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Article

Histopathology of Gills Fish in Rivers Contaminated by Heavy Metals from Artisanal Gold Mining Waste in Lebong Regency, Bengkulu Province

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Abstract. The aquatic environment is crucial for fish survival because fish require appropriate aquatic environment. Artisanal gold mining generates heavy metal waste in Lebong Regency, polluting the river flows. Heavy metals released into the aquatic environment generate water pollution problems due to their toxicity, persistence, and bio-accumulation in organism. The aim of this study was to investigate the histology of fish gill organs in the river contamined by heavy metals. Fish samples were taken by purposive sampling. Histological sections using the paraffin method and hematoxylin-eosin staining. The result of mercury detected in water is 0.01mg/L, exceeded maximum limit in river based on class II water quality standard (0.002mg/L). Histopathology of gills *H. nemurus* and *T. Tricopterus* fish (HAI scores <20) showed slight alterations such as epithelial lifting, secondary lamella fusion, and hyperplasia. *Rasbora sp.*, *M.circumcinctus*, and *O.niloticus* gills fish (HAI scores>100) had severe irreversible damage, marked by telangiectasis at the tips of the secondary lamella. This is due to the presence of heavy metal contaminants in the water, that causes histopathological damage to fish gills. Parasitic infections were also discovered in some fish gills, impairing their function for respiratory and excretory organs which can be caused by low water quality.

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1. Introduction

Healthy aquatic environment is essential for living organisms to function properly. Freshwater ecosystems provide food, habitats, and other services that support life of organism. Heavy metals in water have been an issue in recent decades. Heavy metals are found throughout the environment. They are easily dissolved and transported by water, where they are promptly absorbed by aquatic biota [1]. Mercury, a heavy metal, is a global contaminant prevalent in aquatic habitats and poses a risk to all organism, even at low concentrations of 0.05 µg/g [2]. Natural and anthropogenic sources emit mercury into the atmosphere, water, and soil. Anthropogenic sources of mercury exposure include gold mining activities. Mercury is found in both aquatic and terrestrial habitats in three chemical forms: element, organic, and inorganic. Inorganic forms, such as mercury chloride, accumulate in aquatic environments.

Lebong Regency is one of the regencies in Bengkulu Province where there are still active gold mines that are widely carried out conventionally by the community, so this activity has a high potential for pollution in rivers due to heavy metal pollution, especially since this mining has been going on for decades. Community in Lebong processes gold ore using mercury metal (Hg) for amalgamation (gold separation). According to previous research [3], in air kotok rivers, North Lebong District detected mercury and cyanide, despite amounts of mercury and cyanide were observed remaining below the threshold for class II river water quality, however, were found several alteration in the histological structure of the fish liver. The existence of heavy metal contamination in this river will also be hazardous because heavy metals will flow into the sea and spread to other marine biota, causing broad environmental damage. Heavy metals are severe toxicants that can change quality and aquatic life [4].

Heavy metals, such as mercury, accumulate in tissues and can kill fish [5]. Heavy metals in water, such as mercury, are converted by microorganisms through a transition from organic compounds to inorganic forms, which is the first step in the accumulation process in aquatic biota and is harmful to aquatic biota [6]. Increasing pollution in aquatic ecosystems can cause severe morphological and physiological changes in aquatic organisms, if heavy metal levels in the water rise and exceed the threshold value, resulting in a decrease in water quality and disruption of aquatic biota, including fish [7]. Accumulation of pollutants disrupts the physiology of organism tissues [8].

Fish are a critical component of the aquatic food cycle. Fish in Lebong river are still widely traded and consumed by local community. Fish are important organisms in the study of heavy metal pollution because they move freely and assimilate heavy metals in a variety of ways, including ingestion of suspended particles in water, ion exchange of dissolved heavy metals via lipophilic membranes (gills), and surface adsorption tissues and membranes. The accumulation of hazardous metals in fish and their habitat disrupts communication between fish and their aquatic environment, having a deleterious impact [9]. Biomarkers are biological indicators that showing how a stressor affects the body [10]. Moreover, fish are organisms to be good bioindicators due to their ability to absorb chemicals quickly through their gills and body surfaces, or through direct consumption, making them highly sensitive to environmental pollutants. Changes in fish organs, such as the gills, are good biomarkers of water contamination.

The first organs that are directly exposed to environmental pollutants are the gills [11]. Pollutants in the aquatic environment can alter the state of fish organs, particularly gills. Gills have the most surface area exposed to the aquatic medium because they play an important role in gas exchange, osmoregulation, acid-base regulation, detoxification, and excretion via water [12]. Gills operate as a barrier against poisons, reducing their uptake and magnifications in other organs [13]. Heavy metal pollution can injure fish organs both immediately and chronic. Pollutants can cause damage in fish tissue [14]. Histopathological studies are regarded as an accurate and complete biomonitoring method for assessing fish health and survival in polluted aquatic habitats. Heavy metals are the most common pollutants found in river ecosystems that receive bulk residential waste water

[15]. The aim of this study was to investigate the histology of the fish gill organs in the Amen river contaminated by heavy metals in Lebong Regency. This study is expected to provide information effect of gold mining waste for freshwaters ecosystem in Lebong, as well as potential risks to human health.

2. Experimental Section

2.1. Materials

The materials that used in this study are gills fish, clove oil, NaCl 0,9%, Neutral Buffered Formaline (NBF) 10%, toluol, xylol, alcohol 70%-96%, paraffin, glyserin-albumin, Hematoxylin-Eosin, and entellan.

