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The effect of chlorpyrifos insecticide on the histological structure of wader pari fish intestine (*Rasbora lateristriata* Bleeker, 1854)

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Abstract. *Chlorpyrifos is one of the commonly used insecticides. However, the use of insecticides in agriculture has negative impacts including lethal effects on non-target organisms. Wader pari fish is a type of freshwater fish that lives in rice fields or rivers and can be used as an indicator of water pollution. The purpose of this study was to determine the intestinal histological structure, the histopathological effect of chlorpyrifos on the intestine, and the population number of goblet cells, respectively. The treatment concentration of 0.001; 0.005; and 0.01 ppm were determined by preliminary trial and exposed for 168 hours. Fish behavior and water quality were monitored. The fish were still alive until the last day of exposure were then made intestinal histological preparations using the paraffin method and staining with Haematoxylin Eosin (HE), Mallory Acid Fuchsin (MAF), Periodic Acid Schiff (PAS). The results showed that the histological structure of the wader pari intestine consisted of mucosal tunica, submucosal tunica, muscularis tunica, and serous tunica. Furthermore, chlorpyrifos has a significant effect on the population number of goblet cells. The intestine damages were edema, vacuolization, cloudy swelling, hemorrhage, lysis, and fusion of vili. It could be concluded that chlorpyrifos caused intestinal damage in wader pari fish.*

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INTRODUCTION

Indonesia is a country that has good agricultural prospects. To increase the productivity of agricultural products, farmers use insecticides to control plant pests. However, the use of insecticides in agriculture caused negative impacts such as environmental pollution, accumulation in agricultural products, and toxic effects on non-target organisms (Wisudanti *et al*., 2019). The results of research evidence this by Taufik *et al*. (2003), that there was contamination of endosulfan insecticides in irrigation canals and waters body in Brebes Regency, Central Java with concentrations of 2.7 and 3.2 g/L, respectively.

Chlorpyrifos is a common insecticide that is often used in agriculture. Chlorpyrifos is an organophosphate insecticide, its active ingredient is very toxic to invertebrates. Moreover, chlorpyrifos is considered very toxic and very harmful to non-target organisms (Sutamihardja *et al*., 2015). The water flows in the field wash and will carry the chlorpyrifos residue away and cause pollution to the water body. Polluted waters will greatly affect the aquatic organisms living in them, one of which is fish.

Fish that inhabit contaminated water bodies tend to absorb and accumulate insecticides, which then cause bioaccumulation of active insecticide ingredients such as chlorpyrifos in the fish body tissues. The insecticide

intake into the fish body could occur through the respiratory, digestive, and integumentary systems, respectively. The accumulation concentration of insecticide residues in the fish's tissues tends to increase along with a longer exposure duration (Taufik *et al*., 2002).

The tissues bioaccumulation of chlorpyrifos at certain concentrations could induce the alteration of histological structure on fish's organs (Taufik, 2011). According to Watson *et al.* (2014), exposure to organophosphate pesticides showed physiological changes on Zebrafish and Xenopus embryos, such as a decrease in the number of heartbeats per minute. In addition, Stalin *et al.* (2019) showed that exposure to chlorpyrifos insecticide caused behavioral and histopathological changes in various organs of *Channa punctatus*.

Insecticides also cause structural changes or damage as well as functional disorders of organ systems, one of which is the intestine. The intestine is a part of the digestive organ that plays an important role in food digestion and absorption. Intestine histological structure alteration will result in the cause disruption of nutrient absorption, causing less than optimal fish growth and development (Sulastri *et al*., 2018).

Wader pari fish (*Rasbora lateristriata* Bleeker, 1854) is an Indonesian endemic freshwater fish species that have an important role in wild habitat and high economic value as culinary fish for the community (Figure 1). Moreover, the fish also can be used as a water pollution bioindicator (Retnoaji *et al*., 2016). The fish naturally live in rivers and rice fields with clear and slow water currents. In addition, this fish is also very sensitive to environmental changes (Purwanto *et al*., 2014).

