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Research Article

Evaluation of Acute Toxicity (LC_{50}) and Biochemical Response of *Labeo rohita* (Hamilton 1822) to Crystal Violet Dye Exposure

Archana B. Dhandale[1,2,*], Sachinkumar. R. Patil[1], Surat. A. Manjare[1,*]

[1]Department of Zoology, Jaysingpur College Jaysingpur. Maharashtra, INDIA.
[2]Department of Zoology, Shivaji University, Kolhapur, Maharashtra INDIA.

ABSTRACT

Background: Crystal Violet (CV) dye has been widely used in dye processing industries and also used to provide a deep violet colour to paints and printing ink. It is a topical antimicrobial agent that inhibits cell replication, it can be toxic if swallowed and used in various staining procedures. CV has been reported as a recalcitrant dye molecule, a mitotic poison that persists in environment for a long period and pose toxic effects in environment especially aquatic biome. Fishes are best indicator of water quality and are used for ornamental and food purpose. In the present study, the freshwater fish, *Labeo rohita* fingerlings, were exposed to CV dye and Biochemical changes were recorded (protein, glycogen and lipid). **Materials and Methods:** In the present study Lethal Concentration (LC_{50}) of CV on *Labeo rohita* at 96 hr was determined by using SPSS. Biochemical analysis of tissues like liver, gill, intestine and kidney was done through protein estimation by Lowry's method, glycogen estimation by DeZwaan and Zandee method and lipid estimation by Barnes and Blackstock method. **Results:** The fingerlings of *Labeo rohita* weighing 7±0.4 g shows abnormal behaviour like hyper activity, excess mucus secretion, try to jump outside the tub, changes in swimming pattern in acute exposure, while in chronic exposure fishes become lethargic, swimming at corner and body becomes pale in colour**.** The total biochemical content of protein, glycogen and lipid was decreased after the exposure of acute and chronic level at significance level (p<0.01). Chronic exposure shows more decrement of biochemical composition as compare to acute (p<0.01). The observations show that CV gets easily adsorbed in fish tissue (liver, gill, kidney, intestine) from surrounding thereby reducing the metabolism of fish. **Conclusion:** This study shows that CV alters the total biochemical composition of fish. It also alters the behavioural response after the exposure of CV.

Keywords: Crystal violet, Toxicity, Biochemical changes, Acute, Chronic, *Labeo rohita*.

Correspondence:
Dr. Surat A. Manjare
Research Guide, Department of Zoology, Jaysingpur College, Jaysingpur-416101, Maharashtra, INDIA.
Email: drsuratmanjare@gmail.com

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INTRODUCTION

Exposure of laboratorial and industrial dyes causes severe health related issues in exposed organisms. Large sources of dyes are textile industries, pharmaceutical industries, tanneries etc. They are directly disposed into natural water bodies without any treatment causing serious water pollution.[1,2] Benzidine, naphthalene, and other aromatic compounds are derivatives of dyes which are highly mutagenic and carcinogenic.[3] Mostly azo dyes are toxic, non-degradable, mutagenic and carcinogenic to humans and aquatic ecosystem. Azo dye contains about 70% synthetic dyes which are used in textile and dyeing industries.[4] Around 10% to 15% Dyes are released into aquatic environment during process of dyeing, which make effluent highly colored and unpleasant aesthetically.[5] Due to environmental persistence and ability to accumulation by aquatic organism textile azo dyes are appears to be hazardous pollutant for aquatic environment.[6] Entry of dyes in water stream has serious environmental impact because it lowers the DO of water, light penetration and increases the COD level of water which affects the aquatic fauna and flora.[7] Major source of water pollution is effluents released by dye processing plants and dyes which are used for colouring processes enters the aquatic environment.[8] Pollution due to dye is common problem to human health and aquatic organism since mostly they are highly toxic, allergic, mutagenic, carcinogenic and quickly accumulate in living cell and harmful to entire food chain.[9-12] The Crystal violet is cationic dye having a mixture of methyl pararosaniline dyes (Azo Dyes) and used to colour paper as a component of navy blue and black inks for printing, ball-point pens, and inkjet printers. It is also used in fertilizers, antifreezes, detergents, and leather. The dye has a bright and high

