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# *Labeo rohita*, a bioindicator for water quality and associated biomarkers of heavy metal toxicity

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This study investigated the effect of heavy metals on *Labeo rohita* inhabiting the Yamuna River, India. Levels of heavy metals measured in the water were as follows: Fe>Mn>Zn>Cu>Ni>Cr>Cd. Gill and liver tissue of exposed *L. rohita* showed a high metal pollution index, compared to reference fish collected from the Agra Canal. In the exposed fish, higher levels of creatinine and enzyme activity (alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase) were observed, while the A:G index declined. Additionally, higher TLC, lymphocytes, respiratory burst, and nitric oxide synthase activity indicated a heightened immune response. Levels of superoxide dismutase and lipid peroxidation were elevated, while catalase, glutathione S transferase, and glutathione was reduced. DNA of the exposed fish appeared deteriorated, with a greater mean tail length in comparison to the reference. Our results imply that Yamuna River water generates oxidative stress and DNA damage in *L. rohita*. As this river is a critical source of water and food to the native community, this could pose a threat to public health similar to that in the indicator organism.

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## INTRODUCTION

For many previous decades, heavy metal poisoning of aquatic systems allured the curiosity of environmental researchers. Many industrial applications and intensive agricultural practices have largely contributed to the pollution of freshwater habitats thereby causing detrimental effects on aquatic organisms and the health of human beings[1,2]. Heavy metals are non-degradable, hence can easily accumulate in the environment and the tissues of aquatic biota, and as such accumulation in tissues can be of public health concern to both animals and humans[3,4]. Researchers have developed some early diagnostic biomarkers for predicting the health of the environment and the bio-indicator itself. Biomarkers are interpreted as evaluation of interactivity between body system and environmental agent, which could be physical, chemical, and biological[4–7]. Hence, in vivo introduction of biomarkers is an excellent tool to estimate the vulnerability and negative effect of environmental agents on organisms[5,8]. The response of an organism after uptake could lead to alteration in biochemical level, molecular level, tissue level, cell structure, and function, and in the behavior of organisms[9,10]. Several biomarkers have been used to show the biological effects of various chemical compounds on aquatic fauna, in both experimental and natural conditions. The usefulness and rationality of the biomarkers can be assessed by the ability they impart to exhibit a concrete correlation between simply verifiable biological modifications and pollutant exposure and to provide knowledge on the biological alterations of pollutants rather than on environmental pollution[11]. Mainly biochemical and physiological parameters such as enzyme (leak on tissue damage) activities, quantifiable products, etc., have been in increasing use as significant tools to determine the effect of environmental pollutants[11,12]. For such studies, fishes are used as the endpoint of the food chain, because of their capacity of bioaccumulation of toxicants not only from water but also from the available food[13]. *Clarias gariepinus* was taken as an indicator organism of Orontes River to point out the deteriorated quality of river water along with bioaccumulation, oxidative stress, and DNA damage[14]. Maurya et al.[15] took several fishes like *Cirrhinus mrigala*, *Cirrhinus reba*, *Catla catla*, *Labeo rohita*, and *Crossocheilus latius* to check the pollution status of the Ganga River basin and bioaccumulation of Cr, Cd, Zn, Cu, and Pb. Alshkarchy et al.[16] inspected physiochemical parameters of the Euphrates River and the effects of heavy metals on hematological parameters of *Cyprinus carpio*. Dane and Sisman[17] studied histopathology and adverse effects on vital organs of *Alburnus mossulensis*. Recently, reports on the pollution of the Yamuna River, India showed that it is one of the most polluted rivers in the world. The pollution load is too high at the New Delhi segment where Najafgarh and Shahdara drain discharges heavy loads of pollutants. This segment has turn into a drain where the possibility of life is rare. Therefore, the present concern is related to the contamination by effluents loaded in another segment of Yamuna River at Agra (27°11′2.59″N and 78°1′47.58″E). It receives treated, partially treated and untreated effluents primarily containing heavy metals from numerous point and nonpoint sources. Hence, it poses life threats to inhabitants and users. Therefore, it becomes mandatory to test the water quality, the existence of heavy metals, and adverse effects on inhabitants and/or bio-indicator organisms. With this background, the current research work chose endemic fish *L. rohita* as an indicator of the water quality of the Yamuna River, India. *L. rohita* is one of the Indian major carps. It feeds mainly on plant material like algae and macrophytes, but can also take rotifers, cladocerans, protozoans, etc. It is well known that heavy

