



## Research article

Heavy metal toxicity in Buriganga river alters the immunology of Nile tilapia (*Oreochromis niloticus* L)Zakir Hossain<sup>a,\*,1</sup>, Md. Saddam Hossain<sup>a,1</sup>, Noore Safa Ema<sup>a</sup>, Abdelwahab Omri<sup>b</sup><sup>a</sup> Department of Fisheries Biology and Genetics, Faculty of Fisheries, Bangladesh Agricultural University, Mymensingh, 2202, Bangladesh<sup>b</sup> The Novel Drug and Vaccine Delivery Systems Facility, Department of Chemistry and Biochemistry, Laurentian University, Sudbury, P3E 2C6, Ontario, Canada

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## ABSTRACT

The objective of the current study was to evaluate the biochemical and immunological responses of tilapia, *Oreochromis niloticus* due to heavy metals pollution. Histomorphological alterations in the liver and kidney suggested tissue damages due to this polluted water exposure. The brain acetylcholinesterase (AChE) as an indicator of neurotoxicity was significantly ( $P < 0.01$ ) decreased after 10 days exposure of fish to heavy metal contained river water, while plasma glutamate oxalacetate transaminase and plasma glutamate pyruvate transaminase were significantly increased ( $P < 0.01$ ). Moreover, superoxide dismutase, catalase, glutathione peroxidase, glutathione S-transferase enzyme activities, as well as reduced glutathione and malondialdehyde levels were significantly increased in heavy metals contained river water treated fish compared to the control. Additionally, glucose level and blood serum  $Ca^{2+}$  concentrations were significantly ( $P < 0.01$ ) decreased in fish exposed to heavy metal contained river water compared to the control. Hematological indices such as Hemoglobin, RBC, WBC, MCV etc. of polluted river water treated fish were significantly ( $P < 0.01$ ) different in comparison to that of control fish. The cytokines i.e. IL-1 $\beta$ , IL-6, and TNF- $\alpha$  level were significantly ( $P < 0.01$ ) increased in the fish exposed to heavy metals contained river water in comparison to that of control fish. The present findings explored the detrimental effects of heavy metal contained river water on fish at biochemical and immunological levels.

## 1. Introduction

River pollution is a terrible environmental issue in the world for a long time [1]. Industrialization is the main factor of increasing river pollution and toxicity in aquatic biomes [2]. Heavy metals, such as arsenic, cadmium, chromium, lead, and copper produce toxicity to the aquatic organism [3]. Metal toxicity could cause physiological disorders, which can lead to excessive oxidative stress [4]. Freshwater fishes are susceptible to pollutants comparing to marine water fish [5]. A diverse group of pesticides from the running water of agricultural fields is the main cause for pollution of freshwater bodies [6]. The impact of pollution in freshwater fish depends on environmental or biological factors [7].

The Buriganga river is situated in the southern part of the capital city of Bangladesh. Recently, the water quality of this river has been deteriorating due to a high level of pollution; the main three causes of deteriorating the water of the Buriganga river are waste dumping, riverside encroachment, and improper management [8].

Among the aquatic organisms, fish is an experimental model, which is used as an important bio-indicators of potential risks of contamination with toxicants in the aquatic water body [9, 10, 11]. The changes in the behavior of fishes are used as biomarkers in the polluted aquatic environment [12, 13]. Histopathology of fish vital organs i.e. liver, kidney, and gill are also used as a biomarker to assess health status in stressed conditions [14, 15]. Acetylcholinesterase (AChE) is considered one of the finest neuro-biochemical parameters for the evaluation of environmental stress [16]. Water pollution can produce potential toxicity inhibiting cholinesterase enzyme referred to as "anticholinesterase agents", which plays a role on the neurotransmitter acetylcholine [17]. Besides, glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) are liberated into the blood in pathological terms and therefore are of clinical importance and their presence in blood plasma can give a message on tissue injury or organ dysfunction [18]. GPT and GOT are potential biomarkers playing an important role in transamination reactions and indicate hepatotoxicity and cellular damage in ecotoxicological studies [19]. Glucose is used as the main fuel for metabolism. The

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blood glucose level of fish is an important part of the stress response and stress can alter the level of glucose to cope with stress response [20].

An antioxidant is a substance that prevents the oxidation of chemical compounds [21] and maintains a balance between oxidants and antioxidants. The shift in the balance of oxidant and antioxidant level causes oxidative stress and increase production of reactive oxygen species (ROS) [22]. Antioxidant defense system consists of enzymatic and non-enzymatic antioxidants includes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST), glutathione (GSH), and lipid oxidation system includes malondialdehyde (MDA) [23] which are used as biomarkers as an indication of pollution [24]. Enzymatic antioxidants such as SODs, a group of oxidoreductases, catalyze superoxide into oxygen and hydrogen peroxide [25]; CAT is a primary antioxidant substance that reduces the hydrogen peroxide into water and molecular oxygen [26]; GPx detoxifies hydrogen and organic peroxides and GST breaks down GSH. On the other hand, non-enzymatic antioxidant GSH acts as a reducing agent with xenobiotic conjugation [27]. MDA is considered a lipid oxidation indicator that reduces lipid peroxides [28]. Pollutants such as pesticide, nitrite, nitrate, ammonia, metal ions can alter the antioxidant activity of fish [29]. Cytokines are involved in the generation of cytotoxic T cells and the production of antibodies through binding to their corresponding receptors [30]. The toxicity of heavy metal on the fish immune system is relevant to the release of cytokine [31]. The freshwater ecosystem of rivers has great importance as a source of fish, particularly in developing countries, but very little empirical research on river pollution has been conducted. Therefore, the objective of the current study was to determine the effect of heavy metals on multiple biomarkers i.e. histopathological changes, serum glucose,  $Ca^{2+}$  concentration, enzyme activity, hematological alteration, and cytokine level.

## 2. Materials and methods

### 2.1. Experimental design

The heavy metal contained river water sample was collected in a sterilized plastic container from the Buriganga river during the dry season (Figure 1). After collection, the polluted river water was transported to Mini Hatchery and Laboratory complex, Faculty of Fisheries, Bangladesh Agricultural University, Mymensingh, Bangladesh. In the present investigation, experimental fishes were divided into two groups control and heavy metal contained river water treated group. Ten fishes were kept in the control group and treated group, and exposed for 10 days to normal tap water and heavy metal contained river water,

respectively. An aquarium contained 25 L of water was used for the present experiment each group having three replications.

