

Assessment of Hepatic Damage in *Labeo rohita* (Hamilton, 1822) Exposed to Azo dye C. I. Direct Green 6

Jagruti Barot^{1,*}, Anita Bahadur²

Abstract

Textile industries are one of the major backbones of Indian economy. They are the major reason for contamination of water bodies and their toxicity to ecosystem is an alarming environmental issue. The present study was designed to evaluate the hepatotoxic effects of C.I. Direct Green 6 (DG 6), a commercially used textile azo dye on *Labeo rohita* fingerlings. For this, *L. rohita* fingerlings were exposed to three sublethal concentrations (2.5, 5.0 and 10.0 mg/l) of DG 6 for 15 days. After 15 days of exposure period, liver tissues were dissected out and processed further for histopathological and biochemical observations. For biochemical study, liver alanine amino transferase (ALAT; E.C.2.6.1.2) and aspartate amino transferase (AST; E.C.2.6.1.1) were assayed. Histopathological result showed mild changes such as disposition of hepatic cords, hemorrhages in sinusoids with focal necrosis, depletion of cytoplasmic material from the hepatocytes, karyorrhexis etc. A marked decrease was noticed in ALAT and AST activity in the liver. Present findings revealed that DG 6 exerts toxic effects even in sublethal concentrations. Hence, histopathological and biochemical parameters of liver proved to be sensitive biomarkers to evaluate the effect of any xenobiotics such as textile or other industrial pollutants, on aquatic lives.

Keywords: azo dye toxicity, hepatic histopathology, *Labeo rohita* fingerlings, alanine amino transferase (ALAT; E.C.2.6.1.2), aspartate amino transferase (AST; E.C.2.6.1.1)

INTRODUCTION

Colours are a pleasure of eyes and an unavoidable thing to mankind. Many types of dyes are synthesized and used in textile, cosmetic, food stuff, plastic, paper and pulp and in paint industries. Indian dyes and pigment industry is a growing sector with approximately 370, 000 metric tons of dye production in 2019, and holding the third position in Asia [1]. The synthetic azo dyes (-N=N-) have a great demand (60–70%) in Indian industries owing the properties such as wide ranges of colours, good fastness and ease of application. Mostly all azo dyes are non-biodegradable and are toxic, carcinogenic and/mutagenic to aquatic organisms and human too [2–4]. However, indiscriminate use and discharge of these synthetic azo dyes cause pollution in the aquatic ecosystem and poses a high risk to aquatic biota [5, 6]. Direct Green 6 (DG 6) is one of the highly demanding azo dyes.

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Fish being at the highest trophic level of aquatic food chain is maximally afflicted with azo dyes. Fish population is the reflection of water quality of a particular aquatic ecosystem, and is proved to be a sensitive marker to elucidate the toxic effects of various pollutants [7]. As a food, fish is one of the most important routes by which human beings are exposed to toxic substances via food chain. Hence, an Indian major carp, *Labeo rohita* (Hamilton, 1822)—edible freshwater fish, available throughout the year—was considered for the present study [8].

Liver is the most important and largest glandular organ in fish body and is the chief target of various kinds of xenobiotics [9]. When toxics hit on the liver, they may lead to histological alterations and/or disturbed biochemical functions. Alanine aminotransferase (ALAT) and aspartate aminotransferase (AST) are liver-specific enzymes that are more sensitive measure of hepatotoxicity and histopathological changes; they can be assessed within a short time [10–12]. Thus, hepatotoxic changes are also good indicators of fish health as well as quality of aquatic ecosystem [13–15].

To support the environmental risk assessment and monitoring policies, the present work was designed to elucidate the hepatotoxic effects of C. I. direct Green 6, azo dye on *L. rohita* fingerlings using histopathological and biochemical biomarkers.

MATERIALS AND METHODS

Test Sample and Preparation of Dye Solution

The Direct Green 6 [$C_{34}H_{22}N_8Na_2O_{10}S_2$] dye was obtained and was directly used for the experimental work. Stock solutions of dyes were prepared using distilled water (50 mg/ml) and were stored at 4 °C. Required dilutions of dyes were then prepared by adding stock solution to a measured quantity of water in experimental aquaria [16].

