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Toxicity of Indigo Dye-Contaminated Water on Silver Barbs (*Barbonymus gonionotus*) and Pathology in the Gills

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Abstract

Indigo dye is an organic compound derived from a fermentation process. The rinsing water of the indigo dye is contaminated by the acid and alkaline agents needed for precipitation. It would affect fish or other aquatic animals if the indigo dye-contaminated water is released into a public water body the environment will be impacted. The objective of this study is to investigate the toxicity of indigo dye on silver barb (*Barbonymus gonionotus*) mortality at the concentration that kills 50% of the fish (LC_{50}) and pathology in the gills. The exposure period was 96 hours. The fish were divided into 6 groups of 30 fries (weight 0.4 - 0.5 grams) and each was exposed to indigo dye-contaminated water (except the control group) with the concentration of indigo dye-contaminated water at 0.133, 0.150, 0.160, 0.167 and 0.171 µg/mL respectively. After 96 hours of exposure, the survival rates were lowest for the concentration of indigo dye-contaminated water at 0.160 µg/mL, 0.167 µg/mL and 0.171 µg/mL. The lethal concentration that kills 50% of the fish was 0.137 µg/mL. The pathology in the gills in all groups exposed to the indigo dye-contaminated water had altered. Conclusion, wastewater should be treated before releasing it into water bodies.

Keywords: Indigo dye; Silver barb; Toxicity

1. Introduction

True Indigo (*Indigofera tinctoria* L.) is a shrub in the Leguminosae family and is commonly known as Indigo or Indigotin. Indigo has a dark blue color with light blue tone. *Indigofera* is widely distributed throughout Africa, West America, and Southeast Asia. *Indigofera tinctoria* (Linn) can be found in Northeastern and Northern Thailand. It is the major plant that provides natural blue dye (Chauhan and Pandey, 2014). Recently, many people are interested in fabrics dyed in indigo. The processes of indigo dye-contaminated water have been developed to use indigo dye to generate income to families, communities, and community enterprises. The products are locally sold as well as exported. A report from the Community Development Department at Sakon Nakhon Province showed that there were altogether 120 groups of the indigo dyed fabric producers and enterprises in 2015. Indigo dyed fabric generates more than 48 million Baht per year to people in Sakon Nakhon (Duangbubpa *et al.,* 2015). The examples of indigo dyed fabric are pieces of cloth, apparels, and home decorated products. Though indigo dyeing process most relates to natural products, the fermentation process typically includes chemicals which help precipitate and settle the insoluble indigo pigment.

Consequently, if the indigo dye-contaminated water is directly released to natural water bodies, it will pollute the nearby environment.

Indigo dyeing process involves the extraction of indigo dye from the plant, the fermentation of indigo plants, and textile dyeing process. After indigo dyeing process, the rinsing water is contaminated by the acidity and alkalinity needed for precipitation. Adding calcium oxide (CaO) into the rinsing water increases alkalinity in the water which would affect fish or other aquatic animals if the wastewater is not treated before discharged into natural source of water. Srivastava *et al.* (1994) reported that wastewater released from textile and printing industrial sites was contaminated by suspended solids, organic compounds, heavy metals, and had high acidity and alkalinity. Textile dyeing wastewater is considered as one of the primary causes of environmental problems due to high concentration in water bodies inhibit the reoxygenation ability and cut off sunlight, disrupting biological activity in aquatic life as well as the photosynthesis process of aquatic plants or algae (Gita *et al.,* 2017). This also affected soil properties (Raj *et al.,* 1997; Chia and Musa, 2014), plants (Khandelwal, 1996; Raj *et al.,* 1997) and freshwater microalgae (Chia & Musa, 2014). Commercial textile dyeing process also adds various types of reducing agents such as alkaline solution, sodium dithionite ($Na_2S_2O_4$) or sodium sulfide (Na_2S), to change the indigo blue to leucoo-indigo (Bozic and Kokol, 2008). The reducing agents will impact environment when sodium dithionite reacts with oxygen. The oxidation reaction will change sodium dithionite to sodium sulfide, sulfate ion, and thiosulfate which is harmful to humans and the environment (Aino *et al.,* 2001). Toxic components in untreated wastewater released from textile manufacturers have an impact on freshwater ecosystems (Adeogun & Chukwuka, 2010) and lead to stress and behavioral changes in aquatic organisms, especially in fishes (Ogundiran *et al.,* 2010). The histological changes that occur in fish tissue have been used as biomarkers of various environmental stresses, especially fish gills which are primary site of toxic action due to their direct contact with the effluent concentrations during exposure and therefore are sensitive and suitable for eco-toxicity study for textiles and dyeing effluents (Gavilán *et al.,* 2001; Hutchinson *et al.,* 2006; Abdullahi & Ibrahim, 2021). Abdullahi & Ibrahim (2021) have studies on textile and dyeing effluents on African Catfish (*Clarias gariepinus*) showing inflammation of secondary lamella and deterioration of gill's central hyaline cartilage which exposed to high concentration of dyeing effluent. During the fermentation of indigo dye, some bacteria such as *Bacillus* sp., *Clostridium isatidis*, *Alkalibacterium* sp. can be found in the fermentation process (Park *et al.,* 2012; Li *et al.,* 2015; Padden *et al.,* 2000). These bacteria can cause diseases in fish or other aquatic animals. Consequently, if indigo dye-contaminated water is released to ecosystem, it will affect aquatic animals and the environment.