Figure 1 Wader pari fish (*Rasbora lateristriata*) (Sentosa and Djumanto, 2011)

Research on the effect of the chlorpyrifos on non-target organisms such as *R. lateristriata*, especially on its histological organ structure, has not been carried out, currently. Therefore, the study aimed to determine the effect of chlorpyrifos exposure on the histological structure of the *R. lateristriata* intestine as well as the number of goblet cells in the intestine. The general histological study of species has been conducted in our study (Nita, 2021), while here we want to highlight our deep review and histological analysis.

METHOD

Adaptation and Maintenance of The Fish

This research was conducted for seven months, starting in August 2020-March 2021 at the Laboratory of Animal Histology and Embryology, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta. The larvae were obtained from eggs that resulted from the laboratory cadapted spawning. The eggs then hatched and were kept for one month. Wader fish larvae were raised and maintained in a pond. Feeding was done twice a day in the morning and afternoon in *ad libitum* with high-protein pellets until one month old.

Water Quality Monitoring

In this research, water quality measurements were also carried out. Water quality was monitored including physical parameters such as pH, DO, and temperature. Each of these parameters was measured every day.

Materials

Chlorpyrifos (Dursban 200 EC) with a concentration of 200 000 ppm, distilled water, fish feed (Takari), label paper, Bouin's solution, series of ethanol concentration, distilled water, toluene, xylene, paraffin, Mayer's Albumin, Ehrlich Haematoxylin, Eosin Y 1%, Fuchsin Acid 0.1% solution, PMA (Phosphomolybdic Acid)

1% solution, Mallory's solution, 1% Alcian Blue solution, 1% Periodic Acid solution, Schiff's solution, entelan, glassware, cover glass, and tissue.

Preliminary Test

Preliminary tests were carried out as a range-finding test, with treatment set as follows: fish media only as control and chlorpyrifos solution of 0.001, 0.01, 0.1, 10 ppm, following Leuwol *et al*. (2018). Each concentration was put into 5 plastic cups of 250 ml with two replications. The 1-month-old larvae were randomly selected for a group of 10 larvae and were put into each treatment set and were exposed for 72 hours. The survival rate was calculated on 24 hours basis. At the end of exposure, the fish were euthanized and proceeded for the next analysis.

Chlorpyrifos Treatment

Based on the preliminary result, the chlorpyrifos concentration used in this study was 0.001, 0.005, and 0.01 ppm, respectively, with three replications each. The 1-month-old larvae were selected for 5 larvae, which were put into each treatment, and exposed to the solution for 168 hours. Media were changing on a daily basis for a total of 75% of total media volume. This media change was carried out to maintain a healthy environment, as well as to ensure sufficient oxygen supply and to prevent the accumulation of metabolic waste for fish larvae. The survival rate and fish behavior were observed every day. The percentage of survival rate was calculated using the following formula (Erhana and Retnoaji, 2020).

$$\underset{(\text{SR }\%)}{\textit{Survival Rate}} = \frac{\text{The final number of live fish}}{\text{Initial Number of Fish}} \times 100$$

The survival rate results were analyzed with linear regression to determine the LC_{50} value of 168 hours. In addition, water quality was monitored, including parameters of pH, DO, and temperature. At the end of exposure, the fish were euthanized and proceeded for the next analysis.

Intestinal Histological Preparation

Fish that survived for 168 hours were fixed with Bouin's solution and processed following standard paraffin method, with 5um of thickness and stained with Haematoxylin Eosin (HE), Mallory Acid Fuchsin (MAF) for histological structure analysis, and Periodic Acid Schiff with Alcian Blue (PAS-AB) for goblet cell quantification, under the Leica ICC 50 E series microscope.

Data Analysis

Qualitative data analysis was carried out for the histological structure of intestines and fish behavior. Whereas, quantitative data were presented as the number of goblet cells, which were analyzed with one-way ANOVA using IBM SPSS Statistics 21 software and further tested with Duncan's Multiple Range Test (DMRT) with a level of 5% to see the significance between each treatment and control.

RESULTS AND DISCUSSION

Water is a medium for the life of aquatic organisms. Therefore, its quality will affect and determine the ability of these organisms to perform all metabolism normally (Purwanto *et al*., 2014). In this research, water quality was measured every day including temperature, pH, and DO (Figure 2).