Test Animal and Acclimatization

L. rohita is an Indian major carp and an edible freshwater fish. It has economic importance also. *L. rohita* fingerlings were procured from the Government Fish Farm, Valod, Gujarat, (21.04 °N 73.24 °E), India. They were disinfected with 0.1% $KMnO_4$, and acclimatized to laboratory conditions in dechlorinated tap water at room temperature 25 ± 3 °C for 15 days in 100 l PVC-lined glass aquaria with continuous aeration. The physicochemical parameters of water such as temperature, pH, dissolved oxygen, alkalinity, hardness, etc. were measured and maintained during acclimatization and throughout the experimental period [16] (Table 1).

Fingerlings were fed with standard fish meal, readily available in the market at the rate of 5% of their body weight, once a day. Water along with the nitrogenous excreta was changed after every 48 h. After acclimatization, the healthy fingerlings were selected for exposures.

Table 1. Physicochemical parameters of water and methods used for their estimation.

No.	Physicochemical parameters of water	Method	Range (unit)
1.	pH	By using digital pH meter	7.3 ± 0.3
2.	Temperature	By using thermometer	25.0 ± 3.0 (°C)
3.	Dissolved oxygen (DO)	Winkler's iodometric method	6.8 ± 0.2 (mg/l)
4.	Total hardness (TH)	Complexometric titration	125.0 ± 0.5 (mg/l)
5.	Total alkalinity (TA)	Titrimetry method	130.2 ± 0.4 (mg/l)

(All the values shown here are mean $\pm$ SE)

Sublethal Concentrations of DG 6 and Exposure Details

Mortality tests were carried out for DG 6 following the standard method [17]. Based on the acute LC_{50} values of DG 6 (33.84 mg/l), three different sublethal concentrations (2.5, 5, 10 mg/l) were selected for the experimental work [18]. About 90 healthy fingerlings were categorized into three groups to expose them to above mentioned sublethal concentrations (10 in each concentration and in triplicates) for 15 days. More 10 fingerlings were kept into dye-free dechlorinated tap water as a control group for the same period of time. All these exposed fingerlings were fed daily. They were starved for 24 h prior their sacrifice.

Histopathological Method

Microtomy was performed to study the histopathological details of the target organ—liver. For that, after 15 days of exposure time, the liver were dissected out from all the exposed as well as control

groups and further processed for the microtomy and biochemical assays. The liver were fixed in 10% formaline, dehydrated and embedded in paraffin wax, sectioned (5 µm) and stained with H & E method [19]. All the histopathological observations were made using Carl Zeiss Trinocular microscope, Axio scope A1. Microphotographs were taken and documentation was done. These changes were compared with those of the control group.

Tissue Homogenate Preparation and Enzyme Assays

Collected liver tissues were used to determine enzyme activity. Liver of the individuals from each group were homogenized separately in chilled 0.25M sucrose solution (8 ml/g of wet tissue) in Teflon-coated tissue homogenizer and centrifuged at 15000 g at 4 °C for 20 min. Enzyme activities were measured from supernatants. Alanine aminotransferase (ALAT; E.C.2.6.1.2) was analyzed with 53.68 g of L-alanine and 0.7734 g a-oxoglutarate, 1.2 U/ml LDH and 0.17 g/l NADH in Tris buffer (pH 7.5) and estimated at 340 nm for 3 min. Aspartate aminotransferase (AST; E.C.2.6.1.1) was assayed using 41.62 g aspartic acid, 0.7734 g a-oxoglutarate, 0.42 U/ml MDH and 0.17 g/l NADH in Tris buffer (pH 7.5) and estimated at 340 nm for 3 min [20]. ALAT and AST activities were expressed as:

ALAT activity (IU) = $\Delta A / \text{min.} \times 1015$

and

AST activity (IU) = $\Delta A / \text{min.} \times 1015$

All colorimetric assays were carried out using UV-VIS spectrophotometer (Schimadzu, UV-1800).

Statistical Analysis

All enzyme assays were carried out in triplicates. The data were analyzed by using Student's *t*' test and the significance of the data was approved at $p < 0.05$ level.

RESULTS

No mortality was observed throughout the exposure period in all the exposed groups of fingerlings including the control one. It showed that three selected sublethal concentrations were sublethal though create hepatic damages in the DG 6 exposed fingerlings.