### 2.2. Experimental animal

The average weight of the Nile tilapia, *Oreochromis niloticus* was  $32.75 \pm 0.35$ g. *O. niloticus* were collected from Bangladesh Fisheries Research Institute, Mymensingh, Bangladesh, and dipped in 0.05% potassium permanganate solution for 2 min to escape from infection. Consequently, the fish was acclimatized in the laboratory for 3 days. The use of test animals was approved by the Animal Welfare and Experimental Ethics Committee of Bangladesh Agricultural University, Mymensingh-2202, Bangladesh (AWEEC/BAU/2019).

### 2.3. Physicochemical condition of water

Temperature, pH, dissolved oxygen (DO), total alkalinity, total dissolved solids, biochemical oxygen demand (BOD), chemical oxygen demand (COD), ammonia, ammonium, nitrate, and total suspended solids, of water in each aquarium were recorded during the experimental period. Temperature, dissolved oxygen (DO), pH, ammonia, total alkalinity, and Total dissolved solids (TDS) measured by using a Celsius thermometer, a digital DO meter (multi 340 iset, DO-5509; China), a portable digital pH meter (MICRO-TEMP, pH 500, Romania), the API® ammonia test kit, alkalinity test kit (HANNA instrument HI3811, Rmania) and a portable digital TDS meter (HANNA instrument HI98302, Rmania), respectively. BOD and COD were estimated by using a portable BOD meter (Hach HQ40D, USA) and laboratory kit (COD meter 9585), respectively.

### 2.4. Estimation of heavy metals in river water

For heavy metal analysis, 2 ml water of each aquarium was weighed and placed in a Teflon reactor. After the addition of 3 ml  $H_2O$  Milli-Q water, 5 ml concentrated nitric acid (Merk Suprapur), and 2 ml  $H_2O_2$  at 30%; the reactor was maintained in a microwave digester for 20 min with a maximum temperature of  $210^\circ C$  [32]. Once the sample was cold, the volume adjusted to 25 ml with Milli-Q water and transferred to a low density polyethylene container at  $4^\circ C$  until analysis. Heavy metals (Cr, Fe, Pb, and Cd) were estimated by using atomic absorption spectrophotometer (AA-7000 Shimadzu, Japan) according to APHA [33].

### 2.5. Collection of blood sample

The blood collected in an Eppendorf by disposable syringe and needles from caudal puncture [34] of *O. niloticus* and kept in an icebox and left to coagulate during transferring to the lab. Then the blood was centrifuged at 8000 rpm for 15 min, the serum was preserved in 1.5 ml Eppendorf tubes at  $2-8^\circ C$  for various biochemical analysis.

### 2.6. Study of the haematological indices

For blood cell morphology analysis, fresh non-heparinized blood was smeared on glass slides. It was fixed and stained with methanol and Wright-Giemsa, respectively. Blood corpuscles were then observed under microscope and photographed with Olympus camera (OLYMPUS-CX41, Japan). Other haematological indices were measured following the methods of Tabassum et al. [11] The following formula was used to determine the indices:

Total RBC = (No. of cells  $\times$  dilution factor  $\times$  depth factor) / Total no. of small squares

Hemoglobin (Hb) (g/dL) was determined using hemoglobin strips (Easy Mate® GHb).

Haematocrit (Ht) (%) =  $(L1/L2) \times 100$



Figure 1. Location of the study area.

where

L1 = height of the RBC column

L2 = total length of the column (RBC + Plasma + buffer coat)

Mean corpuscular volume (MCV) ( $\mu\text{m}^3$ ) = {Haematocrit (Ht) (%) / Erythrocyte ( $\times 10^6/\text{mm}^3$ )}  $\times 10$

Mean corpuscular hemoglobin (MCH) (pg) = {Heamoglobin (Hb) (%) / Erythrocyte ( $\times 10^6/\text{mm}^3$ )}  $\times 10$

Mean corpuscular hemoglobin content (MCHC) (%) = {Heamoglobin (Hb) (%) / Haematocrit (Ht) (%)}  $\times 100$

## 2.7. Blood glucose level analysis

The blood samples from both the control and treated group were collected from 10 days exposed fish using anesthetic MS222 and glucose was determined using a blood glucose meter (Health Assure®, Taiwan).

## 2.8. Serum calcium ( $\text{Ca}^{2+}$ ) analysis

Serum  $\text{Ca}^{2+}$  concentrations were measured following the method of Reza et al. [35] with slight modification. Briefly, the centrifuged blood serum was mixed in working reagent contained O-cresolphthalein complexone and 8-hydroxyquinoline for reaction. The absorption was measured with a spectrophotometer (Spectronic Genesys TM5) at 540 nm. The  $\text{Ca}^{2+}$  concentration was expressed as  $\text{mg}/\mu\text{l}$ , which was determined using a standard curve.

## 2.9. Histomorphological study

*O. niloticus* was exposed to polluted river water in glass aquarium for 10 days to observe the changes of histomorphology. In the present study, the method of Akter et al. [36] for histomorphological was followed. Briefly, dehydration, clearing, and infiltration of the preserved liver and kidney tissues were completed using an automatic tissue processor (SHANDON, CITADEL 1000, UK). The wax embedded sample was cut in 5  $\mu\text{m}$  size using a microtome machine, and then stained with hematoxylin (H) and eosin (E) stains, and finally, photographed using a photomicroscope (OLYMPUS-CX41, Japan).

## 2.10. Determination of AChE activity

The activity of AChE of fish brain tissue was measured using the method of Ellman et al. [37] modified by Akter et al. [36]. Briefly, the homogenate fish brain tissue was mixed with ice-cold sodium phosphate buffer and 5,5-dithiobis (2-nitrobenzoic acid) was added, then the mixture was vortexed and incubated at room temperature for 10 min. Two hundred microliters of the mixture were placed in microtitre plate wells with three replications. Immediately after addition of 50  $\mu\text{l}$  acetylthiocholine iodide, the reaction was started. The variations in the absorbance were determined on a microplate reader (Model: SPECTRA max 340PC384) at 412 nm for 10 min at 12s intervals.

## 2.11. Determination of plasma enzymes (GOT and GPT) activity

The GOT and GPT activity were calculated using the method of Reitman and Frankel [38] modified by Tabassum et al. [11]. Fresh blood was collected from the fish and centrifuged at 11000 rpm for 10 min. One milliliter of GOT buffer (Phosphate buffer: 50ml, Alanine: 0.89g,  $\alpha$ -ketoglutarate: 0.0146g) and one milliliter of GPT buffer (Phosphate buffer-50ml, Aspartate-1.33g,  $\alpha$ -keto glutarate-0.0146g) buffer were taken separately into two 15ml screw cap test tube. The buffer was incubated at 40 °C for 10 min. Two milliliter of serum was added to both buffers and mixed by the vortex. The mixture was incubated at 40 °C

temperature for 60 min and 30 min for GOT and GPT, respectively. One milliliter of 2,4 di-nitrophenyl hydrazine was added and incubated at room temperature for 20 min. Ten milliliters of 0.4N NaOH was added to the solutions and mixed by inversion, and incubated for 30 min. The optical density was determined at 505 nm at room temperature. The activities of GOT and GPT were expressed as U/ml.

