

Sublethal Toxicity Effects of the Organochlorine Insecticide Endosulfan on Oxygen Consumption Level and Gill Histopathology of Tilapia (*Oreochromis Niloticus*) Fry

Adista Fadilah^{1*} and Boedi Setya Rahardja² 

¹Aquaculture Department, Faculty of Fisheries and Marine Sciences, Universitas Airlangga, Surabaya

²Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Universitas Airlangga, Surabaya

*Corresponding author: adistafdl11@gmail.com

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Abstract

The uncontrolled use of organochlorine insecticides in the agricultural sector has an impact on water pollution and threatens the lives of organisms in it. Pollutants can reduce water quality and interfere with the performance of fish organ systems, triggering stress and even death. The purpose of the study was to determine the sublethal effects of the organochlorine insecticide endosulfan on the level of oxygen consumption and histopathology of tilapia seed gills. The research was conducted at the Faculty of Fisheries and Marine Science, Universitas Airlangga, from March to May 2021. This study used a completely randomized design (CRD) experimental method. The treatment used was exposure to organochlorine endosulfan doses of 10%, 30%, 50%, and 70% with LC50-96 hours, which was observed for 28 days. Oxygen consumption data were analyzed by Analysis of Variance, followed by DMRT (Duncan's Multiple Range Test). While gill histopathology data were analyzed descriptively by comparing normal and abnormal gill histopathology. The results showed that the sublethal toxicity of the organochlorine insecticide endosulfan had a significant effect ($p < 0.05$) on the level of oxygen consumption and had an impact on the histopathological condition of the tilapia seed gills. The types of gill damage found were oedema, hyperplasia, lamellar fusion, hemorrhage, and necrosis.

Keywords: Endosulfan organochlorine, tilapia, oxygen consumption level, gill histopathology

INTRODUCTION

The use of pesticides in the agricultural sector is inevitable in this era of technological advancement. Because it is considered efficient and easy to use by farmers in Indonesia to control pests on plants (Andesgur, 2019). Organochlorines are a class of chemical pesticides that are dangerous because of the active ingredient endosulfan but are still commonly used. Uncontrolled use of pesticides can pollute rivers and lakes, making fish toxic and fatal (Putri et al., 2017). Fish species from various locations have been contaminated with organochlorines such as pesticides and polychlorinated biphenyls (PCBs) (Ding et al., 2019). Moreover, endosulfan pesticide exposure was found in the deaths of dozens of black reef sharks in Cun Ming fish breeding pond in Jepara

(Wahyu, 2019). *Oreochromis niloticus* is one of the valuable fishery products that is potentially exposed to organochlorine endosulfan effluents that accumulate in water bodies. This is because river water in Indonesia is commonly used to raise *O. niloticus* (Taufik et al., 2009). *O. niloticus* can be used as an environmental bioindicator because it is adaptive to environmental changes (Jamin and Erlangga, 2016). Organochlorine endosulfan residues in water can enter the body of fish through the gills. Where, it will naturally accumulate and cause damage to the structure of the gill tissue, which interferes with the respiration and metabolism of the fish (Damayanty and Abdulgani, 2013; Fernandes et al., 2013). Changes in the aquatic

environment will directly or indirectly affect the structure and function of the gills, resulting in respiratory disorders and death (Saputra et al., 2013). Based on this, research is needed on the measurement of oxygen consumption levels and histopathological examination of the gills of tilapia fry to see more specific impacts of organochlorine endosulfan contamination on tilapia.

MATERIALS AND METHODS

Place and time

The research was carried out in March - May 2021 at the Anatomy and Cultivation Laboratory and Microbiology Laboratory, Faculty of Fisheries and Marine Affairs, Airlangga University, Surabaya.