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colour intensity, even at very low concentrations. The textile dyes are more often found as major water pollutants. The disposal of CV into the hydrosphere can lead to environmental degradation. Therefore, the waste water which contains dyestuff CV, needs to be treated before it is discharged into water bodies. Fish is the best model to study these contaminants which causes carcinogenicity or mutagenicity and alterations in cell.[13] When fish is exposed to any toxic pollutant, the major organs which show effect in detoxification mechanism are gills, liver and kidney.[14] Fish organs like kidneys and liver having a role in detoxification of body fluids and metabolism are essential in determining the health status of fish in the freshwater ecosystem. The biomarkers study of such vital organs has led to wondrous environment risk assessments.[15]

The freshwater fish *Labeo rohita* is native to Indian subcontinent.[16] This species also found in number of countries like Pakistan, Bangladesh and Myanmar.[17] Fish is an excellent model to understand the dye toxicity of oxidative stress in aquatic ecosystems.

MATERIALS AND METHODS

Experimental fish collection

Freshwater fish *Labeo rohita* fingerlings weighing about 8±.05 g and 6 to 7 cm in length were collected from Government fish farm Centre, Chandoli dam, District- Sangli. The *Labeo rohita* fingerlings were Acclimatized in laboratory conditions for 15 days. During acclimatization fishes were disinfected with 0.5% $KMnO_4$ solution. During acclimatization water was changed daily from aquarium. For acute toxicity, fishes were starved about 24 hr. Healthy fingerlings were selected and introduced into water container containing 20 L of water. Healthy fingerlings were selected weighing about 8±2 g and length about 9±4 cm. Water quality parameter were checked during experiment followed by[18] APHA as follows; temperature, 25±2°C, pH 8.3±2, total hardness 122 mg/L, alkalinity118 mg/L and dissolved oxygen 7.4±0.2 mg/L.

Test chemical

Crystal violet was purchased from Qualigens, Mumbai (C.I. 42555, Sp. Absorptivity maximum at 589-594 nm, Transition pH 0.1-2.0, Molecular weight- 407.98). Test solution was prepared by powder dissolved in distilled water as per the toxicity.

Test solution and exposure

10 fingerlings introduced into each container having 20 L of water containing control, 0.1, 0.2, 0.3, 0.4 and 0.5 ppm dye concentrations for determination of LC_{50} value. The LC_{50} value of Crystal Violet Dye was 0.3 mg/L (0.3 ppm) up to 96 hr of exposure. Probit analysis was carried out by SPSS 16.0.

Chronic exposure

Fingerlings were randomly divided into 3 groups, Group I: Control, Group II: $1/10^{th}$ exposure of LC_{50}, Group III: $1/20^{th}$ exposure of LC_{50}. In each group 10 fingerlings were introduced for exposure of 30 days. While fish were feed once a day at the rate of 2% body weight during chronic exposure of toxicant. Behavioural changes was observed during experimentation as compared with control.

Biochemical analysis

Estimation of total protein

The total protein present in selected tissue (liver, gill, kidney and intestine) was estimated by Lowry's method.[19] 100 mg tissue was homogenised into TCA sample. Sample was centrifuged at 3000 rpm around 15 min. Supernatant was discarded and adding 5 mL of D.W. in pellet. 0.1 mL sample was taken from each tissue sample and 0.9 mL D.W was added into it. By adding Lowry's, A and B sample was kept under dark for half an hour until blue colour was developed. Standard was prepared by using BSA and control was prepared by using D.W. Optical density was measured at 660 nm on visible spectrophotometer.