2.2. Water Parameter Quality

This study was conducted in Amen river using three location points with coordinates: S 03°07'18.45" E 102°12'05.28", S 03°07'18.56" E 102°12'04.96", and S 03°07'17.84" E 102°12'04.76" (Fig.1). Measurements of water quality were also carried out on parameters such as: Water temperature, pH, conductivity, TDS, TSS, and DO. Water samples analyzed in the laboratory for heavy metals in the form of mercury, cyanide, and cadmium. The data of water quality parameter compared with the Class II River Water Environmental Quality Standards based on PP RI No. 22 of 2021.

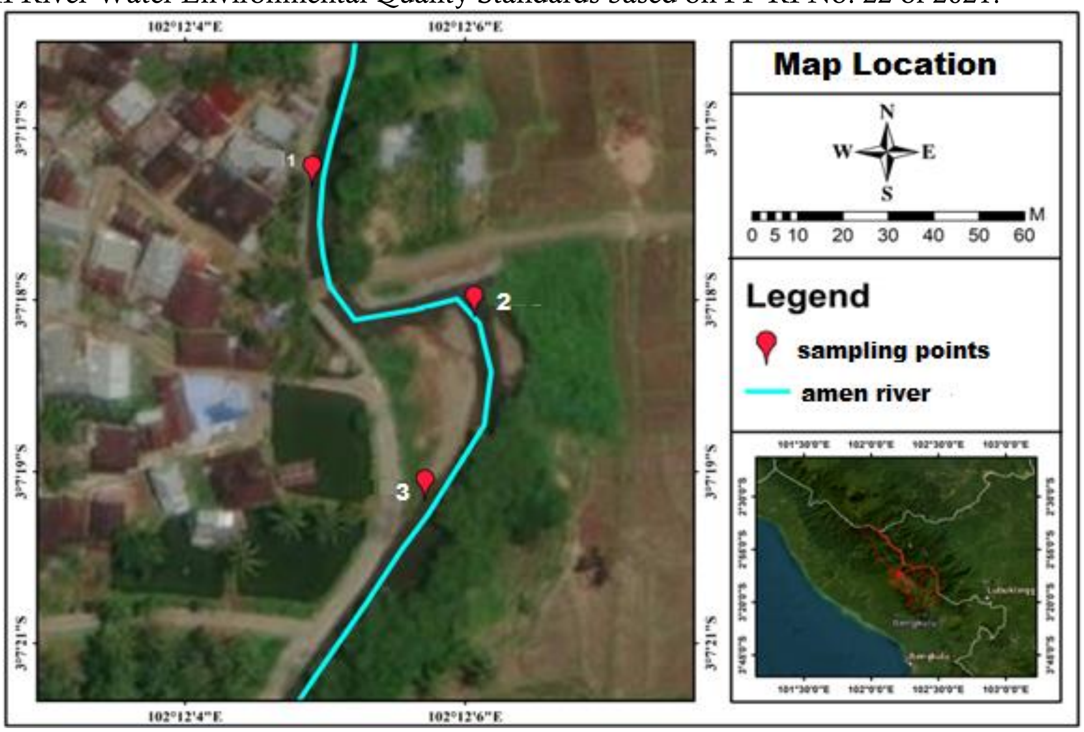

Figure 1. Map location

2.3. Histological Preparations

Fish were caught from three sampling points using fishing traps by purposive sampling. Fish were used as samples, up to three per species, and their types were identified before euthanized and the gill organs removed. Gills organ were rinsed used 0.9% NaCl and then fixed with NBF10% fixative for 24 hours. The fixed tissue were dehydrated in graded alcohol for each 30 minutes at 70%, 80%, 90%, and 96%, then cleared with toluene for 45 minutes. Infiltration with paraffin was performed four times in an oven at 60°C for 45 minutes each. Before sectioning, the tissue is placed in an embedding cassette for 24 hours.

Paraffin sectioning is then performed with a Leica microtome with a thickness of approximately 5 µm. Afterward, the paraffin coupes attached to the slide using glyserin-albumin were spread out in hot plate. Deparaffinize the slides with xylene for 15 minutes, then rehydrate for 3 minutes using graded alcohol (96%-70%). Slides were stained with hematoxylin meyer for 3 seconds, rinsed with running water for 10 minutes, and then stained with eosin for 3 minutes. After staining, dehydrate the slides with graded alcohol for 3 minutes each. The preparation was then submerged in a xylene solution for three minutes before drying. The slides were mounted with entellan. Slides were observed using a Meiji MT-31 Binocular Digital Microscope using the TCapture software. The preparation was observed in 5 fields of view.