Histotoxic Changes

Liver is the largest glandular organ in the fish body. It is the chief target of all kinds of xenobiotics. A typical arrangement of hepatocytes, central veins, sinusoids etc. were observed in liver sections from fingerlings of control group. DG 6 exposed fingerlings liver developed some changes which were dose and time dependent. In 2.5 mg/l concentration of DG 6, liver showed mild changes such as disposition of hepatic cords and rupture of blood capillaries in sinusoids with focal necrosis after 15 days of exposure. Vacuolated cytoplasm in cells, haemorrhage and cytolysis accompanied by dilation of sinusoid, karyorrhexis and complete disintegration of nucleus were seen at some places when fingerlings were exposed to moderate (5.0 mg/l) concentration of DG 6 after 15 days of exposure. When fingerlings from the higher (10.0 mg/l) concentration were sacrificed, liver showed drastic disorganization such as dilated and deformed central vein, necrosis, severe haemorrhage, karyolysis and karyohexis were seen after the experimental period. The changes seen in liver histology are compiled in Figure 1.

Toxic Effects on Aminotransferases Activity

The liver showed noticeable decrease in ALAT level in all concentrations of DG 6 as compared to control that is, 200.2 IU. ALAT activity showed declined trend as the concentration of dye increased. At 2.5 mg/l concentration of DG 6, the liver ALAT decreased by 32.4 IU after 15 days of exposure. Whereas, liver ALAT activity showed progressive decline upto 163.8 IU and 150.8 IU when exposed to 5.0 mg/l and 10.0 mg/l, respectively after 15 days of experimental period.

The liver showed decreased level of AST activity in fingerlings exposed to all the concentrations of DG 6. Similar to the ALAT activity, AST activity also showed decreasing trend with the increased

concentration. At 2.5 mg/l concentration of DG 6, the liver AST decreased upto 209.3 IU after 15 days of exposure as compared to controlled one that is, 250 IU. Whereas, liver AST activity showed significant progressive decline upto 200.3 IU and 199.1 IU when exposed to 5.0 mg/l and 10.0 mg/l, respectively after 15 days of experimental period. All these enzymatic changes are represented in Figure 2.