This research aims to investigate testing the effect of toxicity of indigo dye-contaminated water on silver barbs (*Barbonymus gonionotus*). Silver barb were selected because they are commonly found in natural resource. Their consumption behavior is typically on the water surface area and are quite sensitive to contaminated with toxic pollutants of the environment. The objective of this research is to study the lethal concentration level of the indigo dye-contaminated water that kills 50% of the fish (LC_{50}) and pathology in the gills after 96 hours of exposure.

2. Materials and Methods

The experimental protocols and animals were reviewed and approved by the Institutional Animal Care and Use Committee of Rajamangala University of Technology Isan, based on the Ethics of Animal Experimentation of National Council Research of Thailand (record RMUTI 5323/261 and reference ID proposal 10/63).

2.1 Animal preparation stage

A thousand silver barb fries weighing 0.4 - 0.5 grams obtained from private farms were used in the experiment. The fish fries were conditioned in a 5000 liters fiber tank for 7 days. Air pump was put throughout the experiment. One hundred and eighty fish fries were randomly selected and put into a glass tank of size 18×30×20 centimeters for the experiment with 3 repetitions at 30 fish/tank of stock density.

2.2 The study of the indigo dye toxicity on Silver Barb mortality after 96 hours of exposure

Thirty silver barb fries were used in testing the toxicity of indigo dye. The indigo dye-contaminated water was the tap water from rinsing dyed fabrics process, were obtained from household indigo dye industry at Sakon Nakhon province, Thailand. Since there was no information on concentration toxicity of indigo dye-contaminated water. Hence the preliminary study was carried on the concentrated indigo dye-contaminated water at 0.025 and 0.050 µg/mL, the results was not found the level of fish mortality, but all the fish fries died immediately at 0.02 µg/mL of concentrated indigo dye-contaminated water. The acute toxicity bioassay was conducted by exposing silver barb to concentrated indigo dye-contaminated water at 0.133 µg/mL (dilution ratio 2:1), 0.150 µg/mL (dilution ratio 3:1), 0.160 µg/mL (dilution ratio 4:1), 0.167 µg/mL (dilution ratio 5:1) and 0.171 µg/mL (dilution ratio 6:1) compared with dechlorinated tap water (control). The indigo dye-contaminated water was diluted with dechlorinated tap water for each treatment (dilution ratio of indigo dye-contaminated water:tap water) and thoroughly mixed, and concentrations of the quantity 7 liters. The fish fries in the control group were put in dechlorinated tap water. Fish mortality was checked after 24, 48, 72, and 96 hours of exposure and the toxicity of indigo dye on silver barb mortality at the lethal concentration that kills 50% of the fish (LC_{50}) investigated using Probit analysis.