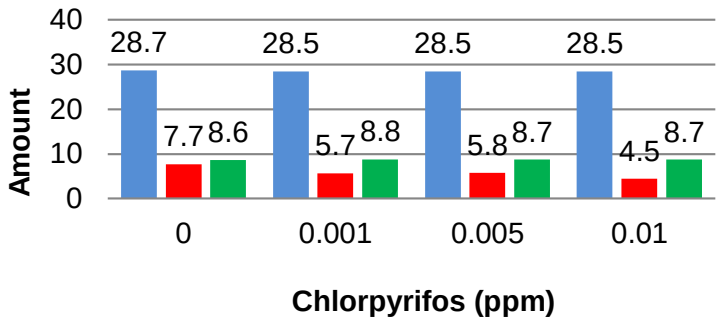

Figure 2 Water quality monitoring result for fish media showed the water physical properties during the experiment. Blue: temperature, Red: DO, Green: pH

The monitoring result showed that all parameters of water quality are always in suitable condition and the normal range for fish optimum need. The optimum temperatures are ranging from 20-30ºC, pH ranges from 6-8.5 and DO levels should be above 5 mg/ for aquatic organisms (Effendi, 2003; Papilon and Effendi, 2017). However, we found that at a concentration of 0.01 ppm, there was a decrease in dissolved oxygen (DO) levels a bit below 5. The Range Finding Test results obtained were presented through the following graph in form of the average percentage of survival fish larvae (Figure 3).

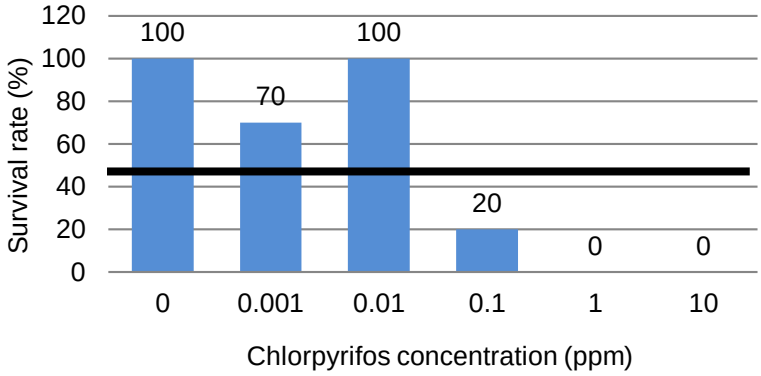

Figure 3 Percentage of the average number of live fish larvae in the preliminary test for 72 hours. The black line indicates the 50% limit of the fish survival rate

The result showed that the survival rate of the control: 0.001 and 0.01 ppm, were above 50%. Whereas concentrations of 0.1, 1, and 10 ppm caused the mortality of fish larvae more than 50% as shown in the graph (Figure 3). This indicates that the LC 50 concentration of chlorpyrifos ranged from 0 to 0.01 ppm. Four sub-lethal concentrations were used in the actual test of 0 (control): 0.001, 0.005, and 0.01 ppm, respectively. The results obtained from this exposure were presented in the following graph (Figure 4).

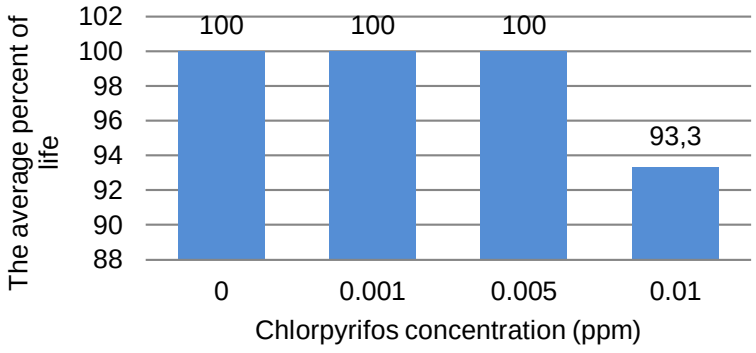

Figure 4 The average percentage of fish larvae that survived in the 168 hours actual test

The control and concentration treatment of 0.001, 0.005 ppm for 168 hours showed a fish survival rate of 100%. Fish mortality began to occur on the 0.01 ppm chlorpyrifos with a percentage SR of 93.3%. We then determined the actual LC_{50} value from these result, by analyzing the survival rate of the actual test with linear regression, and the LC_{50} value of 168 hours of chlorpyrifos on wader fish is 0.078 ppm, which mean that chlorpyrifos with a concentration of 0.078 ppm can kill 50% of the fish population for 168 hours exposure period. These suggest that the actual test concentration of control 0.001, 0.005, and 0.01 ppm were all below the LC_{50} 168 hours value and be considered as sublethal concentration.

