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Effect of untreated sewage water on antioxidant enzymes of fish *Labeo rohita*

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Abstract
The unregulated discharge of untreated municipal sewage water to the natural water bodies is a major threat to the aquatic ecosystems. In the present study, the fingerlings of *Labeo rohita* were exposed to treated sewage water and 1/10th of LC_{50} and 1/20th of LC_{50} of untreated sewage water (UT) obtained from sewage water treatment plant, Ludhiana, India. After determining 96 hr LC_{50} value of UT, fingerlings were divided into four groups: control, treated, 1/10th of LC_{50} and 1/20th of LC_{50} UT and exposed for the period of two months. The oxidative stress parameters viz. proteins, lipid peroxidation (LPO), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S- transferase (GST) were examined in the liver of fingerlings. Results of this study revealed a significant decrease ($p \leq 0.05$) in the levels of proteins and the activity of SOD and GPx in 1/10th LC_{50} and 1/20th LC_{50} UT groups, however, the activity of LPO, CAT, GR and GST was found to increase significantly ($p \leq 0.05$) in 1/10th LC_{50} and 1/20th LC_{50} UT groups in comparison to control and treated group. Hence, the study concluded that untreated sewage water has a potential to alter the activity of antioxidant enzymes in liver, however, clean and treated water can restore the activity of these enzymes in fish.

Keywords: Cytotoxicity, liver, *Labeo rohita*, oxidative stress, sewage water

Introduction
The modern methods of agriculture, urbanization and industrialization involve the increased release of various chemicals, biocides, pesticides in the aquatic resources [1]. The aquatic ecosystems are therefore, increasingly threatened due to unregulated discharge of untreated industrial, agricultural and municipal pollutants throughout the world [2]. Municipal and industrial wastewater toxicants, pose serious risk to aquatic fauna and are regarded to be cytotoxic, mutagenic and carcinogenic [3].

Although, the complex mixture of pollutants in aquatic ecosystems may exert severe damage to the aquatic biota [4] yet it is not financially or technically feasible to evaluate all the organisms in the entire ecosystem at all times. Fish can easily metabolize, concentrate and bioaccumulate water pollutants and hence, it provides an excellent source of material for the study of the pollution in water bodies [5]. Fishes are relatively sensitive to changes in their surrounding environment making them potential indicators of the status of a specific aquatic ecosystem. Early toxic effects of pollution may be evident at cellular or tissue level before significant changes can be observed in fish behaviour or external appearance [6]. Biological communities can integrate the effect of changes in chemical, physical and biological factors of environment and hence, are good indicators of ecosystem health [7].

Physiological state of an organism is a key factor in determining species sustainability, survival and availability because this factor is susceptible to the effects of pollutants at all stages of an organism life cycle [8]. Biochemical responses can be affected by environmental factors, such as water pollution, temperature, age, disease, nutritional status, and seasonal changes [9].

The organ most associated with the detoxification and biotransformation process is the liver and hence, it is one of the organs most affected by contaminants in water [10]. Doherty *et al.* [11] noted that fish species are suitable candidates for the assessment of biomarkers of oxidative stress induced by pollutants because they play a dual role of being on top of the aquatic chain as vertebrates and respond strongly to stress conditions.

Oxidative stress results from disruption of the pro-oxidant/antioxidant balance by reactive oxygen species (ROS) and other radicals or oxidants [12].

While xenobiotics are able to increase ROS levels, the capacity to induce oxidative stress depends on the overwhelming of antioxidant defences [13]. The antioxidant enzymatic system protects organisms from the toxic effects of the activated oxygen species and helps to maintain cellular homeostasis by removing ROS. Aerobic organisms have developed antioxidant defense mechanisms that scavenge ROS or prevent ROS-mediated cellular damage [14], including enzymes sensitive to free radical proliferation such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione S-transferase [15].

Various studies have been conducted to assess water quality of polluted water bodies [16] and morphological alterations in gills, flesh and liver of fish inhabiting polluted water bodies [17-19], however, little work has been done regarding the effect of sewage wastewater on oxidative stress parameters of liver of fish *Labeo rohita*.