2.3 The study of pathology in fish gills after 96 hours of exposure

After 96 hours of indigo dye-contaminated water exposure, the gills of the fish from each group were taken and put into 10% formalin for 24 hours. The calcium ions were removed from fish gills using 0.5 M $AlCl_3.6H_2O$, 2.6 M HCl, 1.325 M formic acid for 4 - 5 hours. The gills were cleaned with tap water and put into 5% Na_2SO_4 for 4 - 5 hours or it could be up to 24 hours (confusing times). The gills were cleaned again with running tap water for 10 minutes. The gills were then put into alcohol to dehydrate the cells and parafin embedded them. Samples with the thickness of 4 - 5 micrometers were cut (Humason, 1979). Finally, the samples were hematoxylin and eosin (H&E) stained to investigate the pathological changes in the gills using microscope and the pictures were taken.

2.4 Checking physical and chemical characteristics of the indigo dye-contaminated water

This study used indigo dye-contaminated water from a community enterprise called "Pha Kram Group" located in Oon Dong village, Pannanikom district, Sakon Nakhon Province. Prior to the experiment, the physical and chemical properties of the indigo dye-contaminated water were analyzed including temperature (°C), acidity and alkalinity, dissolved oxygen (mg/L), electrical conductivity (µS/cm) using multi probe meter and biochemical oxygen demand (BOD), chemical oxygen demand (COD), suspended solids (SS), total suspended solids (TSS) (mg/L), total dissolved solids (TDS) (mg/L), total nitrogen (TN) (mg/L), and alkalinity recorded. The data were analyzed using a standard method (APHA, 1995). Colorimeter was used to analyze the true color. Average values were calculated and compared with wastewater discharge standards and water quality criteria for protecting aquatic animals set by the Pollution Control Department.

2.5 Statistical Analysis

Result obtained was subjected to statistical analysis of variance (ANOVA) at 5% level of significance. For the determination of differences in percentage of mortality of Silver barb between the group were statistically compared by Duncan test of significance.

For the median lethal concentration values (LC_{50}) were estimated with Probit Analysis, using SPSS (version 20.0; SPSS, Chicago, IL, USA) through the dose-response relationship of fish mortality due to the exposure to indigo dye-contaminated water.

3. Results and Discussion

Both natural and synthetic fabric dyes in a pot are not easily degraded or altered (Dogdu and Yalcuk, 2016). Wastewater from indigo dyeing or other dyeing processes can pollute nearby water source (Robinson *et al.,* 2001) which is harmful for human and animal consumption. Besides, it can create an imbalance in food chain in an ecosystem (Rocha, 1992). The traditional biological wastewater treatment is no longer effective because most dye does not degrade (Beydili *et al.,* 2001; Aygün *et al.,* 2012). Recently, there are many studies on how to treat indigo dye-contaminated wastewater and how to remove dye to reduce toxicity of wastewater before releasing it to public water bodies. This research is a novel study of the effect of indigo dye-contaminated water exposure on aquatic animals (silver barb) and organic tissues (gills). The results showed that all concentrated indigo dye-contaminated water at 0.133, 0.150, 0.160, 0.167 and 0.171 µg/mL affected fish mortality if the fish were exposed to the indigo dye-contaminated water long enough. Fish mortality increased with an increase in the concentration of the indigo dye (Table 2, Figure 1A-D).

The analysis percentage of cumulative mortality of the fish showed that the silver barb that had been exposed to the concentration of indigo dye-contaminated water at 0.171 µg/mL started to die after 6 hours (statistically significant at $p < 0.05$). After the fish had been exposed to the indigo dye-contaminated water for 24 hours, the cumulative death of the fish at the concentration of indigo dye-contaminated water at 0.167 µg/mL and 0.171 µg/mL were not different. However, the cumulative mortality of the fish that had been exposed to the concentration of indigo dye-contaminated water at 0.167 µg/mL was statistically different ($p < 0.05$) from the control group. After the fish had been exposed to the indigo dye-contaminated water for 96 hours, the cumulative mortality of the fish was higher in the groups with the concentration of indigo dye-contaminated water at 0.160, 0.167 and 0.171 µg/mL, respectively. The cumulative mortality of the fish in each group was 100.00 ± 0.00, 100.00 ± 0.00 and 100.00 ± 0.00 percent, respectively (not statistically different at ($p > 0.05$). However, they were statistically different from the fish that had been exposed to the concentration of indigo dye-contaminated water at 0.133 µg/mL, 0.150 µg/mL and the control group (found no fish death). The cumulative mortality of the fish in each group were 70.00 ± 10, 40.33 ± 2.51 and 0 percent, respectively (Table 2, Figure 1A-D).

