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Effects of pollutants on fish gills and molecular damages of Mozambique tilapia, *Oreochromis mossambicus* (Peters, 1852)

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ABSTRACT

Aquatic pollution is the most hazardous one and is of great concern. There is always a need for bio-monitoring tools for impact assessment. The study was designed to investigate the influence of water pollution on biological systems by identifying the cellular destructions- direct and reliable evidence. In the present study, two widely accepted parameters like, histopathological alteration of gill apparatus and micronucleus assay from blood, were analyzed for exposure of 120 and 96 hours under 1/5th times diluted pharmaceutical effluents water in *Oreochromis mossambicus*. Gills were extracted from the control and exposed groups and the slides are made after chemical processing and microtomy. Blood samples were also collected, and prepared a smear. Histology slides were stained with haematoxylin and eosin differential staining and blood smear slides were stained with Giemsa staining. The main histopathological alterations observed were lamellar necrosis, lamellar shortening, telangiectasis and lamellar clubbing. The severity of the lesions increased with the time of exposure, with the complete loss of vascular integrity having been observed in the groups exposed for 120h. Inductions of micronucleus formation in blood cells were also observed, and an increase of micronucleus formation observed as time dependent manner. These results indicate that histopathology of gill and micronucleus formation in blood can be used as a biomarker tool to affect aquatic pollution directly.

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1. Introduction

Industrial effluent is a major cause of pollution in developing countries like India and other south Asian counties. Industrial runoff in the river and other wetlands is a common practice in these countries, resulting in direct mixing of pollutants to wetland ecosystems such as rivers, lakes and oceans. Small scale industries especially situated in suburban region, use freshwater to carry out waste which become the major source of environmental pollution. In most cases, the lack of a proper system of filtration and detoxification of these effluents and lack of proper government surveillance increases these problems severalfold. According to Singh, only 60% of industrial effluent may be treated before release and the rest remains untreated [Singh *et al.*, 2021]. Pollutants such as asbestos, lead, nitrates, phosphates, mercury, and other heavy metals are the major source of pollutants to the ecosystem. [Inyinbor *el. al*. 2018, Gheorghe *el.al* 2018, Shah 2017].

Change or disruption in different metabolic pathways of this fish species due to environmental pollution ultimately induces a harmful effect and when eaten by human beings also affects negatively. Accumulating toxic materials and heavy metals within the fish tissue may create huge abnormal conditions in human health, collectively known as bioaccumulation and further biomagnification effects [Chapman 1992].

The formation of Micronucleus (MN) in blood cells and histo-pathological alterations of specific organs like gills can be a good indicators to assess environmental pollution and are accepted globally. Changes in histological architecture in gill tissue indicate the preliminary site of exposure of different toxicants and building up the first line of defense by the gills. Absorbance of pollution at the cellular level also produces DNA alteration and damage which creates micronucleus. Micro nuclei are one of such biomarkers that are cytoplasmic chromatin masses with the appearance of small nuclei that arise from lagging chromosomes at anaphase or from acentric chromosome fragments. Chromosomal fragments lagging behind are excluded from the main nuclei in the daughter cells during division. These fragments form their own membranes and appear as feulgen-specific bodies, termed micronucleus in the cell cytoplasm [Alimba and Bakare 2016, Alimba *et. al*., 2015a, Alimba *et. al*., 2015b, Alimba *et. al*., 2017].

In the present study, two most frequently used bio-monitoring parameters, micronucleus assay and gill histo-pathological alterations were adopted to observe the toxic effect of industrial effluent in aquatic ecosystem. A preliminary water analysis showed the presence of organic acetate and butyrate, inorganic phosphate, and sulfate compounds. *Oreochromis mossambicus*, known as Tilapia, were chosen for that purpose, as experimental models and exposed to that pharmaceutical effluent water in variable times (96 hours, 120 hours) for toxicological assessment.