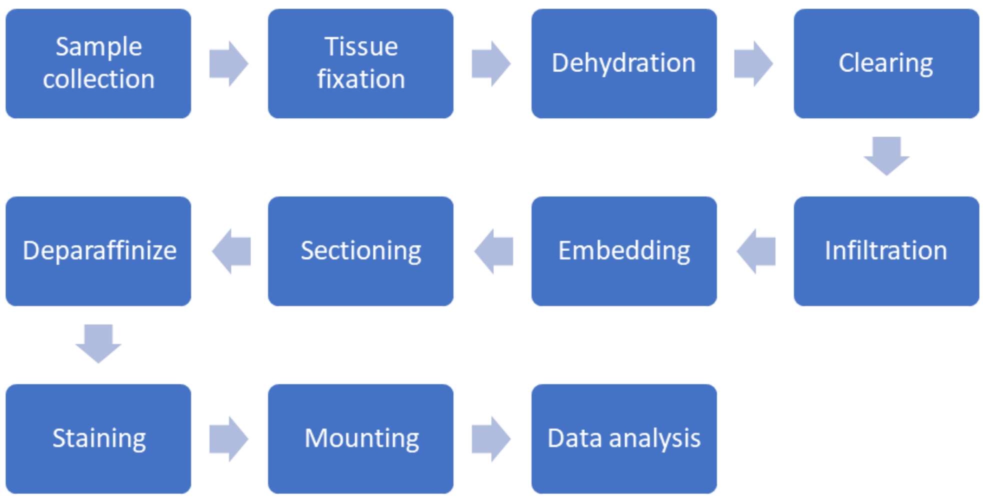

Figure 2. Flow chart of histological procedure

2.4. Data Analysis

The histological images that have been obtained were analyzed descriptive qualitatively. Histological changes in gill tissues were classified by [16] based on scores from 0 to 3, wherein 0 = no changes, 1 = slight changes, 2 = moderate changes, and 3 = severe changes The existence of histopathological changes on gills was assessed semi-quantitatively using the degree of tissue modification (HAI-*Histopathological Alterations Index*) depending on the severity of the lesions. The alterations in each organ were classified in progressive stages of damage in the tissues (Table 1). The HAI value was calculated based on Poleksic & MitrovicTutundzic (1994) using the following formula.

$$HAI = (1xSI) + (10xSII) + (100xSIII)$$

Where,

I, II and III	: the number of stages of alterations
1, 2 and 3, and S	: represents the sum of the number of alterations for each particular stage

HAI values ranging:

0-10	: normal organ functioning,
11-20	: slight changes in the organ
21-50	: moderate changes in the organ
51-100	: severe lesions
>100	: irreparable organ lesions

Table 1. Histopathological alterations in gills

Stage	Histopathological alterations in gills
I	Hypertrophy and hyperplasia of gill epithelium Sanguineous congestion Dilation of marginal vascular channels Lifting of respiratory epithelium Fusion and disorganisation of secondary gill lamellae Shortening of secondary lamellae Leukocyte infiltration of gill epithelium
II	Hemorrhage and rupture of lamellar epithelium Hypertrophy and hyperplasia of mucous cells Empty mucous cells or their disappearance Hypertrophy and hyperplasia of chloride cells
III	Lamellar aneurysm Necrosis and cell degeneration Lamellar telangiectasis

3. Results and Discussion

The results of water parameter measurements (Table 2) shows that the temperature, pH, and dissolved oxygen levels are still acceptable for the survival of fish and other aquatic organism because the value is bellow standard for river. Fish can survive when temperature, salinity, and dissolved oxygen levels are within normal limits. Water pH reveals a tendency towards normal pH, and the water sample shows that TSS, TDS, and conductivity are all below the class II water quality standard value. This shows that river water is still sufficient for the survival of freshwater fish. Heavy metals like mercury, cadmium, and cyanide were detected in river water samples. Mercury levels exceeded the water quality limit until 0.01mg/L, showing that the local community in Lebong is still using mercury in gold processing. Mercury (Hg) is widely used in illegal gold mining (PETI) is known as artisanal mining. The water sample contained 0.006 mg/L of cyanide and <0.01 mg/L of cadmium, which were both below the water quality threshold. Moreover, the community uses cyanide in gold processing as part of the cyanidation process.

Table 2. Water parameters quality

Parameter	Result	River Quality Standard Class II (PP RI No. 22 of 2021)
pH (unit)	6.3 ±0.02	6-9
Temperature (°C)	27.3 ± 0.01	26-30
Salinity (ppt)	1	1
DO (mg/L)	7.58±0.01	>4
TSS (mg/L)	7±0.2	50
TDS (mg/L)	75.47±0.3	1000
Conductivity (µS/cm)	156.3±0.2	200-1000
Cyanide (mg/L)	0.006	0.02
Mercury (mg/L)	0.01	0.002
Cadmium (mg/L)	<0.01	0.01

Heavy metals pollutant in water can be influenced by a variety of activities. Although various factors influence the results of measurements such as the time of water sampling.The timing of water sampling after many days of heavy rain effects the amount of heavy metals in the water. Mercury (Hg) is an active component of the food chain. Mercury (Hg) that enters the water is absorbed and digested by microorganisms via the methylation process, yielding methylmercury [17]. Mercury (Hg), whether in methyl form, that enters the human body on a continual basis causes lasting harm to the brain, liver, and kidneys.

Fish gills are always in direct contact with the surrounding aquatic environment. These characteristics imply that the gill epithelium is the highest exposed to environmental pollutants and pathogens. Histology gill of several fish in the rivers shows damage in the gill organ by alterations in the structure of the tissue, including damage in the primary and secondary lamellae. Wader fish (*Rasbora sp.*) gill structure (Fig. 3) shows alteration such as hyperplasia, lifting epithelium, lamellar fusion, telangiectasis, and clubbed tips in secondary lamellae. Furthermore, histology structure shows the existence of ectoparasites that infect the gills of wader fish (Fig.3c). Lamellar fusion occurs when the blood vessels in the primary lamellae are decreased, leaving only connective tissue. Lamellar fusion, caused by lamella cell hyperplasia, constantly fills the space between secondary lamellae with new cells, resulting in secondary lamellar adhesion [18]. In addition, there is also epithelial lifting which is characterized by the displacement of the epithelial layer which can disrupt oxygen. Abnormalities that experience swelling can cause the epithelial layer to lift. Epithelial lifting is a minor swelling of the gill cells caused by the entry of lead ions, resulting in the formation of secondary flat epithelium that wraps around the secondary lamella and serves as a defense mechanism.