## 2.12. Determination of antioxidant activity and lipid oxidation

Activities of SOD, CAT and GPx of the liver were determined following the methods of commercial kits (Cell Biolabs Inc., San Diego, USA) used by Pervin et al. [39]. The MDA concentration of liver tissues was measured according to the method by Jain et al. [40]. The GST activity was measured using commercial kits (Abcamplc, Cambridge, UK) following the methods of the manufacturer's instruction. Briefly, for GST, liver tissue was washed with PBS contained heparin (0.15 mg/ml) to remove red blood cells and clots. Then it was re-suspended in 500  $\mu\text{l}$  of GST assay buffer. It was homogenized and centrifuged at 10,000  $\times g$  for 15 min at 4 °C and then the supernatant was transferred to a new tube. Ten microliters of samples and positive control were taken in wells and 40  $\mu\text{l}$  of GST assay buffer was added to the wells, and 50  $\mu\text{l}$  GST assay buffer was taken to background control well. Five microliters of glutathione were added to each sample and control well. Fifty microliters of the reaction mixture were added into each sample and control sample wells and the plate was shaken carefully to start the reaction. The absorbance was measured at 340 nm on a microplate reader in a kinetic mode, every 2–3 min, for at least 10 min at RT protected from light, to obtain at least 5-time points.

## 2.13. Cytokine content assay

Fish were anesthetized using MS-222 (FDA inspected Ferndale, Washington, USA) and maintained optimal dose followed by Rairat et al. [41], and then the liver of fish was separated. The liver tissues were washed with 0.9% NaCl for homogenization in PBS buffer (pH 7.4). The homogenate was centrifuged at 15,000  $\times g$  for 10 min at 4 °C. The supernatant was collected to analyze the cytokines. The cytokines i.e. Interleukin-1 beta (IL-1 $\beta$ ), Interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF- $\alpha$ ) level were assayed by ELISA kit (MyBioSource, Inc. San Diego, CA, USA). The level of cytokine was expressed as pg/ml.

## 2.14. Statistical analysis and data presentation

The statistical significance of the differences was calculated between the control and the treated groups via t-test analysis using SPSS (version 23) computer program. All the data were expressed as means  $\pm$  standard error (SE). The significance was tested at  $P < 0.01$  or  $P < 0.05$ .

## 3. Results

### 3.1. Physicochemical condition of water

Water quality parameters are very crucial factors, which influence the growth, reproduction, immunity, and other biological activities of fish. During the entire experimental period, water quality parameters such as pH, dissolved oxygen (DO), total alkalinity, total dissolved solids, biochemical oxygen demand (BOD), chemical oxygen demand (COD), ammonia, ammonium, nitrate, and total suspended solids of heavy metals contained river water was significantly ( $P < 0.01$ ) different from the control water except for temperature (Table 1).

### 3.2. Estimation of heavy metals in river water

Heavy metal concentrations in the water of the Buriganga river are given in Table 2. World health organization represents the acceptable limit for the studied heavy metals in drinking water [42]. The Among all

the metals studied in the Buriganga river water, zinc (Zn) concentration ( $35.5 \pm 6.1$  ppm) was the highest, while, cadmium (Cd) concentration ( $2.8 \pm 0.2$  ppm) was the lowest. The chromium (Cr), iron (Fe), lead (Pb), copper (Cu), Zn, and Cd in the water of the Buriganga river were significantly ( $P < 0.01$ ) different from the control water. In the present study, Cr, Fe, Pb, Cu, Zn, and Cd did not cause any fish mortality after 10 days of exposure of fish to heavy metals contained river water but found several physiological and immunological changes in the fish body, which were compared with the control group.

### 3.3. Observation of fish behaviors

The behaviour of tested *O. niloticus* was observed during the experiment period. Several irregular behaviours like sluggish movement, restlessness, extreme opercula movement, and settle on the bottom with imbalance swimming have been found throughout the experimental period in heavy metals contained river water treated fishes. On the other hand, control fishes were not shown any abnormal behaviour.

### 3.4. Histopathological changes

It has been observed that after 10 days of exposure of fish to heavy metals contained river water and found several structural changes in liver and kidney tissues, which were compared with the control group. The liver is the vital metabolic organ of living organisms. After the experimental period, the control group has not shown any histopathological changes in the liver (Figure 2a). The liver of the test animal in polluted river water treated, severe hemorrhage, vacuoles, pyknotic space, and evidence of lipid accumulation has been observed (Figure 2b). The liver plays a main role in the detoxification and degradation of pollutants. Additionally, in the control group, kidney tissues showed normal appearance (Figure 2c), while polluted river water exposed fish showed aggregation of melano-macrophage, pyknotic area, haemorrhage, glomerular enlargement, dilation of Bowman's space, abnormalities in kidney tubules, hypertrophy, hyaline and vacuolation (Figure 2d). High concentrations of heavy metals in water cause fish tegument discoloration, histological changes in liver and kidney, slow growth, and low efficiency in feed conversion.

### 3.5. Hematological alteration

After 10 days of exposure of fish heavy metals contained river water, the experimental fish showed changes in blood cells morphology such as isocytosis and formation of rouleaux in erythrocytes, an increase of binucleated cells, dead cells, hemolyzed cells, swelled cells, senile cells, sickle cells, teardrop like cells and vacuoles in the cells (Figure 2f). An

**Table 1.** Physicochemical conditions of water.

Parameters	Control (Mean $\pm$ SE)	Polluted river water (Mean $\pm$ SE)
Temperature	$27.5 \pm 2.1$ °C	$28.1 \pm 1.7$ °C
pH	$7.4 \pm 0.1$	$8.6 \pm 0.3^{**}$
Dissolved oxygen (DO)	$6.7 \pm 0.5$ ppm	$1.1 \pm 0.2$ ppm**
Total alkalinity	$120.3 \pm 0.4$ ppm	$155.2 \pm 0.4$ ppm**
Total dissolved solids (TDS)	$175 \pm 12.2$ ppm	$632 \pm 11.2$ ppm**
Total suspended solids (TSS)	$17 \pm 1.3$ ppm	$43 \pm 1.5$ ppm**
Biochemical oxygen demand, (BOD)	$11 \pm 0.7$ ppm	$24 \pm 1.2$ ppm**
Chemical oxygen demand (COD)	$22 \pm 2.1$ ppm	$76 \pm 1.6$ ppm**
Ammonia (NH <sub>3</sub> -N)	$2.7 \pm 0.2$ ppm	$13 \pm 0.7$ ppm**
Nitrate (NO <sub>3</sub> -N)	$3.5 \pm 0.9$ ppm	$17 \pm 5.1$ ppm**
Nitrite (NO <sub>2</sub> -N)	$3.0 \pm 0.6$ ppm	$18.3 \pm 4.9$ ppm**

In same row of each parameter with asterisk are significantly different (\*\* $P < 0.01$ ).