2. Materials and Methods

2.1 Experimental Animals

Oreochromis mossambicus is a fresh water fish belongs to the family Cichlidae and order percipormes. All fishes for experimental purpose were collected from a local fish firm and acclimatized in filtered dechlorinated water in the oxygenated facility two weeks before the experiment. They were acclimated to laboratory condition of 25.0^{o} ± 3.0^{o} C and 12/12 hours dark/light modes. The fishes were fed balanced nutritious food (HERONS Freeze Dried Tubifex Worm, supplemented with vitamin and minerals). The fecal matter and other waste materials were siphoned off daily during the acclimatization and exposure periods. All fishes were of similar weight and size (length 12.01±0.37

cm and weight 18.77±0.21gm). Fish without any structural, clinical and behavioral symptoms were chosen for experiment. Care and use of laboratory animals follow the guideline published by the US National Institute of Health (NIH Publication No. 85–23, 1996) and approved by the institutional ethical committee [CIOMS 2012, Gad 2007].

2.2 Collection of the sample

An urban small scale pharmaceutical company was selected to study the effect of effluent water on *Oreochromis mossambicus*. This pharma company is located in the district of north 24 parganas, state West Bengal, India. It was chosen because it is situated in a densely populated area and discharges huge amount of effluent untreated into the locality. The sample was collected into five, 1.5L capacity polypropylene sterile containers from the selected site just one day before the experiment.

2.3 Experimental Design

The acclimatized fishes were divided into three groups and 10 fishes were taken in each group. The first group (Gr-I) was considered as negative control group and maintained in de-chlorinated water. Other two groups were exposed in the pharmaceuticals effluent water, diluted five times ($1/5^{th}$) with de-chlorinated tap water (v/v; effluent/ de-chlorinated water). The second exposed group (Gr-II) was used for micronucleus assay and third exposed group (Gr-III) for histo-pathological study. All three groups were subdivided into two sub-groups containing five fish into each sub-group. Subgroup-A (Gr-IA, Gr-IIA, Gr-IIIA) exposed for 96 hours exposure and sub-group B (Gr-IB, Gr-IIB, Gr-IIIB) for 120 hours exposure of pharmaceutical effluent water, containing 5liters/tank of pure as well as diluted effluent water.

2.4 Micronucleus Assay

The fish was anesthetized; peripheral blood was collected by cardiac puncture in heparinzed syringe and a clean smear was drawn onto grease free glass slide. The slides were fixed with 85% ethanol for 20 min and then air dried for 24 hours. Slides were immersed into freshly prepared 10% giemsa solution diluted with phosphate buffer (ratio 2:3) respectively for 20 minutes, rinsed with double distilled water and finally air dried. Six slides per fish were prepared and coded. Cells were scored under microscope (Olympus) at 1000X magnification. Slides of poor quality due to thick and uneven smear or inefficient puncture were discarded before scoring. A mean of 1000 cells were scored from each fish. The $1/3^{rd}$ size of the main nucleus, non refractive, circular or ovoid chromatin bodies displaying similar staining and focusing pattern were considered only as micronucleus [Alink *et. al*., 2007, Obiakor *et. al*., 2010].

2.5 Histo-Pathological Study

Gills were removed from the opercular chamber of anesthetized exposed fish and immediately fixed in 10% neutral buffered formalin for 24 hours. The major steps involved in histopathological analysis are fixation, tissue processing, decalcification, section cutting and staining [Raphael 1976]. Decalcification has been done in 10% nitric acid. Tissue processing steps involves dehydration in increasing concentration of alcohol and then clearing and infiltration of the tissue with paraffin. Sections were cut at 5 µm thickness and were floated in a water bath between 38-49°C. The sections from water were then mounted on clean glass slides smeared with Mayer's egg albumin. They were then dried on a hot plate at about 50°C for 30 minutes. The sections on the slides were stained with Haematoxylin and Eosin (H.E) differential staining. [Luna 1968]. The slides were observed under microscope (Olympus) and digital photograph had been taken. For the histopathological study, eight to ten images were analyzed per fish and also ten secondary lamellae were viewed per photograph and any occurrences of the histological alterations were noted [Ramudu *et al.*, 2020]:

2.6 Statistical Analysis

All result was presented as mean ± standard error. Data were processed using a crossed two way analysis of variance (ANOVA) where factors were pharmaceutical effluent water exposure and duration of exposure. The dependent variable was micronucleus frequency. The micronucleus induction rate was calculated by the ratio of MN frequency between exposed and control samples.

