniles of Nile tilapia were maintained in an aquarium with 15 L of water. During the research, the water quality examinations were carried out twice a day, in the morning and evening. The temperature, dissolved oxygen (DO) (mg L^{-1}), pH, ammonia (mg L^{-1}) and salinity (ppt) were measured in the morning and evening. The optimal temperature for Nile tilapia is between 25-30°C.

Statistical analysis. The histopathological results of the study were analyzed using a Kruskal-Wallis test (one-way ANOVA) to find out the differences between the samples. The Mann-Whitney U test followed.

Results and Discussion

Survival rate. The survival rate of Nile tilapia in water with mercury chloride decreased (Table 2). No fish survived in treatment A after 30 days.

Table 2
Survival rate of juvenile Nile tilapia (*Oreochromis niloticus*) exposed to mercury chloride in different salinities

Treatment	*Survival rate in all repetitions (%)*					*Σ*	*×±SD*
	1	*2*	*3*	*4*	*5*		
A	0	0	0	0	0	0	0
B	60	40	50	40	10	40	16.7
C	50	30	50	60	60	50	10.95

Test results of histopathological scoring for gills in juvenile Nile tilapia. It can be concluded that there were significant differences between all treatments in terms of hyperplasia, hemorrhage and necrosis (Table 4). There was no significant difference in the treatments for edema. A normal score has values between 0 and 0.75. A low damage score has values between 0.75 and 1.5. Higher scores, between 1.5 and 2.25, show a medium damage, while high damage presents scores ranging from 2.25 to 3.

Table 3
Histopathological damage of gills in Nile tilapia (*Oreochromis niloticus*) exposed to $HgCl_2$ in different salinities

Treatment	*Edema*	*Hyperplasia*	*Hemorrhage*	*Necrosis*
Control (0 ppt)	1.1^{a}	0.9^{a}	1.2^{a}	0.4^{a}
A (0 ppt + 0.016 mg L^{-1} $HgCl_2$)	1.7^{a}	2.4^{b}	2.1^{b}	1.1^{ab}
B (10 ppt + 0.016 mg L^{-1} $HgCl_2$)	1.7^{a}	1.8^{b}	1.6^{ab}	1.3^{ab}
(20 ppt + 0.016 mg L^{-1} $HgCl_2$)	2.0^{a}	2.1^{b}	2.1^{b}	1.4^{b}

Note: different superscripts in the same column show significant differences ($P<0.05$).

The average damage scores regarding hyperplasia in different treatments show that the control treatment was significantly different from treatments A, B and C, which did not present differences among themselves. There were no differences regarding the edema scores between treatments. In terms of hemorrhage, it can be seen that treatments A and C showed no differences, but were significantly different from the control treatment, while treatment B was similar with all other situations. There were no differences in necrosis scores among A, B and C treatments, but the control treatment was significantly different from the C treatment, while showing similar results with the ones from A and B. Based on the damage observation on juvenile Nile tilapia due to mercury chloride exposure in 0 ppt, 10 ppt and 20 ppt salinity, there were visible damages to the gills. The gill damage can be seen in Figure 1.

Edemas were found in the form of perfect spheres with fluid inside. Hyperplasia was found in all treatments, however, in different amounts and with different levels of damage. In the control, there was no necrosis in the gills. It can be said that the damage caused by exposure to mercury chloride is relatively mild. In addition to hyperplasia and edema, the presence of hemorrhages in the gills was also observed in all treatments, but with a different degree of damage.