Epithelial lifting, hyperplasia, and hypertrophy in chloride cells are frequent pathologies in the gills of fish that have been exposed to copper [19]. Epithelial lifting lamella is a tissue alteration that can effect as a result of severe edema. Telengiectasis in this study also found in (Fig.3d) and clubbed tips of secondary lamellae can be seen in (Fig.3b). Telengiectasis in this study can be seen at the tips of the secondary lamellae, which are enlarged and rounded, resembling balloon bubbles. Telangiectasis occurs when capillary blood vessels dilate, due to damming or blood clotting. Clubbed tips is a histological abnormality discovered in fish gills that can be induced by exposure to diesel oil, mercuric chloride, or lead. Gill fish from environment experiencing slight alteration such as epithelial lifting, hypertrophy and fusion and also severe alteration occurrence of aneurysms of various sizes in some secondary lamellae depending on the amount of pollutants in the water [16]. In this study (Fig.3c) also showed cyst of parasite. The occurrence of the illnesses identified in previous research appears to be related with other variables such as water quality or toxicants. Epitheliocystis is an emerging infectious illness that affects fish gills and skin worldwide [20].

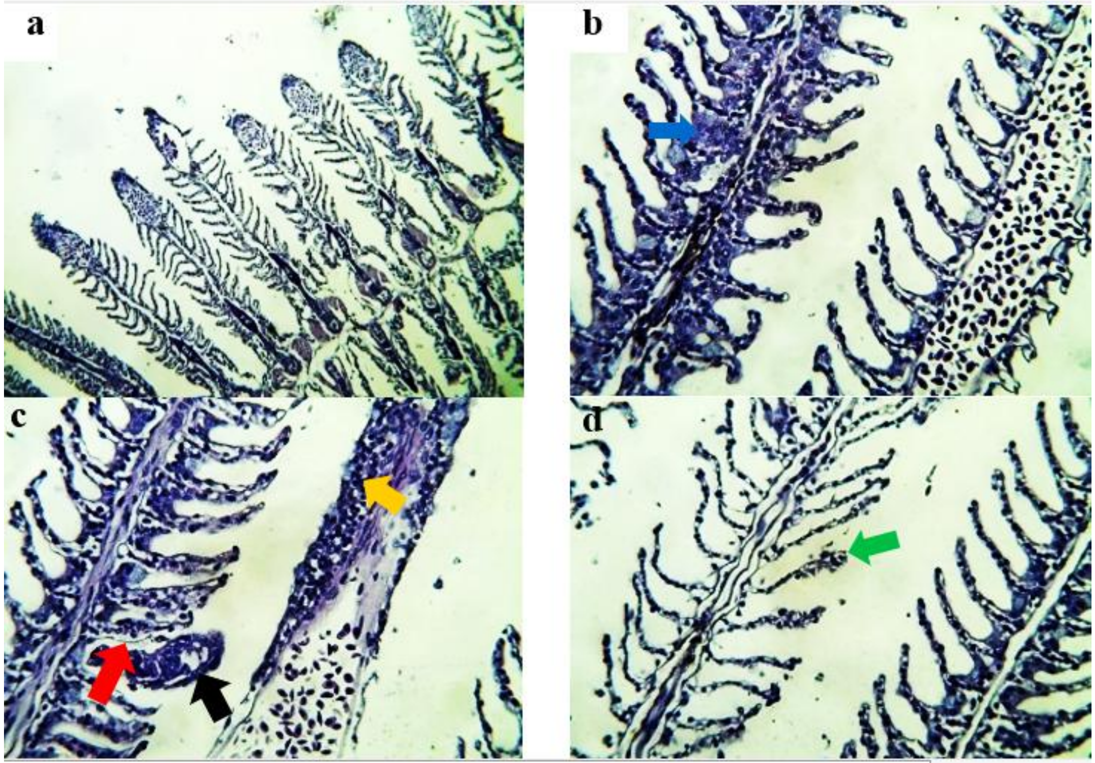

Figure 3. Photomicrograph of *Rasbora sp.* gills stained with Hematoxylin&Eosin. Mag. 100x (a), 400x (b-d). blue= hyperplasia, red= epithelial lifting, yellow= lamellar fussion, black= ecto-parasite, green= telengiectasis.

Photomicrograph of baung fish *(Hemibagrus nemurus)* gill structure (Fig. 4) is undergo alteration such as proliferation and hyperplasia, secondary lamella fusion, and epithelial lifting. Abnormalities in the gills can be noticed by the amount of cells that grow and become irregular. Excessive tissue development as a result of cell proliferation. Hyperplasia occurs when the number of cells increases with time, causing thickening and narrowing of the filaments in the secondary lamella, causing them to fuse between lamella.The baung fish showed less damage than the wader fish, with hyperplasia being the most common alterations. Hyperplasia is recognized from swollen tissue and the shape of the cell structure within is no longer evident, but still has epithelial tissue [21]. Hyperplasia that occurs constantly will produce lamella fusion, as a result, in this study, there was complete secondary lamellar fusion in baung fish. Heavy metals that accumulate in the gills can disrupt respiration and osmoregulation processes, resulting in cellular damage to gill cells. *H. nemurus* exposed to lead and iron metals developed hyperplasia, lamella fusion, and necrosis, all of which were indicative of severe tissue damage [22].