3. Results

3.1 Micronucleus assay

Micronuclei (MN) induced by the exposed industrial effluent water in erythrocytes were appeared dot-like structure close to the main nucleus with size and shape and varied among cells. Mostly each affected erythrocyte observed single micronucleus while more than one micronucleus within the same cell was also observed in minimal number. The induction rates of micronucleus were observed in peripheral erythrocytes after 96 and 120 hours of exposure were 4.44 and 4.45 respectively. There was only 0.58±0.47 occurrence of micronucleus frequency were recorded in control group. MN frequency in fish exposed to pharmaceutical effluent water was much higher, 2.58± 0.43 for 96 hours exposure. The frequency of micronucleus scored in control and pharmaceutical effluent water (96 hours and 120 hours) in exposed fish and MN induction rate have been clearly depicted in Table 1. [Fig. 1 A-C)].

The results of two-way analysis of variance (ANOVA) for pharmaceutical effluent exposed water (Table 1) showed a significantly high micronucleus induction in both 96 and 120 hours exposure with pharmaceutical effluent ($1/5^{th}$ dilution) when compared with its respective control sample for each duration of exposure ($p‹0.05$). However, there was no time dependent change in micronucleus induction between 96 hours and 120 hours of exposure.

Table 1. Frequency of micronucleus scored in control and pharmaceutical effluent water exposed fish and MN induction rate

Exposure (Hours)	Experimental Groups	MN Frequency	Induction Rate
96	Exposed	2.58±0.43*	4.44
	Control	0.58±0.47	
120	Exposed	2.80±0.36*	4.45
	Control	0.63±0.79	

*Significant difference from the corresponding control group p‹0.05 level.

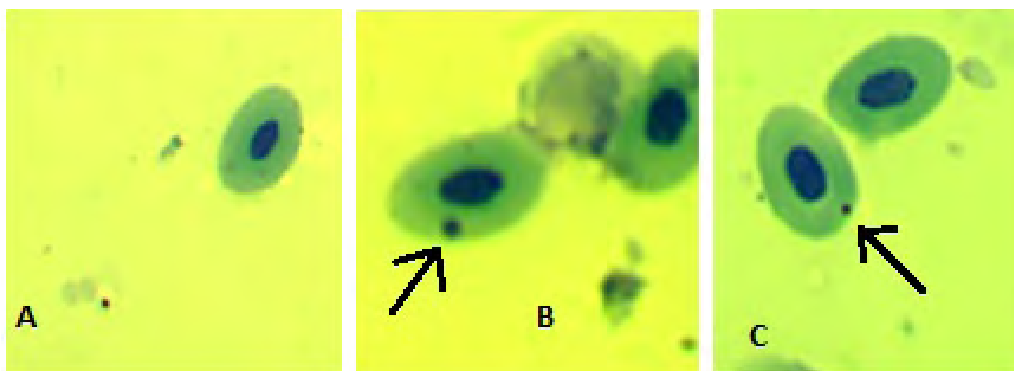

Fig. 1. Micronucleus formation observed in the gills of Oreochromis. mossambicus exposed to pharmaceutical effluent water for 96 hours and 120 hours time intervals and compared with control fishes A: Erythrocytes in control fish, B: Micronucleus observed in fish with 96 hours pharmaceutical effluent water exposure, C: Micronucleus observed in fish with 120 hours pharmaceutical effluent water exposure.