Keeping in view the aforesaid, the present investigation was designed to evaluate oxidative stress parameters in liver of *Labeo rohita* fingerlings after rearing fingerlings in different concentrations of untreated sewage water which was collected from sewage water treatment plant, Bhattian, Ludhiana, Punjab, India.

Materials and Methods

Fingerling collection and acclimatization

Fingerlings of *Labeo rohita* (Hamilton 1822) (7.62 ±0.25 cm in length, 8.25±0.32 g in weight) were procured from Guru Angad Dev Veterinary and Animal Sciences University (GADVASU) Ludhiana, Punjab, India. The fingerlings were acclimatized to the laboratory conditions for 10 days in tanks (35 litres capacity) containing chlorine-free tap water with adequate values of temperature, conductivity, dissolved oxygen and biochemical oxygen demand. A normal photoperiod (12-h light: dark cycle) was maintained during acclimatization period and during experimentation. Fingerlings were fed *ad libitum* with commercial fish food throughout the acclimatization period. The feeding was suspended 1 day prior to the experiment. The tubs were continuously aerated with electrically operated aerators (2 aerators/35 lts. tub) and filters (2 filters/tub). The experimental protocol met the Organisation for Economic Co-operation and Development (OECD) guidelines (1992) [20].

Collection and analysis of municipal sewage water

Municipal sewage waste water was collected from Sewage Water Treatment (STP) plant located at village Bhattian district Ludhiana, Punjab, India. This collection site (30º57'57''N and 75º49'54''E) is within the municipal limits of Ludhiana located at the distance of 10.4 Kms from Punjab Agricultural University, Ludhiana, Punjab, India. In the present study, the fingerlings were reared in the water obtained from the plant using SBR technology with capacity of treating 50 MLD with 90% efficiency of effluent removal. The control, untreated and treated water was analyzed for analysis of physico-chemical parameters viz. pH, temperature, Biochemical oxygen demand (BOD), Dissolved oxygen (DO) and free carbon-dioxide (CO_2), using standardized methods given by American Public Health Association, American Water Works Association, and Water Environment (APHA) [21].

Acute toxicity test

Two batches, each of seven healthy fingerlings (n=7), were exposed to dechlorinated tap water (taken as control) and different concentrations (10%, 25%, 50%, 75% and 100%) of untreated sewage water for the period of 24, 48, 72 and 96 hours. Per cent mortality rate of the fingerlings was recorded after 24, 48, 72 and 96 hours of exposure in control as well as in exposed groups. None of the fingerling was found to dead in control and treated water, however, mortality was observed in the fingerlings exposed to different concentrations of untreated sewage water. The fish was considered dead when it did not respond to the probing with a glass rod. The dead specimens were removed from the tubs at the earliest after being noticed. POLO software [22] was used to calculate the lethal concentration (LC_{50}) value of untreated sewage water to *Labeo rohita* for 96 hrs at different concentrations of untreated sewage water.

Chronic Bioassay

The untreated sewage water was supposed to be toxic as depicted from the value of lethal concentration (LC_{50}) of untreated sewage water obtained from acute toxicity test. For the chronic experimentation, two concentrations of this LC_{50} i.e. $1/10^{th}$ LC_{50} and $1/20^{th}$ LC_{50} were taken. Four batches, each of nine healthy fingerlings (n=9), were divided into four groups: control group, treated group, $1/10^{th}$ LC_{50} group of untreated water (UT) and $1/20^{th}$ LC_{50} group of untreated water (UT). The fingerlings were exposed for the duration of two months and six fingerlings (n=6) from each group were dissected at an interval of 15, 30, 45 and 60 days.

Analysis of oxidative stress parameters

After the interval of every 15 days fingerlings from control, treated and $1/10^{th}$ LC_{50} and $1/20^{th}$ LC_{50} group were dissected and 0.5 g of liver was homogenized in 2ml of phosphate buffer (PBS: 0.1M, pH 7.4) and centrifuged at 3000 r.p.m for 10 mins. After centrifugation, the supernatant was taken and stored at a specific temperature (-20ºC) for conducting analysis of parameters viz. proteins, lipid peroxidation (LPO), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S-transferase (GST) using standardized methods given by Lowry *et al.* [23], Stocks and Dormandy [24], Marklund and Marklund [25], Aebi [26], Hafeman *et al.* [27], Carlberg and Mannervik [28] and Habig *et al.* [29], respectively.