Exposure to high concentrations of chlorpyrifos insecticide can cause fatality to fish. Chlorpyrifos is a neurotoxic organophosphate insecticide that affects the central nervous system impulses. Specifically, the pesticide makes nerve impulses from one synapse to another to be cut-off and no longer able to an impulse. Impulse cut-off was caused fish to experience paralysis and later will die.

The observation was conducted to determine the effect of chlorpyrifos exposure on the fish intestine. Histological structure of intestinal was observed following paraffin method with HE stains. According to Fizikri *et al*. (2018), the histological structure of the fish intestine has four layers, namely tunica mucosa, submucosa, muscular layer, and serous layer (Figure 5).

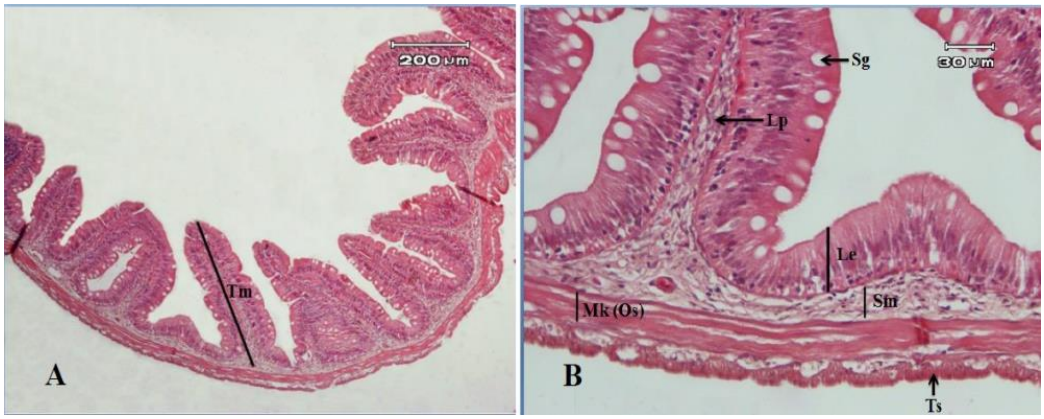

Figure 5 Histological structure of the intestine of *Tor tambroides*, namely: A, B. Proximal intestine. Image captions: Tunica mucosa (Tm), Tunica submucosa (Sm), Tunica muscularis (Mk), Tunica serosa (Ts), Lamina epithelium (Le), Lamina propria (Lp), Circular muscles (Os), Lymph nodes (Ln), Goblet cells (Sg). HE staining, 100 and 400x magnification (Fizikri *et al*., 2018)

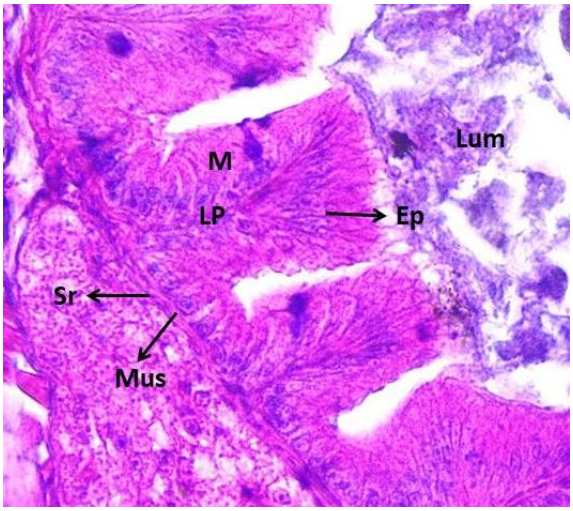

Figure 6 Histological structure of the intestine of control. Showing: Lum (Lumen), M (Tunic Mucosa), Mus (Tunic Muscularis), Sr (Tunic Serosa), LP (Lamina Propria), Ep (Columnar Epithelium). HE staining and 40x magnification