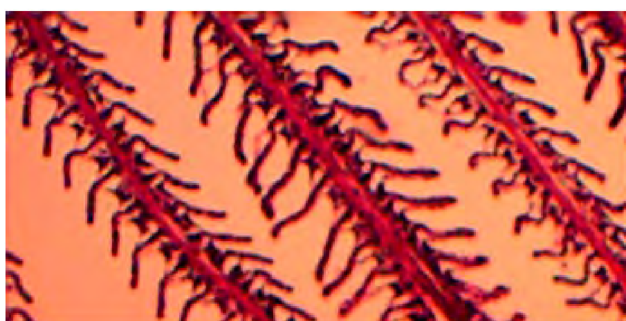

Fig. 2. Gills of the control fish *Oreochromis mossambicus*

3.2 HHistopathologicalstudy

A normal pattern of gill filament was observed in control fish. The gills are present in gill chamber and supported by gill arches. Each gill consists of two rows of slender filaments. The filaments are made up of vertically placed lamellae which are lined by squamous epithelium composed of non differentiated cells. Below that epithelium are lamellar blood sinuses separated by pillar cells. A thick stratified epithelium constituted by chloride, mucus and pavement cells separates filaments and lamellae of the gill**.** The structural details of the gill of control *O. mossambicus* are shown in Fig. 2. In comparison to control, in the pharmaceutical effluent water treated for 96 hours group has been observed complete or partial architectural loss, necrosis of secondary lamellae, desquamation of epithelial layer, hyperplasia of primary filament, lamellar telangiectesis, degenerated lamellae, lamellar cubing, fused lamellae and lamellar shortening**.** The most significant gill histopathological alterations observed in 120 hours exposed groups were lamellar bending and necrotic lamellae. Other alterations observed in 96 hours exposed group were also observed in the group of 120 hours but the severity varies as per the increase of exposure time. [Fig. 3 (A-L)]. The severity of gill histopathological alterations with varied exposure time was shown in Table 2.

Table 2. Histo-pathological alterations observed in the gills of *Oreochromis. mossambicus* exposed to pharmaceutical effluent water for 96 hours and 120 hours time intervals and compared with control fishes

Histopathological Alterations	Exposure Time		
	Control	96 hours	120 hours
Necrosis of secondary lamellae	-------	+	+ +
Detachment of epithelium	-------	+ +	+ + +
Hyperplasia of primary filament	-------	+ + +	+ + +
Epithelial desquamation	-------	+ +	+ + +
lamellar shortening	-------	+	+ +
Lamellar bending	-------	-------	+ +
Lamellar telangiectesis	-------	++ +	+ + + +
Degenerated lamellae	-------	+ +	+ + +
Lamellar cubing	-------	+	+ + +
One sided fused lamellae	-------	+ +	+ + +
Necrotic lamellae	-------	-------	+ +

Note: The gill histopthological alteration in the gill morphology were divided based on the severity into five grades such as (-) no alteration, (+) alteration in < 25%, (++) alteration in < 25-50%, (+++) alteration in < 50 to 75%, (++++) alteration in > 75%.

4. Discussion

In the present study, effect of the pharmaceutical effluent water at 1/5th concentration was severe in both histological and genomic level in fish model but did not lead immediate mortality of the exposed fish. The exposure caused a significant induction of micronucleus in blood. The MN assay is a reliable, sensitive and cost effective assay technique and has been used more than two decades as biomarker of toxicology for testing toxic agents in animal model especially in fish. The induction of MN in fish blood cells proves the clastogenic nature of the effluent water [Al-Sabti 1986]. The high frequencies of both single and double micronucleus together are in agreement with previous studies of micronucleus frequencies in fishes from the polluted habitat. The literature review revealed that the pharmaceutical effluent water increases frequency of MN in polychromatic erythrocytes and chromosomal aberration in bone marrow cells in mice and rat species [Bakare *el. al.,* 2009, Adeoye *et. al.,* 2015]. Disruption in DNA synthesis during hematopoiesis and cell cycle [Udroiu 2006], altered karyokinesis and cytokinesis during cell division [Fenech *et. al*., 2011], alteration of p53 protein expression , activation of antioxidant genes associated with apoptosis [Verlhac and Gabaudan 1994, Pulido and Parrish 2003], collectively or individually, may play an important role for the induction of the cytogenetic abnormality like micronucleus formation in blood cells of fish. This current result also proved that these cyto-genotoxic modifications mainly occur in response to