**Table 2.** Estimation of heavy metals in river water.

Parameters	Control (Mean $\pm$ SE)	Polluted river water (Mean $\pm$ SE)	World health organization limit [42]
Chromium (Cr)	ND	$3.5 \pm 0.4$ ppm	0.05 ppm
Iron (Fe)	$1.5 \pm 0.3$ ppm	$6.2 \pm 0.6$ ppm**	-
Lead (Pb)	ND	$10.1 \pm 1.2$ ppm	0.01 ppm
Copper (Cu)	ND	$8.5 \pm 1.1$ ppm	2 ppm
Zinc (Zn)	$1.6 \pm 0.4$ ppm	$35.5 \pm 6.1$ ppm**	3 ppm
Cadmium (Cd)	ND	$2.8 \pm 0.2$ ppm	0.003 ppm

ND: Not detectable.

In same row of each parameter with asterisk are significantly different (\*\* $P < 0.01$ ).

oval-shaped cell with a concentric nucleus in the erythrocyte was found in control group (Figure 2e). Moreover, the blood hemoglobin (Hb) of polluted river water treated fish was significantly decreased by the control group at  $4.7 \pm 0.5$  g/dl and  $6.8 \pm 0.4$  g/dl, respectively. A significant decrease ( $P < 0.01$ ) of RBC count was found as  $0.8 \pm 0.1$  ( $10^6$  mm<sup>3</sup>), whereas it was  $1.4 \pm 0.1$  ( $10^6$  mm<sup>3</sup>) for the control group. The WBCs count of polluted river water treated fishes was found as  $18.5 \pm 1.1$  ( $\times 10^3$ /mm<sup>3</sup>), whereas it was significantly different from the control group. Additionally, Hematocrit/PCV (%), MCV, MCH and MCHC values of heavy metals contained river water treated fishes were significantly different with the control values and also observed at  $18.4 \pm 1.6$  (%),  $271.1 \pm 9.6$   $\mu$ m [3],  $69.4 \pm 4.4$  pg and  $11.5 \pm 0.7$  (%), respectively (Table 3).

### 3.6. Blood glucose concentration

Glucose level in the blood of treated fish were significantly ( $P < 0.01$ ) decreased with the control group (Figure 3a). The lowest glucose level was found at the heavy metals contained river water after 10 days of exposure.

### 3.7. Serum Ca<sup>2+</sup> ion concentrations

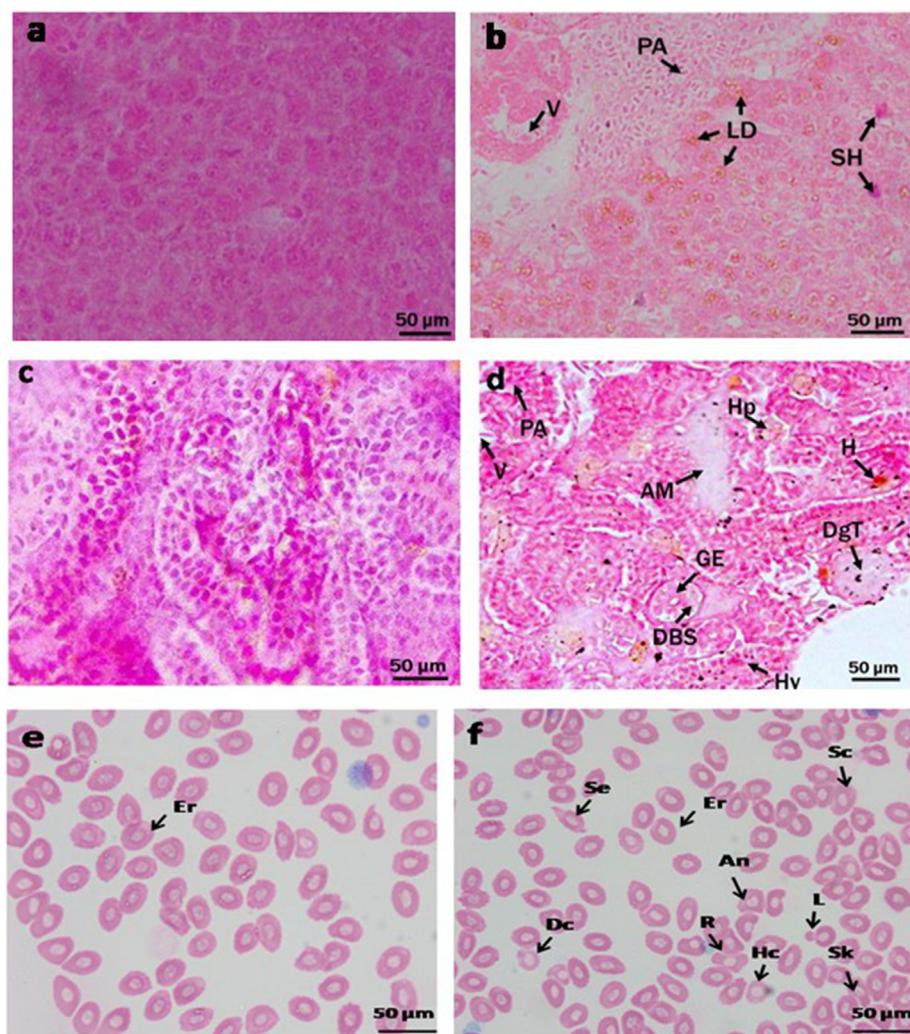
It has been observed that fish exposed for 10 days to heavy metals contained river water, the Ca<sup>2+</sup> ion concentrations in the blood serum of treated fish was significantly ( $P < 0.01$ ) lower compared with the control group (Figure 3b).

### 3.8. Brain AChE, plasma GOT and GPT enzyme activity

It has been observed that after 10 days of exposure of fish to heavy metals contained river water, the AChE activity in the brain of treated fish was significantly ( $P < 0.01$ ) decreased in comparison to the control group (Figure 4a). The GOT and GPT enzyme activities in blood were significantly ( $P < 0.01$ ) increased in the heavy metals contained river water treated fish in comparison with control group (Figures 4b and 4c).