In the control treatment, the histological structure of the intestines consisted of mucosa, submucosa, muscular, and serous layers, respectively (Figure 6). The mucosa is directly adjacent to the lumen and consists of a layer of simple columnar epithelium, the lamina propria, and the muscularis mucosa. The propria layer is a loose connective tissue located on the inside of the basement membrane. There were intestinal villi, a mucosa projection towards the lumen, and intestinal crypts in the form of wells or valleys of the mucosa layer. The next layer is the submucosa which consists of loose connective tissue enriched with blood and lymph vessels. Moreover, the muscular layer was consisted of smooth muscle that is arranged circularly on the inside (stratum circulare) and longitudinally on the outside (stratum longitudinale). The outermost layer, the serous, consists of loose connective tissue, innervated with blood vessels and wrapped with adipose tissue The results suggest that wader fish intestinal structure follow the common intestine histological structure as described by Kierszenbaum (2002), Ereschenko (2008), Wallace *et al*. (2005) and Manganang *et al.* (2020).

Treatment with chlorpyrifos 0.001ppm caused histological structure change in fish's intestine, such as edema, hemorrhage, cloudy swelling, and lysis of cells, respectively. Cellular edema occurs due to an exccesive increase of intracellular fluid compartments in interstitial spaces of tissues organs, or body cavities, which causes the volume to increase, and swelling to occur (Guyton and Hall, 1996; Sulastri *et al*., 2018).

The cloudy swelling is the mildest form of cell degeneration in fluid accumulation on cells caused by fluid regulation mechanism disruption in the cells. The cell nucleus persists on cloudy swelling, while the cytoplasm usually appears cloudy and rough (Figure 7). The cloudy swelling may progress to cellular vacuolization if the situation gets worse. Meanwhile, cell lysis is a condition in which the cell ruptures and the cell nucleus disappears. Usually, the cell lysis stage will continue in the occurrence of necrosis (Sulastri *et al*., 2018). Overall, cell degeneration can cause cell function to be disrupted or altered (Rahayu *et al*., 2013).

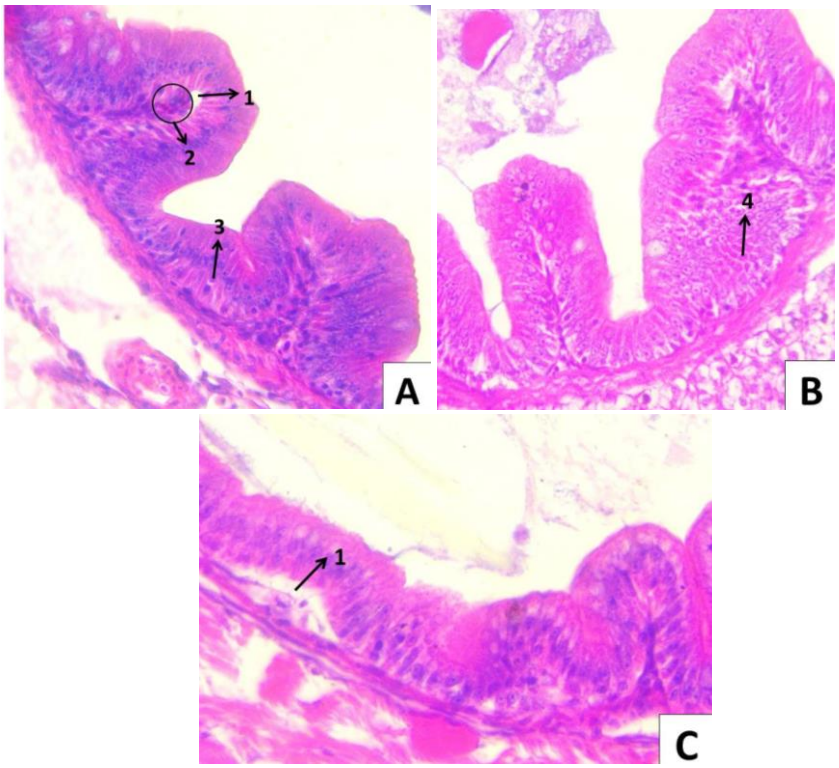

Figure 7 Histological structure of the intestine in 0.001 ppm treatment. Showing: 1. Edema, 2. Hemorrhage, 3. Swelling Cloudy, 4. Cellular Lysis. HE staining and 40x magnification