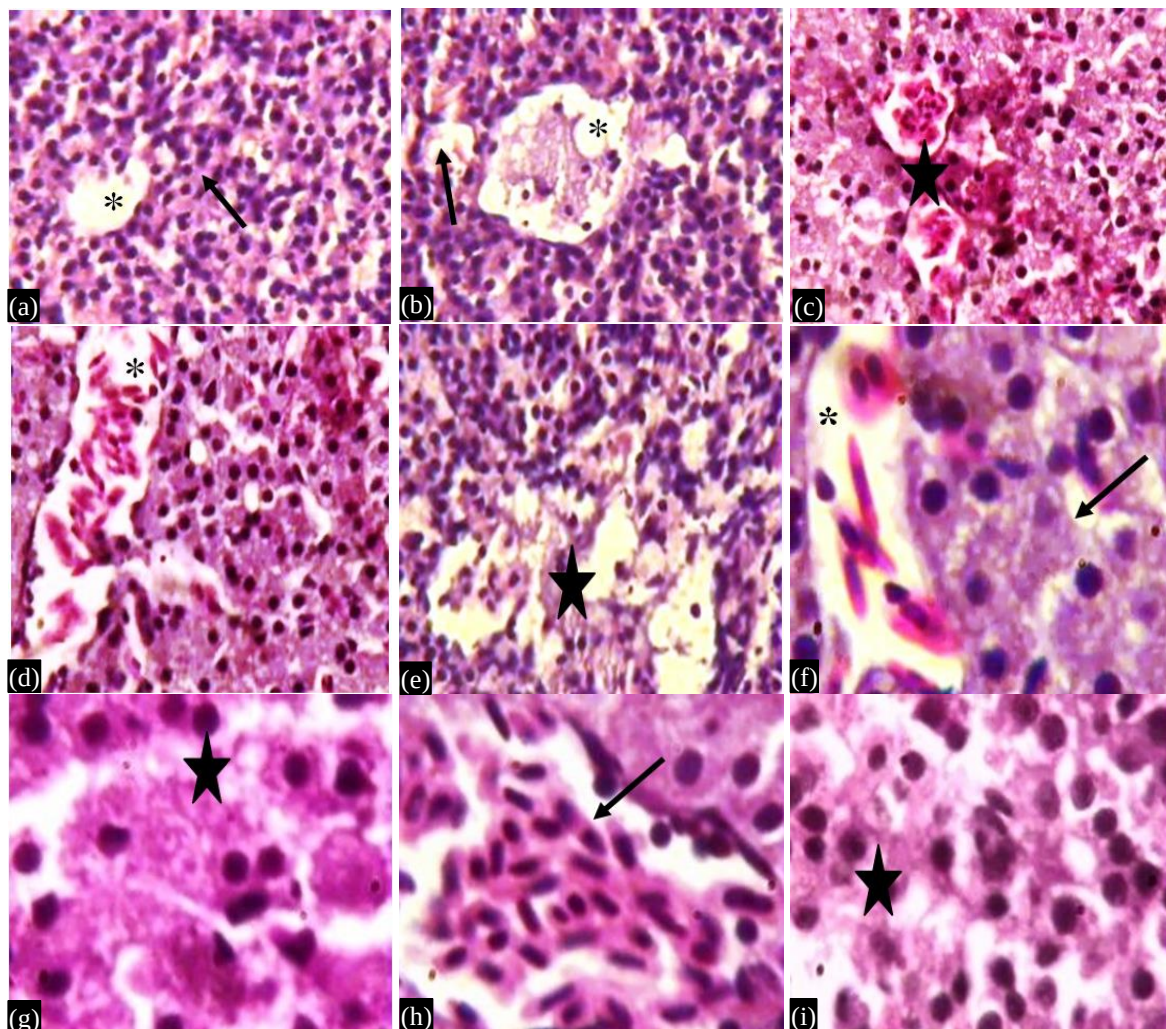


Figure 1. Various histological changes in the liver of all DG 6 exposed fingerlings as compared to control. **(A)** Normal histology of liver from the control group of fingerlings; well-arranged hepatocytes and sinusoids (arrow) with well-defined central canal (*) [H & E; 450X]. **(B, C)** Liver sections from fingerlings exposed to 2.5 mg/l of DG 6 showing damaged central canal (*); disoriented sinusoid (arrow); two hemorrhagic spots (Star) [H & E; 450X]. **(D-F)** Liver sections from fingerlings exposed to 5.0 mg/l of DG 6 showing dilated sinusoids with hemorrhage (*); severely damaged central canal (star); necrosis (arrow) (H & E; 450X; 1000X). **(G-I)** Liver sections from fingerlings exposed to 10.0 mg/l of DG 6 showing severe necrotic effect including karyolysis and karyorrhexis (Star); severe hemorrhage in the hepatic tissue (arrow) [H&E; 1000X].

DISCUSSION

The present work suggested that the selected concentrations of DG 6 were sublethal as no mortality was recorded throughout the experimental period. It was found that the liver of fish had a limited ability to repair the damages occurred due to the DG 6 exposure. But, as all the histological changes were dose dependent, they lead to disturbed metabolic/enzymatic activities of the liver. Our previous study also supported the present findings when *L. rohita* fingerlings were exposed to azo dye Acid Orange 7 [21]. A recent study also documented extensive histopathological lesions in *L. rohita* fingerlings when exposed to Malachite Green dye [22].