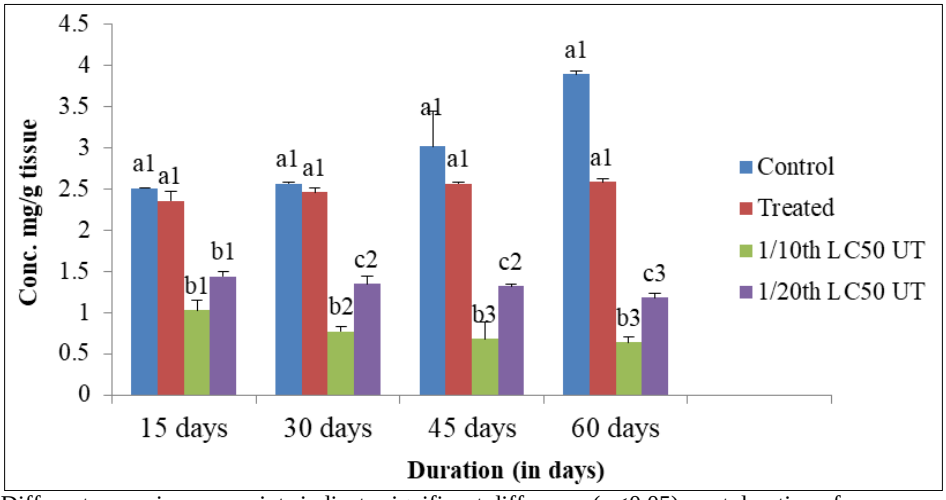
Statistical analysis

One-way and multifactor analysis of variance (ANOVA) were used to determine significant difference among different groups viz. control, treated, $1/10^{th}$ LC_{50} untreated and $1/20^{th}$ LC_{50} untreated group, using CPCS I software. Significant differences at $p<0.05$ were determined using Tukey's test as the post hoc analysis. Statistical analysis was done using Statistical Package for Social Sciences (SPSS) version 20.0 in consultation with the Department of Mathematics, Statistics and Physics, PAU, Ludhiana. Values were expressed as Mean±S.E.

Results

Proteins

The results of the present study revealed that the levels of protein decrease significantly ($p<0.05$) in $1/10^{th}$ LC_{50} and $1/20^{th}$ LC_{50} groups in comparison to control and treated groups (Fig. 1). Moreover, the levels of protein decrease significantly ($p<0.05$) in $1/10^{th}$ LC_{50} UT group in comparison to $1/20^{th}$ LC_{50} UT groups. It has been observed that the protein content decreases significantly ($p<0.05$) in $1/10^{th}$ LC_{50} UT and $1/20^{th}$ LC_{50} UT group with the increase in duration of exposure (Fig. 1).



Different numeric superscripts indicate significant difference ($p<0.05$) w.r.t duration of exposure
Different alphabetic superscripts indicate significant difference ($p<0.05$) among groups

Fig 1: Protein content (mg/g tissue) in liver of *Labeo rohita* fingerlings after rearing in control, treated and different concentrations of untreated water (UT)

Lipid peroxidation

The results of the present study revealed significant increase ($p<0.05$) in LPO in 1/10th LC_{50} UT and 1/20th LC_{50} UT groups in comparison to control and treated group (Table 1). Significant increase ($p<0.05$) was also observed in 1/10th LC_{50} UT group in comparison to 1/20th LC_{50} group. It has been observed that with the increase in duration of exposure the levels of LPO increase significantly ($p<0.05$) in 1/10th LC_{50} UT and 1/20th LC_{50} UT groups (Table 1).