Estimation of total glycogen

Glycogen estimation was carried out by DeZwaan and Zandee method[20] (1972). The selected tissues viz. liver, gill, kidney and intestine were removed from fish. 100 mg tissue was homogenised into 1 mL of 30% KOH. Keeping it in boiling water bath for 3-5 min to dissolve the tissue. Adding 2 mL of 96% of ethyl alcohol after the cooling. The samples were kept in refrigerator overnight. Sample was centrifuges at 3000 rpm for 15 min. Again, sample was kept at water bath at 70°C for 5 min. 0.1 mL sample was taken into sample and adding 0.9 mL D.W. into it to make volume 1 mL. Adding Anthrone reagent into it keeping in boiling water bath for 10 min. After cooling absorption was checked at 660 nm on visible spectrophotometer.

Estimation of total lipid

Total lipid estimation was carried by Barnes and Blackstock (1973) method.[21] 100 mg selected tissue was homogenised into folsch mixture. Samples were incubated at 40°C in water bath for 2 hr. Add 1 mL conc. H_2SO_4 and heated into boiling water bath for 10 min. After cooling add 2 mL of vanillin reagent. The optical density was measured at 530 nm on visible spectrophotometer.

Statistical analysis

Probit analysis was carried out by SPSS 16. Statistical analysis was carried out by using one-way ANOVA.

RESULTS

Physicochemical parameter

Water quality parameter were checked during experiment followed by APHA as follows; temperature, 25±2°C, pH 8.3±2, total hardness 122 mg/L, alkalinity 118 mg/L and dissolved oxygen 7.4±0.2 mg/L.

Behavioural changes

Table 5 shows the behavioural changes in Labeo rohita fingerlings in control (without treatment) and treated groups.

Probit analysis

It was carried out by SPSS- 16.0 (Figure 1). The LC_{50} value of Crystal violet was 0.3 ppm. (Table 1).

Protein estimation

Protein estimation was carried by Lowry's *et al.*, (1951) method. Concentration of protein was changed as duration and concentration of exposure. Gill, liver, kidney and intestine were exposed for protein concentration. The maximum decrease of protein was seen in liver and gill (Table 2). The values of protein concentration were decreased due to increasing exposure period. The protein concentration in liver at acute exposure was 121±0.22, in gill was 74.7±1.47, in intestine was 12.8±0.51 and in kidney was 15.7±0.40. While at chronic exposure ($1/10^{th}$) in liver was 42.6±0.76, in gill was 74.7±1.47, in intestine was 5.71±0.24 and in kidney was 6.9±0.16. At $1/20^{th}$ chronic exposure protein concentration in liver was 81.3±0.37, in gill was 28.95±1.87, in intestine was 11.95±2.02 and in kidney was 12.8±0.51 (Table 2). The order of protein concentration in acute exposure (0.3 ppm) was liver>gill>kidney>intestine. The order of protein concentration is similar in chronic exposure but concentration is different ($1/10^{th}$ and $1/20^{th}$) as compare to control and acute toxicity test (96 hr). Maximum decrease was observed in gill and liver and minimum in kidney and intestine (Figure 2).

Glycogen Estimation

Glycogen concentration in all tissues of fish were decreased as compare to control (liver, gill, intestine, kidney). Liver shows maximum effect of CV, there is decreasing trend of liver concentration from control to acute and chronic exposures. Kidney and intestine shows less alteration from control to treated (Table 3). Gill also shows decreasing trend of glycogen. The decreasing order of glycogen is liver>gill>intestine>kidney. Liver and gill shows maximum alteration in glycogen estimation (Figure 3). There is a slight alteration in decreasing trend of intestine and kidney into sublethal and chronic effect, these may be due to the kidney may have mechanism that reduce the accumulation of toxicant or detoxify the dye before affecting glycogen metabolism, thus impact on glycogen level might be less. Similarly, intestine has a role in digestion and absorption of nutrient, not in glycogen metabolism or storage.

Total lipid

A decrease in total lipid content during the study in liver, gill, kidney and intestine. Maximum lipid concentration seen in liver and minimum is in kidney of control set. All tissues show greater fluctuations in total lipid concentration in exposed group. The bellow Table 4. Shows Greater fluctuations in acute and chronic exposure as compare to control. The decreased total lipid content of tissues in affected *Labeo rohita* was found to be in the order of liver>gill>intestine>kidney (Figure 4).