The analysis of physical and chemical characteristics of indigo dye-contaminated water from rinsing water using as a stock water in the experiment showed that suspended solids (SS), total nitrogen (TN) and true color (PCU) exceeded the standard criteria for wastewater discharge set by the Pollution Control Department. The average values were equal to 1,528 ± 42 mg/L, 45.82 ± 0.91 mg/L and 783.33 ± 38.19 PCU, respectively (Table 1). Besides, when the water quality values of the indigo dye-contaminated water were compared with the water quality criteria for protecting aquatic life set by the Pollution Control Department, it was found that the average values of dissolved oxygen (DO) and suspended solids (SS) were 1.65 ± 0.01 mg/L and 991 ± 21 mg/L, respectively, which exceeded the standard criteria. Dissolved oxygen of the indigo dye-contaminated water was below 3 mg/L which would affect fish mortality. One of the reasons of low dissolved oxygen could be that microorganisms in the water used oxygen in the fermentation process which help alter indigo dye. High suspended solids were found in the indigo dye-contaminated water which clogged the fish gills affecting the flow of oxygen throughout their bodies. This could impact fish mortality. Furthermore, high alkalinity was found which was 520 ± 0.00 mg/L (Table 1). This was unsuitable for fish and aquatic animal survival. Natural water bodies usually have suitable alkalinity at 50 - 300 mg/L.

High alkalinity reduces nutrients in water because it is a source of carbon dioxide in the form of carbonate and bicarbonate. These nutrients are used for growth and photosynthesis of phytoplankton and aquatic plants which are fish food. It can be seen that irregular values of physical and chemical characteristics could affect mortality of fish and aquatic animals. This is aligned with the study of Srivastava *et al.* (1994) which reported that the wastewater released from textile and printing industrial sites was contaminated by suspended solids, organic compounds, heavy metals, and had high acidity and alkalinity which were unsuitable for aquatic animal livelihood in water sources.

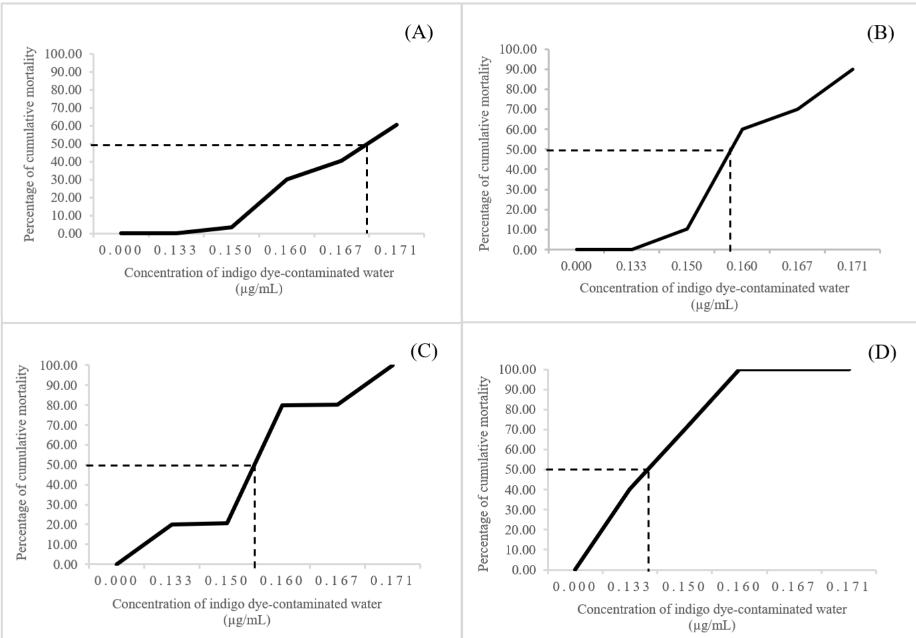

Figure 1. Dose-response curves of percentage of cumulative mortality survival silver barbs that were exposed to the indigo dye-contaminated water for 24 hours (A), 48 hours (B), 72 hours (C), and 96 hours (D)

Table 1. Physical and chemical characteristics of the indigo dye-contaminated water from rinsing water using as a stock water in the experiment.