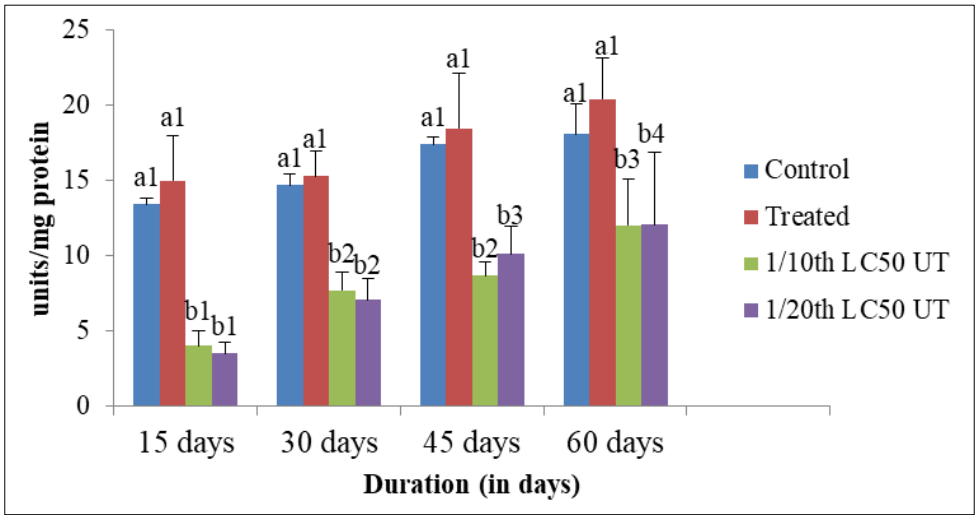
Table 1: Lipid peroxidation (LPO) level in *Labeo rohita* fingerlings after rearing in control, treated and different concentrations of untreated water (UT)

Groups Duration	Control	Treated	1/10th LC_{50} UT	1/20th LC_{50} UT
15 days	1.44±0.30^{a1}	1.70±0.65^{a1}	6.78±0.13^{b1}	4.65±0.05^{c1}
30 days	1.78±0.57^{a1}	1.96±0.47^{a1}	7.61±0.05^{b1}	5.60±0.51^{c1}
45 days	2.49±0.29^{a1}	2.69±0.57^{a1}	9.01±0.21^{b2}	7.03±0.45^{c1}
60 days	3.00±0.30^{a1}	3.26±0.41^{a1}	11.33±0.50^{b23}	10.27±0.07^{c23}

Values are Mean±S.E
Values with different numeric superscript (1-3) in columns differ significantly ($p<0.05$)
Values with different alphabetic superscript (a-c) in rows differ significantly ($p<0.05$)
Units: nM MDA/100 mg tissue

Superoxide-dismutase (SOD)

In the present study, significant decrease ($p<0.05$) was observed in SOD in 1/10th LC_{50} UT and 1/20th LC_{50} UT group in comparison to control and treated groups (Fig. 2). Non-significant increase ($p<0.05$) was observed in 1/10th LC_{50} UT group in comparison to 1/20th LC_{50} group. It has been observed that with the increase in duration of exposure the levels of SOD increase significantly ($p<0.05$) in 1/10th LC_{50} UT and 1/20th LC_{50} UT groups (Fig. 2).



Different numeric superscripts indicate significant difference ($p<0.05$) w.r.t duration of exposure
Different alphabetic superscripts indicate significant difference ($p<0.05$) among groups

Fig 2: The specific activity of superoxide-dismutase (units/mg protein) in *Labeo rohita* fingerlings after rearing in control, treated and different concentrations of UT

Catalase (CAT)

The results of the present study depicted significant increase ($p<0.05$) in CAT in 1/10th LC_{50} UT and 1/20th LC_{50} UT groups in comparison to control and treated group (Table 2). Non-significant increase ($p>0.05$) was observed after 15 days of exposure in 1/10th LC_{50} UT group in comparison to 1/20th LC_{50} group, however, significant increase ($p<0.05$) was observed after 30 days of exposure in 1/10th LC_{50} UT group in comparison to 1/20th LC_{50} group. It has been observed that with the increase in duration of exposure the levels of CAT increase significantly ($p<0.05$) in 1/10th LC_{50} UT and 1/20th LC_{50} UT groups (Table 2).