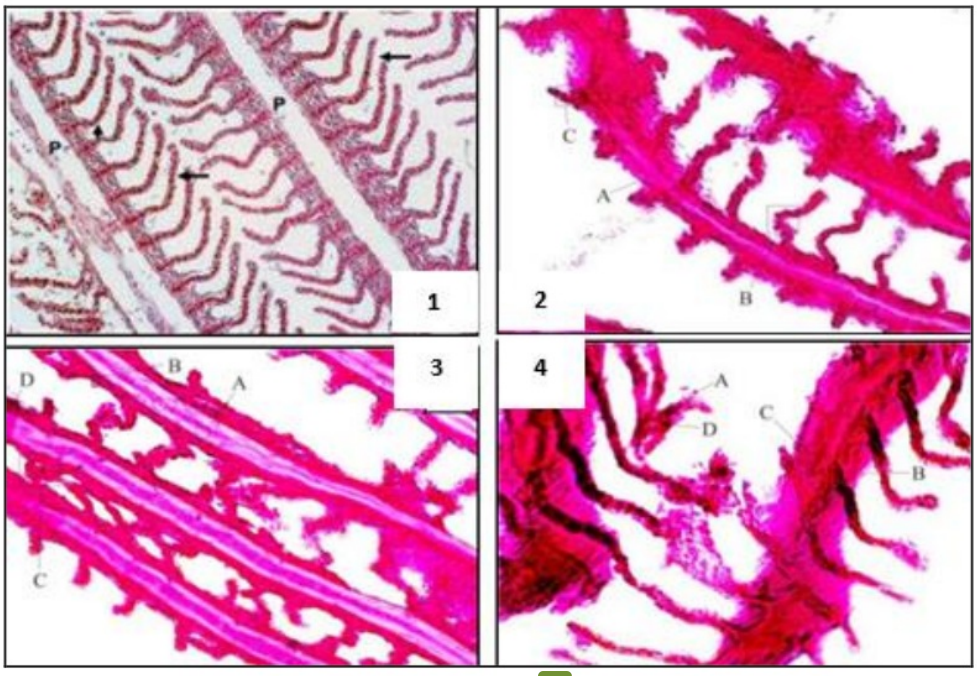

Figure 1. The histopathological section of gills in Nile tilapia (*Oreochromis niloticus*) exposed to $HgCl_2$ in different salinities. 1 - normal gills (Sharon & Zilberg 2012); 2 - treatment A (0 ppt salinity + 0.016 mg L^{-1} $HgCl_2$); 3 - treatment B (10 ppt salinity + 0.016 mg L^{-1} $HgCl_2$); 4 - treatment C (20 ppt salinity + 0.016 mg L^{-1} $HgCl_2$). A - edema; B - hyperplasia; C - hemorrhage; D - necrosis.

Damage in the skin of Nile tilapia. It can be concluded that there was no significant difference between treatments (Table 4). A normal score has values between 0 and 0.75. A low damage score has values between 0.75 and 1.5. Higher scores, between 1.5 and 2.25, show a medium damage, while high damage presents scores ranging from 2.25 to 3.

Table 4

Histopathology of skin damage of juvenile Nile tilapia (*Oreochromis niloticus*) exposed to $HgCl_2$ in different salinities

Treatment	*Edema*	*Hyperplasia*	*Necrosis*
Control (0 ppt salinity)	1.5^a	1.5^a	1.2^a
A (0 ppt salinity + 0.016 mg L^{-1} $HgCl_2$)	1.5^a	2.1^a	1.8^a
B (10 ppt salinity + 0.016 mg L^{-1} $HgCl_2$)	2.1^a	1.7^a	1.3^a
C (20 ppt salinity + 0.016 mg L^{-1} $HgCl_2$)	1.7^a	1.3^a	0.7^a

Note: the same superscript in the same column shows no significant difference ($P>0.05$).

Table 4 shows that the average scores of edema, hyperplasia and necrosis damage in the skin in all situations presented no significant differences. Histopathological observations determined that there was damage to the skin of juvenile Nile tilapia exposed to mercury chloride in all treatments and control. The skin damage produced by mercury chloride exposure in different salinities can be seen in Figure 2.