DISCUSSION

Behavioural changes

Behavioural changes in fishes such as boldness, action, movement and their interaction were linked together in conditions with important suggestion for fitness and evolutionary development.[22,23] Exposed fish shows erratic swimming, loss of equilibrium, secretion of excessive mucus and become hypoactive, change in body pigmentation and submerged into bottom while change in concentration and duration as compare to control.[24] Same studies were observed by as gulping air, excess

Table 1: Acute toxicity (LC_{50}) of Crystal Violet to the freshwater fish *Labeo rohita*.

Conc. in ppm	No. of fishes	Mortality No.				% Mortality
		24 hr	48 hr	72 hr	96 hr	
Control	10	0	0	0	0	0
0.1	10	0	0	0	0	0
0.2	10	0	0	1	1	20
0.3	10	1	1	1	2	50
0.4	10	1	1	2	2	60
0.5	10	2	2	2	1	70
0.6	10	2	2	2	2	80
0.7	10	3	2	2	2	90

Table 2: Effect of Crystal Violet on Protein estimation at acute exposure are as follows (µg/100 mg tissue).

Organ	Control	LC_0	LC_{50}	$1/10^{th}$	$1/20^{th}$
Liver	145.7±1.41	129±0.45	121±0.22	42.6±0.76	81.3±0.37
Gill	96.5±0.41	82.6±0.69	74.7±1.47	8.05±1.06	28.95±1.87
Intestine	25.8±0.33	22.6±0.57	12.8±0.51	5.71±0.24	11.95±2.02
Kidney	29.35±0.63	25.3±0.33	15.7±0.40	6.9±0.16	12.8±0.51

Table 3: Effect of Crystal Violet on total Glycogen content at acute exposure (µg/100 mg tissue).

Tissue	Control	Subacute (96 hr)	Acute (96 hr)	Chronic (30 days)	
				$1/10^{th}$	$1/20^{th}$
Gill	53.85±0.38	25.3±0.25	14.2±0.71	20.8±0.36	21.4±0.1
Liver	75.25±0	54.1±0.45	27.1±0.55	24.7±0.12	23.8±0.26
Intestine	28.34±0.94	21.4±30	3.65±0.48	18.5±0.76	17.3±0.3
Kidney	8.05±0.16	5.65±0.21	6.15±0.66	11.6±0.16	15.3±0.31

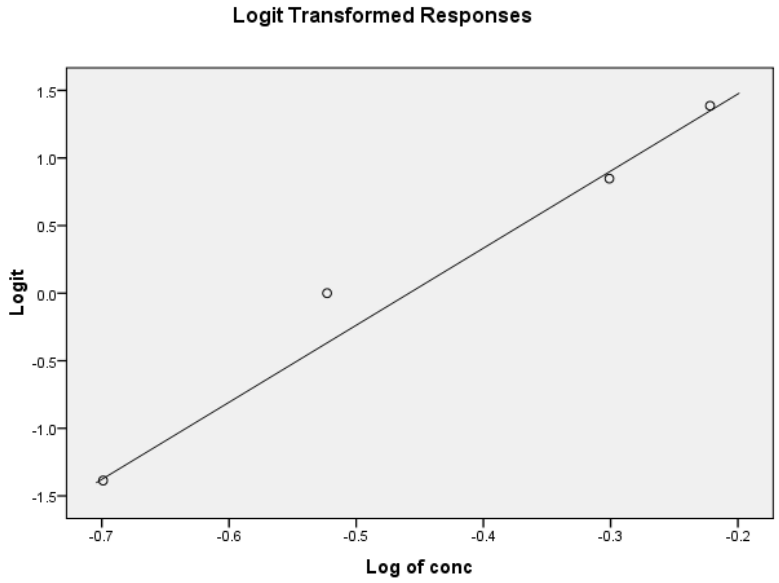

Figure 1: Probit analysis of *Labeo rohita* exposed to Crystal violet dye.