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**Table 1.** Concentrations of heavy metals in *Labeo rohita* tissues (mg/kg.dw) and in water (mg/L).

| | | Cr | Mn | Fe | Ni | Cu | Zn | Cd |
|---|---|---|---|---|---|---|---|---|
| Exposed *L. rohita* | Gill | $_{a}31 \pm 0.21^{e}$ | $_{d}4.55 \pm 0.01^{f}$ | $_{b}38.4 \pm 0.8^{d}$ | $_{b}46.4 \pm 0.35^{d}$ | $_{a}109.45 \pm 2.3^{a}$ | $_{a}70.6 \pm 0.79^{b}$ | $_{a}63.25 \pm 0.3^{c}$ |
| | Liver | $_{b}24.85 \pm 0.16^{d}$ | $_{a}19.8 \pm 0.41^{de}$ | $_{a}64.25 \pm 1.9^{bc}$ | $_{a}105.45 \pm 0.74^{a}$ | $_{c}1.85 \pm 0.001^{f}$ | $_{ab}67.82 \pm 1.5^{b}$ | $_{a}64 \pm 1.4^{bc}$ |
| Reference *L. rohita* | Gill | – | $_{b}9.7 \pm 0.01^{c}$ | $_{c}18.45 \pm 0.9^{a}$ | – | $_{c}1.5 \pm 0.001^{d}$ | $_{d}12.8 \pm 0.97^{b}$ | – |
| | Liver | – | $_{bc}7.3 \pm 0.01^{c}$ | $_{d}15.93 \pm 0.89^{ab}$ | – | $_{b}6.6 \pm 0.01^{c}$ | $_{c}16.9 \pm 0.01^{a}$ | – |
| Yamuna water | | $_{a}3.5 \pm 0.01^{e}$ | $_{a}19 \pm 0.32^{b}$ | $_{a}48 \pm 0.45^{a}$ | $_{a}11.9 \pm 0.12^{c}$ | $_{a}7 \pm 0.01^{d}$ | $_{a}13 \pm 0.92^{c}$ | $2.4 \pm 0.01^{e}$ |
| Reference Agra canal | | – | $_{b}0.02 \pm 0.01^{c}$ | $_{b}5.2 \pm 0.1^{a}$ | – | $_{b}3.6 \pm 0.01^{b}$ | $_{b}3.9 \pm 0.01^{b}$ | – |

All values are provided as mean ± SEM ($n = 15$); Statistical analysis done by ANOVA (2 way) and DMRT. Different superscripts and subscripts indicates significant difference at $p < 0.05$ in respective rows and columns; Blank cells indicate below detection limits; Statistical analysis done separately for fish and water.

metals tend to bind with proteins, enzymes, DNA, and several other biomolecules resulting in the distortion of their structures. Many investigators have described abnormal changes in the activities of enzymes like superoxide dismutase (SOD), catalase (CAT), peroxidases, glutathione-S-transferase (GST), oxidases, dopamine β-monooxygenase, etc. in bio-indicator organisms in response to bioaccumulation of heavy metals[18–20]. Body fluid components like leukocytes, macrophages, and mast cells form an important part of the innate immunity of fish and also actively contribute to attacking antigens. To survive in disturbed environments, organisms have evolved defense mechanisms to modulate metal ions such as globulins and metal-binding metallothioneins[11,21,22]. Besides, respiratory burst and nitric oxide synthase (NOS) are also employed as good immunological indexes in fish[23,24]. Hence, the current study aimed to assess the quality of Yamuna River water, bioaccumulation of heavy metals, and their adverse effects on target organs (gills and liver), pathology marker enzymes like aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and creatine kinase (CK). For immunotoxicity, biomarkers used were serum proteins (Albumin, globulin, and their ratio), total leukocyte count (TLC), differential leukocyte count (DLC), respiratory burst, and NOS function. Moreover, oxidative stress caused was measured with the help of SOD, CAT, glutathione (GSH), GST, and lipid peroxidation (LPO). Furthermore, genotoxicity was also assessed by comet assay.