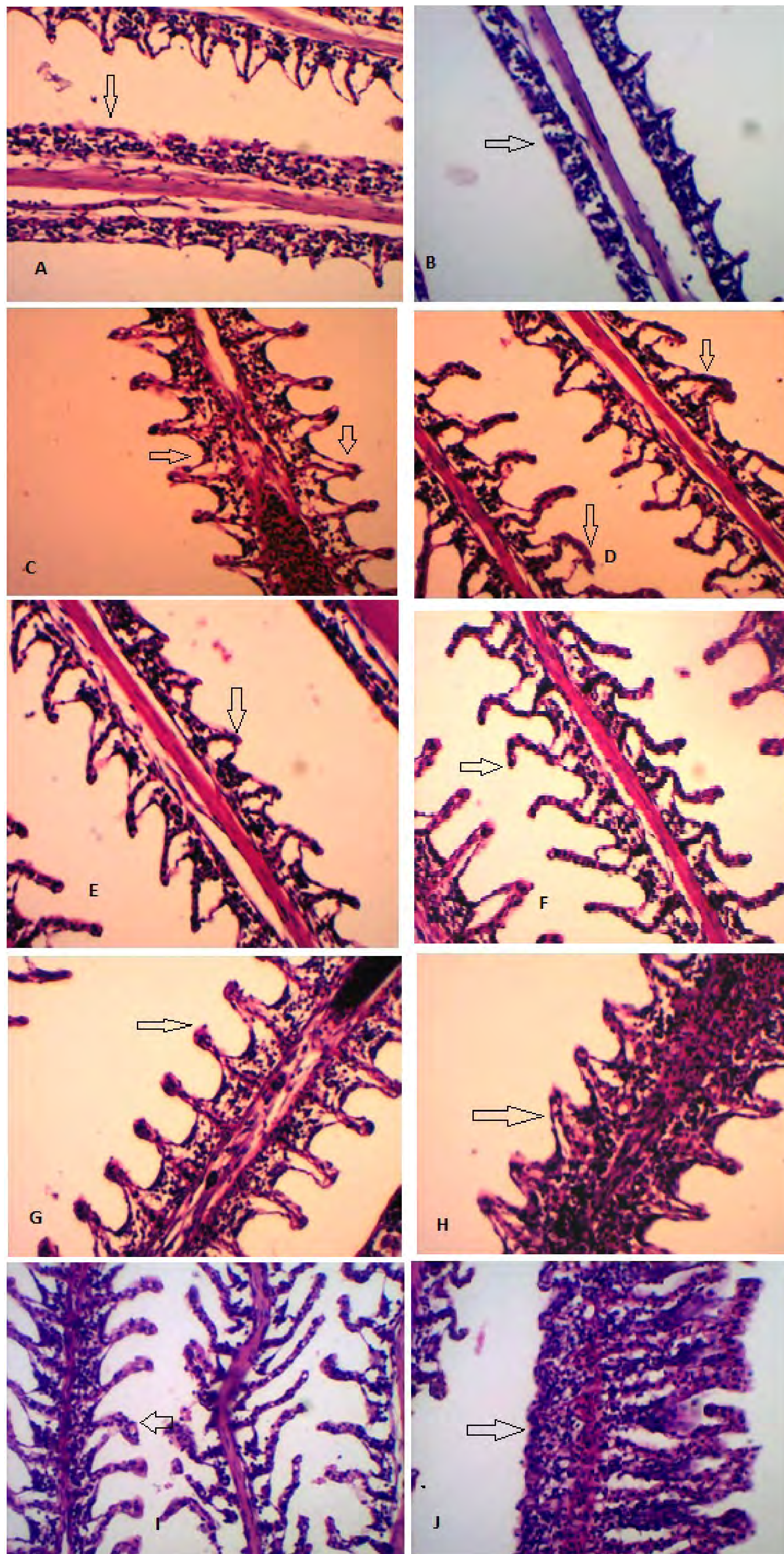

Fig. 3. Histopathological alterations observed in the gills of Oreochromis. mossambicus exposed to pharmaceutical effluent water. A & B: Complete necrosis of secondary lamellae C: Detachment of Epithelium, D: Hyperplasia of primary filament, E: Epithelial desquamation and lamellar shortening, F: Lamellar bending, G: Lamellar telangiectesis, H: Degenerated and necrotic lamellae, I: Lamellar telangiectesis and Lamellar cubing, J: Degenerated and necrotic, K: One sided degenerated and necrotic lamellae, L: One sided fused and necrotic lamellae

the toxic stress from the environmental exposure and thus can be used as an early monitoring of water inhabitants using simple techniques like micronucleus assay.

Histopathological changes in fish tissues especially in gills have been widely used for toxicological testing in field study as well as laboratory experiments [Mela *et. al*., 2007]. It is well known that changes in fish gill are among the most commonly recognized responses to environmental pollutants [Mallatt. 1985, Laurent and Perry 1991, Au DWT 2004]. In the present experiment extensive loss of gill morphology and architecture as a time dependant manner was observed. The observable gill abnormalities were hyperplasia, lamellar telangectiasis, lamellar fusion, lamellar cubing and lamellar shorting, lamellar necrosis, complete desquamation of the gill epithelium.

Increase in the number of normal cells of a gill tissue was referring as hyperplasia increases the distance between blood and external environment thus reducing the toxicant-blood diffusion distance which in turn serve as a barrier to the entry of toxicant into the body. This kind of fish response may help to block the stimuli generated by the toxicant has been found in the present study [Worolynska *et. al*., 2017]. Lamellar telangiectasis or localized dilation of the blood vessel was exhibited