Original Research Article

Effects of textile dye, Basic Red-18 on Tilapia fish and reduction of these effects by bioremediation with a novel bacterium, *Mangrovibacter yixingensis* strain AKS2 isolated from textile wastewater

ABSTRACT

Due to rapid industrialization and market demand of vibrant textile products, the natural textile dyes have been replaced by the synthetic textile dyes. These synthetic dyes are released in environment with textile wastewater resulting in a major environmental pollution, especially in aquatic ecosystem. Hence, aquatic organisms like fish are highly vulnerable to the pollution caused by dyes of textile wastewater. This study was designed to evaluate the deleterious effects of Basic Red-18 (BR-18) dye on behavior, survivability, haematology and histology of Tilapia fish (*Tilapia mossambica*) and to minimize these deleterious effects of BR-18 dye by bioremediation with the novel bacteria isolated from textile wastewater. The isolated novel bacteria was identified as *Mangrovibacter yixingensis* strain AKS2 by 16s rRNA sequencing (Accession no. OM189530). The 30% and 70% mortality rates were observed in fish exposed to untreated BR-18 dye at concentrations of 100 and 200 ppm respectively. Interestingly, the mortality rate of fish was decreased significantly to 10% and 20% when fish were exposed to 100 and 200 ppm BR-18 dye respectively after treatment with *M. yixingensis* strain AKS2. Fish exposed to tap water and treated dye solution exhibited typical behavioral responses, whereas fish exposed to untreated dye solution exhibited anomalous behavior. Fish subjected to untreated dye solution displayed decreased RBC, Hb, but increased WBC levels, demonstrating the dye's haemotoxicity. Contrary, no remarkable haematological toxic effect was found when fish were exposed to treated dye indicating the non-toxic character of the degraded dye metabolites. Similarly, extensive histological abnormalities in the gill, liver, intestinal, stomach, and heart tissues were seen when fish was cultured in untreated BR-18 dye, but the abnormalities were less significant when fish were raised in treated dye. Altogether, it can be concluded that BR-18 dye are toxic to fish, but this toxicity can be minimized by bioremediation with *M. yixingensis* strain AKS2.

Keywords: Decolourization, Bioremediation, Azo dye, *Mangrovibacter yixingensis* strain AKS2, Fish

1. INTRODUCTION

The textile manufacturing industry is one of the oldest and most complex industries worldwide. Textile production was also increased due to the increasing demand of the people. Rapid industrialization and market demand have replaced natural dyes with synthetic dyes due to

better durability, brightness, wide color gamut and ease of application and cost savings. Textile factories and their wastewater have increased accordingly, causing a major pollution problem in the world [1]. It is recognized as the root cause of environmental pollution. Many chemicals used in the textile industry cause environmental and health problems [2-4]. In textile dyeing wastewater, dyes are considered as important pollutants. It greatly reduces the oxygen concentration in the water due to the presence of hydrosulfide and blocks the transmission of light through the water body, which is harmful to the aquatic ecosystem. About 40% of the dyes used worldwide contain organo-bonded chlorine which is a carcinogen. It constitutes an emerging threat to ecological disturbances and is the cause of many physiological and biological variations of aquatic animal resources. Untreated wastewater is filled with many organic and inorganic substances that are mutagenic and carcinogenic during human interactions [5]. It also causes behavioral changes, especially in fish in the ecosystem, leading to stress. Fish is an ideal model for toxicology studies as it is very sensitive to any physicochemical or biological changes in water quality. An organism's behavior represents a unique perspective that links its physiology to its environment [6]. The fish's altered behavior is the first reflex response to any toxic substance. It is a good biomarker for determining the impact of toxic substances on aquatic ecosystems [7].

Fish can serve as bioindicators of environmental pollution and can therefore be used to assess water quality [8, 9]. Due to chemical contamination, the normal functioning of cells is disturbed, which can lead to deterioration of physiological and biochemical mechanisms of animals leads to impairment of important functions such as respiration, osmotic regulation, reproduction and even death [10, 11]. Hematology pools are useful in assessing the health and general condition of animals under changing environmental conditions.

The toxicity of dyes depends on the physico-chemical composition of the water as well as other substances present in the water. Fish live close to the aquatic environment and are very sensitive to many types of toxic substances. They serve as valuable models for assessing the effects of different pollutants. A series of hematological and histological responses in fish were used as biomarkers of various environmental stresses [12].

About 10-15% of dyes are released into the environment during the dyeing process [13]. Therefore, textile azo dyes have certainly attracted attention for their properties such as toxicity, non-biodegradation, carcinogenicity, mutagenicity etc [13, 14].

Basic Red 18 is a cationic azo dye used to color textiles. *T. mossambica* is widely grown due to consumer preference and commercialization. Knowledge of the hematological and histopathological effects of fish exposure to dyes to determine their mode of action. Characterization of fish blood components was used to measure the response of the selected dye (BR -18) to *T. mossambica* . *T. mossambica*, a benthic species that mainly consumes algae and aquatic plants, was selected as the experimental animal in this study due to its fast growth rate, high endurance, tolerance to high population densities and high population density and the ability to survive in oxygen-deficient waters. Studies regarding the exposure of *T. mos*sambica to BR -18 treated with the strain *Mangrovibacter yixingensis* AKS2 are few.

Toxic stress can be assessed by analyzing hematological parameters such as red blood cells, white blood cell counts, and Hb. Studies regarding exposure to BR -18 dye on *T. mossambica* are very limited. Therefore, this study aimed to evaluate the exposure of BR -18 dye activity on hematological parameters such as %Hb, total white blood cell count, leukocyte differentiation

and red blood cell count (total number of red blood cells) and histopathology of *T. mossambica* after untreated exposure and treated textile dyeing waste water.

Exposure to untreated dyes in water bodies can cause behavioral changes, biological and physiological changes, allergy, mutagenicity, carcinogenicity and harmful effects on animals, especially fish [15, 16]. Therefore, this study becomes important with an animal toxicity test that will provide a complete and effective solution to perform in contaminated river systems without harming aquatic fauna.

Various physical methods can be used to remove azo dyes from wastewater. Some of these methods are effective but quite expensive because they produce significant amounts of chemicals. So in such situations, bioremediation can be a real hope. Therefore, this study was designed to efficiently isolate strains of azo bleaching bacteria from textile dyeing wastewater. Therefore, these bacteria can be used to develop biological treatment systems for wastewater contaminated with azo dyes. Treated wastewater from dyeing industries has been used in aquaculture practices around the world for the production of fish biomass, which is a primary purpose with primary concern for waste water reclamation [17, 18]. Therefore, this study was performed to evaluate the effect of exposure of freshwater fish to textile dyeing wastewater with reference to its histological and hematological parameters under laboratory conditions.

2. MATERIALS AND METHODS

2.1 Collection and Storage of the Sample

Wastewater is collected from different textile dyeing industries in Sathia, Sirajgonj, Gazipur, Madhapdi, Narshingdhi, Bangladesh. Samples were taken from a variety of locations, such as drainage channels for stagnant textile dyeing wastewater. Samples were in the form of untreated liquid wastewater and untreated sludge. All samples were collected in sterile plastic bottles and sterile polyethylene bags and stored at 4°C in the refrigerator for 24 h to avoid changes in their physicochemical properties.

2.2 Dyes and Culture Media

Basic Red-18 dye purchased from DysinChem limit, Dhaka. All media components and chemicals used in studies of analytical quality.

The Basic Red -18 dye used in this study is of industrial origin.

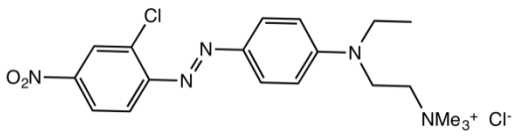

Fig 1: Basic Red-18

2.3 Isolation and Screening of Dye Decolorizing Bacteria

All samples (untreated textile wastewater) were used to Isolation of cultured bleaching bacteria from dyes by LuriaBertani. enrichment culture technique (LB) medium modified with 20 ppm of test dye Malachite Green for microbial adaptation. LB agar plate prepared by Dissolve 0.5g of NaCl, 1 g of peptone and 0.5 g of yeast Extract in 100/ml distilled water, adjust pH to 7 then 1.5 g of agar was added to 250 ml flasks. The medium was autoclaved at 121°C and 15 . lbs/inch2 pressure for 15 minutes. Bacterial colonies shows a clear discolored area around them on LB agar was collected and cultured for 24 h in MS medium modified with 1 ml/1 TE Dissolution. The growth of bacterial colonies is observed after 24 h of incubation at 35°C. Effect dyes on the growth of bacterial strains have been determined in minimum salt medium (MSM) that content (g/l): K2HPO4, 2; (NH4) 2SO4, 0.5; KH2PO4, 0.2; MgSO4.7H2O, 0.05 modified with 1 ml/1 spot Elemental solution (ET) contains (g/l): FeSO4.7H2O, 0.4; ZnSO4.7H2O, 0.2; MnSO4, 0.4; CuSO4, 0.04; KI, 0.3; Na2MoO4, 0.05; CoCl2, 0.04; (pH 7) and added with 20 ppm of azo Malachite Green dye. After certain incubation period from discoloration Test tube strains of dye-degrading bacteria are stored on LB agar plate at 4°C for further testing.