## RESULTS AND DISCUSSION

### Freshwater quality, bioaccumulation, and Metal pollution index

The physicochemical properties of test river water such as conductivity, dissolved oxygen, pH, and temperature were 55 Us/cm, 6 mg/L, 7.5, and 28 °C respectively. Whereas, in reference water, the parameters were recorded as 56 Us/cm, 6.5 mg/L, 7.0, and 26 °C respectively. All these parameters in the test and the reference water were found to be within the permissible limits set by BIS[25], and WHO (UNEPGEMS)[26]. The detected heavy metals concentrations in the test river water were in the order of Fe> Mn> Zn> Ni> Cu> Cr> Cd (Table 1). The concentrations of these metals in the test water were significantly higher than the specified limits set by BIS[25] and WHO[26]. This creates stressful conditions for freshwater fish species for their survival, growth, and health in the Yamuna River. However, in reference water, only Fe, Mn, Cu, and Zn were detected and that too were below the specified standards (Table 1). The overall heavy metal concentration (Metal pollution index, MPI) in fish pointed out that the gill act as a target organ as compared to the liver in exposed *L. rohita* (Table 2). MPI calculation becomes mandatory to study the accumulation of multiple heavy metals in distinct tissues/species of fishes. MPI helps in understanding the total metal load in a tissue. Pengfei et al.[27] reported the bioaccumulation of heavy metal concentrations especially As, Cd, Pb, and Hg in three fish species specifically *Carassius auratus*, *Hypophthalmichthys nobilis*, and *Pelteobagrus fulvidraco* from Nansi Lake of China. Recently, Ahmed et al.[28] investigated the accumulation of Cu, As, Cd, Pb, and Cr in six commercially important fishes in the Karnaphuli River estuary, Bangladesh. Several studies including these had reported that Hg, Pb, and Cd were greatly toxic metals, followed by Cu, Zn, Cr, Mn, and Ni[27–30]. Chronic exposure of organisms to these metals even at very low concentrations could result in biochemical alterations, damage to immunity, and DNA in distinct tissues of fishes[15,30]. Therefore, investigations are required to verify the toxicities associated with these heavy metals at least once in a while. The comparative account of water quality of different river systems of the world and the present study is shown in Table 3.

**Table 2.** MPI calculation in exposed and reference fish.

| Fish groups | Tissues | MPI |
|---|---|---|
| Exposed *L. rohita* | Gills | 38.38 |
| | Liver | 30.87 |
| Reference *L. rohita* | Gills | 7.6 |
| | Liver | 10.6 |

### Effect of heavy metals on enzyme activities

Enzyme biomarkers are widely used indicators to assess physiological alterations in target organisms resulted from toxic substances in the environment. Heavy metals affect the activities of enzymes by distorting their structures and influence their action as they act on the specific substrates for metabolism[31]. In the present investigation, serum ALT (+247%), ALP (+386%), AST (+513.7%), and CK (+607.5%) activities were found to be elevated in exposed *L. rohita* than the reference (Table 4). This elevation in enzyme function might be due to tissue damage in the gill and liver resulted from the accumulation of heavy metals. Recently, Barisic et al.[32] noticed an exposure-duration-related alteration in serum ALT, ALP, and AST of salmon fish as the higher the activity the more was liver damage. ALT, ALP, and AST are serum markers, mainly used to interpret damage in the liver and thus, they are known as serum aminotransferases[33,34]. Many investigators revealed an increased measure of serum ALT, ALP, and AST in *Tilapia zillii*, *Mugil cephalus*, *Oncorhynchus mykiss*, and *Barbus luteus* due to heavy metals exposure[18,35,36]. CK converts creatine to adenosine diphosphate and phosphocreatine after utilizing adenosine-triphosphate. The excess CK activity the excessive will be the tissue damage indicating myocardial infarction, rhabdomyolysis, autoimmune myositides, and muscular dystrophy[37,38].