Materials and tools

A total of 400 tilapia fry measuring 10 - 12 cm were obtained from the UPT Balai Benih Ikan Penataan Pasuruan, East Java, and used in this study. Organochlorine endosulfan Akodan 35EC™ with active ingredient endosulfan 350 g/L, chlorine as disinfectant, pelleted feed, fixative (10% Formalin Neutral Buffer Solution), desalination solution (nitric acid), graded alcohol (70%, 80%, 96%, absolute alcohol), clarifying agent (Xylol), paraffin, Haematosilin and Eosin dye, adhesive (gelatin), and distilled water were the materials used. Meanwhile, the equipment used consist of 20 aquariums with dimensions 50x30x30 cm, aerator set, acclimatization tank, catches, measuring jug, dropper pipette, 1 ml syringe and spatula. Furthermore, tools to measure the level of oxygen consumption include DO meter, ruler, digital scales, Aquarium with a capacity of 1 L (as respire meter tube), plastic wrap, duct tape, and scissors. Histopathological examination of the gills using Sectio set, tray, sample bottle, object glass, cover glass, water bath,

tissue cassette, base mold, tissue embedding, microtome, staining tube, binocular microscope, label paper, stationery, and Optilab camera. The tools used to measure water quality are DO meter and pH meter.

Research design

The method used in this study is a completely randomised design (CRD), with 5 treatments and 4 replicates. Tilapia seeds were treated with exposure to different concentrations of endosulfan organochlorine insecticide to determine the level of oxygen consumption and histopathological picture of the gills. The endosulfan concentrations given to each treatment were P0 as control (0%), P1 (10%), P2 (30%), P3 (50%) and P4 (70%) with LC50-96 hours (12,795 µg/L) observed for 28 days.

Media Preparation

Twenty-sized aquariums used for tilapia fry testing were cleaned using soap by brushing the bottom and edges, then rinsed with clean water, and then soaked for 24 hours in chlorinated water. After that, the soaking water is discarded, and the aquarium is rinsed with clean water until it is odorless. Next, the aquarium is dried in the sun for 24 hours in an upside-down position (Ausia, 2020; Lutfiyah et al., 2020). The dried aquariums were then labeled for each treatment. After drying, each aquarium was filled with 20 L of water from the treatment tank before use to minimize contaminants (Abidin et al., 2006). Then aeration was installed in each aquarium to provide an oxygen supply during the period of rearing tilapia fry.

Sample preparation

Before use, the seeds are selected based on size, healthy and without defects to avoid cannibalism and susceptibility to disease (APHA, 2014).

The seeds are also acclimatized first for 7 days in a large tank equipped with aeration. The seeds are also given food in the form of pellets 2 times a day every morning and evening at ad satiation. Next, the seeds are ready to be put into the testing aquarium.

Solution test generation

The dose of endosulfan used must be adjusted to the desired concentration in each treatment. For this reason, it is necessary to dilute first by making a stock solution and then determine the volume of the stock solution taken to be added to each treatment aquarium using the following dilution formula (Zarkasi, 2019).

$$V1 \times N1 = V2 \times N2$$

Information:

V1 = Volume of diluent solution (water)

N1 = Concentration of the stock solution resulting from dilution

V2 = Volume of pesticide required

N2 = Initial concentration of pesticide

The test solution in this research was made by diluting a concentrated solution of endosulfan 350 g.L⁻¹ with the addition of water. Dilution is carried out to obtain a smaller solution concentration, namely in µg.L⁻¹.

Toxicity Test

In this test, fish fry were exposed to the organochlorine insecticide endosulfan for 28 days at a sub lethal dose (Zarkasi, 2019). All labeled aquariums were filled with fresh water that had been settled with a volume of 20 L of water each. Then diluted to make a stock solution, followed by taking insecticides from the stock solution using a syringe according to the calculated dose and put into each aquarium according to the treatment label. Next, 20 tilapia fry that had been

acclimatized and selected were put into the test aquarium at a density of 1 fish.L⁻¹ (SNI: 01-6141-1999). During exposure, the aquarium was aerated and the fish were fed pellets twice a day in the morning and evening on an ad satiation basis. Water changes were carried out every 2 days at 100% using a semi static water change system, which is an alternative that can be used to improve the accuracy of toxicity test results (Taufik and Setiadi, 2012).