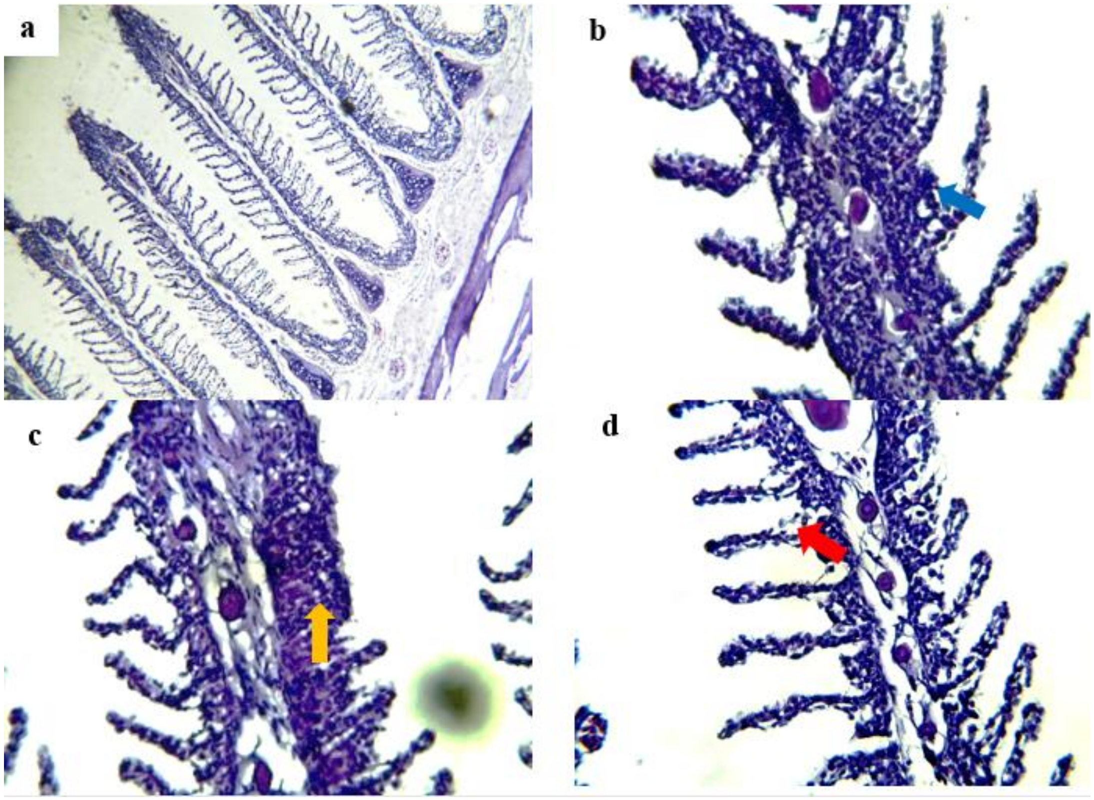

Figure 4. Photomicrograph of *H.nemurus* gills stained with Hematoxylin&Eosin. Mag. 100x (a), 400x (b-d). blue= hyperplasia, yellow= lamellar fussion, red= epithelial lifting.

The gill histological structure of the sili fish (*Macronagtus circumcinctus*) (Fig. 5) showed telangiectasis, hyperplasia, lifting epithelium, lamellar fusion and erosion. In this study investigation, histological section of sili gills fish free from parasitic manifestation. Moreover, pathological alteration in mucous, as shown in (Fig. 5c) can indicate the mucous over secretion as empty spaces that cannot absorb color or uncolored. Several lamella in this study were found incomplete and complete fusion, as well as rupture and peel from the gill filament epithelium. The most common lesions were gill epithelial lesions, which were followed by lamellar epithelial lifting and hyperplasia. The lamellar epithelium ruptured and peeled, and the gill filament epithelial hyperplasia occurred often. Furthermore, some researchers have described the lifting of fish gill epithelial cells as a result of exposure to metals, organic pollutants, and acute pesticide exposure [23]. Moreover, rapid and profuse cell proliferation leads to subsequent lamella fusion and hyperplasia. Telengiectasis in histological gills of sili fish also reported (Fig. 5b). Telangiectasis, stating that faster blood circulation can lead to erythroid buildup. Erythrocytes commonly accumulate at the secondary lamella's tip [24].