**Table 3.** Comparative account of water quality status of present study with other water bodies of the world.

| Site | Heavy metals (ppm/mg/L) | | | | | | | References |
|---|---|---|---|---|---|---|---|---|
| | Cr | Mn | Fe | Ni | Cu | Zn | Cd | |
| Yamuna River | 3.5 | 19 | 48 | 11.9 | 7 | 13 | 2.4 | Present study |
| Gediz River | – | 0.13 | 0.232 | 0.02 | 0.008 | 0.05 | – | [68] |
| Manyame River | 42 | – | 46 | 3.4 | 17 | 542 | – | [69] |
| Ganga River | 29 | – | 0.27 | – | 0.24 | 0.09 | – | [70] |
| Nansi Lake | – | – | – | – | – | – | 0.002 | [27] |
| Ganaga basin (Kanpur) | 0.32 | – | – | – | 1.35 | 4.74 | 0.54 | [15] |
| Karasu River | 13.1 | 7.7 | 7.8 | 1.01 | 0.82 | 0.61 | – | [17] |
| Orontes River | 1.08 | 7.23 | 89.90 | – | 2.2 | – | 0.03 | [14] |
| Reservoir | 16.73 | – | – | – | – | – | 0.66 | [71] |

**Table 4.** Pathology marker enzymes and immune parameters of serum/blood.

| variables | Reference *L. rohita* | Exposed *L. rohita* | % change over reference |
|---|---|---|---|
| AST (U/L) | 4.87 ± 0.01 | 16.9 ± 0.3* | +247 |
| ALT (U/L) | 8.31 ± 0.1 | 51 ± 1.2* | +513.7 |
| ALP (U/L) | 7.2 ± 0.01 | 35 ± 0.74* | +386 |
| CK (U/L) | 10.6 ± 0.03 | 75 ± 0.92* | +607.5 |
| Alb (mg/mL) | 1.2 ± 0.001 | 0.41 ± 0.001* | −65.83 |
| Glo (mg/mL) | 0.7 ± 0.001 | 1.5 ± 0.01 | +114 |
| A/G ratio | 1.7 ± 0.01 | 0.27 ± 0.02* | −84 |
| TLC ($10^3$/mm$^3$) | 30.5 ± 0.42 | 56.12 ± 0.51* | +84 |
| Neutrophils (%) | 13 ± 0.01 | 85 ± 0.11* | +553.8 |
| Lymphocytes (%) | 24 ± 0.16 | 79 ± 0.13* | +229 |
| Eosinophils (%) | 1.2 ± 0.01 | 9 ± 0.02* | +650 |
| Basophils (%) | 0 | 2 ± 0.01 | – |
| Monocytes (%) | 3.6 ± 0.01 | 15 ± 0.03* | +316.6 |
| Respiratory burst | 0.76 ± 0.001 | 13.82 ± 0.01* | +1718 |
| Nitric oxide synthase (mol/ml) | 4.39 ± 0.01 | 19.4 ± 0.02* | +341.9 |

All values are given as mean ± SEM ($n = 10$).
*Indicates significant difference at $p < 0.05$.

### Serum albumin and globulins

Albumin and globulin make up the major part of the total proteins and modification in their levels leads to the perturbation of the A:G ratio[39]. Therefore, the impact of concerned metals has also been observed on these serum proteins. In the current work, a significantly low level of albumin (−65.83%) with an increase in globulin (+114%) content leading to low Albumin to Globulin (A:G) ratio (−84%) was noticed in exposed *L. rohita* (Table 4). This observation corroborates well with a previously reported result of total protein contents in *M. cavasius*[40]. Recently, Barisic et al.[32] observed a decreasing trend in total serum protein and albumin in a salmonid. The ideal values of the A:G ratio need to be between 0.8 and 2.0, which assist in detecting the variation in the framework of serum or plasma[6]. In exposed *L. rohita*, the A:G ratio was found to be much lower than 0.8 with the decreased value of 0.27 showing a −84% decline which indicated higher liver damage.