Parameter	Average of water quality of indigo dye contaminated water (Mean ± SD)
Indigo concentration (µg/mL)	0.0200 ± 0.0001
Temperature (°C)	28.82 ± 0.00
Dissolved oxygen (mg/L)	1.65 ± 0.01
Acidity and alkalinity (pH)	7.21 ± 0.02
Electrical conductivity (µS/cm)	600 ± 0.00
True color (PCU)	783.33 ± 38.19
BOD (mg/L)	1.12 ± 0.15
COD (mg/L)	10.67 ± 0.00
Suspended solid (SS) (mg/L)	1,528 ± 42.00
Total suspended solid (TSS) (mg/L)	483 ± 25.00
Total dissolved solid (TDS) (mg/L)	974 ± 24.00
Total nitrogen (TN) (mg/L)	45.82 ± 0.91
Alkalinity (mg/L)	520 ± 0.00

Table 2. Percentage of cumulative mortality are percentage of silver barb at different concentration ratios of the indigo dye contaminated water and exposure period (Mean ± SD)

Concentration of indigo dye-contaminated water (µg/mL)	Percentage of cumulative mortality of silver barb fries at different exposure periods							
	Exposure period (hr)							
	1	3	6	12	24	48	72	96
0 (Control)	0	0	0^{b}	0^{c}	0^{c}	0^{c}	0^{c}	0^{d}
0.133	0	0	0^{b}	0^{c}	0^{c}	0^{c}	20.00 ± 1.00^{bc}	40.33 ± 2.51^{c}
0.150	0	0	0^{b}	0^{c}	3.33 ± 0.00^{c}	10.33 ± 0.57^{c}	20.67 ± 0.57^{b}	70.00 ± 1.00^{b}
0.160	0	0	0^{b}	10.33 ± 0.00^{b}	30.00 ± 0.00^{b}	60.00 ± 0.10^{b}	80.00 ± 2.00^{a}	100.00 ± 0.00^{a}
0.167	0	0	0^{b}	20.00 ± 0.00^{ab}	40.33 ± 0.00^{ab}	70.00 ± 2.64^{ab}	80.33 ± 1.52^{a}	100.00 ± 0.00^{a}
0.171	0	0	10.33 ± 0.00^{a}	20.67 ± 0.00^{a}	60.67 ± 0.57^{a}	90.00 ± 1.73^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}

Note: a, b, and c indicate the statistical significance ($p < 0.05$), vertically.

The percentage of survival silver barb that had been exposed to the indigo dye-contaminated water for 96 hours was lowest (0%) for the groups that were exposed to the concentration of indigo dye-contaminated water 0.160, 0.167 and 0.171 µg/mL (Figure 2D). In addition, it was found that the fish exposed to the concentration of indigo dye-contaminated water at 0.171 µg/mL for 24, 48, and 72 hours had lowest survivals compared to the control group (Figure 2A-D).

The analysis of toxicity of indigo dye-contaminated water at the exposure period of 96 hours showed that 50% of the fish died when exposed to 0.137 µg/mL of the contaminated water. With the exposure periods of 24, 48, and 72 hours, 50% of the fish died when exposed to 0.167, 0.160, and 0.150 µg/mL of the contaminated water, respectively (Table 3). This showed that the mortality of the fish depended on the concentration of the indigo dye-contaminated water and time that the fish were exposed to. Hence, wastewater from indigo dyeing or other dyeing processes can pollute nearby water source (Robinson *et al.,* 2001) which is harmful for consumption (Rocha, 1992). The indigo dyeing process involves the fermentation process which generates many types of microorganisms such as *Amphibacillus* sp., *Oceanobacillus* sp., *Pseudomonas* sp. (Aino *et al.,* 2010; Park *et al.,* 2012). These bacteria cause diseases which impact mortality of fish and other aquatic animals. Moreover, indigo dyeing process consumes a lot of water to wash the indigo dyed fabric. This can significantly affect environment broadly.