2.4 Genomic DNA Extraction and 16S rDNA Gene Amplification

Genomic DNA was extracted from dye decolourizing bacteria using CTAB method [17].The PCR primers used to amplify 16S rDNA fragments were the bacteria-specific primers a forward primer 8F (5'-AGA GTT TGA TCC TGG CTC AG-3'; Tm: 61°C); and a reverse primer 806R(5'-GGA CTA CVS GGG TAT CTA AT-3'; Tm: 67.4°C). A total of 25 µl of reaction mixture consisted of – water 15µl, MgCl2 2.5µl, buffer 2.5, dNTPs 0.5µl, template 1µl, primer (forward 2 µl and reverse 2 µl). The PCR amplification was performed by Swift™ Minipro Thermal Cycler (Model: SWT-MIP-0.2-2, Singapore) using the following program: Denaturing at 95°C for 5 minutes, followed by 40 cycles of 40 seconds of denaturing at 95°C, 60 seconds of annealing at 65°C and 2 minutes of elongation at 72°C with a final extension at 72°C for 10 minutes. Then, the PCR products were subjected to 1% agarose gel electrophoresis, stained with ethidium bromide and visualized on a UV transilluminator for the presence of about 1500 bp PCR products.

The amplified PCR product was purified using AccuPrep® Gel Purification Kit (Bioneer Company, Korea) according to the manufacturer's protocol. PCR amplified 16s RDNA of the isolates screened as submitted for automated sequencing (Applied Biosystems 3130) at the Center for Advanced Scientific Research (CARS) of the University of Dhaka, Bangladesh. The sequence generated from the automatic sequence of PCR amplified DNA analyzed by NCBI BLAST Program (http://www.ncbi.nlm.nih.gov) for discover a similar organism possible through association of similar sequences. Finally, the isolates were determined based on partial sequence alignment of 16S rDNA with sequences available in database.

2.5 Sequencing of 16S rDNA & BLAST Analysis

The nucleotide sequence of the 16S rDNA gene has been sequenced on both sides through the BigDye chain termination cycle sequencer (ABI) and the sequence is decoded on Dideoxy Sanger 3130XL String Genetic Analyzer (ABI). The final method is then assembled by the Cap3. program for genetic sequencing. Gene sequence has been determined by looking for similarities in the database via BLASTn for 16S rDNA.

2.6 Effect of Different Parameters on Process of Dye Decolorization

Effect of initial dye concentration on discoloration of Basic Red- 18 dye by isolated bacteria was tested after 96 h incubation as described previously [2]. In short, for examined the effect of different dye concentrations on color change, MS medium is added 100 and 200 ppm Basic Red- 18 dye was adjusted to pH 7. Then, the medium was inoculated with bacterial strains incubated at 35 °C for 192 h.

2.7 Measurement of Decolorization Efficiency

The decrease in absorbance at absorption maxima(λmax) was monitored using a UV-Visible spectrophotometer to evaluate decolorization activity in terms of % decolorization. Uninoculated MS medium supplemented with Corresponding dyes were used as a reference. At different times interval, 2 ml of sample was taken from reaction mixture and centrifuged at 10000 rpm for 10 min for biomass separation. The concentration of dye in the supernatant is determined by monitoring the absorbance at maximum absorption wavelength (λmax) at 660 nm. Bleaching dosage is calculated according to the following formula

$$Dye\ Decolourization\ (\%) = \frac{Initial\ absorbance - Final\ absorbance}{Initial\ absorbance} \times 100$$

2.8 Collection of Experimental Fish

Live, T. mossambica of size (weight 37.58±0.79 g, length, 15.01 ± 0.27 cm) were collected from fish landing centre of Saheb bazar, Rajshahi. The experimental fishes were carried to the laboratory for conducting the experiment and kept in 60 L tank with natural pond water.

2.9 Acclimatization

Experimental fishes were acclimatized to aquaria filled with pond water under laboratory condition for one week. Same temperature (26±0.1^{0}C) was maintained during the acclimatization. The water in aquaria was properly aerated to avoid any contamination. The water in the acclimatization tank was renewed daily to remove the excess amount of feed and excretory materials. Dead fishes were removed immediately to prevent the contamination and to maintain desired concentration of oxygen. Prior to the investigation of experiment, healthy fishes were collected randomly and transferred into the respective treatments. The experimental fishes were fed twice a day (at 8:00 a.m. and 6:00 p.m.).

2.10 Experimental Procedure

Five test glass aquaria were taken for conducting the experiment. The test glass aquaria were washed with detergents and 0.1% potassium permanganate solution to prevent from fungal infection. The test aquaria were dried under sunlight and each aquarium was filled with 30 liters

of pond water and contained 10 *T. mossambica* fishes. The collected fishes were divided into control group and treatment group. Test water was changed every 48 h. Fishes were sampled and dissected for hematological and histological determination after 15 and 30 days following to dye exposure. They were not fed for 24 h before being sacrificed for hematological as well as histological studies.

2.11 Preparation of Treated Dye Solution

Mangrovibacter yixingensis strain AKS2 was introduced into the aqueous solution amended with Basic Red-18 dye and the decolourization efficiency was recorded at room temperature. The physico-chemical characterization (color, odor, pH, electrical conductivity, total dissolved solids (TDS), total suspended solids (TSS), total solids (TS), biological oxygen demand (BOD), chemical oxygen demand (COD), total hardness, total alkalinity, chloride, sulphate and nitrate) of untreated and treated Basic Red-18 dye solution was assessed and the decolorized/treated dye solution is used for the zootoxicity study.

2.12 Dose Exposure of Experimental Fish

The dye Basic Red 18 used for this investigation was obtained commercially from local source and was directly used for the experiment. Stock solutions were prepared by dissolving dye in water and diluted further to obtain desired concentrations in test aquaria.

100 and 200 ppm doses of Basic Red 18 were estimated for conducting the experiment to which the experimental fishes were exposed. During conducting the experiment, both control and treated fishes were fed with commercial diet. Here the control group was maintained properly. The experimental fishes were treated with different concentrations, viz. 0 (control) 100 and 200 ppm of Basic Red 18 dye.

2.13 Mortality Rate

Behavioural changes like movement, feeding style, frequency of excretion, opercula movement were observed very carefully in both control and dye exposed groups at regular intervals throughout the experiment.The mortality of L. rohita was recorded for every 24 h during the observation period. Fish are considered dead if there is no visible movement (e.g. gill movements) and if touching of the caudal peduncle produces no reaction, they were removed and mortality was recorded. At the end of the experimental period, the fishes from each treatment and control were collected for toxicological studies.

2.14 Collection of Samples for Toxicological Studies

The blood was collected from the experimental fishes by cardiac puncture using heparinized syringes and transferred into heparin-coated vials. The whole blood was used for hematological studies. For RBC and WBC count, 20µL blood was mixed with 3,980 µL of RBC diluting fluid and WBC diluting fluid respectively in pipettes. Then, these mixtures were shaken well

separately and then poured onto hemocytometer to count the cells [19]. The hemoglobin content was estimated by cyanmethaemoglobin method using Drabkin's fluid (SPAN Diagnostics, India). 20µL blood was mixed with 5 ml of Drabkin's working solution.The absorbance was measured at 540 nm using a spectrophotometer and was expressed as g/dl [20].

2.15 Haematological Analyses

RBC and WBC were counted by haemocytometer [21], and the hemoglobin concentration was estimated by cyanmethemoglobin method [22].

2.16 Histological Examination

For histological investigation, The tissues of liver, intestine, stomach & gills were dissected from control, untreated and treated exposed fishes for histological studies. The histological changes in the tissues were examined under light microscope and photomicrography. The changes in the tissues of the treated fishes were observed and compared with the control fish. The dissected tissues were immediately fixed in 10% formalin and washed in distilled water, dehydrated in graded ethanol series (30%, 50%, 70%, 85% and 100%), infiltrated with xylene and embedded in paraffin wax at 56–60 °C. The tissues embedded in paraffin wax were sectioned using a rotator microtome (5 µm) sections were placed on glass slides and treated with xylene to remove paraffin and subsequently washed in 90%, 70%, 50% and 30% alcohol (Bancroft and Cook 1994). Finally, paraffin-free sections were washed with distilled water, stained with haematoxylin for 3 min and washed in running tap water for 1 min. Finally, the tissues were stained in eosin for 45 s, examined under microscope (Labomed, California) and photographed. Histopathological changes in these tissues were recorded and compared with controls.