Oxygen consumption

Oxygen consumption data was conducted once every 7 days during the test period. The oxygen consumption rate of fish was measured using the closed respire-meter method (Novita et al., 2011). A closed respire-meter is made from an aquarium filled with water of a certain height (Lukman, 2016). Fish oxygen consumption was calculated from the ratio of dissolved oxygen in the respire-meter tube at the beginning (DO₀) and at the end (DO_t) of the observation after the fish were put into it (Pratiwi, 2013). Dissolved oxygen measurements were carried out using a dissolved oxygen meter. The oxygen consumption rate was calculated by considering the net weight of the fish, the volume of water in the tube, and the length of observation. The respire-meter tube used in this study was a one litre capacity aquarium. Oxygen from outside the tube must be avoided by covering the top with plastic wrap according to the area of the lid and taping the edges until they are airtight. Each measurement of fish oxygen consumption requires an hour of observation time (Edwin et al., 2018). The oxygen consumption data that has been obtained is then calculated using the equation according to (Kadarini, 2009) to obtain the oxygen consumption value as follows.

$$OC = \frac{V \times (DO_o - DO_t)}{W \times T}$$

Information:

OC = Oxygen consumption ($\text{mgO}_2 \cdot \text{g}^{-1} \cdot \text{hour}^{-1}$)

V = Volume of water in the container (L)

DO_o = Dissolved oxygen concentration at initial observation ($\text{mg} \cdot \text{L}^{-1}$)

DO_t = Dissolved oxygen concentration at final observation ($\text{mg} \cdot \text{L}^{-1}$)

W = Weight of test fish (g)

T = Observation period (hours)

Gill Histopathology

Preparations and histopathological observations of the gills were made at the end of the study by randomly sampling the gills of tilapia fry from each treatment. Gill sampling was done by dissecting the operculum using a section set. Then the gill samples were preserved by immersing them in 10% Neutral Buffered Formalin (BNF) fixative solution for two days. After fixation, decalcification is done to soften the tissue by soaking in nitric acid solution for several days. After that, it is rinsed with running water to remove the fixation material. Next, the tissue was cut into ± 5 mm thick using a scalpel and inserted into tissue cassettes. A number of tissue cassettes containing gill tissue were put into an automatic processing device for a gradual dehydration process for 2 hours using graded alcohol, namely 70%, 80%, 96% alcohol. After that, the tissues were placed in a clarifying solution (xylol) to remove the alcohol so that the tissues became transparent. The tissue sample is then infiltrated (filling the pores) by placing it in liquid paraffin in an incubator at a temperature of 56° - 60°C for 2 hours. Followed by embedding using a tissue embedding tool with the help of a stainless steel

prints (base prints) filled with hot liquid paraffin, then covered with a tissue cassette and labelled. Then the paraffin block is placed on the ice board until the paraffin freezes.

After freezing, the paraffin block is removed from the prints, then the tissue is sliced using a microtome (4-5 μm thickness). The results of the tissue incision are placed briefly in warm water in a water bath to expand the paraffin, then taken and placed on a glass object, and dried briefly. Continue tissue staining using the HE (Hematoxylin and Eosin) staining technique for several minutes. Then the gluing (mounting) process involves dripping entelan directly onto the thin slice of tissue preparation that has been colored and then covering it with a cover glass. After that, the results of the preparations were observed under a light microscope with a magnification of 40x - 400x connected to an Optilab camera to determine the changes and types of damage that occurred in the gill tissue of tilapia fry due to exposure to the organochlorine endosulfan.

Data analysis

Data from oxygen consumption calculations analyzed using Analysis of Variance and Duncan's Multiple Range Test. While, histopathology data on the gills were analyzed descriptively by comparing the histology of normal gills and gills with abnormalities.

RESULTS AND DISCUSSIONS

Oxygen consumption rate

The average level of oxygen consumption of tilapia fry in each treatment obtained from observations is presented in (Table 1). Based on the research results and statistical test analysis (ANOVA), it was shown that the sub-lethal toxicity of the organochlorine insecticide endosulfan exposed for 28 days had a significant

effect ($P < 0.05$) on the increase in oxygen consumption levels of tilapia fry. Where, on days 7, 14 and 21, it showed that treatment P0 was significantly different ($p < 0.05$) from all treatments. Likewise, P3 is significantly different from treatments P0, P1, P2 and P4.