### 3.9. Determination of antioxidant activity and lipid oxidation

The activity of GST enzyme was significantly ( $P < 0.01$ ) increased in the blood of heavy metals contained river water treated fish in comparison with control group. After the exposure of fish in polluted water for 10 days, the antioxidants (GPx, SOD, CAT) and lipid oxidation (MDA) were significantly ( $P < 0.01$ ) increased from the control group (Figure 5).



**Figure 2.** Images of liver, kidney and blood smears of *O. niloticus* exposed to heavy metal contained river water for 10 days. In liver, (a) control and (b) exposed to polluted river water, PA-pyknotic area; SH- severe hemorrhage; LD-lipid droplet; V- vacuolation. In kidney, (c) control and (d) exposed to polluted river, AM-aggregated melanomacrophage; H- hemorrhage; PA-pyknotic area; GE-glomerular expansion; DBS- dilation of Bowman's space; DgT-degenerating kidney tubule; Hp-hypertrophy; Hy-hyaline; V- vacuolation. In blood smears, (e) control and (f) exposed to polluted river, An-anisocytosis of erythrocyte cells; Dc-dead cell; Er- erythrocytes; Hc-hemolyzed cells; L-leucocytes; R-rouleaux formation of erythrocyte cells; Sc-swelled cells; Se-senile cell; Sk-sickle cell.

**Table 3.** Haematological indices of *Oreochromis niloticus* under the control and polluted river water exposure for 10 days.

Parameters	Control	Polluted river water
Hemoglobin (g/dl)	6.8 ± 0.4	4.7 ± 0.5**
RBC (x 10 <sup>6</sup> /mm <sup>3</sup> )	1.4 ± 0.1	0.8 ± 0.1**
WBC (x 10 <sup>3</sup> /mm <sup>3</sup> )	21.2 ± 1.0	18.5 ± 1.1**
Hematocrit/PCV (%)	25.1 ± 1.6	18.4 ± 1.6**
MCV (μm <sup>3</sup> )	186.6 ± 18.1	271.1 ± 9.6**
MCH (pg)	50.8 ± 4.6	69.4 ± 4.4**
MCHC (%)	15.13 ± 0.4	11.5 ± 0.7**

In same row of each parameter with asterisk are significantly different (\*\*P < 0.01).

RBC, red blood cell; PCV, packed-cell volume; WBC, white blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration.

### 3.10. Cytokine levels

After 10 days of exposure of fish to heavy metals contained river water, the IL-1β, IL-6, and TNF-α level were significantly increased in the liver of the treated group in comparison to the control group (Figure 6). IL-1β, IL-6, and TNF-α level in the experimental animal can be considered as a reliable bioindicator in determining the pollution of the aquatic ecosystem.

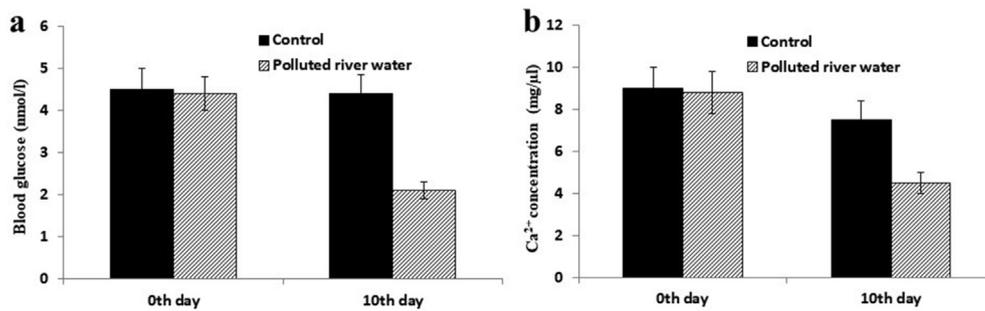
## 4. Discussion

### 4.1. Water quality parameters during the study period

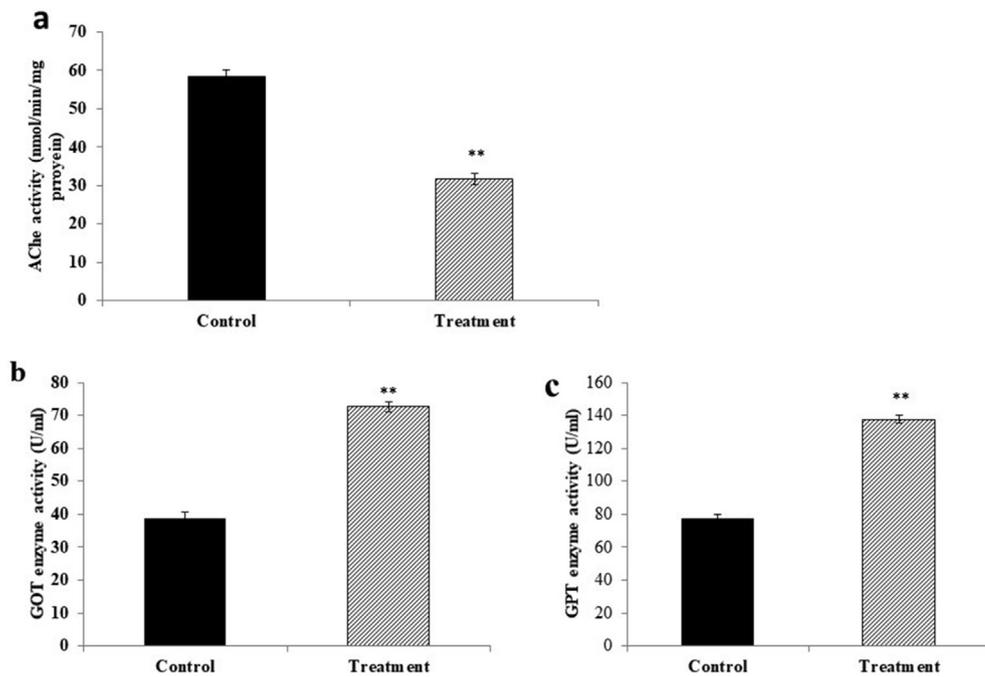
The physicochemical data of water has been recorded during the experimental period, which gives appropriate information about the present status of the heavy metal polluted river water. Based on these results, polluted river water was significantly different from the control group and also indicates polluted river water was not suitable for living organisms as recommended by the American Public Health Association [33]. The Buriganga river water is a threat to the contamination from untreated municipal wastes, industrial discharges, runoff from organic and inorganic fertilizers, and pesticide emission around the river and leading to the extinction of aquatic life and breakdown of the river ecosystems [43]. Fatema et al. [44] found that the Buriganga river was highly polluted with heavy metals during the dry season, which is harmful to the river ecosystem. The results from the present study showed that heavy metal pollution in river water was not safe for living organisms.