3. RESULTS

3.1 Physico-Chemical Characteristics of Textile Dye

In the present study, physic-chemical characteristics of the collected textile dye was analyzed and the results were showed in chart-1

Chart -1

Sr. No:	Sample	Nature of Sample	TS (mg/l)	TDS (mg/l)	pH	COD (mg/l)	BOD (mg/l)	Temp (℃)	Color	Odor
1	Sample 1	Water	3700	2300	6.3	710	270	35	Black	Foul
2	Sample 2	Sludge	4800	2100	6.8	750	280	35	Black	Foul
3	Sample 3	Water	4300	2500	7.4	620	250	35	Black	Foul

4	Sample 4	Sludge	4100	2700	6.0	785	310	35	Black	Foul

3.2 Isolation and Identification of Dye Decolorizing Bacteria:

The bacteria isolated from textile wastewater were subjected to morphological and biochemical tests, and 16S rRNA gene sequence analysis. The analysis of 16S rRNA gene sequence by NCBI BLAST Program revealed that the isolate was *Mangrovibacter yixingensis* strain AKS2 (Accession no. OM189530).

3.3 Effect of Textile Dye Concentration on Decolorization:

It was found the decolorization rate was dependent on the initial dye concentration and time of incubation. The maximum decolorization rates, 76.57% & 71.34%, were observed after 192 hours of incubation period at 100 & 200 ppm dye concentration respectively (Fig. 2).

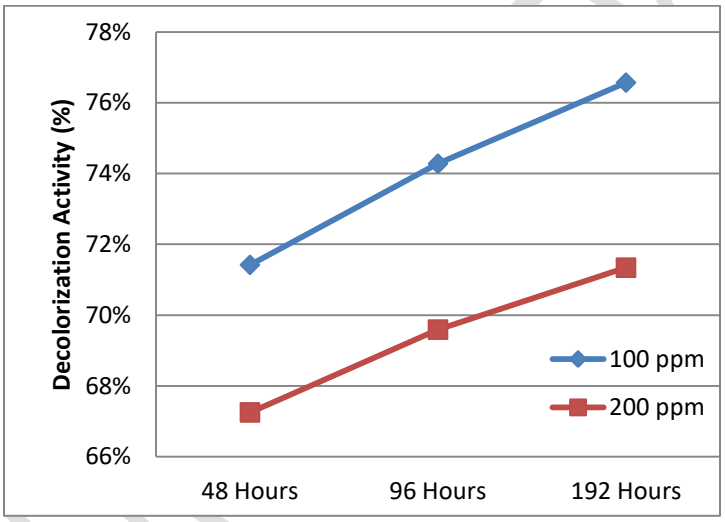

Fig 2: Effect of Textile Dye Concentration on Decolorization Activity of Basic Red-18

3.4 Behavioral and Morphological Alterations:

Upon exposure to control and two different treatments, fishes showed abnormal behavioral changes. The behavioral responses of the test fish to the textile dye BR-18 at different concentrations at the period of 96 h (Table 1) indicated high secretion of mucus, uncoordinated movement, restlessness, and caudal fin movement, loss of balance surfacing, loss of buoyancy, escaping tendency, hyperactivity and discoloration of the skin. Mortalities were observed in all the treatments except control. The fish exhibits stressful behavior which increased with the BR-18 concentration.

3.5 Mortality percentage

The high mortality rate (200ppm dye- 70%) in the fishes exposed to untreated BR-18 dye was found in this study indicating the toxic nature of dye. Whereas fishes grown in control and

treated BR-18 dye showed less mortality rate (Control-0%) and (200ppm treated- 20%) which depicts the less toxicity of BR-18 dye after bioremediation with *Mangrovibacter yixingensis* strain AKS2.

Table 1: Behavior of *T. mossambica* to Textile Dye

Behavioral & morphological response	Control	100 PPm Dye	100 PPm Treated Dye	200 PPm Dye	200 PPm Treated Dye
Mucus Secretion	None	Mild	Moderate	Strong	Very Strong
Restlessness	None	Mild	Moderate	Strong	Very Strong
Loss of balance	None	None	Moderate	Strong	Very Strong
Hyperactivity	None	None	Mild	Moderate	Maximum
Decolorization of skin	None	None	Mild	Moderate	Maximum

Table 2: Mortality Percentage

Concentration % (v/v)	No of fish	24 hrs		48 Hrs		72 Hrs		96 Hrs	
		M	M%	M	M%	M	M%	M	M%
Control	10	0	0	0	0	0	0	0	0
100 PPm Dye	10	0	0	1	10	2	20	3	30
100 PPm Treated Dye	10	0	0	0	0	1	10	1	10
200 PPm Dye	10	4	40	5	50	7	70	7	70
200 PPm Treated Dye	10	0	0	1	10	1	10	2	20

3.6 Hematological Analysis

Hematological parameters are related to response of the organisms to changing environmental conditions and therefore can be used to screen the health of fish exposed to toxicants. Hence the study was carried out to determine various hematological parameters like Hb%, total WBC count, Differential count of WBC and total RBC count after exposure of T. mossambica to 100 ppm & 200 ppm untreated and treated textile dye for a period of 15 & 30 days. In this study, changes observed in hematological parameters of the exposed fishes showed remarkable influence when exposed. The significant reduction in the RBC count and Hb content indicates partial anemia, hypoxic condition, erythrocytopenia, decreased hemopoiesis or damage in the hematopoietic tissues due to intoxication of BR-18. These changes probably lead to the structural damage in RBC membranes resulting in hemolysis and impairment in hemoglobin synthesis. Due to toxic action of dye on the erythropoietic tissues affecting the cell viability leads to decreased RBC number and Hb level. On the other hand, WBC count increased in all Basic Red-18 exposed fish. This is an indication of disruptive effects of azo dyes on erythropoietic tissues as well as cells viability. Hematological parameters of the blood of fish such as Hb%, total WBC count, Differential count of WBC, and total RBC count after exposure of *T. mossambica* to 100 ppm & 200 ppm untreated and treated textile dye for a period of 15 & 30 days showed different changes.

3.6.1 Estimation of Hemoglobin (Hb)

The results of the study revealed that the Hb% has low value in *T. mossambica* exposed to 100ppm untreated dye (2.1±0.06) compared to treated dye (3.1±0.12) and 200ppm untreated dye (1.2±0.07) compared to treated dye (2.9±0.15) but both the values of Hb were found to be low when compared with that of control (4.7±0.08) of fish (Fig 3).

3.6.2 Total count of WBC (thousand/cu.mm)

The results of the study showed that total number of WBC was found to be low in 100ppm untreated dye (4.2thousand/cu.mm ±0.20) compared to treated dye (3.3thousand/cu.mm ±0.22) and 200ppm untreated dye (4.5thousand/cu.mm ±0.18) compared to treated dye (3.7thousand/cu.mm ±0.29) but both the values of Hb were found to be low when compared with that of control (3.0thousand/cu.mm ±0.25) (Fig 4).

3.6.3 Total count of RBC (million/cu.mm)

The results of total count of RBC's (Fig 5) in the blood of *T. mossambica* in control was found to be 3.5million/cu.mm±0.04 whereas number of RBC's in the blood of fish exposed to 100ppm untreated dye was 2.5million/cu.mm±0.08, treated dye was 2.9million/cu.mm±0.10 and in the blood of fish exposed to 200ppm untreated dye was 1.40million/cu.mm±0.06, treated dye was 2.70million/cu.mm±0.08. Which was recorded thereby indicating that total number of RBC's were found to be low in the blood of fish exposed to untreated and treated samples when compared with that of control values.