### Innate immunity

Innate immunity variables of serum/blood are presented in Table 4. TLC is the most valued character to check the host's immune status and resistance to infection or disease[41]. For foreign invaders', leukocytes play a major role in defending the cells against infection. The present investigation reported a significant increase in TLC (+84%) levels than the reference *L. rohita*. This elevation, observed in TLC could be due to heavy metal overload, which led to the hampering of immune response and scavenging of ROS, thereby causing tissue damage[42]. Neutrophils, lymphocytes, and monocytes are most commonly present in fish blood whereas, the presence of eosinophils and basophils are very rare or may be unavailable in a healthy state[43]. In affected fish, the percent change of neutrophils (+553.8%), eosinophils (+650%), lymphocytes (+229%), and monocytes (+316.6%) were depicted higher in contrast to the healthy fish (Table 4). This observation shows notable immune stimulation in exposed *L. rohita*. Increased percentage of lymphocytes in affected fish in contrast to healthy fish possibly ascribed to the substantial effort of T-cells, natural killer cells, and B-cells, which responded and recognized antigens consequently generated antibodies and encountered the target cells which were triggering the damage. Furthermore, a few basophils were also seen in the smear of exposed fish which were completely absent in reference. A significant association between heavy metals and the multiplication of lymphocytes was described by Lawrence[44]. The higher percentage of neutrophils could be attributed to the activity of the enzyme myeloperoxidase. Myeloperoxidase is found in neutrophils and they remove invaders from the body of the organism[23,45]. Furthermore, the innate immunity of fish was examined by the respiratory burst and NOS enzyme. Generally, it is assumed that the sensitized phagocytes in fish discharge superoxide anion and its derivatives during a phase of vigorous oxygen consumption, called the respiratory burst[24,46]. Elevated respiratory burst (+1718%) in exposed *L. rohita* than reference fish implies intensified phagocytosis to check the toxic agents (Table 4). A few investigators have also found an increase in a respiratory burst in fish[24,46,47]. In the present study, higher levels of NOS (+341.9%) showed the efficient immune system of exposed *L. rohita* as compared to the reference (Table 4). NOS causes the production of nitric oxide, a cell-signaling molecule that participates forcefully in the defense mechanism of fish[23,48]. Thus, higher respiratory burst and NOS reflect the activated defense system of *L. rohita*.

### Oxidative stress

The enzymatic and non-enzymatic parameters such as SOD, CAT, GST, GSH, and LPO in the gill and liver of *L. rohita* are shown in Fig. 1. In exposed fish a significant ($p < 0.05$) increase in quantities