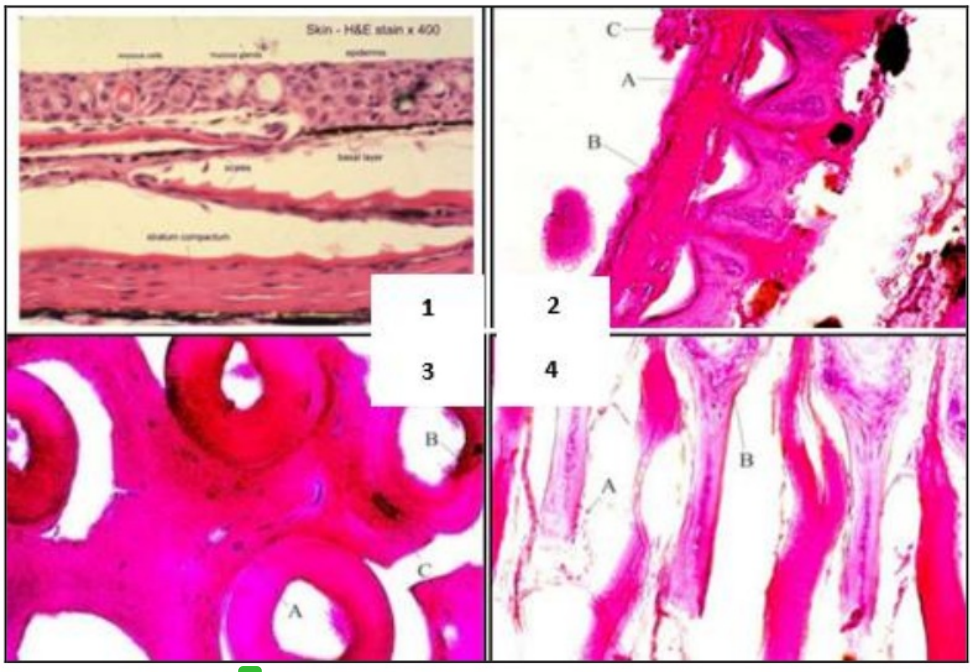

Figure 2. Skin section of juvenile Nile tilapia (*Oreochromis niloticus*) exposed to $HgCl_2$ in different salinities. 1 - normal skin (Poleksic et al 2010); 2 - treatment A (0 ppt salinity + 0.016 mg L^{-1} $HgCl_2$); 3 - treatment B (10 ppt salinity + 0.016 mg L^{-1} $HgCl_2$); 4 - treatment C (20 ppt salinity + 0.016 mg L^{-1} $HgCl_2$). A - edema; B - hyperplasia; C - necrosis.

Based on Figure 2, the results of observations regarding skin damage due to $HgCl_2$ exposure in different salinities showed that edemas, hyperplasia and necrosis were present. In treatment C no necrosis was found, meaning that no cell death occurred in the skin of juvenile Nile tilapia. However, edema was found in the form of increased fluid in the tissue. The lack of salinity triggered cell damage until necrosis in treatment A, with $HgCl_2$ exposure to a salinity of 0 ppt and 10 ppt. In treatment C there were only changes or damage in the form of edemas and hyperplasia.

Water quality. Water quality parameters include temperature, DO, pH, ammonia, and salinity. The parameter values are presented in Table 5. The range of the parameters of water quality during the exposure period was still in the optimal range for the life of juvenile Nile tilapia.

Table 5

Water quality during the experiment

Water Quality Parameter	*Control*	*A*	*B*	*C*
Temperature (°C)	25.5-30	25.5-30	25.5-30	25.5-30
Salinity (ppt)	0	0	10	20
pH	5.8-8.9	5.5-9.1	5.7-9.2	5.8-9.2
Ammonia (mg L^{-1})	0-0.5	0-0.5	0-1	0-0.5
Dissolved oxygen (mg L^{-1})	4.8-5.6	4.14-5.09	4.88-5.06	4.6-6

Histopathological damage was observed in all treatments, including the control. This is presumably due to water having no exchange during the maintenance period. This is in line with the results of a previous study, stating that the accumulation of heavy metals

from water in the fish is influenced by water quality (Purnomo & Muchyiddin 2007). However, the water quality during the experiment was generally in the optimal range for the maintenance of Nile tilapia.

The results of the histopathological examinations of the gills and skin showed damage. Damage in the gills included edemas, hyperplasia, hemorrhage and necrosis. Gill damage is in normal to low levels. The initial damage that occurs is hyperplasia, which develops into edemas and hemorrhages. However, the treatments did not cause severe necrosis in the gills of juvenile Nile tilapia. When water flows in through the gills, the brachial filaments stretch, so that water and pollutants have a direct contact with the lamellas, entering the blood vessels and damaging the other organs, even in low levels (Gerking 1974). The initial damage that occurs to the gills continues to grow during the exposure to mercury (Yuniar 2009). Another factor that can cause damage to the gills, namely hyperplasia, which then develops into hemorrhage and necrosis, is ammonia derived from excretion (Zeitoun et al 2016).