Meanwhile, between P1 and P2 there is no significant difference ($p > 0.05$) but both are significantly different ($p < 0.05$) with P0, P3 and P4. Meanwhile, on day 28 it showed that P0 was significantly different ($p < 0.05$) from all treatments. Meanwhile, between P1 and P2 there is no significant difference ($p > 0.05$) but both are significantly different ($p < 0.05$) with P0, P3 and P4. Likewise, between P3 and P4 there is no significant difference ($p > 0.05$) but both are significantly different ($p < 0.05$) with P0, P1 and P2. The higher the dose of the

organochlorine insecticide endosulfan given, the higher the level of oxygen consumption of tilapia fry, this occurred on observation days 7, 14, 21 and 28.

The increase in oxygen consumption each week occurred on P1, P2, P3 and P4. This is different from the control treatment which can be said to be normal because it tends to be more stable, where there is only a slight increase or decrease in oxygen consumption which is not significant. Under normal conditions fish maintain oxygen consumption at a constant level according to the environmental oxygen concentration gradient (Neelima et al. 2016). This is because there is no exposure to the organochlorine endosulfan in the control treatment so that the water quality is quite good and the fish can live normally.

Table 1. Average value of Oxygen Consumption Level for Tilapia fish seeds in each treatment

Treatment	Oxygen consumption ($\text{mgO}_2 \cdot \text{g}^{-1} \cdot \text{hour}^{-1} \pm \text{SD}$)			
	Day -7	Day -14	Day -21	Day -28
P0	0,504 ^a ± 0,052	0,536 ^a ± 0,043	0,547 ^a ± 0,064	0,496 ^a ± 0,034
P1	0,578 ^b ± 0,029	0,657 ^b ± 0,056	0,738 ^b ± 0,053	0,778 ^b ± 0,044
P2	0,624 ^b ± 0,022	0,716 ^b ± 0,029	0,763 ^b ± 0,118	0,828 ^b ± 0,068
P3	0,688 ^c ± 0,011	0,834 ^c ± 0,040	0,901 ^c ± 0,087	1,052 ^c ± 0,117
P4	0,749 ^d ± 0,049	0,932 ^d ± 0,041	1,080 ^d ± 0,106	1,184 ^c ± 0,150

Description: P0 (control), P1 (10%) P2 (30%), P3 (50%), P4 (70%). The dose of material in the form of organochlorine endosulfan was observed from LC50-96 hours. Different superscripts in the same column indicate significant differences ($p < 0.05$).

The increase in oxygen consumption of tilapia fry in all treatments exposed to the organochlorine endosulfan in sub-lethal concentrations is thought to be due to the fish experiencing environmental shock and then stress. As a result of the accumulation of insecticide poisons in the water, it becomes more active to combat stress, resulting in increased energy needs. To meet this need, there is an increase in oxidative metabolism in the fish's body, where this reaction involves the role of oxygen so that the need for oxygen increases. An increase

in metabolic rate means an increase in oxygen demand by tissues which causes the gill respiration process to become faster, resulting in increased oxygen consumption (Budiardi et al., 2005). Under stress conditions, fish oxygen consumption tends to increase until a critical oxygen concentration limit is reached, and after that oxygen consumption decreases gradually (Neelima et al., 2016). Continuous fluctuations in the level of oxygen consumption over a long period of time disorganize the work of the respiratory

system due to damage to the structure of the gill tissue and end in death. However, in this study the level of gill damage caused by exposure to the organochlorine insecticide endosulfan was still relatively mild so it did not immediately reduce tilapia oxygen consumption but was still at a gradual increasing stage.