### 4.2. Histomorphological alterations in treated fish

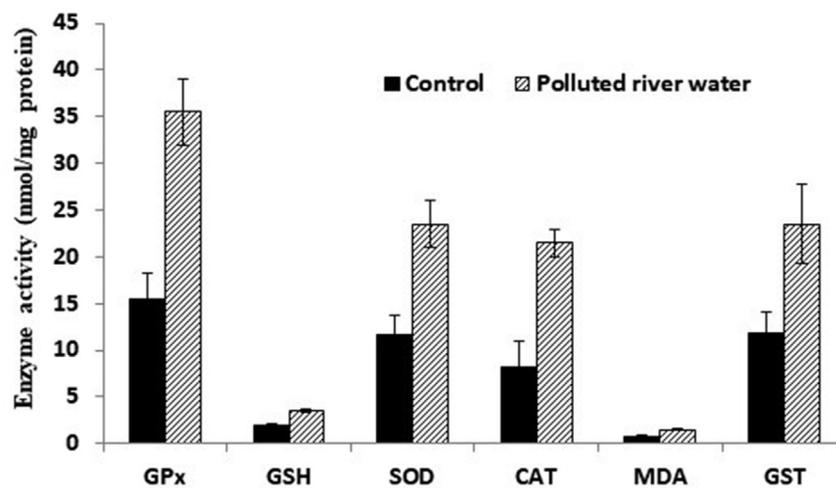
From the histomorphological observations, the alterations have been identified in the liver and kidney of the test fish. The prominent sign of pathology was found in fish exposed to the heavy metal contained river water for 10 days. The liver is the most important



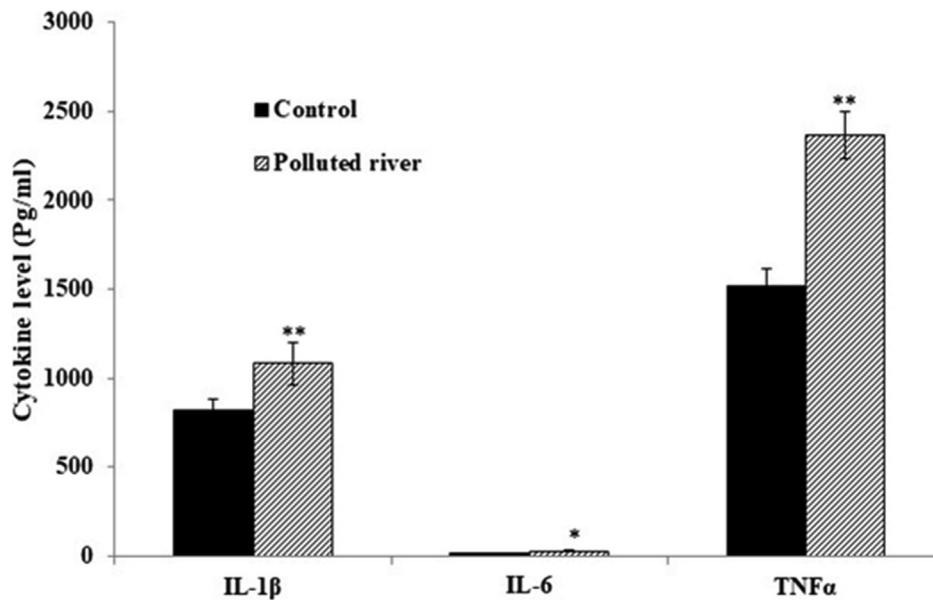
**Figure 3.** Blood glucose and Ca<sup>2+</sup> ion concentration in *O. niloticus* exposed to the heavy metal contained river water for 10 days. a. Blood glucose concentration; b. serum Ca<sup>2+</sup> ion concentration. Asterisks are indicated significant difference (\*\*P < 0.01).



**Figure 4.** Alteration of enzymatic functions in *O. niloticus* after 10 days exposure of heavy metal contained river water. a. Brain AChE activity; b. Glutamate-oxalacetate transaminase (GOT) enzyme activity; c. Glutamate pyruvate transaminase (GPT) enzyme activity. Asterisks are indicated significant difference (\*\*P < 0.01).



**Figure 5.** Effects on the levels of antioxidant activity of *O. niloticus* after 10 days of exposure of heavy metal contained river water. Asterisks are indicated significant difference (\*P < 0.05 and \*\*P < 0.01 vs control).



**Figure 6.** Alteration of cytokine IL-1 $\beta$ , IL-6 and TNF- $\alpha$  levels in *O. niloticus* after 10 days exposure of heavy metal contained river water. Asterisks are indicated significant difference (\*P < 0.05 and \*\*P < 0.01 vs control).

metabolic fish organ and it plays an important role in the uptake, accumulation, biotransformation, and excretion of toxic elements [45]. The results of the present experiment are similarly supported by the outcome of previous studies of Mahboob et al. [46] where due to the exposure to heavy metal polluted water, remarkable alterations in the kidney of Nile tilapia have been observed. Rakhi et al. [9] was also found the structural changes in liver and kidney with mentionable pathological signs in *Heteropneustes* treated with river water contained pollutants including heavy metals. Puntoriero et al. [47] observed structural changes of fish due to heavy metal toxicity effects, such as hemosiderin, congestion in hepatic sinusoidal, loss of contact between hepatocytes and pancreaticocytes, cellular degeneration, focal necrosis areas, and cellular apoptosis during the study period. Omar et al. [48] reported that Fe toxicity specifically effects the fish liver. Copper exposure caused vacuolization, necrosis, shrinkage, nuclear pyknosis and increase of sinusoidal spaces of hepatic cells in fish [49, 50]. Arsenic-exposed rainbow trout showed significant renal histopathological changes [51]. It has been also reported that Arsenic caused intercellular oedema [52] and vacuolation [53] in the kidney cells of *Clarias batrachus*. Changes in the histomorphology in the study has occurred due to the combined effects of different heavy metals presence.

#### 4.3. Hematological alteration of treated fish

The stress-induced cells could leak the hemolytic enzymes as a result the morphology of RBC has been altered [54]. Consequently, the necessary oxygen circulation could be inhibited through alterations of RBCs and WBC which leads the fish death [55]. In the current study, decrease of the PCV and MCHC; and increase of the MCV and MCH may cause the reduction of healthy red blood cells of the body which could cause for the hemorrhagic, hemolytic, and hypoplastic conditions [11]. Different studies showed that containment of metals in water showed significantly higher RBC counts, haematocrit (Hct), MCHC and, MCH while it showed significantly lower MCV in fish blood [56]. Cyriac et al. [57] reported that acute Cu exposure in *Oreochromis* showed a rise in both haematocrit and hemoglobin content in blood, also caused an increased rate of red blood cells (RBCs) catabolism [58]. Arsenic exposure lowered the number of lymphocytes and melano-macrophage in *Clarias batrachus* [53].