The histopathological observation of the skin showed no significant differences between treatments. $HgCl_2$ exposure caused damage to the skin in the form of edemas, hyperplasia and necrosis. The skin was damaged by $HgCl_2$ exposure through the diffusion process. The cells from an organ affected by toxic substances will degenerate and eventually die (Roberts 2012). $HgCl_2$ enters the skin through pores. $HgCl_2$ binds to proteins and forms metallotioneins, which are carried by blood and released into the body (Darmono 1995). The blood vessels in the skin are located under the bottom layer of the skin, so that the process of heavy metal absorption through the skin becomes slower and the damage is usually not very severe. Only a few heavy metals that enter through the skin accumulate in the body, mainly because the skin has mechanisms through which it can carry out detoxification and excretion processes. Thus, the influence of toxic properties can still be tolerated where xenobiotics in the body will stimulate physiological resistance to minimize the effects of toxins (Connell & Miller 1984). Similar results are observed in studies conducted by Poleksic et al (2010) in *Acipenser ruthenus* L., suggesting that heavy metals can affect skin histopathology. Samples from *A. ruthenus* showed changes in the epidermal layer, without changes in the dermis and hypodermis layers. Damage of 25% was only found in the outer part of the skin, with severe hemorrhage.

$HgCl_2$ exposure had the most harming effects in the gills compared to the skin in treatment A, especially in hyperplasia damage. Hyperplasia can cause blood vessels to narrow and blood will accumulate in the constriction area. More blood accumulates in the blood vessels, which will rupture due to not being able to resist the pressure, resulting in hemorrhage (Flores-Lopes & Thomaz 2011). This is presumably due to exposure to heavy metals and because of high levels of salinity resulting in disruption of respiration and osmoregulation. $HgCl_2$ can result in cell swelling or edema in the gills or excessive accumulation of fluid in the body tissues, characterized by the release of the base membrane and narrowed lacuna cells. These cause the gills to experience deficiencies in function, further causing breathing difficulty. The metabolism is also disrupted (Fitriawan et al 2011). This lack of oxygen results in hypoxia. Fish will stimulate the binding of red blood cells, hematocrit and hemoglobin to increase the mechanism of oxygen transfer in the body (Affonso et al 2002), but in the presence of mercury, the binding process will be disrupted. Previous studies showed a faster mercury uptake in killifish fish (*Fundulus heteroclitus*) under higher salinity. Hg concentrations accumulate more at higher salinities (Dutton & Fisher 2011).

Treatments B and C showed a decreased damage due to the higher salinities. It is suspected that salinity is able to suppress damage caused by $HgCl_2$ in the gills of Nile tilapia. Decreasing salinity will increase the toxicity of heavy metals (Sullivan 1977). In fresh water and water with low salinity, uncharged hydroxide complexes and chloride ions are formed, while in high salinity, Hg will form a negative complex with chloride ions, where molecules that do not change are more easily delivered to the membrane. Bioavailability from mercury to methyl increases with decreasing salinity levels (Ullrich et al 2001). High salinity causes an increase in chloride ion formation, which results in a decrease in the concentration of heavy metal ions in the waters due to the reaction of

these metal ions with chloride ions (Mance 2012). When compared with treatment B and C, treatment A experienced a decrease in the survival rate of fish in the second week of maintenance. *Clarias bathracus* exposed to mercury for 30 days presented changes in the form of necrosis and degeneration of the gill epithelium, with hyperplasia, hemorrhage and death (Selvanathan et al 2013).

The survival rate of juvenile Nile tilapia in the treatments decreased. The highest survival rate was 80%, in the control aquarium. As for the lowest survival rate, a 0% survival rate was observed in treatment A with a salinity of 0 ppt and mercury chloride.

Conclusions. It can be concluded that exposure to $HgCl_2$ (0.016 mg L^{-1}) in different salinities had an effect on the histopathology of the gills and skin of juvenile *O. niloticus*, damages appearing in the form of edemas, hyperplasia, hemorrhage and necrosis. The exposure to 0.016 mg L^{-1} mercury chloride at 0 ppt salinity affected the skin, while gills were damaged in both 0 ppt and 10 ppt salinity. At 20 ppt salinity, the decrease in damage occurrs along with the increasing salinity. In general, Hg adsorption decreases significantly when salinity is high. Thus, salinity is able to suppress toxic levels and damage caused by mercury chloride exposure.

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