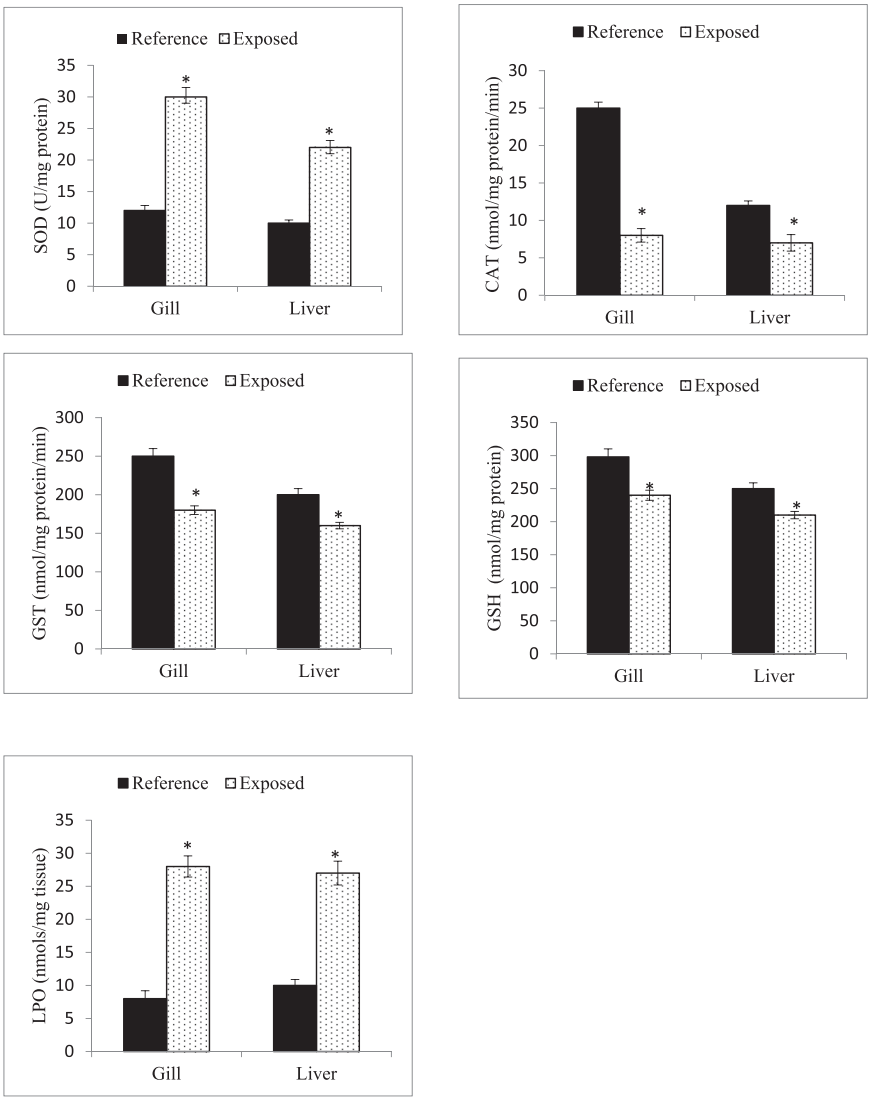


**Fig. 1 Biomarkers of oxidative stress in *Labeo rohita* dwelling in reference and polluted water.** Significance was established at $p < 0.05$; symbol (*) indicates significant difference.

of SOD and LPO was observed whereas, lower levels of CAT, GST, and GSH were found in the gills and livers juxtapose to the healthy fish. The SOD activity was observed to be 2.5 and 2.2 folds higher, respectively, in gill and liver as compared to the reference. SOD activity was elevated in *C. punctatus* on exposure to heavy metals overload of thermal power plant effluents[18]. The SOD belongs to the family of metalloenzyme which catalyzes the removal of $O^{\bullet-}_2$ to $O_2$ and $H_2O_2$. Further, CAT converts $H_2O_2$ into $H_2O$ and $O_2$ in an energy-efficient way. A significant decline ($p < 0.05$) in CAT activity, 3.12 and 1.7 folds, respectively, was depicted in the exposed gill and liver. CAT suppression can also happen due to the over-production of SOD or due to its substrate $H_2O_2$[49]. Due to heavy metal exposure, a significant decline in CAT activity was also confirmed by other groups of researchers in different fish species[50,51]. GSTs belong to a family of cytosolic biotransformation enzymes. They play an essential role in the transport and fight against oxidative stress which causes unavoidable damage to DNA, cell membrane, and proteins[52]. Significant ($p < 0.05$) reductions of 1.38 and 1.25 folds in GST activity and 1.24 and 1.19 folds in GSH contents were observed in gills and livers of exposed fish than the reference. The fall in activity of GST and GSH could be due to quick utilization of both GST and GSH, resulting in oxidative stress[18,53]. The increase in LPO levels was 3.5 folds in gills and 2.7 folds in livers as compared to the respective control groups. Our results are in agreement with the results of Lopez et al.[54] and Francisco et al.[55]. This increase in LPO levels may alter the physiological function of cell membranes due to excess production of reactive oxygen species, which may lead to cell membrane damage. Therefore, we suggest that exposure to these heavy metals effluents causes disturbances in oxidative stress parameters. Hence, it may suppress the activity of the antioxidant system and consequently could compromise the compensatory processes.

## Genotoxicity

The single-cell gel electrophoresis method (SCGE) is the most reliable, simple, and accurate technique for the measurement of genotoxicity in the form of DNA damage. SCGE was used to determine the damage in gill and liver cells of exposed individuals of *L. rohita* in comparison with their respective reference fish. Figure 2 shows the representative images of SCGE of reference and exposed cells of gills and livers of *L. rohita*. Significantly higher damage was seen in both the organs of exposed fish. In gills of affected fish, a significant ($p < 0.05$) mean tail length (25.8 µm) was

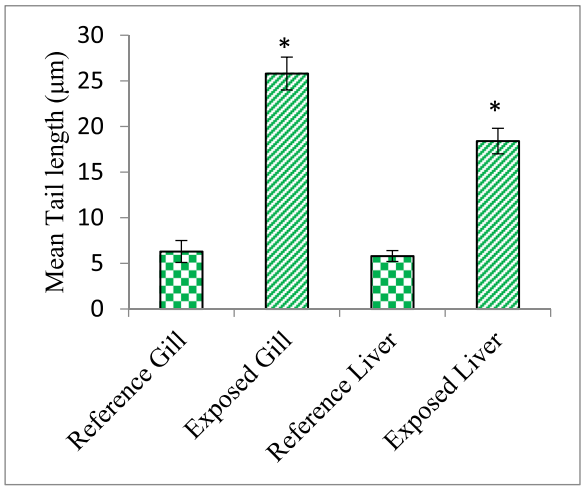


**Fig. 2 Mean tail length (µm) of DNA comets in *Labeo rohita* in gill and liver tissues collected from reference and exposed samples.** Significance was established at $p < 0.05$; symbol (*) indicates significant difference.