Gill Histopathology

Exposure to the organochlorine insecticide endosulfan causes changes in the structure of the gill tissue of tilapia fry. Based on the results of observations, several gill damages were found in the primary lamella and secondary lamella, including edema, hyperplasia, lamella fusion, haemorrhage and necrosis (Figure 1-5). There was a visible difference in the appearance of the gill tissue of tilapia fry between the control treatment (P0) and the treatment exposed

to the organochlorine insecticide endosulfan (P1, P2, P3, P4). In the control treatment (P0) without administration of the organochlorine endosulfan, it showed that there was no damage to the gill tissue of tilapia fry. So, the gill parts are still intact and clearly visible. Meanwhile, the condition of the gills in the treatment exposed to organochlorine endosulfan with concentrations of P1 (10%), P2 (30%), P3 (50%) and P4 (70%) from LC50-96 hours showed an abnormal picture of gill tissue with the presence of several damage to the primary lamella and secondary lamella of the tilapia gills. In P1 there was tissue damage in the form of hyperplasia and lamella fusion, in P2 there was edema and lamella fusion, in P3 there was edema and haemorrhage, in P4 there was necrosis, lamella fusion and hyperplasia

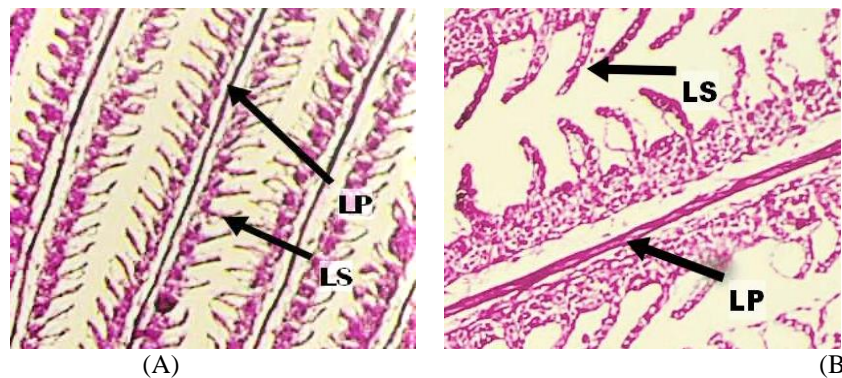


Figure 1. Histology of gills of Tilapia fry in the control treatment. HE is staining. (A) Normal gills, 40x magnification. (B) Normal gills, 100x magnification. LP = Primary lamella; LS = Secondary lamella.

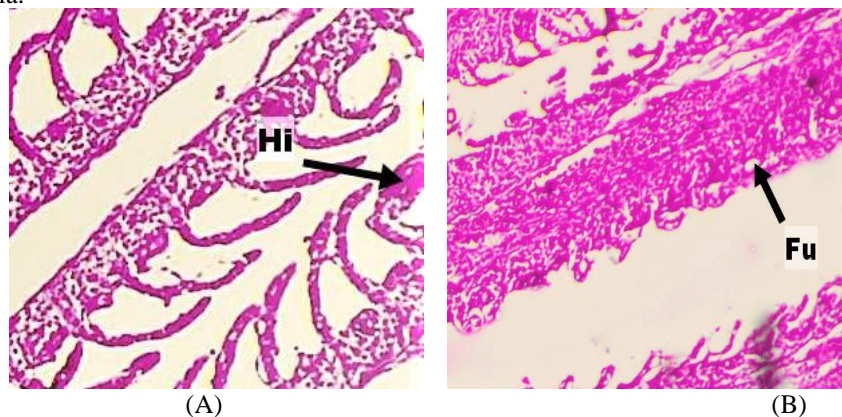


Figure 2. Histopathology of gills of Tilapia fry in treatment 1 (10% x LC50-96 hours). HE is staining. (A) Hi = Hyperplasia, 100x magnification. (B) Fu = lamella fusion, 100x magnification.