in pharmaceutical effluent water exposed group in both 96 hours and 120 hours time durations. The complete loss of vascular integrity observed in pillar cell system of secondary lamellae help to release huge amount of blood which push the lamellar epithelium outward resulting Lamellar telangiectesis [Worolynska *et al*., 2017, Hassaninezhad *et. al*., 2014]. Extensive epithelial desquamation was also observed in the 96hours and 120hours pharmaceutical effluent water exposed group. Decrease or shortening of the size of gill lamellae and the bulbous enlargement at the end of the gill lamellae and also of adjacent lamellae, known as clubbing, were well marked in the present study in the Pharmaceutical effluent water exposed group. Gaseous exchange mainly occurs through the surface of the gill lamellae. Fusion of gill lamellae reduced the total surface area results decrease of gaseous exchange. Excessive mucous secretion and epithelial lifting together increase the water blood barrier which may reduce the oxygen uptake drastically. The reduction in the oxygen consumption disrupts the osmoregulatory functions of gills leading ionic imbalances and decreased bronchial ATPase activity. The observed lamellar necrosis and complete desquamation of the gill epithelium in the present study have been seemed to be direct responses induced by the action of toxic compounds presents in pharmaceutical effluent [Dutta *et. al*., 1997].

The toxic effect of the polluted water has been clearly demonstrated in aquatic fish species tilapia. The present study shows that the histo-pathological biomarkers of toxicity in fish gill are quite visible effect and can be easily detected. The frequency of tissue alterations like hyperplasia, hypertrophy of epithelial cells and telangiectesis along with variety of other deformities shows a direct link to aquatic pollution.

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