recorded than the reference (6.3 µm) *L. rohita* (Fig. 2). Similarly, in the liver, a significant ($p < 0.05$) tail length of 18.4 µm was recorded in exposed *L. rohita* against 5.8 µm in reference fish (Fig. 2). The current results are in agreement with the previous research revealing damage to DNA in terms of tail length in gills and liver due to heavy metals pollution overload in different fish species like *B. barbus*[55,56], *A. testudineus*[57], and mullet and sea bass[58]. It has been reported that when ROS production surpasses the fish defense system due to excessive overload of heavy metals/xenobiotics and comes in contact with DNA and form adducts, it ultimately leads to cellular lesions or DNA damage[6,59]. Moreover, innate immunity also failed to protect against damage. Furthermore, the higher SOD and LPO levels reflected cellular damage. It has also been verified in this study that despite the response of thiol-containing antioxidants GST and GSH, the ROS cause damage to the DNA.

The results of current research work furnish a comprehensive report on the water quality status of Yamuna at Agra, and effects of heavy metals' burden on *L. rohita* in target tissues (gills and liver). Observed bioaccumulation and hematological findings are pointing towards respiratory problems and poor immune health to counter the toxicants. This investigation provides plausible evidence that heavy metals bring about extreme tissue damage in the gills and the liver. Significant increase in LPO and SOD coupled with a marked reduction in CAT, GST, and GSH, demonstrating an imbalance in oxidative and anti-oxidative agents leading to DNA damage as well as an adaptive response of fish against the heavy metal overloads. The indiscriminate and injudicious use of heavy metals should be avoided because these metals will find their way to humans through the food chain and will cause similar effects in them. To develop healthy freshwater fish conservation and fishing industries and to prevent heavy metals risks to human health in the Yamuna River, the allowable limits of heavy metals in the water column must be regularly monitored. In addition to this, some remedial measures can be taken from time to time to minimize the problem.

## METHODS

### Ethical statement

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. It was permitted by Ministry of Environment and Forests, Government of India under registration no. 714/02/a/CPCSEA issued and approved by the institutional ethical committee of Department of Biochemistry, Aligarh Muslim University, Aligarh, India.

### Sample procurement and analysis of water quality

The fish (*L. rohita*) [$n = 15$] were captured from the River Yamuna near waterworks (Agra). Another group of fish of *L. rohita* for reference was collected from the Agra canal. The average full body length and weight of both groups were 16.5 ± 2.3 cm, 150 ± 3.6 g, and that of reference 19.5 ± 0.2 cm and 155 ± 0.16 g, respectively. Gills and liver of both groups of fish were digested in $HClO_4$ and $HNO_3$, taken in 4:1 ratio, and used for the analysis of heavy metals[60,61]. The instrument was calibrated before analysis using standards. In addition to this, the metal pollution index (MPI) was determined as follows:

$$MPI = (Cm_1 \times Cm_2 \times \_\_\_Cm_n)^{1/n}$$

where $Cm_1$, $Cm_2$ ….. up to $Cm_n$ were the amounts for the metal '*n*' in the specimen[18].

Heavy metals (Cr, Mn, Fe, Ni, Cu, Zn, and Cd) in water and fish tissues were quantified in duplicates through atomic absorption spectrophotometer[26]. The physicochemical parameters like temperature, dissolved oxygen, pH, and electrical conductivity were found out on the spot by using digital meters.

### Enzyme assay

Fishes ($n = 10$) were anesthetized and blood samples drawn from the heart were allowed to stand for 20 min. Serum obtained after centrifugation at 4000 × *g* was utilized to estimate the action of ALP, AST, ALT, and CK using kits from RANDOX Limited and assayed by following producer's guidelines.