The study of pathology in the fish gills that had been exposed to the indigo dye-contaminated water for 96 hours showed that fish gills in all groups except the control group had altered. The gill tissue of the silver barb that had been exposed to the concentration of indigo dye-contaminated water at 0.133, 0.150, 0.160 µg/mL experienced both hyperplasia and hypertrophy around gill lamellae causing fusion (Figures 3A, B and C). However, hematopoietic elements could still be seen. When compared to the control group that had normal gill tissues, chloride cells were found around gill lamellae base and pillar cells inside gill lamellae (Figure 3F). In addition, oedema was found in the gill of the fish that had been exposed to the concentration of indigo dye-contaminated water at 0.160 µg/mL (Figure 3C) which was caused by fluid buildup in the tissues. Smaller size of gill lamellae tissues or atrophy were found in the fish that had been exposed to the concentration of indigo dye-contaminated water at 0.167 µg/mL and 0.171 µg/mL. This was because the blood could not flow to the gill lamellae tissues. Curling or irregular shape in gill lamellae were also found (Figures 3D and E). It can be concluded that the gill of the fish that was exposed to highly concentrated indigo dye-contaminated water long enough were more impacted than those that had been exposed to less concentration and for a shorter exposure time period. These findings are similar to the study by Santos *et al.* (2014) that reported the indicators of the effect of unmet water quality on fish gill tissues. They found that the effect could be in the form of curling, hyperplasia, hypertrophy and lamellae fusion, as well as *Centropomus undecimalis* and *Sardinella* sp.

Waste minimization is important for lowering water pollutant loads. Therefore, there is a need to implement effective treatment of indigo dye-contaminated water by dilution with tap water according to the dilution ratio in this study prior to discharge into the environment.

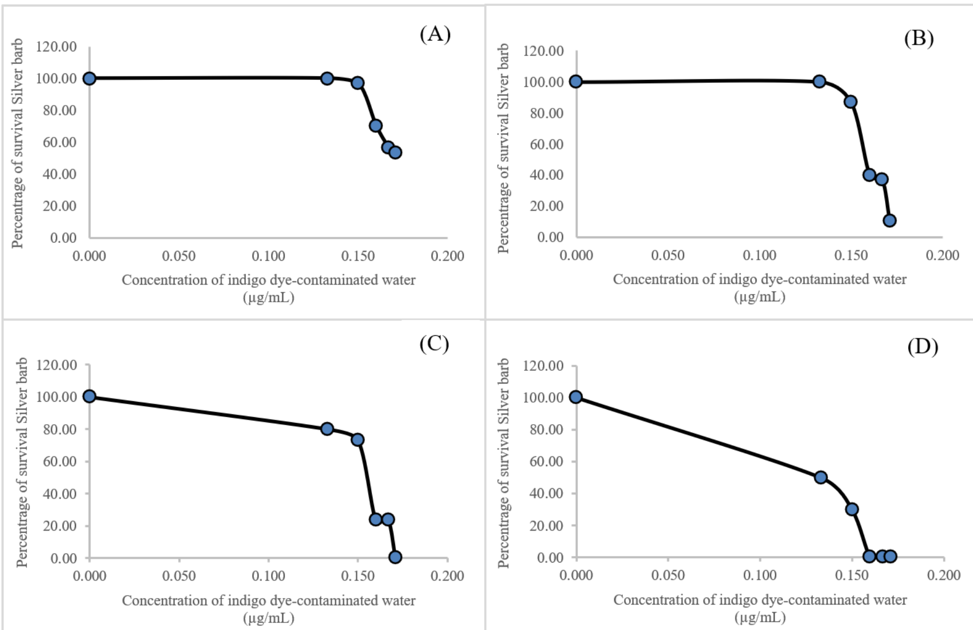

Figure 2. Dose-response curves of percentage of survival silver barbs that were exposed to the indigo dye-contaminated water for 24 hours (A), 48 hours (B), 72 hours (C), and 96 hours (D)

Table 3. Lethal concentration that kills 50% of the silver barb fries (LC_{50})

Exposure period (hr.)	Lethal concentration that kills 50% of the silver barb fries (µg/mL)
24	0.167 (0.162 - 0.177)
48	0.160 (0.154 - 0.164)
72	0.150 (0.142 - 0.156)
96	0.137 (0.124 - 0.144)