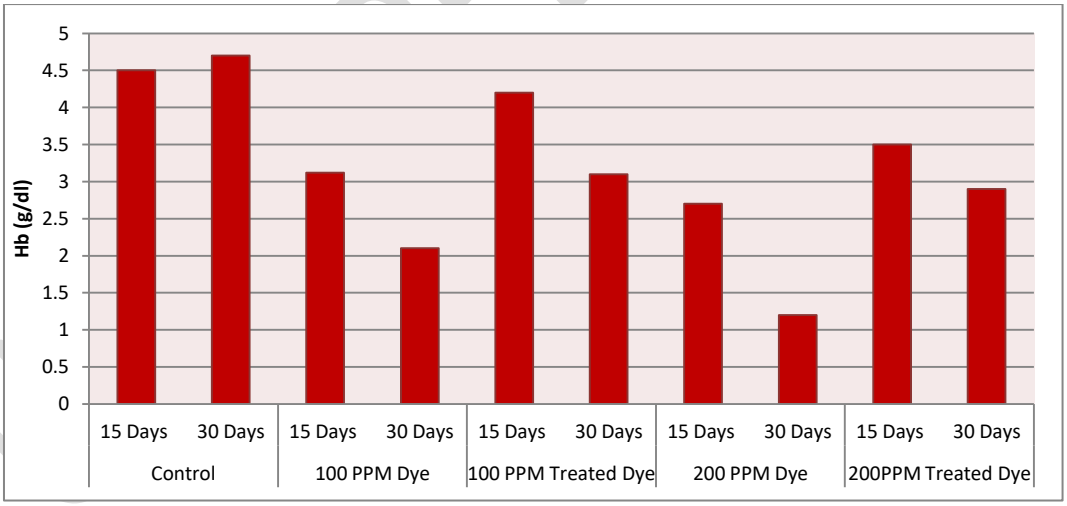

Fig. 3 Hemoglobin count of *T. mossambica*

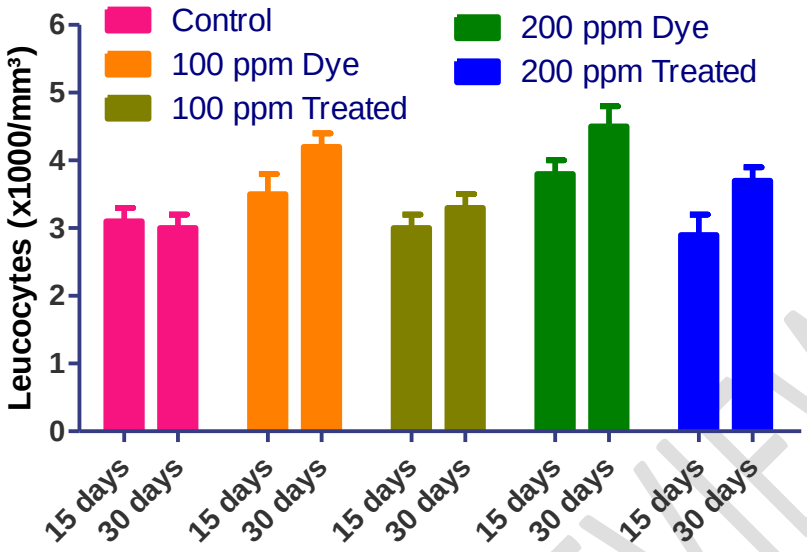

Fig. 4 Total Count of WBC

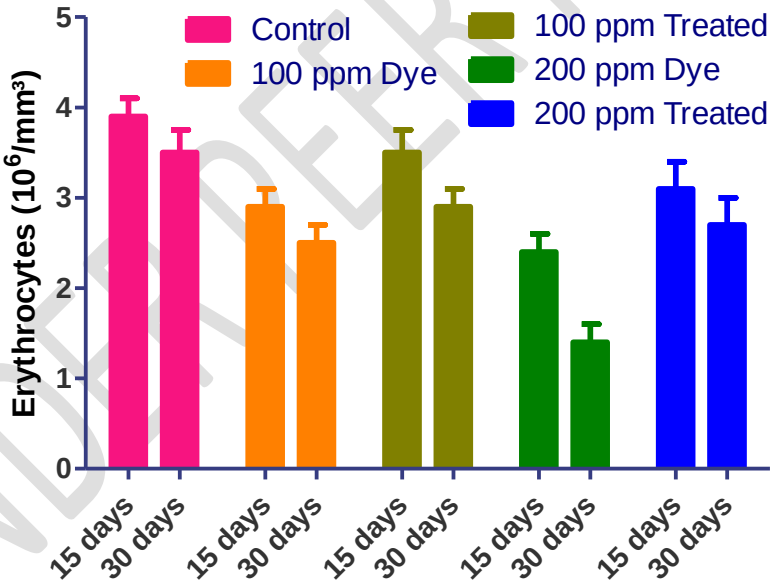

Fig. 5 Total Count of RBC

3.6.4 Differential count of WBC (million/cu.mm)

In the differential count of WBC of blood of fish, neutrophils, eosinophils, lymphocytes and monocytes of fish, *T. mossambica* was found to be as in table 3. The results showed that the differential count of WBC of blood in fish, *T. mossambica* exposed to untreated and treated dye was low when compared with that of control values of neutrophils, eosinophils, lymphocytes and monocytes. Basophils were completely absent.

Table 3 Differential Count of WBC (Data, Mean±SD)

Parameters	Control	100 PPM dye	100 PPM Treated Dye	200 PPM Dye	200 PPM Treated Dye
Neutrophils (%)	54.5±1.5	29.5±0.3	38.2±0.03	15.1±0.1	24.8±0.07
Basophils (%)	**Absent**	**Absent**	**Absent**	**Absent**	**Absent**
Eosinophils (%)	4±0.06	2±0.05	3±0.06	**Absent**	**Absent**
Lymphocytes (%)	43.0±1.9	30.1±1.3	39±1.8	20±1.2	30.1±0.9
Monocytes	2.0±0.04	1.0±0.03	2.0±0.03	**Absent**	**Absent**

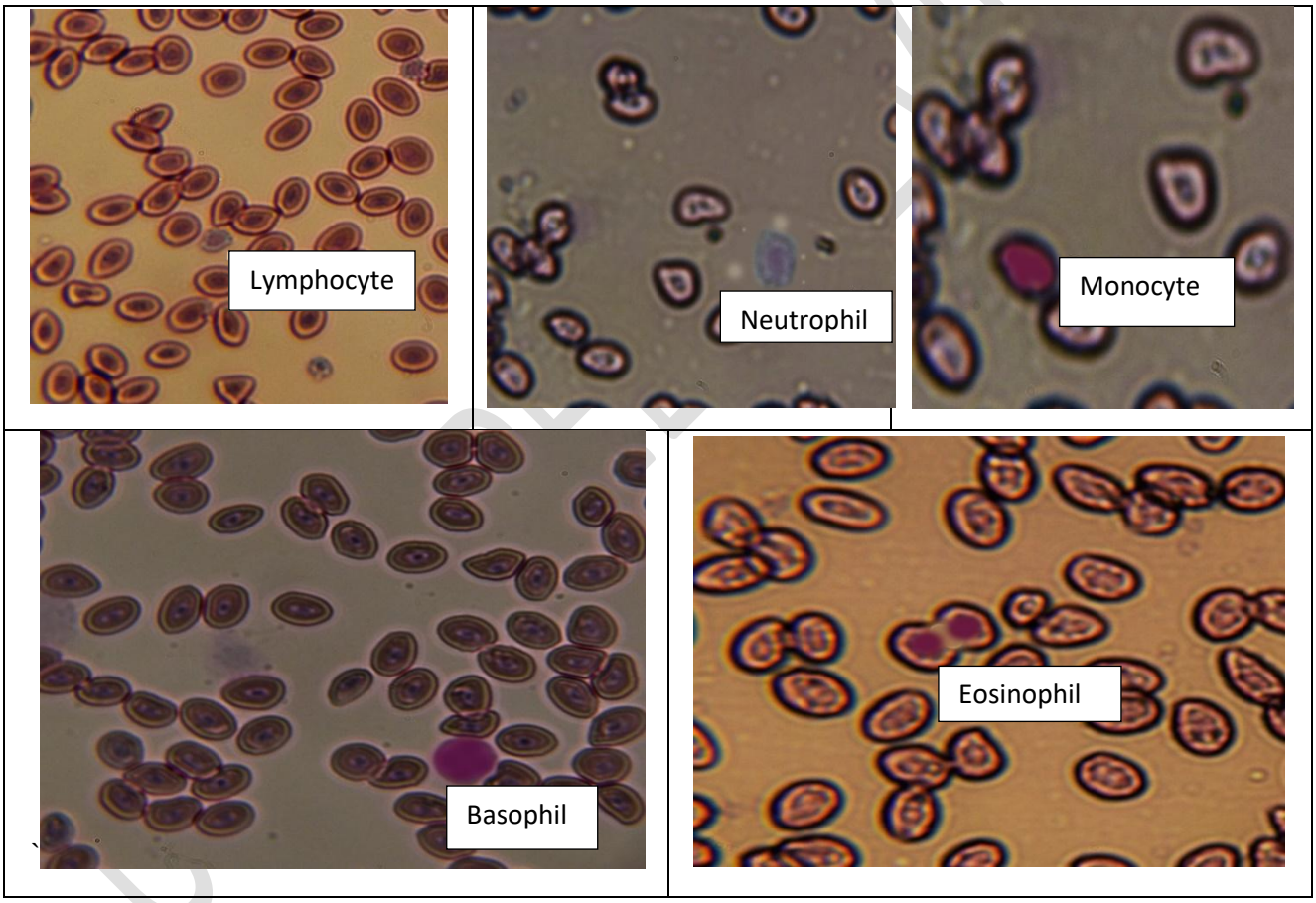

Fig. 6 Differential Count of WBC (representative images of WBC)

3.6.5 Morphological Changes of Red Blood Cells (Erythrocytes)

The mature erythrocytes in the blood of *T. mossambica* in control group are elliptical in shape, the nucleus is also elliptical and centrally located (Fig. 7 a). The morphological changes of Red Blood Cells (RBCs) in *T. mossambica* were observed at the treated groups of 100 and 200 ppm concentrations of BR-18 dye. At 100 ppm concentration of BR-18 dye after 15 days exposure period erythrocytes were found to be swollen and spherical (Fig. 7 b). After an exposure period

of 30 days at the same concentration of BR-18. Erythrocytes exhibited swollen, oblong and shrinked (Fig. 7 f). When the experimental fish, *T. mossambica* was exposed to 200 ppm of BR-18 after 15 days exposure, erythrocyte showed irregular boundary, nuclear hypertrophy and cytoplasmic enlargement in the cell wall (Fig. 7 d). An exposure period of 30 days at 200 ppm of BR-18, the erythrocyte of experimental fish, exhibited different lobopodial projections, discocytes, keratocytes, fusiform of red blood cells. Membrane disruptions, cytoplasmic enlargement and ultimately the oozing out of the cytoplasmic content of red blood cells (Fig. 7 h). But in treated dye of both concentration the results were almost similar to the control.