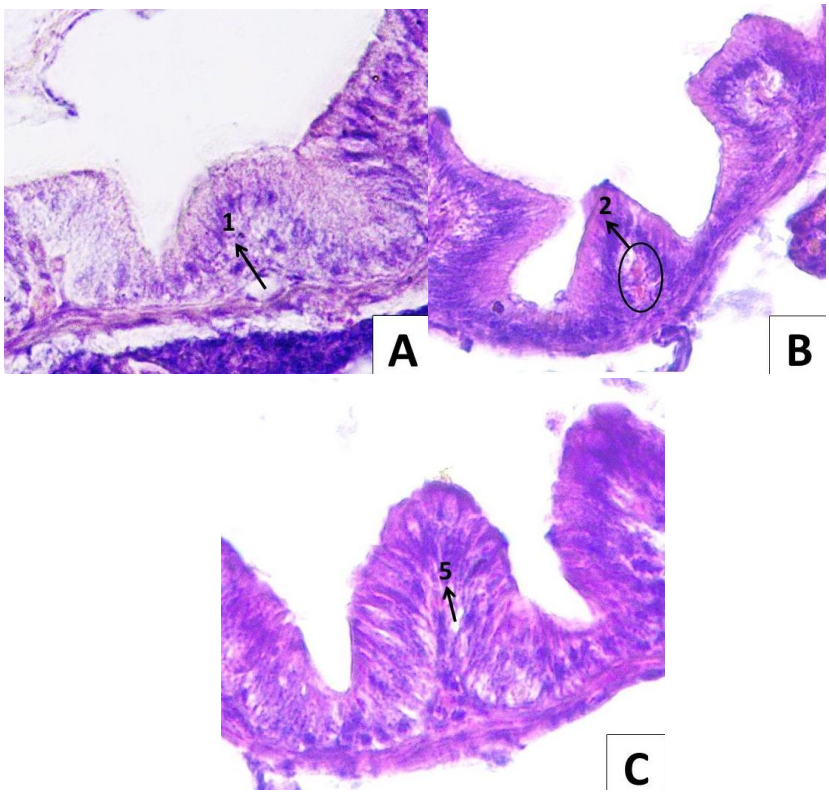

Figure 8 Histological structure of the intestine in treatment A, B, C (0.005 ppm). Showing 1. Edema, 2. Hemorrhage, 5. Vacuolization. HE staining and 40x magnification

Treatment with chlorpyrifos 0.005 ppm treatment result corresponds to 0.001ppm, only with greater effect with the occurrence of vacuolization on 0.005 ppm treatment (Figure 8). Vacuolization is one type of cells degeneration, caused by the intracellular accumulation of fluid as a result of disruption of the mechanism of fluid regulation in cells. Cell nuclei in vacuolization were absent or undergo lysis so that only the cytoplasm is visible, which appears clearer compared to the normal one (Rahayu *et al*., 2013). The Hemorrhage, tearing of blood vessel walls occurrence, was marked by the release of erythrocytes from the blood vessels to the tissue was also observed in this treatment. The presence of hemorrhage causes disruption of blood supply to epithelial cells and affects the organ normal function. The exposure of tissue to trauma or chemicals such as toxic substances could cause damage to capillary endothelium, as well as cause weak vascular walls so that blood vessels are prone to leaking or causing lesions in the intestine (Smith and Jones, 1961; Yudhi, 2014).