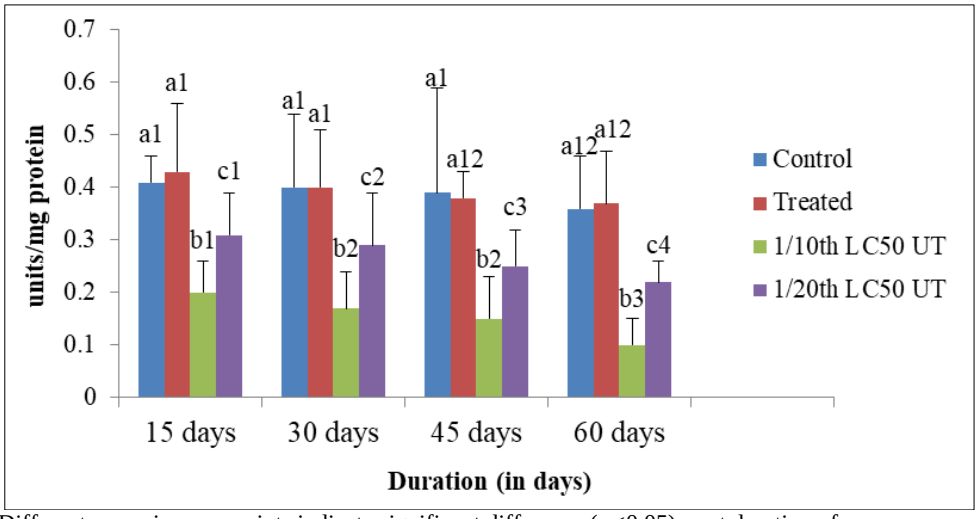
Table 2: The specific activity of catalase (CAT) in *Labeo rohita* fingerlings after rearing in control, treated and different concentrations of untreated water (UT)

Groups Duration	Control	Treated	1/10th LC_{50} UT	1/20th LC_{50} UT
15 days	12.62 ± 0.46^{a1}	11.85 ± 0.64^{a1}	19.90 ± 0.46^{b1}	17.62 ± 0.64^{b1}
30 days	15.81 ± 0.42^{a1}	14.81 ± 0.62^{a1}	26.72 ± 0.69^{b2}	21.66 ± 0.57^{c2}
45 days	16.68 ± 0.02^{a12}	16.80 ± 0.43^{a12}	29.70 ± 0.82^{b3}	23.02 ± 0.88^{c3}
60 days	17.30 ± 0.64^{a12}	18.25 ± 0.90^{a12}	32.25 ± 0.19^{b4}	26.65 ± 0.78^{c4}

Values are Mean±S.E
Values with different numeric superscript (1-4) in columns differ significantly ($p<0.05$)
Values with different alphabetic superscript (a-c) in rows differ significantly ($p<0.05$)
Units: µ moles H_2O_2 decomposed/min/mg protein

Glutathione-peroxidase (GPx)

In the present study, significant decrease ($p<0.05$) was observed in the activity of GPx in 1/10th LC_{50} UT and 1/20th LC_{50} UT groups in comparison to control and treated group (Fig. 3). Significant decrease ($p<0.05$) was also observed in 1/10th LC_{50} UT group in comparison to 1/20th LC_{50} group. It has been observed that with the increase in duration of exposure the activity of GPx decrease significantly ($p<0.05$) in 1/10th LC_{50} UT and 1/20th LC_{50} UT groups for 60 days (Fig. 3).



Different numeric superscripts indicate significant difference ($p<0.05$) w.r.t duration of exposure
Different alphabetic superscripts indicate significant difference ($p<0.05$) among groups

Fig 3: The specific activity of glutathione peroxidase (GPx) (units/mg protein) in *Labeo rohita* fingerlings after rearing in control, treated and different concentrations of UT

Glutathione reductase (GR)

It has been examined in the present study that the activity of GR indicated significant increase ($p<0.05$) in 1/10th LC_{50} UT and 1/20th LC_{50} UT groups in comparison to control and treated group (Table 3). Significant increase ($p<0.05$) was also observed in 1/10th LC_{50} UT group in comparison to 1/20th LC_{50} group. The results of the present study indicate that with the increase in duration of exposure the activity of GPx increase non- significantly ($p>0.05$) in 1/10th LC_{50} UT and 1/20th LC_{50} UT group (Table 3).