Probit analysis It was carried out by SPSS- 16.0. The LC_{50} value of Crystal violet was 0.3 ppm.

mucus secretion on body and abnormal swimming patterns in fish *Clarias batrachus* after the exposure of copper sulphate.[25]

Protein

Protein are involved in physiological events and hence the estimation of protein content can be considered as main diagnostic method to determine physiological condition of organism. Decrease in protein content under stress of toxins was observed by different coworkers.[26-31] Protein is an important biochemical parameter, which has been used to understand the general state of health and biological mechanism and metabolism under the stress of toxicants.[32] The Protein concentration showed decreasing trend on exposure to Crystal violet dye at sub lethal and acute exposure as compare to control. The decrement of total protein is may be due to abnormalities in protein metabolism. The decrease in protein content may be due to its stress induced accumulation for fulfilling the increased energy requirement by organism to survive adverse environmental condition.[33] In *Labeo rohita* and *Oreochromis niloticus* Protein level decreased in muscle after the exposure of organophosphate.[34,35] Decreased protein content has been observed in the muscle, intestine and brain of the fish *Catla catla* due to mercuric chloride toxicity.[36] Exposure of Cypermethrin on freshwater fish *Cyprinus carpio* at sublethal concentration shows remarked changes in protein concentration of Liver, Muscle and brain tissue.[37] Proteolysis was enhancing the role of proteins in the energy production during Cadmium exposure.[38] Effluents at Sublethal and lethal concentration alters the biochemical composition (glycogen, protein and lipid) of various organs of fish due to utilization

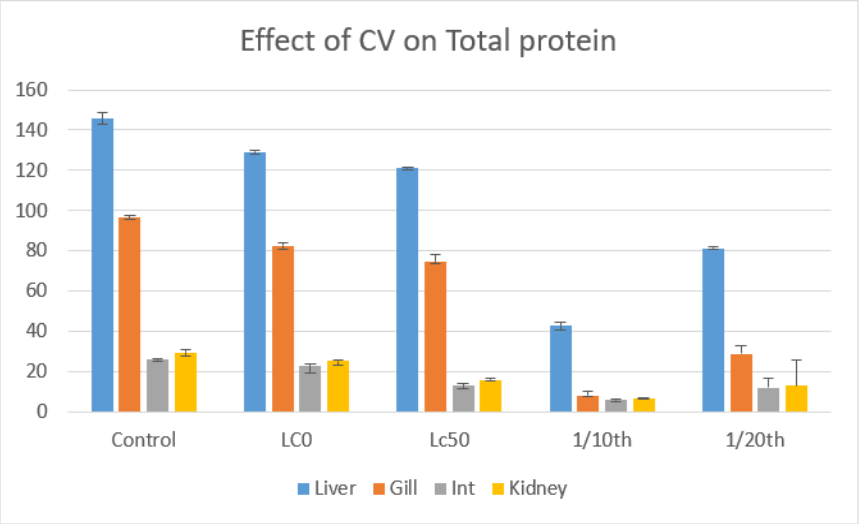

Figure 2: Crystal violet induced changes in total protein in control, Acute and chronic exposure of tissues like gill, liver, intestine and kidney of *Labeo rohita* in µg/100 mg tissue. The values are Mean±S.D. of n=5 (p<0.01).