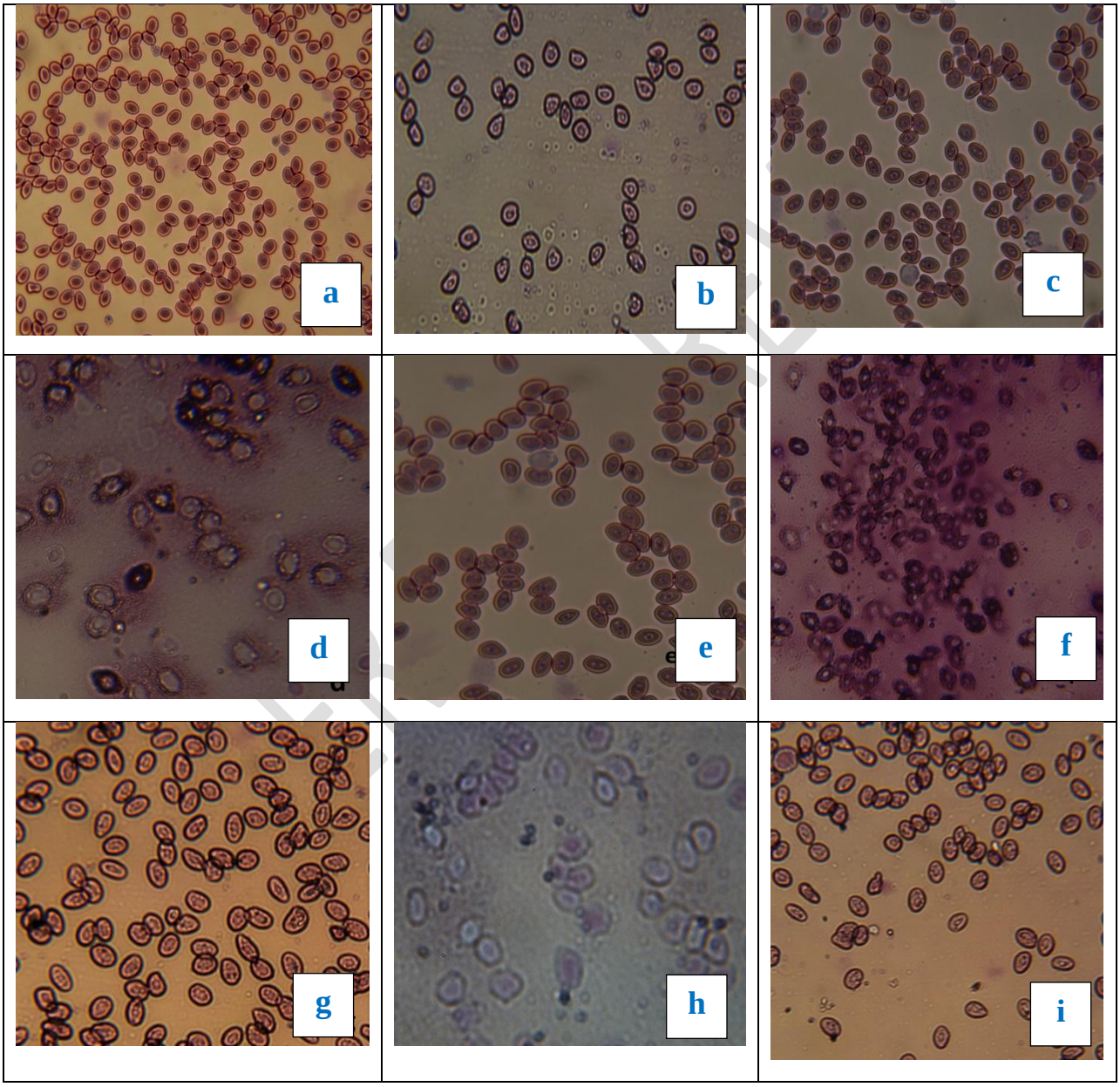

Fig. 7 : Morphological changes of RBC a. Control b. 100 ppm dye solution in 15 days c. 100 ppm bacteria treated dye in 15 days d.200 ppm dye solution in 15 days e. 200 ppm bacteria treated dye in 15 days f. 100 ppm dye solution in 30 days g. 100 ppm bacteria treated dye in 30 days h.200 ppm dye solution in 30 days i. 200 ppm bacteria treated dye in 30 days.

3.7 Histological Studies

After 15 and 30 days of treatment, fish were dissected to examine the histological alterations in the gills, liver, intestine, stomach, and heart. Hepatocyte vacuolation and swelling, as well as congestion and sinusoidal space enlargement, were seen in the liver. In the intestine and stomach, it was seen that the columnar epithelium was deteriorating, the villi's tips were necrotic, and the basement membrane and goblet cells were deformed.

3.7.1 Intestine

Each villus facing the lumen showed cell degeneration and the cells did not show distinct nuclei and cytoplasmic boundaries. There was a distortion of basement membrane of the villi and blood vessel, and lymphocytes were fully distorted . There was a degeneration of columnar epithelium of the intestine. Circular muscle fibers were loosely arranged and degeneration of submucosal tissue was also observed (Fig. 8).

3.7.2 Stomach

The submucosal tissues were fully vacuolated and serosa layer degenerated. Degeneration of columnar epithelium, goblet cell and basement membrane were found in the stomach. The secretary cells were damaged and fully distorted. No necrosis of the stomach cells was observed (Fig 9

3.8.3 Liver

Parenchymal vacuolation and focal coagulative necrosis were observed in the liver treated with dyes after 29 days. Vacuolation in the cytoplasms with moderate degeneration of hepatic mass were noticed in the liver cell. Hydropic degenerations and fatty infiltration in the liver tissue of C. punctatus were also detected. Full congestion of central vein, diffusion of hepatic cells and dilation of sinusoids were also observed (Fig.10).

3.7.4 Heart

The histopathology of heart showed the normal arrangement of cardiac muscular layers (CM). In control group, when fishes were treated with 100 ppm and 200 ppm dye the following abnormalities were recorded. Brown atrophy fragmentation of myocardial muscle fibers, dislocation of nucleus, oedema, infiltration thickening, separation of mussel bundles due to intramuscular oedema and splitting of longitudinal mussels (Fig 11).

3.7.5 Gills

Gills of *T. mossambica* exposed to 100ppm of dye showed aneurism and mild degenerated central axis. 200 ppm of dye concentration produced remarkable changes like epithelial lifting,

hyperplasia, fusion and curling of secondary gill lamellae, enlarged and vacuolated cartilage cells (Fig 12).