Edema is the swelling of cells due to the accumulation of excess fluid in body tissues due to changes in the cell membrane permeability system (Susanah, 2011). The appearance of oedema is caused by blockage of blood flow to the gill lamellae due to direct contact with contaminants, thus disrupting gill function (Wulan, 2017). In this study, edema was found in the gill tissue of tilapia seeds, namely in the P2 and P3 treatments. The presence of edema in the gill tissue is included in the category of mild damage which indicates that contamination has occurred but no

contamination has occurred (Edwin et al., 2018). Hyperplasia is the formation of excess tissue due to an increase in the number of cells in the tissue so that the secondary lamella thickens. Cases of hyperplasia or thickening of the gill tissue in this study were found in the P1 and P4 treatments. Hyperplasia found in P1 was at the end of the secondary lamella, while in P4 hyperplasia was found in almost all secondary lamella. Hyperplasia makes the gill lamellae appear larger than normal, which is a symptom of contamination (Wulan, 2017).

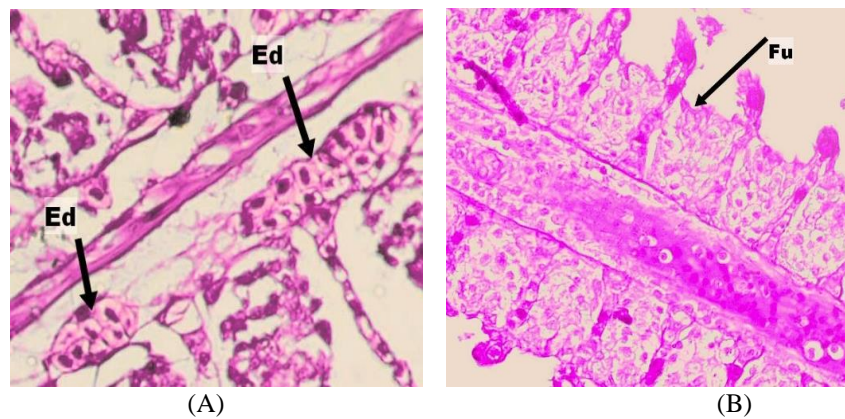


Figure 3. Histopathology of gills of Tilapia fry in treatment 2 (30% x LC50-96 hours). HE is staining. (A) Ed = Edema, 400x magnification. (B) Fu = lamella fusion, magnification 400x.

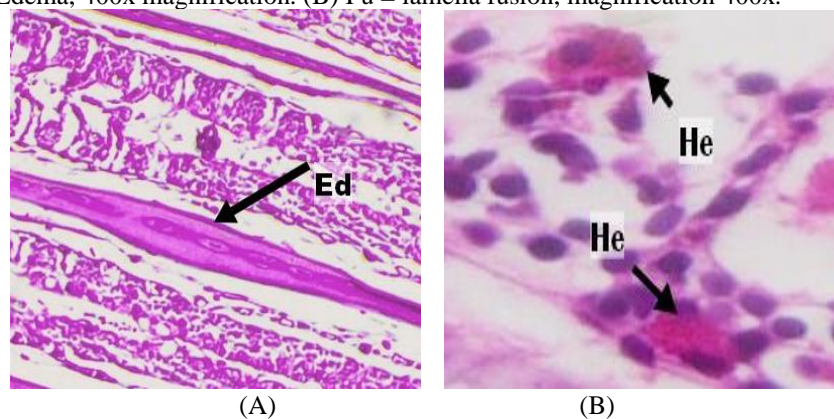


Figure 4. Histopathology of gills of Tilapia fish fry in treatment 3 (50% x LC50-96 hours). HE is staining. (A) Ed = Edema, 100x magnification. (B) He = Haemorrhage, magnification 400x.

Lamella fusion was the most frequent type of tissue damage found in this study. Where it was found in treatments P1, P2 and P4. Fusion is caused by an increase in the number of mucus cells which is a defense reaction due to interference from

toxic substances or parasites (Edwin et al., 2018). Fusion between two secondary lamellae occurs due to a continuous increase in hyperplastic pathology and causes the space between the secondary lamella to be filled with new cells which then triggers the

attachment of the two sides of the lamellae (Aliza et al., 2013). This results in disruption of the function of the secondary lamella in the gill respiration process because the space containing erythrocyte cells for gas exchange is closed by pathological secondary lamella epithelial cells (Wulan, 2017).