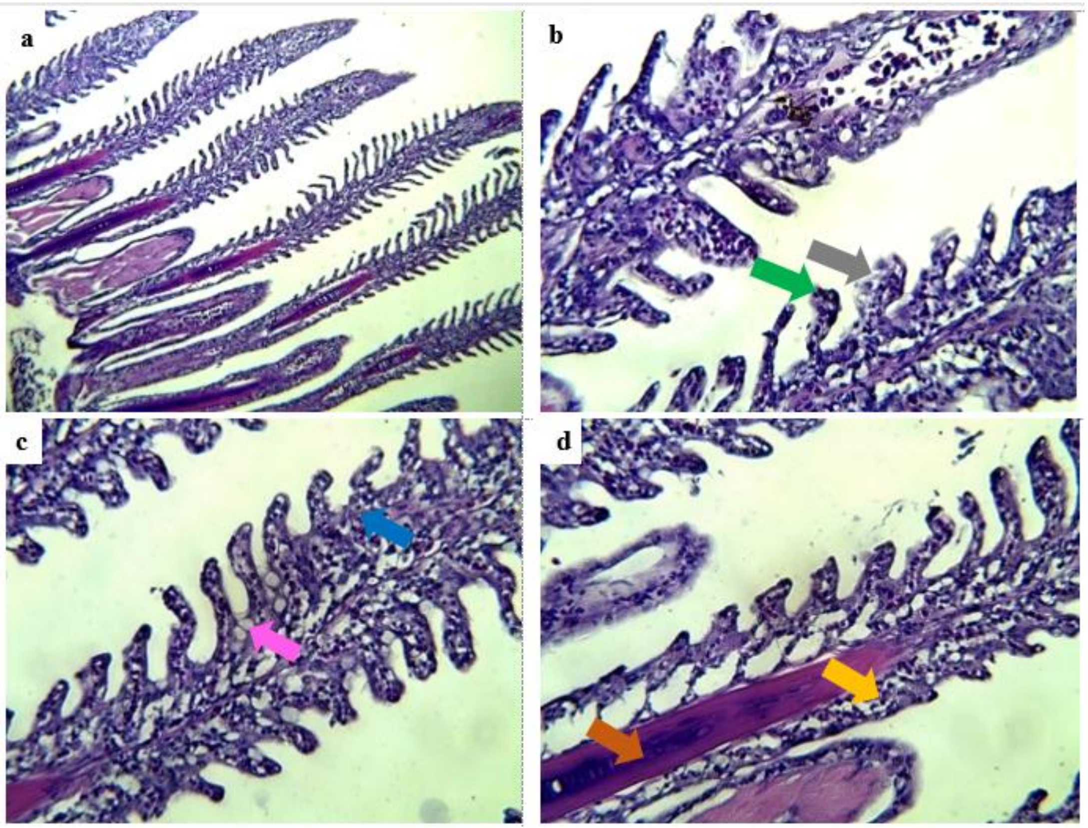

Figure 5. Photomicrograph of *M. circumcinctus* gills stained with Hematoxylin&Eosin. Mag. 100x (a), 400x (b-d). green= telengiectasis, grey= lamellar erosion, blue= hyperplasia, pink= mucous, yellow= lamellar fussion, brown= congestion in primary lamellae.

The histological structure of the gills of three-spotted gourami (*Tricophodus tricopterus*) (Fig. 6) revealed fusion secondary lamellae, congestion, and lifting epithelium. Furthermore, this study was also Furthermore, this study discovered that the gills of *T. trichopterus* fish were infected with parasites which is characterized by the presence of parasite cyst that embedded in gill tissue and the presence of monogenean parasites on the gills. Monogeneans are flatworms that infect fish's gills and cause tissue damage. They are a significant cause of death in fish populations. According to [25], the pathological alterations generated by the presence of parasites were mainly restricted to mild inflammation around the parasite cyst. Edema (swelling of cells) around blood vessels is a common symptom of blood flow blockage in the lamella [18]. This study discovered numerous proliferative alteration in the respiratory lamella epithelium, which could increase epithelial thickness and slow or prevent harmful metals from entering the blood stream. Proliferative changes caused secondary lamella fusion, which disrupted gas exchange and ion regulation [25]. Meanwhile, dilation of blood vessels in the lamella and the presence of edema fluid in the secondary lamella could be attributed to increased permeability caused by prolonged metal exposure.

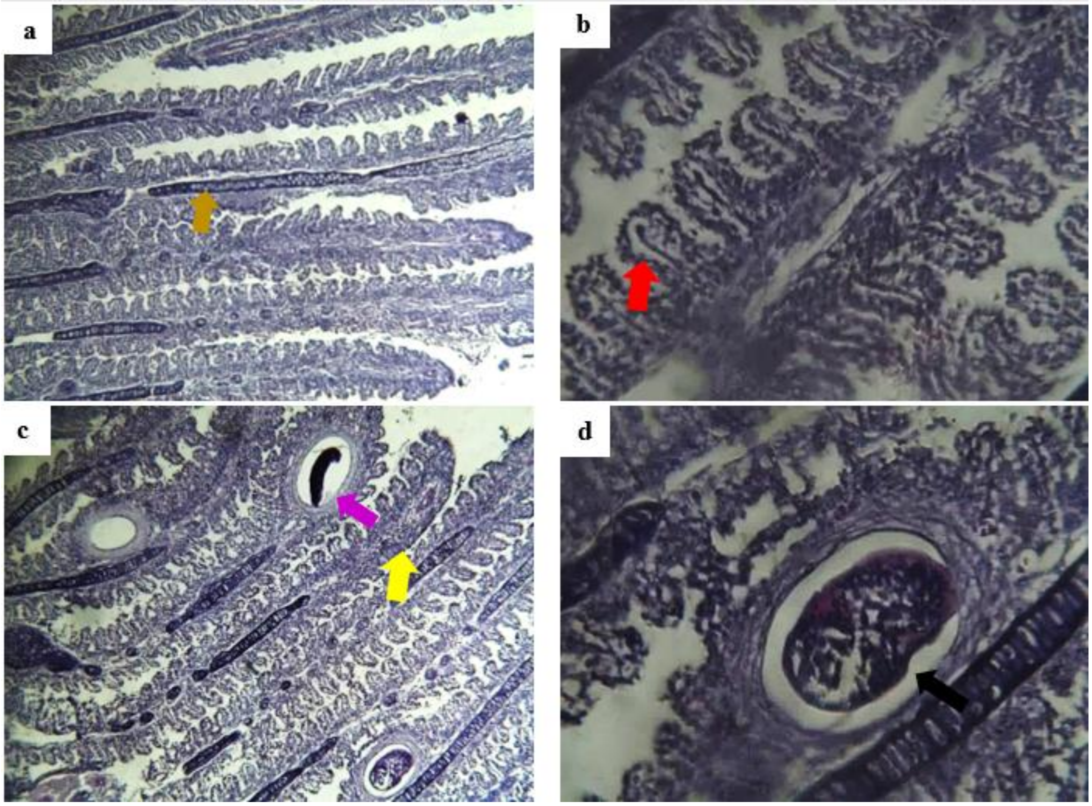

Figure 6. Photomicrograph of *T. tricopterus* gills stained with Hematoxylin&Eosin. Mag. 100x (a), 400x (b-d). purple = parasite infection, brown = congestion in primary lamellae, red = epithelial lifting, yellow = lamellar fussion, black = parasitic cyst.