Table 3: The specific activity of glutathione reductase (GR) in *Labeo rohita* fingerlings after rearing in control, treated and different concentrations of untreated water (UT)

Groups Duration	Control	Treated	1/10th LC_{50} UT	1/20th LC_{50} UT
15 days	0.22 ± 0.05^{a1}	0.23 ± 0.02^{a1}	0.45 ± 0.03^{b1}	0.43 ± 0.09^{b1}
30 days	0.25 ± 0.04^{a1}	0.26 ± 0.04^{a1}	0.49 ± 0.16^{b1}	0.45 ± 0.01^{b1}
45 days	0.29 ± 0.06^{a1}	0.29 ± 0.02^{a1}	0.51 ± 0.03^{b1}	0.48 ± 0.09^{c1}
60 days	0.32 ± 0.03^{a1}	0.31 ± 0.02^{a1}	0.53 ± 0.11^{b1}	0.49 ± 0.19^{c1}

Values are Mean±S.E
Values with same numeric superscript (1) in columns differ non-significantly ($p>0.05$)
Values with different alphabetic superscript (a-c) in rows differ significantly ($p<0.05$)
Units: µ moles NADPH oxidized/min./mg protein

Glutathione-S-transferase (GST)

In the present study, the activity of GST indicated significant increase ($p<0.05$) in 1/10th LC_{50} UT and 1/20th LC_{50} UT group in comparison to control and treated groups (Table 4). Non-significant increase ($p<0.05$) was also observed in 1/10th LC_{50} UT group in comparison to 1/20th LC_{50} group for 45 days of exposure, however, after 60 days of exposure, the activity of GST indicated significant increase in 1/10th LC_{50} UT group in comparison to 1/20th LC_{50} UT group. The results of the present study indicate that with the increase in duration of exposure the activity of GST increase non-significantly ($p>0.05$) in 1/10th LC_{50} UT and 1/20th LC_{50} UT group for 45 days of exposure, however, significant increase ($p<0.05$) was observed in 1/10th LC_{50} UT and 1/20th LC_{50} UT group after 60 days of exposure (Table 4).

Table 4: The specific activity of glutathione-S-transferase (GST) in *Labeo rohita* fingerlings after rearing in control, treated and different concentrations of untreated water (UT)

Groups Duration	Control	Treated	1/10th LC_{50} UT	1/20th LC_{50} UT
15 days	0.08±0.03^{a1}	0.07±0.02^{a1}	0.11±0.01^{b1}	0.10±0.04^{b1}
30 days	0.10±0.02^{a1}	0.08±0.05^{a1}	0.13±0.01^{b1}	0.11±0.05^{b1}
45 days	0.12±0.03^{a2}	0.11±0.01^{a2}	0.18±0.05^{b2}	0.15±0.07^{b2}
60 days	0.14±0.01^{a23}	0.13±0.21^{a2}	0.46±0.19^{b3}	0.36±0.28^{c3}

Values are Mean±S.E

Values with different numeric superscript (1-3) in columns differ significantly ($p<0.05$)

Values with different alphabetic superscript (a-c) in rows differ significantly ($p<0.05$)

Units: µ moles GSH-CDNB conjugate formed/min./mg protein

Discussion

Exposure to different concentrations of untreated sewage water has been found to induce significant alterations in the levels of protein and oxidative stress parameters of fingerlings.

Likewise the results of the present study, the significant decrease was observed in the protein content of liver of the freshwater fish *Tor putitora* collected from the polluted portion of River Kabul, Pakistan [30]. Deficiency of proteins in the vital organs of the body may cause several clinical and sub clinical syndromes viz. impaired health, decreased resistance to infection and vulnerability to diseases [31]. Proteins are the major constituents of the cell architecture, moreover, they are the primary source of nitrogenous metabolism and acts as reservoirs of energy during the period of chronic stress [32].

Oxidative stress enzymes are considered as biomarkers of oxidative stress in marine or freshwater organisms and their induction indicates a specific response to the pollutants [33-35]. In the present study, elevated level of LPO in fingerlings indicates that cellular damage might have occurred as a result of exposure to untreated sewage water. The present decrease in the activity of SOD indicates inefficiency of liver to generate defence against superoxide radicals which may results in cellular injury [36]. Superoxide dismutase (SOD) plays an important role in catalysing the conversion of superoxide radical into either molecular oxygen or hydrogen peroxide (H_2O_2) and hence, protects the cell from oxygen toxicity which could be caused by superoxide radical [36].