#### 4.4. Blood glucose analysis

A decreased level of glucose has been observed in this experiment treated with polluted river water. In Fish, plasma glucose level can be altered due to health status, scarcity of food, poor culture system, and stress condition (physiological, environmental, and pharmacological) [59]. Stress causes primary and secondary responses by the central nervous system of fish and releases stress hormones eventually altered blood parameters including glucose level and stabilize emergency energy demand [60]. According to Nevarez et al. [61] treatment with chemicals showed hypoglycemia with disrupted hepatic expression in channel catfish. Tabassum et al. [11] and Ghelichpoura et al. [62] reported an alerted glucose level due to bio-physiological damages as an indicator of pesticide treated stress. Stimulation of glycogenolysis has been found in *Labeo rohita* on exposure of Copper [63].

#### 4.5. Serum Ca<sup>2+</sup> ion concentration

The Ca<sup>2+</sup> concentration has the vital role to maintain the body fluids in living organisms. After hatching of tilapia, the Ca<sup>2+</sup> concentration has increased rapidly as it is a euryhaline fish [64]. Alteration of kidney tissue might affect the epithelial ion transportation in a fish species, which could cause the hypocalcemia due to change of the mineral concentration in the blood [65]. Freshwater fish larvae i.e. tilapia, goldfish, and zebra fish acclimate less Ca<sup>2+</sup> to fit their growth [66]. According to Tabassum et al. [11], pesticide elevated stress decreases calcium concentration in the blood of *Cyprinus carpio* fingerlings and stinging catfish, *Heteropneustes fossilis* respectively causing hypocalcemia condition. Hypoxic (low oxygen supply) stress in juvenile rainbow trout increases the serum Ca<sup>2+</sup> concentration in blood, unlike our study as sufficient oxygen was supplied. In Eyckmans et al. [67] Ca<sup>2+</sup> in the blood plasma of rainbow trout was decreased by the exposure of waterborne copper. Significant decrease in serum Ca<sup>2+</sup> concentration in fish has also been reported due to Copper exposure in water [68].

#### 4.6. Brain acetylcholinesterase (AChE) activity

In fishes, the brain, heart, liver, and muscle tissues maximally contain AChE [69]. Han et al. [70] described the neurotoxicity biomarker, AChE as a synaptic transmitter of nerve instinct. A decrease in AChE activity is a

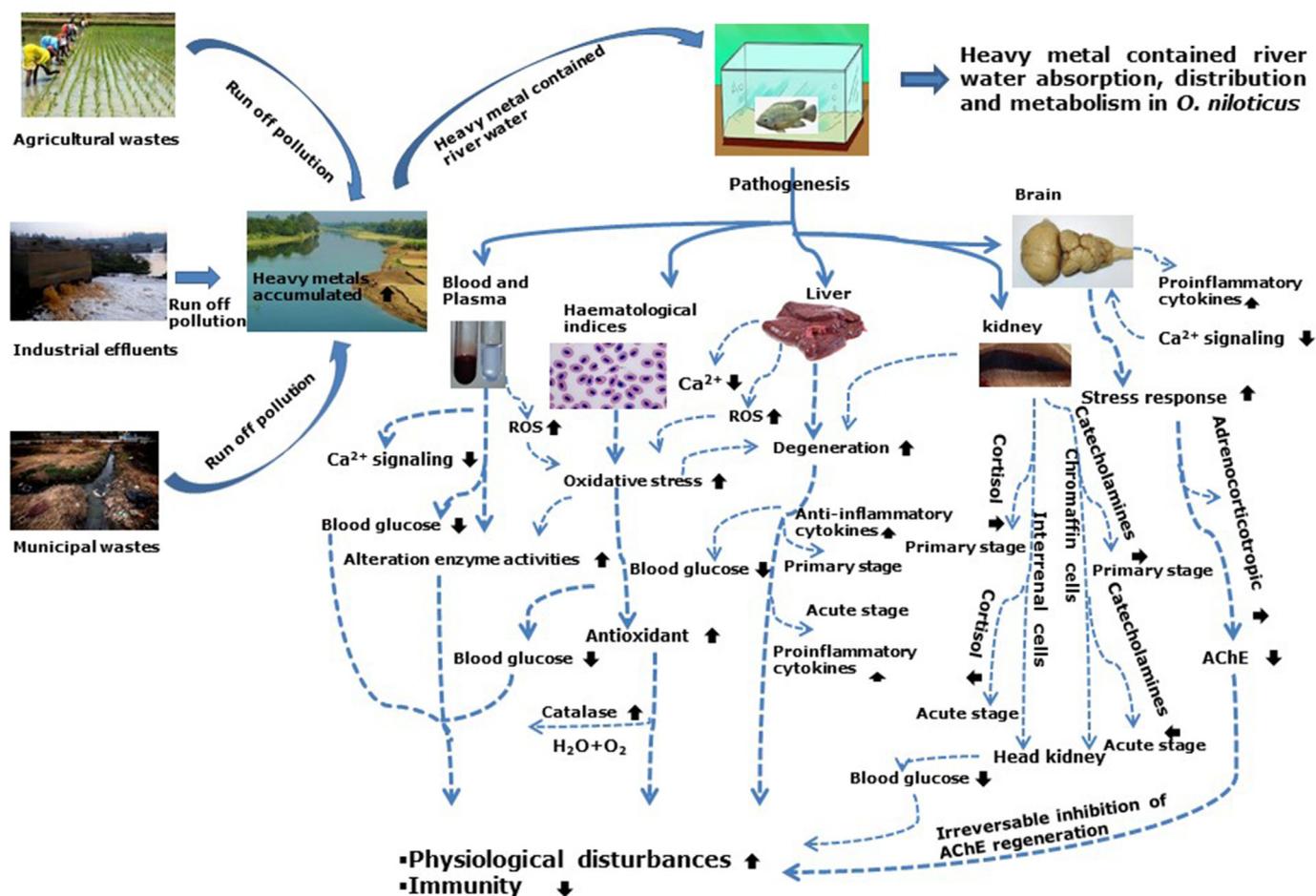


Figure 7. The potential mechanism of heavy metal contained river water effects on fish, *O. niloticus*.

biomarker for organo phosphorus and carbamate pollution in water [71]. Pollutants like heavy metals, aromatic hydrocarbons, and other pesticides also decline the AChE activity in fish [11]. In *Oreochromis mossambicus* brain AChE decreased due to trace element contamination in river water [72]. Tabassum et al. [11] also reported that AChE activity can be altered in *Cyprinus carpio* fingerlings for insecticide induced pollution in water. Heavy metal, specifically lead, inhibited AChE activity in fish to cause pathological changes in tissue and organs [73].