The histological structure of the gills of tilapia (*Oreochromis niloticus*) (Fig. 7) was damaged like proliferation secondary lamella, hyperplasia, lamellar fusion, lifting epithelium, and telengiectasis. In this study showed that several fish species suffer from edema in gills tissue. Heavy metal exposure can cause an increase in edema fluid in secondary lamellar arteries. In *O.niloticus* also found mucous oversecretion (Fig. 7c). This is similar with the previous study [26] that metal toxicity in the histology of the gills of *O. niloticus* fish, showed significant edema and effective mucus secretion, increased in size but decreased in number, and the majority of them experienced vacuolization or almost necrosis. The secondary lamella also showed damage, either to the epithelial cells or some curved lamellae, which caused congestion in the gills. The lifting epithelium in *O.niloticus* has been recorded more frequently than in other species, that can causing interrupting oxygen and increasing capillary blood vessels at the terminals of the secondary lamella. In a previous study [15], found that metal exposure causes lamellar degeneration, bleeding, and aneurysms that are more severe in 28-day-old fish. From several research, exposure of heavy metals can caused alteration of gill tissue that showed degeneration[27], fusion of lamellae [28][29], oedema [30], inflammatory cellular infiltration [31], hyperplasia and necrosis [7][32]. Histopathological alterations in the gills could be attributed to the rapid and increasing development of oxidative stress that impact for hormonal in fish [33].

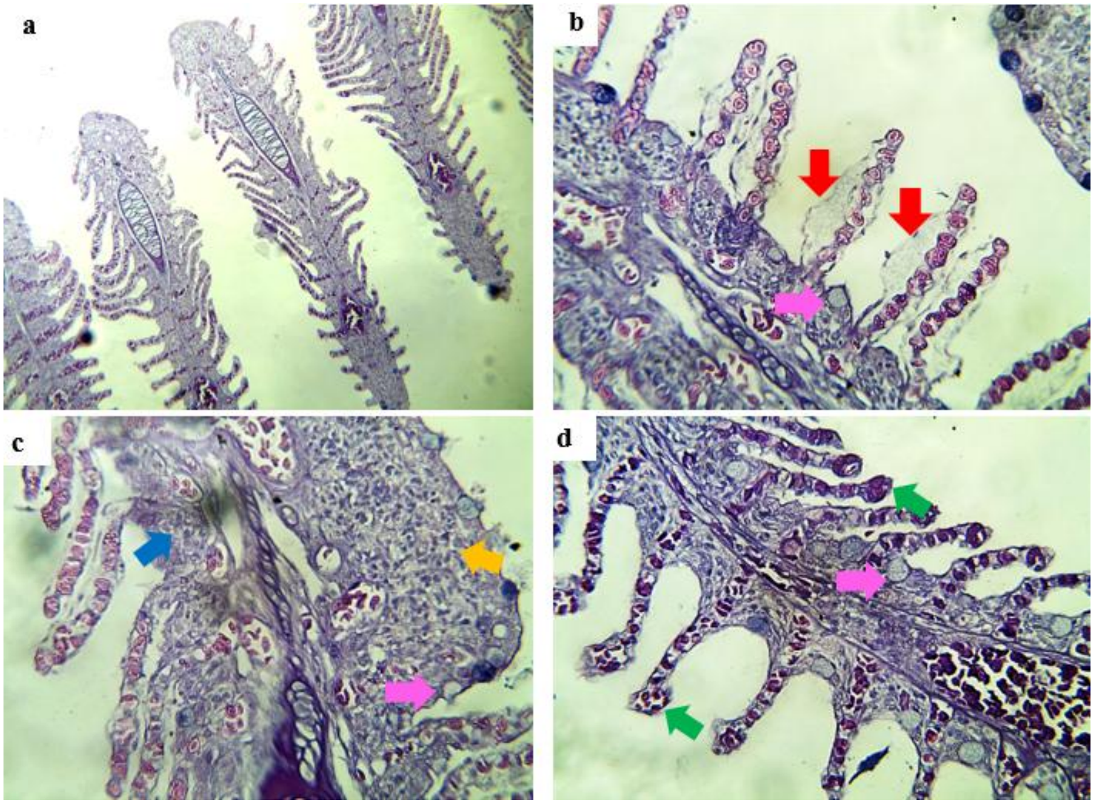

Figure 7. Photomicrograph of the *O.niloticus* gills stained with Hematoxylin&Eosin. Mag. 100x (a), 400x (b-d). green = telengiectasis, blue = hyperplasia, red = epithelial lifting, yellow = lamellar fussion, pink = mucous.