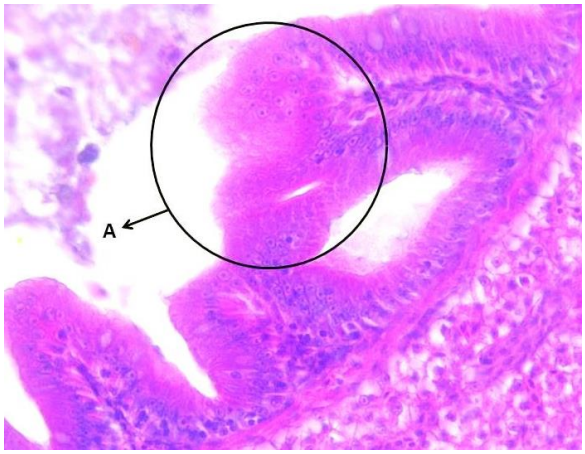

Figure 9 Histological structure of the intestine at 0.01 ppm treatment showing intestinal villi fusion (A), with HE staining and 40x magnification

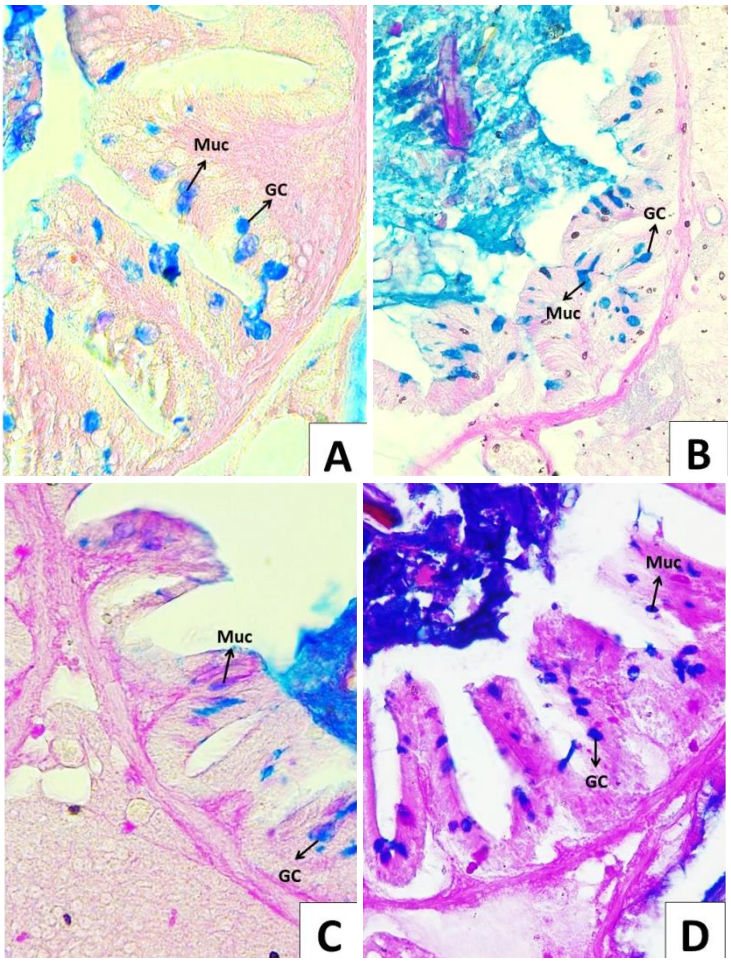

Figure 10 Histological structure of the intestine in each treatment (A) Control; (B) 0.001 ppm; (C) 0.005 ppm; (D) 0.01 ppm. Showing: Muc (Mucin), GC (Goblet Cell). PAS staining and 40x magnification

Treatment with chlorpyrifos 0.01 ppm caused severe intestine histological structure alteration such as fused villi on the intestinal mucosa (Figure 9). The villi fusion in the intestinal mucosa can interfere with physiological function, such as food absorption in fish, which causes the growth and development of fish to be impeded (Kumari and Ahirrao, 2018). The result is in line with the report of Stalin *et al.* (2019) on intestinal damage in villi fusion in freshwater fish *Channa punctatus*, which was exposed to chlorpyrifos for 20 days. The intestinal goblet cells number was counted with Periodic Acid Schiff (PAS) staining following Ng *et al*. (2005) (Figure 10).