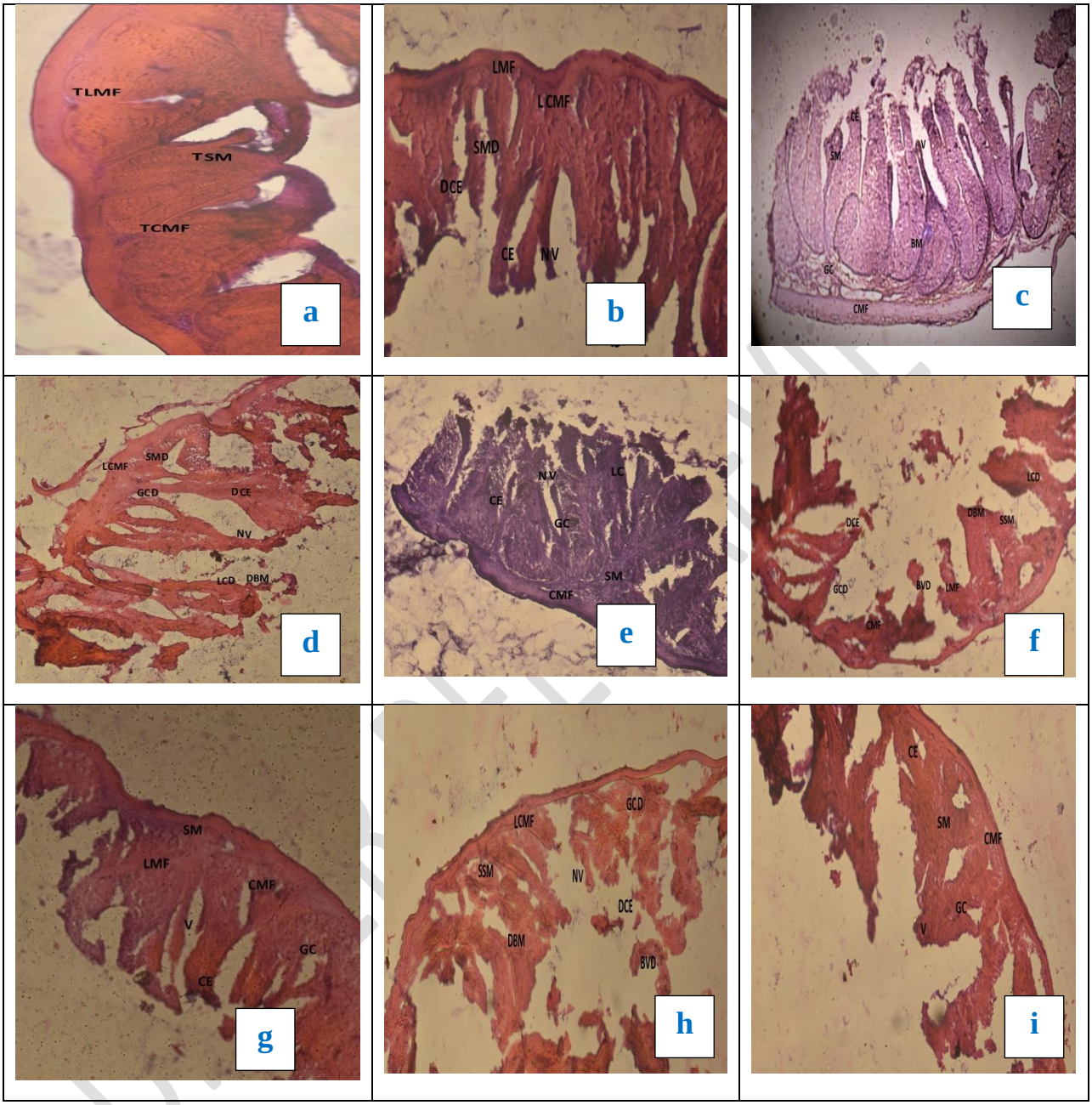

Fig. 8 Histology of Intestine a. Control b. 100 ppm dye solution in 15 days c. 100 ppm bacteria treated dye in 15 days d.200 ppm dye solution in 15 days e. 200 ppm bacteria treated dye in 15 days f. 100 ppm dye solution in 30 days g. 100 ppm bacteria treated dye in 30 days h.200 ppm dye solution in 30 days i. 200 ppm bacteria treated dye in 30 days. CMF- Circular muscle fibers, LMF- Longitudinal muscle fibers, SM- Submucosal tissue, SMD- Submucosal tissue degenerated, LCMF-Loosely arranged of circular muscle fibers, DCE-Degeneration of columnar epithelium, NV- Necrosis at the tip of the villi, GCD- Goblet cell distorted, CE- Columnar epithelium, GC- Goblet cell, SSM- Slight shrinkage of submucosa, BVD- Blood vessel distorted, LCD- Lymphocyte fully distorted.

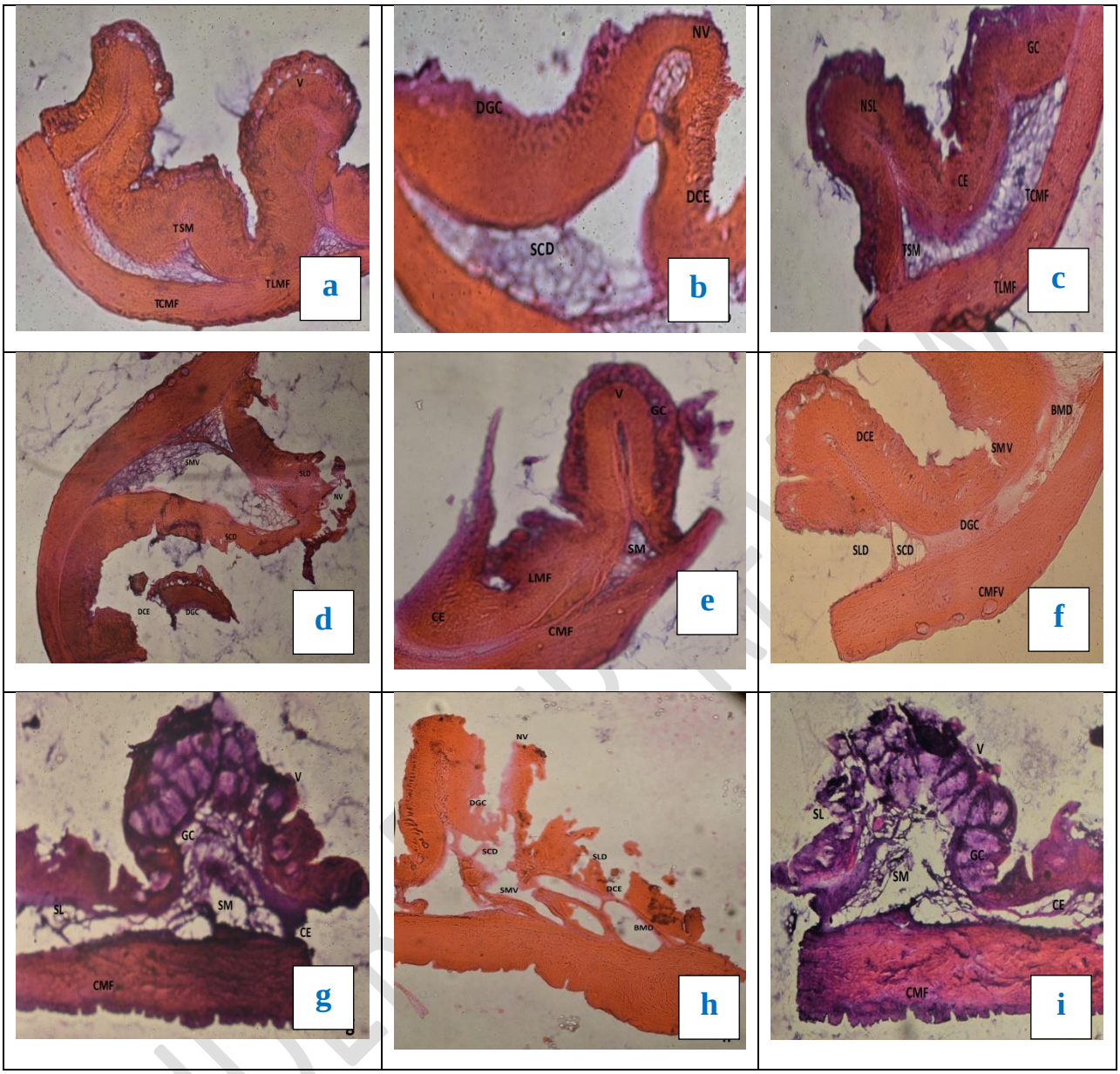

Fig. 9: Histology of Stomach a. Control b. 100 ppm dye solution in 15 days c. 100 ppm bacteria treated dye in 15 days d.200 ppm dye solution in 15 days e. 200 ppm bacteria treated dye in 15 days f. 100 ppm dye solution in 30 days g. 100 ppm bacteria treated dye in 30 days h.200 ppm dye solution in 30 days i. 200 ppm bacteria treated dye in 30 days. CMF- Circular muscle fibers, LMF- Longitudinal muscle fibers, SM- Submucosal tissue, LCMF-Loosely arranged of circular muscle fibers, SMV- Submucosal tissue fully vacuolated, SLD-Serosa layer degenerated, DCE-Degeneration of columnar epithelium, DGC-Degeneration of goblet cell, BMD- basement Membrane distorted, SCD-Secretary cell fully distorted.