HAI stage (Table 3) shows alterations in all histological structures of the fish gills, indicating hyperplasia and secondary lamella fusion. However, the type and severity of histopathological damage to this fish vary depending on the species. There is damage ranging from mild to severe. Mild damage in *H. nemurus* and *T. Tricopterus* fish with HAI scores less than 20. Histological changes in both gill fish species were only identified in stage I and included increased epithelium, hyperplasia, and complete secondary lamella fusion This is assumed to be due to the fact that each species of fish has a unique adaptation and different tolerance level in the environment. *T.tricopterus* is one of fish species that highly tolerant to environmental changes [34]. There were no fish deaths during acute toxicity tests. Metallic contamination caused the bodies of these animals to secrete more mucus. Likewise *H. Nemurus* that can survive in poor aquatic environments because it has well-developed gills that allow to breathe in low-oxygen water environments. In low-water environmental conditions, these fish can slow their metabolism to a minimum level that allows them to survive without food for few time. In this condition, they can take nutrients from the energy reserves stored in their bodies [35]. *H. nemurus* has strong skin that allows them to survive in muddy and murky water environments. Their skin is equipped with mucus that prevents water from entering their bodies and maintains moisture in their bodies and also able to adapt to environments rich in organic waste.

Table 3. Histopathological alterations in fish gills

Stage	Histopathological alterations in gills	*Rasbora sp.*	*H. nemurus*	*M. circumcinctus*	*T. Tricopterus*	*O. niloticus*
I	Hypertrophy and hyperplasia of gill epithelium	+	+	+	+	+
	Sanguineous congestion	-	-	+	-	-
	Dilation of marginal vascular channels	-	-	-	-	-
	Lifting of respiratory epithelium	+	+	-	+	+
	Fusion and disorganisation of secondary gill lamellae	+	+	+	+	+
	Shortening of secondary lamellae	-	+	+	-	-
	Leukocyte infiltration of gill epithelium	-	-	-	-	-
II	Hemorrhage and rupture of lamellar epithelium	-	-	+	+	-
	Hypertrophy and hyperplasia of mucous cells	-	+	-	-	-
	Empty mucous cells or their disappearance	-	-	+	-	+
	Hypertrophy and hyperplasia of chloride cells	-	-	-	-	-
III	Lamellar aneurysm	-	-	-	-	-
	Necrosis and cell degeneration	-	-	-	-	-
	Lamellar telangiectasis	+	-	+	-	+

(+) alteration, (-) no alteration

Table 4. Score of Histopathological Alterations Index in gill fish

No	Species	HAI score
1	*Rasbora sp.*	103
2	*H. nemurus*	14
3	*M. circumcinctus*	124
4	*T. Tricopterus*	13
5	*O. niloticus*	113

Rasbora sp., *M.circumcinctus*, and *O.niloticus* fish had severe damage levels, with HAI scores exceeding 100, because in three species of fish in this study, telangiectasis was found at the end of the secondary lamella which was marked by enlargement at the end of the secondary lamella. This is considered irreparable damage. The research on the microanatomy of fish gills as an indicator of heavy metal pollution in the waters of Kaligarang Semarang, with a HAI score >100 indicating very severe damage due to edema, lamella fusion, hyperplasia, and necrosis caused by the heavy metal mercury (Hg) [36]. This study was also reported by [37], that if *A.latus* gills were exposed to sub-lethal concentrations of $HgCl_2$, they showed lamellar fussion and epithelial lifting, whereas higher concentrations showed vascular alteration such as congestion and aneurysm, indicating critical water conditions. Another study found that *Clarias batrachus* gill tissue exposed to mercury and cadmium at levels below lethal thresholds showed lamellar disintegration, vacuolization, secondary lamellar fusion, and epithelial surface hyperplasia [38].

The current study found that mercury in various *C. batrachus* sublethal concentrations had severe effects on tissues, such as the gill, which lost its normal structure, including club shape, erosion lamellae, complete necrosis of gill filaments, and fusion of lamellae at several points. *C. gariepinus* fish exposed to effluent water including aluminum, lead, cadmium, zinc, and heavy metals showed alteration of lamellar atrophy, telangiectasia, and necrosis of lamellar epithelial cells [39]. Other research reported the gills of tilapia exposed to $HgCl_2$ showed necrosis of the gills as a result of prolonged exposure (30 days) to irritants include suspensions found in water [40].

Alteration in the gills can result in fish death due to the process of anoxemia, which is the suppression of respiratory function as a respiratory organ, disrupting the gill circulation and excretion systems. Damage to the gills can result in fish death due to the process of anoxemia, which is the suppression of respiratory function as a respiratory organ, disrupting the gill circulation and excretion systems. The most severe alterations were discovered to be associated to the most influenced environment, indicating the existence of stressors in the water such as inorganic or organic pollutants, which are detrimental in long term.

According to the findings of this study, additional and more in-depth research into the amount of heavy metal accumulation in fish organs and its impact on organism health is required to provide a more complete data of effect of heavy metal pollution in Lebong Regency. The objective is to increase environmental awareness and make it a priority for the local government to provide solution the gold mining waste problem.

4. Conclusion

Histopathological alteration in fish gill tissue from the river common experience hyperplasia, epithelial lifting, hyperplasia, and lamella fusion. Alteration in stage I include slight damage with a HAI score less than 20. While the presence of telangiectasis is one of the signs of irreversible damage to fish gills with HAI score more than 100. The presence of parasites in some fish gills implies an infection, which disrupts normal gill function for respiratory and excretory organ. Alterations in the structure of fish gills can be used to assess the quality of waters contaminated by heavy metal contaminants from artisanal mining waste disposal.

5. Acknowledgement

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