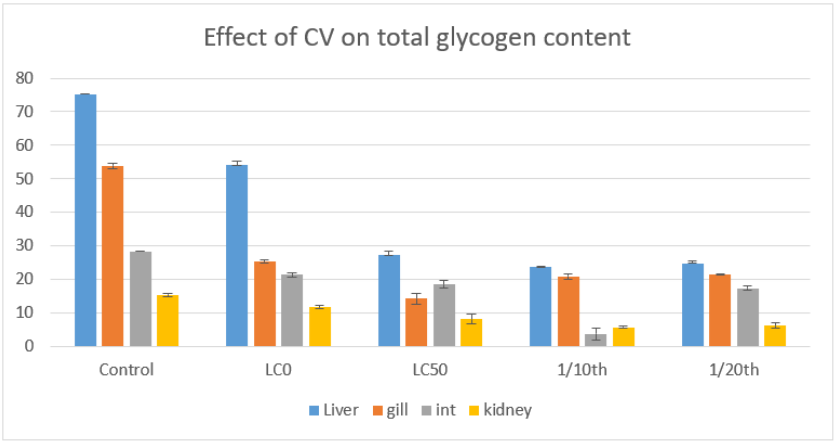

Figure 3: The given table shows values of total glycogen in control, Acute and chronic exposure of tissues like gill, liver, intestine and kidney of *Labeo rohita* in µg/100 mg tissue. The values are Mean±S.D. of n=5 (p<0.01).

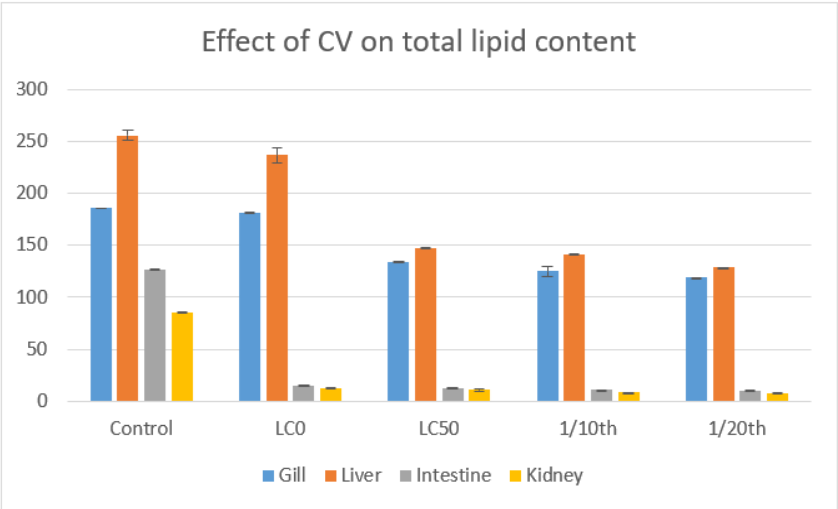

Figure 4: The total lipid content in control, Acute and chronic exposure of tissues like gill, liver, intestine and kidney of *Labeo rohita* in µg/100 mg tissue. The values are Mean±S.D. of n=5 (p<0.01).

Table 4: Effect of Crystal Violet on total Glycogen content at acute exposure (µg/100 mg tissue).

Tissue	Control	subacute	Acute	Chronic	
				1/10th	1/20th
Gill	185.51±17.48	181.69±0.1	133.86±0.10	124.86±2.02	118.94±0.9
Liver	255.16±2.16	236.55±3.24	147.22±0.07	141.51±0.07	128.32±0.05
Intestine	126.57±3.55	14.39±0.11	12.92±0.10	10.73±0.21	10.39±0.071
Kidney	84.96±0.25	12.46±0.20	10.73±0.48	8.37±0.09	7.75±0.18

Table 5: Behavioural changes.

Control	Treated
Normal secretion of mucus	High secretion of mucus
Increase in weight during chronic test	Decrease in weight during chronic exposure
Normal swimming pattern	Abnormal Swimming pattern, Loss of balance
Opercular movement normal	High opercular movement
Interested in food	No interest in food
Live in water container	Escaping outside the water container
Escaping tendency fast	Less escaping tendency
Normal colour pattern	Discoloration of skin
Co-ordinated movement	Uncoordinated movement
Normal activity	Hyperactivity during 24 hr exposure
No mortality	Increase in mortality with increase in dose