Fusion of the secondary lamella indicates initial level pollution (Susanah, 2011). Haemorrhage or bleeding is a condition where erythrocytes come out of the capillaries and collect at the base of the gill filaments which can be characterized by the presence of reddish spots on the blood vessels (Novalia et al., 2013). The occurrence of haemorrhage in the gill lamella is due to direct contact with toxic substances in the water, resulting in irritation which causes high osmotic power of blood vessels and the release of blood capillary fluid (Ramdhani, 2017). Toxic substances such as pesticides that enter the fish's body cause limitations in ion transport in the blood due to rupture of blood cells due to damage to the blood capillary

walls (Wulan, 2017). The hemorrhage cases found in this study were in the P3 treatment.

Necrosis in a simple sense is the death of cells in living body tissue (Susanah, 2011). Cells that experience necrosis will separate from their supporting tissue after the blood supply is lost and encourage proliferation to replace new cells (Haqqawiy et al., 2013). The characteristics of necrotic tissue are that the color is paler than normal, loss of stretchability and faded cells where the core shape becomes smaller and the cytoplasm is lost so that it does not absorb the dye given in the process of making histology preparations (Edwin et al., 2018). According to Ramdhani (2017), necrosis can be caused by exposure to toxic pollutants that enter the gills and pass through the lamella, resulting in disrupting the function of the gills in the respiration process due to damage to the gill tissue so that it no longer looks intact. Damage in the form of gill necrosis in this study was found in the P4 treatment.

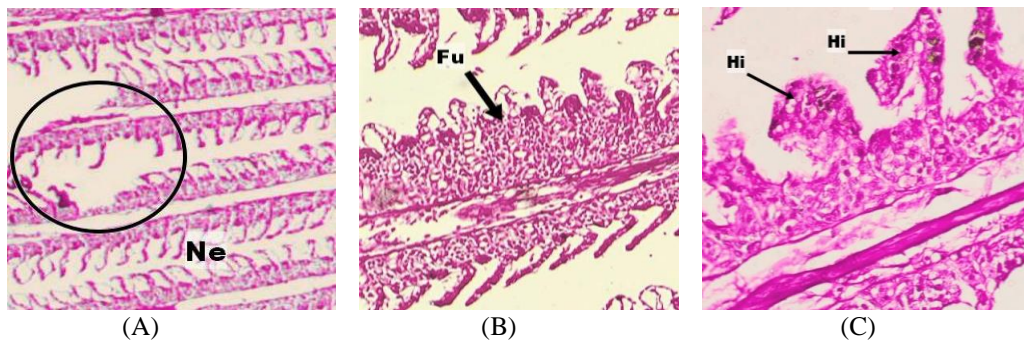


Figure 5. Histopathology of gills of Tilapia fry in fourth treatment (70% x LC50-96 hours). HE is staining. (A) Ne = Necrosis, 40x magnification. (B) Fu = lamella fusion, 100x magnification. (C) Hi = Hyperplasia, magnification 400x.

The changes that occur in the aquatic environment directly or indirectly have an impact on the structure and function of gills (Wulan, 2017). Polluted waters affect the condition of the gill lamellae, namely causing inflammation and swelling due to the presence of foreign

objects (Ramdhani, 2017). This is because the gills are the main respiratory organs which work by the mechanism of oxygen and carbon dioxide diffusion between blood and water so that they are very sensitive to environmental changes, therefore, changes in the micro

anatomical structure of the gills can be used as an indicator of environmental pollution, starting from the occurrence of contamination, mild level to severe level (Wulan, 2017).

CONCLUSION

There is a sub-lethal toxicity effect from administering different concentrations of the organochlorine insecticide endosulfan on the level of oxygen consumption and changes in the histological condition of the gills of tilapia fry. Where at P1 with the lowest concentration of 10% of LC50-96 hours (0.07 ml) it was able to gradually increase tilapia oxygen consumption and cause damage to gill tissue, namely Edema, hyperplasia, lamella fusion, hemorrhage and necrosis.

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