#### 4.7. Plasma enzymes activity

The GOT and GPT activities in the stress conditions of animals could cause the impairment of muscle and liver cells [74]. Li et al. [75] suggested that the organism increased the metabolic rate to mitigate the stress condition. The liver in fish metabolizes the essential biomolecules and repair any physiological and biochemical disorder which include GOT and GPT activities [76]. The findings of the current study have showed increased GOT and GPT activity in *O. niloticus* which is presumed due to the alteration of the muscle and hepatic tissues. The prolonged exposure of toxic chemicals and heavy metals to fish may cause death by hampering the GOT and GPT activities [76]. Koul et al. [77] observed sub-lethal concentration of dichlorous increased the level of GPT and GOT activity in *Channa gachua*. Tilak et al. [78] reported exposed to alachlor increase the GPT and GOT level in *Channa punctatus*. Saravanan et al. [79] observed an increasing level of GPT and GOT in the blood and liver of *Labeo rohita* after treating with endosulfan. An increase in GOT and GPT have been found in *Clarias gariepinus* exposed to the mercury contained water that caused heavy metal stress [80]. A significant

increase in cholesterol, alkaline phosphate, cortisol, GOT and GPT has been found in *Clarias lazera* caused by Vanadium toxicity to fish [81].

#### 4.8. Antioxidant enzyme activity and lipid oxidation

Antioxidants are the first line of defense against oxidative stress that catalyzes the reactive oxygen compound into oxygen and water. In this study, we have shown the effects of polluted river water on the antioxidant activity of *O. niloticus*. Oxidative parameters (SOD, GPx, GST, CAT) and lipid oxidation (MDA) activity have been significantly different in treated fish with polluted river water. Khalil et al. [82] showed that SOD, CAT, GST, GPx activities had significantly increased in *O. niloticus* in winter when the water remains more polluted than the summer. It also showed the impact of heavy metal pollution on the MDA (lipid peroxidation) levels in *O. niloticus* tissues. According to Lin et al. [83] the activity of GPx, GST, CAT had increased significantly in nitrite pollution. The study also observed that nitrite pollution increased the accumulation of MDA but it showed the decreasing activity of SOD unlike our result of this experiment. Vaseem and Banerjee [84] found the increased activities SOD, CAT, and increased level of MDA in the fish collected from the heavily polluted river because of uncontrolled anthropogenic activities. They concluded that the result was indicating the pollutant-induced oxidative stress in the fish. Continuous metal pollution induced the cellular oxidation that ultimately resulted in the increased SOD activity and significantly different antioxidant defense system of *Cirrhina mrigala* collected from the Indus river [85]. Many types of research show that heavy metal pollution in the water can change the antioxidant activity in fish. In the present study, CAT

activity has been increased after the exposure of *O. niloticus* in polluted river water for 10 days. According to Nunes et al. [86] pesticide treatment in carp and zebra fish showed increased activity of GST but the same treatment showed a decreasing result in CAT activity. Gharred et al. [87] observed that MDA and CAT increased in a heavy metal contaminated water body, but SOD and GPx decreased due to the metal pollution. It also said that different types of environmental pollutants like metals, organic compounds negatively affect the antioxidative enzymes. In Karadag et al. [88] they assessed the pollution using several oxidative stress biomarkers in the blood of *Cyprinus carpio*. In their experiment, fish collected from the polluted area contaminated with municipal, agricultural, and industrial waste has shown relatively increased activity of CAT and level of MDA, but decreased activity of SOD compared to the clean water fish sample. Cu contamination in water inhibited CAT activity in liver, gill and muscle in carp, *Cyprinus carpio* L [89]. Sanchez et al. [90] has also reported that Cu induces oxidative stress in fish (*Gasterosteus aculeatus*). Lead decreases major antioxidant activities causing oxidative stress that leads to various dysfunctions in lipids, proteins and DNA [91].

#### 4.9. Cytokine levels

Cytokines are playing a very crucial role in fish fitness, disease, and also regulate the fish immune system [92]. Cytokines are sensitive to stress conditions and react quickly to the unfavourable situation for the aquatic environment [93]. As important cytokines, especially IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$  can quickly initiate macrophages for aquatic contaminants and activate the immune system [94]. In the present study, the IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$  level were significantly up-regulated in the experimental animal, *O. niloticus* of the treatment groups. In stress conditions, cytokines stimulate macrophage interceded phagocytosis process and enhanced the immune response [95]. Ma et al. [96] found the increased cytokines level in fish serum and these cytokines take action to injure tissues under the stress condition. Carfi et al. [31] assessed the heavy metal pollution by means of increased cytokines levels in fish body which were involved of the immune responses. Yildirim and Danabas [97] found the increased immune-modulator biomarkers especially TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 level in fish liver during the heavy metals pollution experiment. So, the cytokine levels act as an important indicator of the fish's immune system to heavy metal exposure.

Due to the heavy metal effects of river pollution on *O. niloticus*, the mechanistic pathways have been proposed in Figure 7 based on the above discussions. This pathway was developed based on whole research work. Heavy metals contained river water was absorbed, distributed, and metabolized into the fish body. When polluted water metabolized in a fish body, it affects different organ and alteration of different physiological function such as release the AChE from the brain and induces an inflammatory reaction, increased the GOT and GPT enzymes level in the blood, decreased the blood glucose and Ca<sup>2+</sup> ion in the fish body, increased in antioxidant enzyme activity and cytokine level. Over the studied period, significantly compared to the control group, biochemical physiological changes have been observed with the heavy metals contained river water treated fish. Finally, these reactions cause the death of exposed fish.

## 5. Conclusion

The present study demonstrated that heavy metal polluted river water can lead to biochemical and enzymatic alterations of freshwater fish. Any alterations in the fish body consequently result in physiological disturbances, which might affect seriously the normal function, growth rate, and survival in nature. These results explore the detrimental effects of heavy metals contained river water on the river ecosystems, especially for freshwater living organisms. It is clear that heavy metals alter both structure and function of different organs including enzymatic changes, also affecting the innate immune system of exposed fish.

## Consent to participate (Ethics)

The fish was used as a test animal in the present study.

## Consent to publish (Ethics)

All authors agreed to submit to the journal for publishing the article.

## Plant reproducibility

Present work was not related to plant reproducibility.

## Declarations

### Author contribution statement

Zakir Hossain: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Md. Saddam Hossain: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Noore Safa Ema: Performed the experiments; Wrote the paper.

Abdelwahab Omri: Analyzed and interpreted the data.

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### Data availability statement

Data will be made available on request.

### Declaration of interests statement

The authors declare no conflict of interest.

### Additional information

No additional information is available for this paper.

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