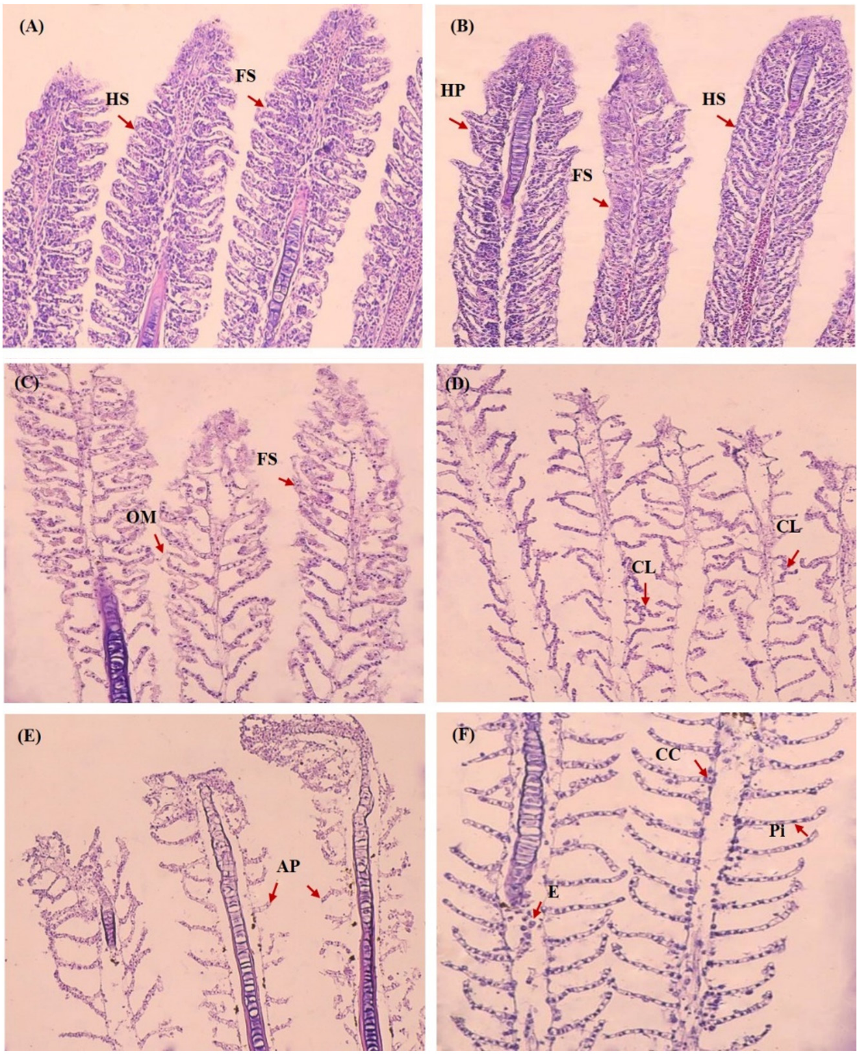

Figure 3. Pathology in silver barbs' gills after 96 hours of exposure. Hyperplasia and hypertrophy were found which led to fusion in silver barbs' gills. These fish were exposed to the concentration of indigo dye-contaminated water of (A) = 0.133 µg/mL (B) = 0.150 µg/mL (C) = 0.160 µg/mL (D) = 0.167 µg/mL (E) = 0.171 µg/mL and (F) = the control group. Note: AP = Atrophy, CC = chloride cell, CD = chondrocytes, CL = curling, E = erythrocytes, FS = Fusion, HS = Hyperplasia, HP = Hypertrophy, OM = Oedema, Pi = pillar cell (H&E staining, 400 × magnification)

4. Conclusion

From this experiment, it can be concluded that the indigo dye contaminated water could affect mortality of silver barbs after 96 hours of exposure. All fish died when they were exposed to the concentration of indigo dye-contaminated water at 0.160, 0.167 and 0.171 µg/mL. The toxicity of indigo dye-contaminated water that killed 50% of the fish (LC_{50}) was 0.137 µg/mL. Irregularities including hypertrophy, hyperplasia, fusion, oedema, atrophy and curling were found in the gills of the fish that were exposed to the indigo dye-contaminated water. In addition, fish cannot survive in waters that do not meet water quality standards. Therefore, water contaminated with indigo dye should be treated before being discharged into natural waters to avoid contamination.

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