Also, in the present study, an increase in the activity of catalase in the liver cells of 1/10th LC_{50} and 1/20th LC_{50} group indicated the stimulation of defensive mechanism in fingerlings to protect cells against H_2O_2 production. The study conducted by Carvalho *et al.* [37] also showed a decrease in the activity of SOD in liver and white muscle cells and an increase in the activity of CAT in gill cells of fish *Oreochromis niloticus* which was collected from the polluted sites of Monjolinho river, Brazil. Catalase (CAT) is required for catalysing the conversion of H_2O_2 into water and oxygen and increase in the activity of catalase is an indicative of high levels of H_2O_2 concentration in cell [37].

The observed decline in the activity of GPx could be because of the presence of certain molecules in wastewater which have the potential to bind with the cysteine (SH groups) at the active sites of enzyme molecules and hence, causing increase in the production of ROS which suppress the activity of GPx. Glutathione (GSH) is an antioxidative enzyme consisting of single cysteine residue and the activity of GSH is dependent on the levels of cysteine [38]. Glutathione peroxidase (GPx), Glutathione reductase (GR) and Glutathine-S-transferase (GST) are GSH dependent enzymes and any change in concentration of GSH may also decrease or increase the activity of GPx, GR and GST [4]. Al-Ghais *et al.* [39] observed increase in the activity of GSH in the liver and muscle cells of freshwater fish *Tilapia mossambica* after rearing fish in untreated sewage water obtained from sewage water treatment plant, Ras Al Khaimah, United Arab Emerates. Furthermore, glutathione reductase (GR) is required for catalysing the reduction of GSSG to GSH, therefore, in order to maintain sufficient levels of GSH in cells, the activity of GR is required to be normal. Moreover, GR acts as a substrate for GPx and GST which are required for the detoxification of peroxides and removal of xenobiotics from an organism, respectively [4].

In the present study, the enhanced activity of GST could be related to increased synthesis and secretion of GST proteins in liver cells. This enzyme plays an important role in preventing oxidative damage to the cells by catalysing the conversion of breakdown products of lipid peroxides to GSH enzymes which are harmless and hydrophilic molecules [40-42]. Moreover, this metabolic pathway of GST also protects nucleophilic groups in macromolecules such as nucleic acids and proteins. In a study conducted by Lopez-Lopez *et al.* [4], the activity of various antioxidative stress parameters viz. SOD, CAT, GPx, LPO and Na+/K+-ATPase was observed in the liver cells of the fish *Goodea atripinnis* after exposure to highly polluted water of Lake Yuriria and the activity of CAT and LPO levels showed significant increase while GPx and SOD activities decreased significantly. Na^+/K^+-ATPase activity varied according to the month of the year. Hence, exposure of fish to all water samples collected from different locations of Lake Yuriria exerted alterations in hepatic LPO levels, oxidative stress parameters and Na^+/K^+-ATPase activity which could substantially impair the mechanisms of fish defences against oxidative stress (Lopez-Lopez *et al.* [4]. These findings are correlated with the findings of the present study.

The observed alterations in the antioxidant profile of liver cells of fingerlings may indicate potential health hazards

induced by the wastewater in the aquatic organisms. Moreover, it is also possible that the toxicants or pollutants present in the water bodies may not only pose threat to the inhabiting organisms but also affect the people who are consuming fish by direct catch from these water bodies and utilizing this polluted water for agriculture or other domestic activities [43].

Conclusion

Keeping in view the findings of the present study, it can be interpreted that untreated sewage water at the level of $1/10^{th}$ LC_{50} and $1/20^{th}$ LC_{50} has a potential to induce cellular toxicity in liver by causing alterations in antioxidant profile of liver of fish *Labeo rohita*. Hence, liver cells of fish could be utilized for biomonitoring program in potentially polluted water bodies to assess the health of aquatic ecosystem. These findings also emphasizes the treatment of untreated sewage wastewater via sewage water treatment plants before its discharge into water bodies to protect the health of aquatic fauna. Since, no significant alterations were observed in the fingerlings reared in treated sewage water obtained from sewage water treatment plant, hence, treated sewage water could be utilized for aquaculture.

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Conflict of Interest

The authors declare no conflict of interest regarding the publication of this paper.

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