of biochemical energy to neutralize the toxic stress due to the heavy metals present in effluents.[39] Proteins serves as energy stores and nutrient in all biological processes but in severe stress condition they provide energy in metabolic and biochemical reactions according to the need of organism.[40] Proteins are the most important energy source to consume during chronic period of stress. Decrease in plasma protein in test fishes was due to necrosis or liver cirrhosis or changes in enzyme involved in the biosynthesis of protein.[41] Animal exposed to toxicant even at sub lethal concentration experiences huge stress at the metabolic level during the period of detoxification of toxicants.[42] The decrease in protein concentration in *Clarias gariepinus* and *Oreochromis niloticus* exposed to textile industry waste water,[43] is coincide with *Oncorhynchus mykiss* treated with malachite green. The decreased Protein concentration was an indication of increased proteolysis,[44] results into shift in metabolism of nitrogen,[45] decreased ribosomal activity,[46] results in protein degradation. The depletion of protein concentration of fish tissues on exposure to sublethal concentration of pesticides may be due to use of protein as a main energy source during long term pesticide exposure.[47]

Glycogen

Liver act as largest reservoir for glycogen and it is major source of glycogen synthesis followed by muscles.[48] Glycogen in liver mainly act as storage and export of hexose unit for regulation of blood sugar level.[49] Glycogen forms great energy source for animals. Chlorpyrifos exposure has adverse effect on carbohydrate metabolism, due to these decrease in glycogen level in all tissues due to toxicant induced hypoxia in *Labeo rohita*.[50] The disruption in glycogen reflects on enzyme pathway of glycolysis or hexose monophosphate pathway in rat[51] and may be the inhibition of hormone which were responsible for glycogen synthesis.[52] Stress due to chemical toxicant induces neuroendocrine disturbances hence disruption in carbohydrate metabolism.[53] Decreased glycogen level in gill, liver, muscle suggest excessive utilization of carbohydrate to withstand chlorpyifos induced toxicosis in small fishes.[54] The decrease in glycogen concentration in freshwater fish *Labeo rohita* was may be due to its increased utilisation as a quick source to meet energy demand under metallic stress and also due to the impact of anoxic or hypoxic conditions which normally increases glycogen utilization.[55] More energy is required for cope up stressful condition, these energies may obtain from carbohydrates, proteins and lipids.[56]

Lipid

Decrease in lipid concentration observed in the pollution affected Anabas testudineus from Buckingham canal attributed to its utilisation in cell organelle and cell repair.[57,58] Chronic exposure of textile effluent decreases food consumption, results in protein, lipid and carbohydrate content and overall lowers the growth rate.[59] Under acute exposure of MG shows significant alterations ($p<0.05$) in haematology, protein, lipid and glucose and also in CAT, GST, LPO activities of tissues.[60] The decrement in the total lipid of liver with the increase in the concentration of pesticides PRO may be due to the usage of energy storage to attain more energy demands for the detoxification process and also to balance the alteration in normal metabolism.[61] The decrement in lipid content in tissues indicated that the lipid has been channelized to attain the metabolic requirement for extra energy needed to overcome the toxic stress.[62]

CONCLUSION

The present study revealed that CV dye shows negative impact on biochemical composition and behavioural response of freshwater fish *Labeo rohita*.

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ABBREVIATIONS

ANOVA: Analysis of Variance; **CV:** Crystal Violet; **Ppm:** Parts per million; **SPSS:** Statistical Package for Social Sciences; **LC_{50}:** Lethal concentration 50.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

Under the toxicological assessment, present study investigates the acute toxicity (LC_{50}) and biochemical effect of crystal violet dye on the freshwater fish *Labeo rohita*. The median Lethal Concentration (LC_{50}) was determined through a 96 hr exposure (0.3 ppm). The results revealed acute toxic effect with alterations in behaviour, total protein, total glycogen, and total lipid content in fish tissues (liver, gill, kidney and intestine) as compare to control. Results demonstrates a concentration dependent decline in biochemical disturbances and stress induced response in exposed fish. These findings highlight the environmental risks posed by CV dye to aquatic organisms and emphasize the need for regulations and treatment for industrial dye effluent before releasing the aquatic ecosystem.

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