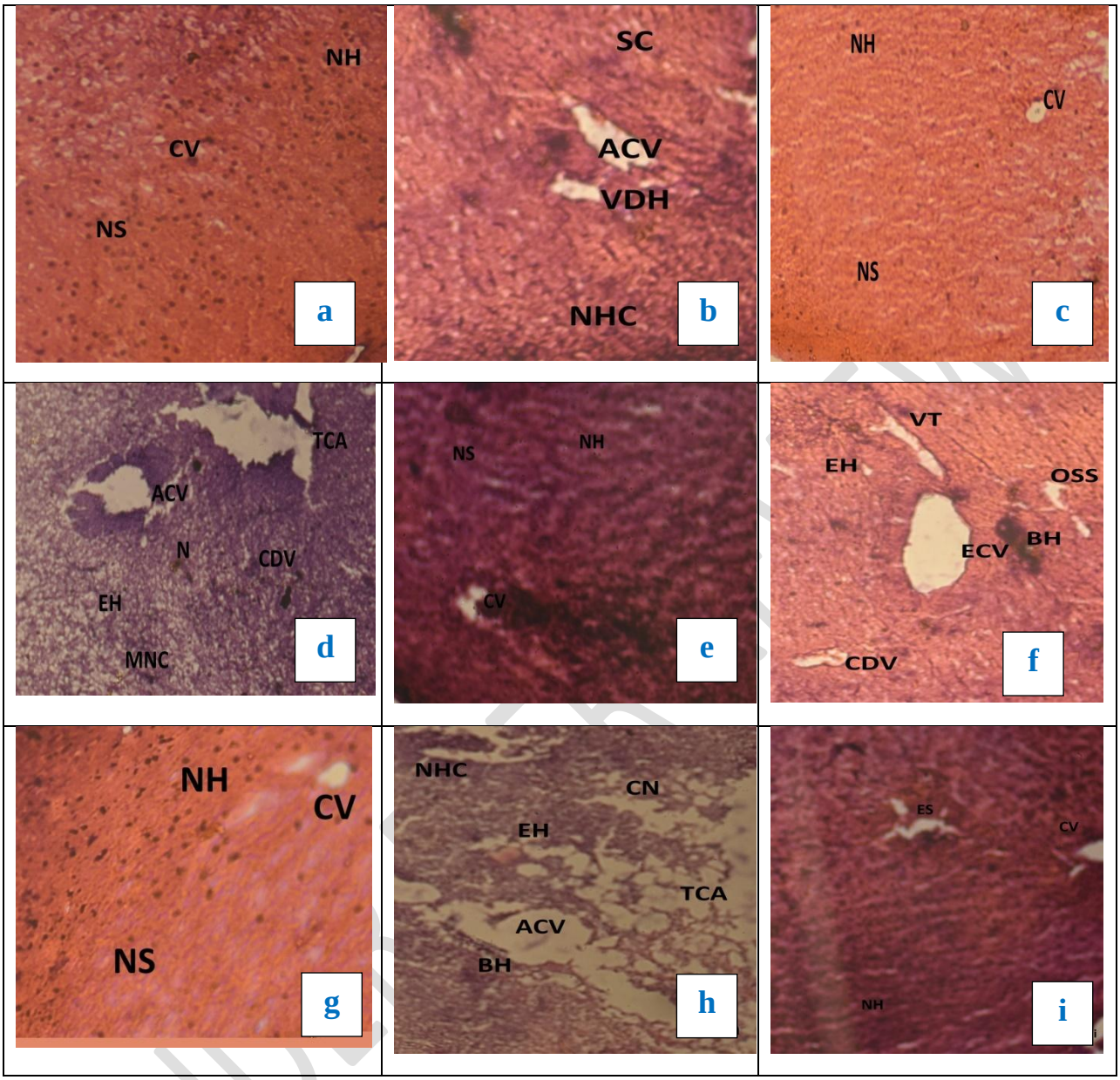

Fig. 10: Histology of Liver a. Control b. 100 ppm dye solution in 15 days c. 100 ppm bacteria treated dye in 15 days d.200 ppm dye solution in 15 days e. 200 ppm bacteria treated dye in 15 days f. 100 ppm dye solution in 30 days g. 100 ppm bacteria treated dye in 30 days h.200 ppm dye solution in 30 days i. 200 ppm bacteria treated dye in 30 days. CV-Central vein, NH-Normal hepatocytes, NS- Normal sinusoids, VT-Vacuolated tubules, EH- Enlarge hepatocytes, BH-Blood hemorrhage, ECV-Enlarge central vein, OSS- Obliterated sinusoidal space, CDV- Cell debris inside the central vein, SC-Sinusoidal congestion, ACV-Abnormal shape of central vein, VDH-Vacuolar degeneration of the hepatocytes, NHC-Necrosis of hepatic cells, N-Necrosis, MHC- Mononuclear cells, TCA- tissue lost its attachment, ES-Enlarge sinusoids, CN-Coagulation necrosis.

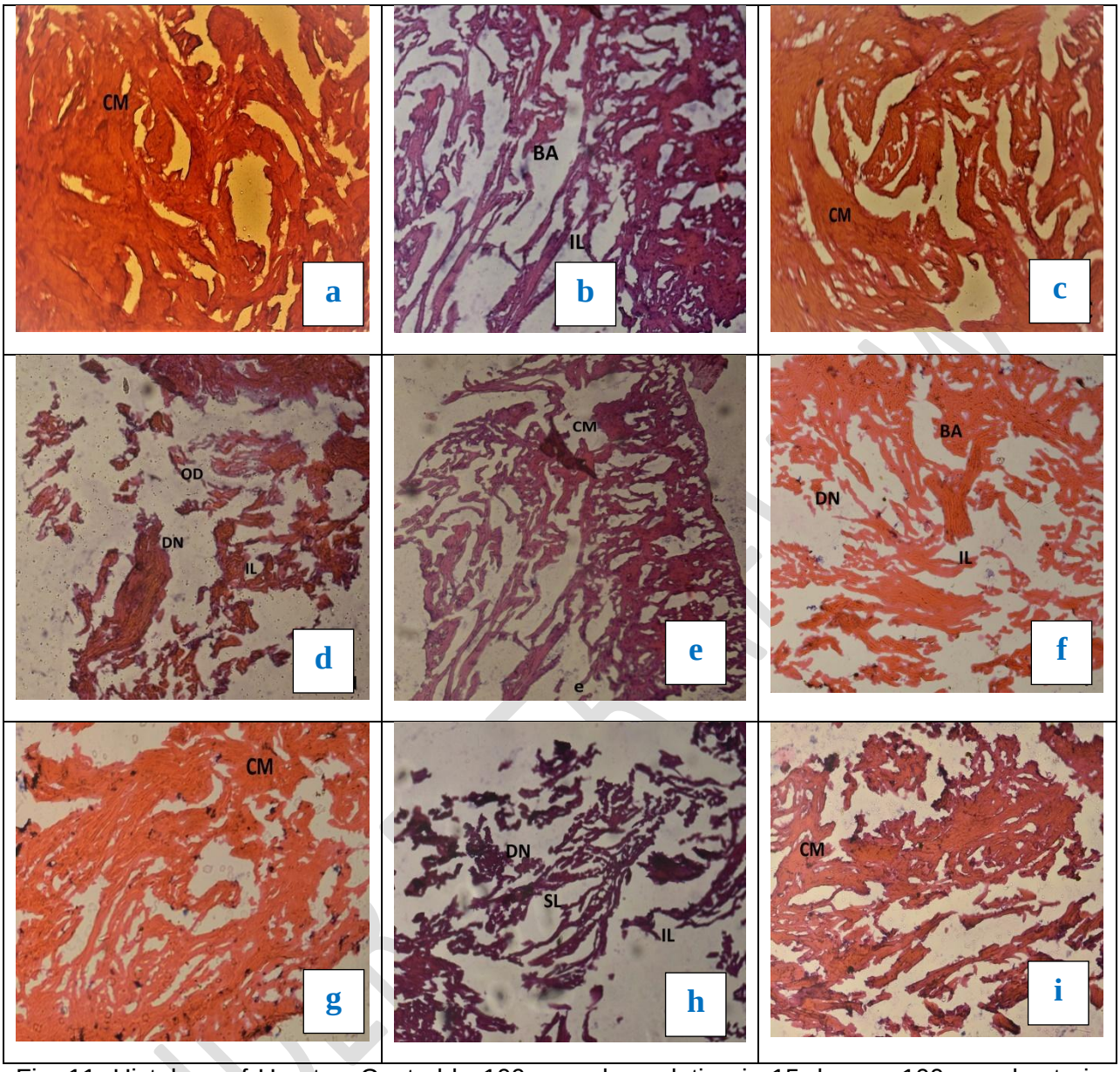

Fig. 11: Histology of Heart a. Control b. 100 ppm dye solution in 15 days c. 100 ppm bacteria treated dye in 15 days d.200 ppm dye solution in 15 days e. 200 ppm bacteria treated dye in 15 days f. 100 ppm dye solution in 30 days g. 100 ppm bacteria treated dye in 30 days h.200 ppm dye solution in 30 days i. 200 ppm bacteria treated dye in 30 days. CM-Cardiac muscular layer, BA- Brown atrophy, IL- fragmentation of myocardial muscle fibers with extensive infiltration, OD-Oedema, DN-Dislocation of nucleus, SL- Splitting of longitudinal muscle.