### Albumin and globulin in serum

Albumin concentration was measured via commercial kits from Siemens Limited and samples were read at 628 nm through a spectrophotometer (UV–VIS Systronics, 118). Total protein was estimated at 595 nm as per the modified method of Javed et al.[18]. The amount of globulin was determined by subtraction of albumin from total protein concentrations. Moreover, an albumin to globulin (A:G) ratio was also obtained.

### Non-specific immune parameters

*TLC and DLC.* TLC ($10^3$ mm$^{-3}$) was determined by neubauer hemocytometer and DLC via blood smear which was stained with giemsa. Lymphocytes, neutrophils, monocytes, eosinophils, and basophils were recorded in percentage.

*Respiratory burst and NOS.* The blood containing EDTA was used for the determination of nitroblue tetrazolium (NBT) as per the instructions and the extent of NBT reduced was determined at 540 nm[23]. Concisely, 0.2% NBT was blended with blood and incubated at 25 °C for 30 min then dimethylformamide was added. NOS was assayed using the method of Chakrabarti et al.[24] with slight modifications. Homogenate was made in phosphate buffer, centrifuged and finally, supernatant was collected and utilized for assay. This was followed by the addition of Griess reagent and incubation at room temperature for 10 min. The absorbance was noted down at 540 nm.

*Oxidative stress assays.* SOD was measured by autoxidation of pyrogallol[62]. To 100 µl of the sample, 2.80 ml of tris–succinate buffer (0.05 M, pH 8.2) was mixed followed by incubation at 25 °C for 20 min. To this mixture, 100 µl of 8 Mm pyrogallol was added and read at 412 nm. CAT activity was assayed by decomposition of $H_2O_2$[63]. Concisely, 100 µl of the sample was taken and 1.90 ml of potassium phosphate buffer (50 mM, pH 7.0) was added to it. 1 ml of hydrogen peroxide ($H_2O_2$) was added to the reaction mixture and absorbance was taken at 240 nm. LPO was assessed by the production of thiobarbituric acid reactive substances and calculated as malondialdehyde equivalents through the procedure of Buege and Aust[64]. GSH was determined by following the methodology of Jollow et al.[65] with some modifications. An equal volume of sulfosalicylic acid was put into the sample homogenate, incubated at 4 °C for 1 h, centrifuged at 12,000 × *g*. The supernatant was collected and 2.2 ml (0.1 M, pH 7.4) of potassium phosphate buffer was mixed, followed by the addition of 0.4 ml 5,5′-dithiobis-2-nitrobenzoic acid, and absorbance was read at 412 nm.

GST was determined according to the procedure of Habig et al.[66]. To a 100 µl of the sample, 2.7 ml of GSH was mixed and the reaction was started by adding 1 Mm 1-chloro-2,4-dinitrobenzene, and absorbance was read at 340 nm.

*Single cell gel electrophoresis*. For comet assay, alkaline conditions were maintained as per the procedure of Singh et al.[67]. Scoring was done by utilizing Komet 5.5, a kinetic imaging system, connected with Olympus fluorescent microscope (CX41). The tail-length of a comet (µm) was taken as index of the nuclear DNA damage.

### Quality assurance

All the reagents utilized in conducting the present study were of analytical grade. All the glassware that were used were rinsed twice, first in 10% $HNO_3$ and then with double-distilled water. For the precision and accuracy of the instruments, standard reference materials and blanks were used. For the atomic absorption spectrophotometer, the recoveries of metals were in the range of 99–101%. All the analysis was performed in duplicates.

### Statistical analysis

Data are given as mean ± SEM (standard error of the mean). Statistical analysis was done using *t*-test, ANOVA (two way), and Duncan's multiple range test through the SPSS software. Significance was established at $p < 0.05$.

### Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

## DATA AVAILABILITY

All data generated or analyzed during this study are included in this published article.

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## AUTHOR CONTRIBUTIONS

M.M., M.J., M.I.A., F.Z., and A.K.S. performed different sets of experiments. M.J., M.I.A., and S.S.A designed and conceptualized the work. M.J. and M.I.A. contributed equally to this work. All authors approved the final submitted manuscript.

## COMPETING INTERESTS

The authors declare no competing interests.

## ADDITIONAL INFORMATION

**Supplementary information** The online version contains supplementary material available at https://doi.org/10.1038/s41545-021-00107-4.

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