The result showed that the intestinal goblet cells population in the intestine, which was stained blue with PAS staining, were affected by the chlorpyrifos exposure (Table 1). The result showed that the intestinal goblet cells number was significantly affected by the chlorpyrifos treatment. The result suggests the number of goblet cells was increased along with the increase in the concentration of chlorpyrifos, where the highest number of goblet cells was found in the 0.01 ppm chlorpyrifos treatment. The increase of goblet cells number in all chlorpyrifos treatments indicates the occurence of digestive system structural and physiological disturbance. Goblet cells are unicellular glands that play a role in secreting mucin. (Bloom and Fawcett, 1962). Mucin is a large glycoprotein that functions to protect and lubricate the epithelial tissue surface, assist food absorption, and transport molecules through the membrane (Andrianifahanana *et al*., 2006).

Table 1 Effect of chlorpyrifos insecticide on goblet cell count

Treatment	Goblet cells number
	Mean±SD
Control	13±2.64[a]
0.001 ppm	23±2.00[bc]
0.005 ppm	20±2.64[b]
0.01 ppm	25±2.00[c]

Remarks Numbers in columns with different letters showed a significant difference at $p < 0.05$ using Duncan's test

The results suggest that the goblet cells number increased in line with the concentration of chlorpyrifos, and the highest number of goblet cells was found in the 0.01 ppm treatment. The fluctuation of goblet cells population at both low and high concentrations of chlorpyrifos treated fish, indicates the occurence of the digestive system disturbance. Therefore, goblet cells number increase in the wader intestines is a defense response to protect epithelial cells and microvilli from chlorpyrifos exposure. According to Hernandez *et al*. (2009), an increase in the number of goblet cells is associated with intestinal activity, and in response to disturbances, to protect the epithelial layer and microvilli from mechanical damage, so that the food absorption could normally functional. Exposure to chemicals orally can disrupt the epithelial barrier which can cause inflammation and intestinal disorders (Gillois *et al*., 2018).

Behavioral change is the most sensitive indicator of potential toxic effects on fish (Mohammed *et al*., 2018). Result showed that chlorpyrifos exposed caused fish behavioral changes (Table 2). The result showed that fish swimming movements and feed responses were affected by chlorpyrifos exposed at the lowest (0.001 ppm) to the highest concentration (0.01 ppm). Chlorpyrifos is an organophosphate pesticide that is a neurotoxin, which inhibits the action of the acetylcholinesterase enzyme by reducing the activity of the acetylcholinesterase enzyme (AChE), caused nerve no longer able to transmit nerve impulses from one synapse to another and will cut off nerve impulses (Leuwol *et al*., 2018). Acetylcholine is hydrolyzed by acetylcholinesterase to choline. The inhibition of acetylcholinesterase can cause impaired impulse delivery resulting in decreased muscle coordination, tremors, convulsions, and incoordination (Rahayu *et al*., 2013). According to Arfiati *et al*. (2018), fish that were exposed to toxicants initially exhibit hyperactive movements, are paralyzed, and cause fatality. Moreover, animals contaminated with poison showed symptoms of stress, which were characterized by decreased appetite, less stable movements, and tended to be on the bottom.

Table 2 Behavior of ray wader fish (*Rasbora lateristriata*) during rearing

Treatment	Fish Behavior
Control	Moves active and normal, active (shortly after being fed fish immediately goes to the surface to eat) when fed
0.001 ppm	Moves aimlessly, body tilts to the side, looks weak, convulsions, fish waiting for food that sinks to the bottom of the water
0.005 ppm	Moves aimlessly, body tilts to the side, looks weak, convulsions, fish waiting for food that sinks to the bottom of the water
0.01 ppm	Moves aimlessly, the body tilts to the side, and sometimes swims upside down, looks weak, convulsing, fish waiting for food that sinks to the bottom of the water

CONCLUSION

This study concludes that the exposure of wader pari fish to chlorpyrifos insecticide disrupted the intestinal histological structure of the fish, in form of cellular edema, hemorrhage, cloudy swelling, vacuolization, lysis, and fusion of intestinal villi, in dosage dependant manner. In addition, chlorpyrifos

exposure also increased the population of the intestinal goblet cells of wader fish in a dosage-dependent manner.

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