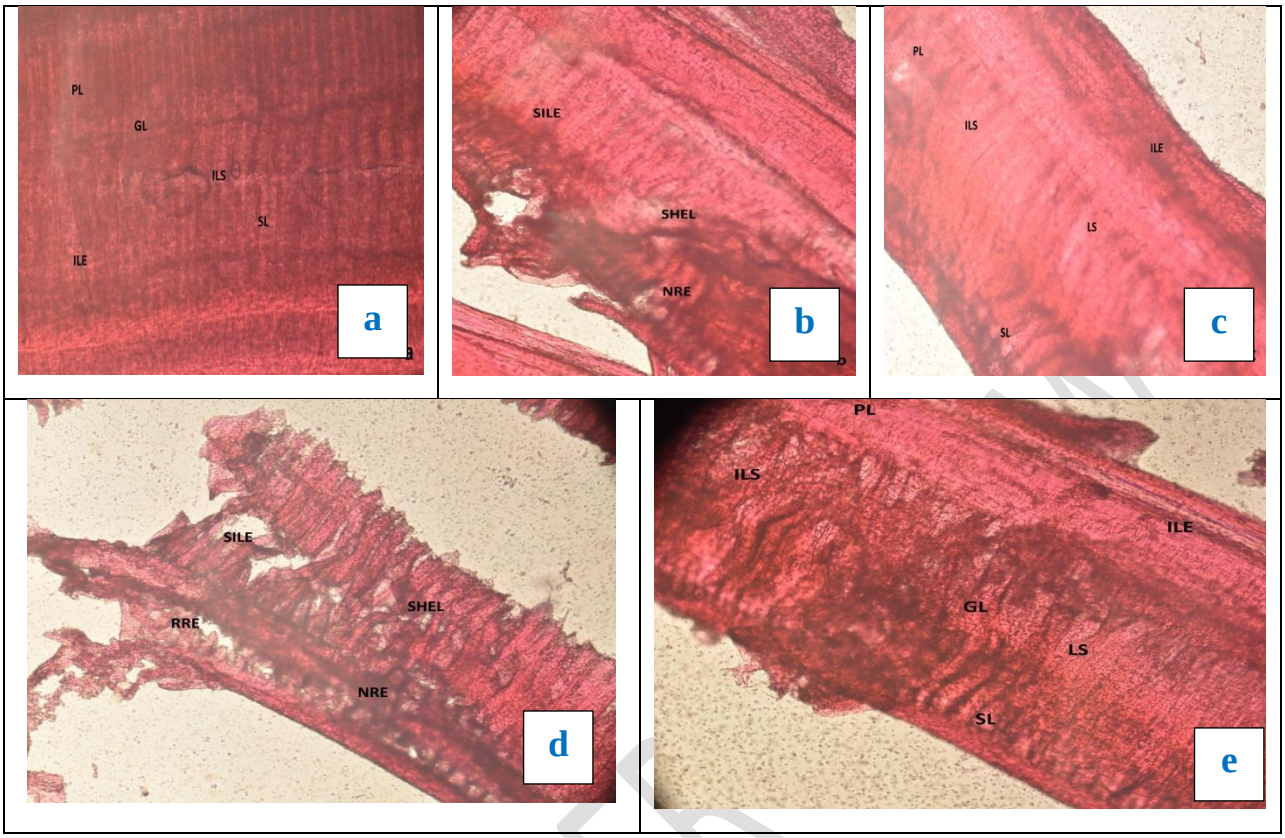

Fig. 12: Histology of Heart a. Control b.200 ppm dye solution in 15 days c. 200 ppm bacteria treated dye in 15 days d.200 ppm dye solution in 30 days e. 200 ppm bacteria treated dye in 30 days. PL- Primary Lamellae, GL- Gill filament, ILS- Inter lamellar space, SL-Secondary lamellae, ILE- Inter lamellar epithelium, SILE- Swelling of inter lamellar epithelium, SHEL- Severe hemorrhage & erosion in the lamella of gills, NRE- Necrosis of respiratory epithelial cells, LS- Lamellar space, RRE- Regeneration of respiratory epithelium.

4. DISCUSSION

In this study, azo dye decolourizing bacteria *Mangrovibacter yixingensis* strain AKS2 was isolated and characterized. This bacterial strain was selected after being grown in an enrichment medium supplemented with dye as the sole carbon source as well as in mineral salt medium which confirm the ability of the isolated bacterial species to survive in the presence of the dye.

At lower dye concentration, bacteria was showed maximum decolourization activity, 76.57% & 71.34%, decolorization occur after 192 hours of incubation period in 100 & 200 ppm dye concentration. This decrease in decolourization ability at high dye concentration might be due to the toxicity of the dye and the finding is comparable to other studies [2, 3, 14, 19]. Azo dyes generally contain one or more sulphonic-acid groups on aromatic rings, which might act as detergents to inhibit the growth of microorganisms [14]. Another reason of the toxicity at higher concentration of dye may be due to the presence of heavy metals and non-hydrolyzed reactive groups which may retard the bacterial growth [14, 20].

Due to internal damages fishes different kinds of abnormal behavior in untreated dyes but the results is comparatively better in treated dyes. Similarly, mortality rate was better in treated dyes. Prior reports demonstrated that the mortality percentage was high in fish grown in untreated textile effluent and in contrast, the mortality rate was low in fishes when grown in treated effluent as the degradation product of the effluent did not induce any hazardous to fishes [21]. Hysteric swimming and loss of balance in exposed fishes were due to the dyed medium. Excess mucus secretion over the body and gills was an immunologic response to avoid the direct contact of toxic medium. It could lead to disfunctioning of gills and might create respiratory distress, resulting into the gulping of air to get oxygen from the air [22, 23]. Loss of appetite might be due to the gradual accumulation of dye in the alimentary tract of exposed fishes. Dye particles absorbed by the skin might have damaged the dermis that resulted in darkening of skin and scale erosion [23, 24].

Hematological parameters of the blood of fish such as Hb%, total WBC count, Differential count of WBC, and total RBC count after exposure of *T. mossambica* to 100ppm & 200ppm untreated and treated textile dye for a period of 15 & 30 days showed different morphological changes. The morphological change of the blood cells was more prominent in the untreated sample than that of treated blood samples due to the high toxicity of untreated effluent [8].

In this present study, changes observed in hematological parameters of the exposed fishes showed remarkable influence when exposed. When *T. mossambica* were exposed to dye Basic Red-18 total count of RBC's reduced compare to control. Alterations in RBC count of fishes exposed to various toxicants have been reported by many researchers [25, 26]

The significant reduction in the RBC count and Hb content indicates partial anaemia, hypoxic condition, erythrocytopenia due to intoxication of BR-18. These changes probably lead to the structural damage in RBC membranes resulting in hemolysis and impairment in hemoglobin synthesis. Due to toxic action of dye on the erythropoietic tissues affecting the cell viability leads to decreased RBC number and Hb level. Such changes in Hb, RBC and WBC counts were also support the previous study when *L. rohita* fingerlings were exposed to C. I. Direct Green 6 azo dye [27]. These changes were also observed in some fish exposed to azo dye methyl red [28].

The malachite green is also able to produce such kind of variations in hematological parameters of *Nile tilapia [29]*.

In the present study, WBC count increased following exposed dye BR-18. Significant increase in the total WBC count was observed in *Heteropneustes fossilis* exposed to malachite green and pyceze which supports the findings of the present investigation [30]. Same results were also observed in *Catla catla* exposed to acid red-97 [16].

Hb seems to be reliable and best blood indicator of environmental stress. Lower level of hemoglobin in fishes might decrease the ability to enhance the activities required to meet demands such as seeking of food and escaping from predators. The depletion or reduction in Hb content in fishes could also be attributed to the production of reactive oxygen species under the influence of toxicant resulting in the destruction of red blood cell membrane and its function. Similar decrease in the amount of RBC, Hb and PCV could be corroborated with the findings of earlier investigations in *Tilapia mossambica* exposed to textile dyeing effluent [8], *Catla catla* exposed to acid red 97 [16]. The decreased level of Hb and packed cell volume were noticed in *Catla catla* fishes [16].

The mature erythrocytes in the blood of *T. mossambica* are elliptical in shape; the nucleus is also elliptical and centrally located. But, morphology of erythrocytes is one of the most specific and sensitive indicators of the effect of various environmental factors and pollutants on fish [15, 31, 32]. In this study, erythrocytes exhibited different shapes according to different concentration of treated and untreated textile dye. Thus, our results are in close conformity with those reported above.

Swelling and vacuolated formation of the hepatocytes and congestion and dilatation of sinusoidal space were observed in the liver in this study. Degeneration of columnar epithelium, necrosis at the tips of the villi and distortion of basement membrane and goblet cells were recorded in the intestine and stomach. Similar results of vacuolation and necrosis in the hepatocytes of *Channa punctatus* after exposure to hybrid pesticide has been reported in a study [33].

The gills are among the most vulnerable structures of the fish because of their external location and intimate contact with the water [16]. The gills, which participate in many important functions in the fish, such as respiration, osmoregulation and excretion, remain in close contact with the external environment and particularly sensitive to changes in the quality of the water are considered the primary target of the contaminants [34]. Haemorrhage and damages in gills were also observed in *Catla catla* and *Labeo rohita* exposed to textile dyes [15, 16].

5. CONCLUSION

Due to the influence of textile dye BR-18 the behavioral, hematological and histopathological changes were found in the gills, liver, intestine, stomach and heart. The amounts of RBC and Hb have been also decreased in the blood of fish exposed to BR-18 contaminated water. But the amount of WBC has been increased as an immunological defense to survive against the toxic substance in the dye contaminated water. Moreover, after treating of the dyes with isolated novel bacterium, the fishes survive for longer periods. Taken together, it can be concluded that the isolate *Mangrovibacter yixingensis* strain AKS2 could be used as a novel bacterium for

decolourization and detoxification of textile effluents in industrial treatment plant to ensure sustainable environment and development.

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