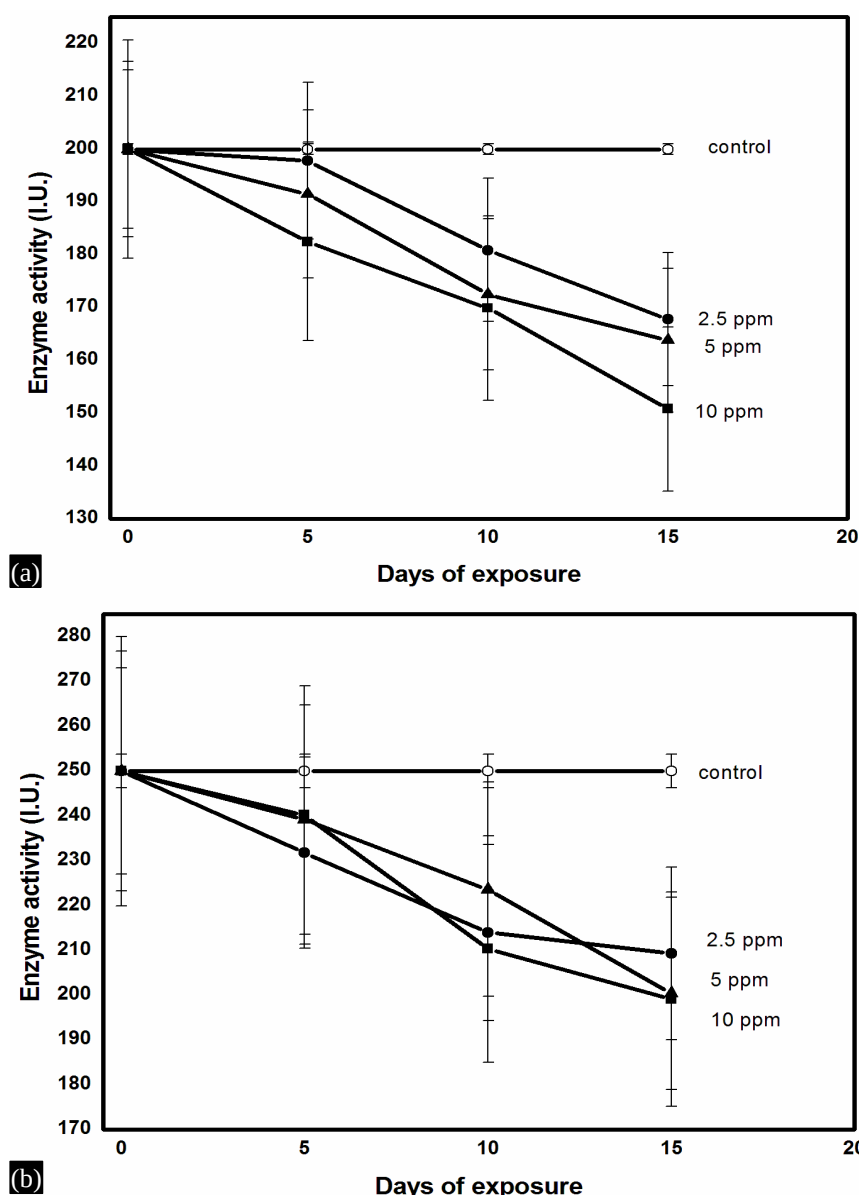


Figure 2. Effect of three sublethal concentrations of DG 6 on liver enzymes of *Labeo rohita* fingerlings **(A)** decreased ALAT activity at 15 days of exposure; and **(B)** decreased AST activity at 15 days of exposure [Results are expressed as mean $\pm$ SE].

Malachite Green dye and Red 195 dye also induced similar changes such as vacuolation, pyknotic nuclei, focal necrosis and sinusoidal congestion in Nile tilapia *Oreochromis niloticus* [23, 24]. Various environmental stressors were also capable of inducing such types of histological alterations in fishes [25]. Many pesticides have been reported to trigger analogous characteristic nuclear alterations, necrosis, erythrocytic congestion in Indian major carp *Catla catla*, *O. niloticus* and *Clarius gariepinus* [26–28]. Moreover, *Oreochromis mossambicus* and *Onchorynchus mykiss* livers showed above mentioned histological changes induced by silica and cerium dioxide nanoparticles [29, 30].

Liver is considered as an important organ for many metabolic processes. Exposure to xenobiotics leads to their accumulation in liver and causes pathological alterations. This results in the release of tissue-specific enzymes. The high level of serum aminotransferases indicates inflammation or death of hepatocytes and thus acts as sensitive marker [31]. Decreased activities of both transaminases indicated lesser transamination processes. A declined transamination might affect amino acid input in the

tricarboxylic acid (TCA) cycle and might lead to energy crisis [32]. With increasing concentration of DG 6, AST and ALAT activities fell significantly below the control levels, probably related to cytolysis and enzyme leakage into the blood. Hepatocellular changes and necrosis noticed in the histopathological sections were in conformity with the present biochemical results [33].

Furthermore, our previous study also supported the present findings when *L. rohita* fingerlings were exposed to azo dye Acid Orange 7 [34]. This declined liver AST and ALAT activities were in agreement with the findings of other workers. Acid black azo dye also has toxic potentials to damage the liver of *L. rohita* and showed declined activities of liver-specific enzymes [35]. On the other hand, heavy metals such as arsenic and chromium greatly increased serum ALAT and AST in *L. rohita* as compared to those of the control group [36]. Zebrafish liver showed gradually decreased aminotransferases level on exposure of acetaminophen [37]. Even seasonal variations also induced such changes in liver enzyme activities of *Tilapia zillii* and *Mugil capito* collected from the Qarun Lake, Egypt [38]. Similar changes were reported in *Channa punctatus* following exposure to azo dye Bismark brown and Monocrotophos [39, 40].

CONCLUSION

The present study concluded that the exposure of azo dye affects the structural, physiological and biochemical status of the target organ (liver) of the exposed fingerlings as seen by the various histological changes and significantly decreased levels of ALAT and AST activity. Hence, DG 6 is a toxic azo dye and is able to disturb the aquatic flora and fauna even at low or sublethal concentrations. However, the present study suggested that industries can minimize the use of such dyes as well as an efficient effluent treatment methodology should be implicated to conserve the aquatic ecosystem, food chain and food web of the nature.

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