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Chronic toxicity of hexavalent chromium affects the morphology and behaviour of *Ctenopharyngodon idellus* (Cuvier and Valenciennes)

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Abstract
Chromium is one of the most common elements in the earth's crust and is used in more than 50 different industries. The discharge from these industries pollute the waters and affects the biota. In the present study, the 96 h LC_{50} of Cr (VI) was determined for *Ctenopharyngodon idellus* and was 53.08 mg/L. chronic toxicity of Cr on the morphology and behaviour of fish was assessed on exposing the fish to sublethal concentrations for 45 days. Acetylcholinesterase activity was also examined. Cr toxicity altered the morphology of the fish with cryptic colouration, loss of scales and a thick coating of mucous on the body. Fish displayed mild hyperactivity, loss of balance, erratic swimming and lethargy; followed by a decrease in fin and opercular movements. The activity of acetylcholinesterase also witnessed modification, thus confirming the behavioural changes. It can be concluded that chromium is toxic to *C. idellus* even at sublethal levels and affects its normal survival in the polluted waters.

Keywords: Chromium, *ctenopharyngodon idellus*, lc_{50}, acetylcholinesterase, behaviour, morphology

1. Introduction
Chromium exists in several oxidation states [1], out of which elemental, trivalent and hexavalent states are stable. Hexavalent chromium is used in a variety industries, ranging from electroplating, dyeing, metal refining, fungicide, wood preservatives, ceramic glass, pigment production and leather tanneries [2, 3]. The improper disposal of effluents from these industries has resulted in its undue presence in the air, water and soil, resulting in environmental pollution [4, 5]. The permissible concentration imposed by EPA on hexavalent chromium is 100 µg/L [6], but the underground water has depicted a surge in the chromium levels to more than 12 mg/L [7, 8]. The hexavalent state is carcinogenic [9] and produces oxidative stress on entering the cell [4, 8].

Fish being at a higher trophic level in the food chain serves as a suitable bioindicator of environmental pollution [10, 41]. The sensitivity of experimental organisms regulates the lethal and sub-lethal concentration of the metal [11]. Chromium enters the body of fish either by ingestion of food or water or with the ion-exchange of dissolved metals through lipophilic membranes [12]. There is limited information on the neurotoxicity of Cr, although its toxicity on the brain of *Drosophila melanogaster* has been established [13], but is not known in fish.

Acute toxicity assessment determines the adverse effects of a toxicant that might occur with accidental or short-term exposure [37] which helps in dosage selection for long term studies [38]. Identification of biological alterations related to cholinergic systems during metal exposure provide insight into the neurochemical and molecular targets involved in neurotoxicity promoted by heavy metals [39]. *Ctenopharyngodon idellus* is an exotic fish, cultured in integrated fish farming. It tolerates a wide range of physicochemical characteristics of water.

In the present study, acute toxicity of Cr (VI) on *C. idellus* was determined and using sublethal concentrations, chronic toxic effects were studied on the morphology and behaviour of the fish.

2. Materials and Methods
2.1 Procurement and acclimatization of fish
Healthy fingerlings of *Ctenopharyngodon idellus* (12 gm±5; 11 cm±2) were procured from a local fish farm (Karnal, Haryana) and were acclimatized for 15 days in a glass aquarium (145

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x 43 x 32 cm) fitted with aerators and filters containing dechlorinated water at 23-25ºC under natural photoperiod. Fish were fed with commercial feed pellets (Tetra Bits Complete, Germany) containing 47.5 % crude protein twice daily. Feeding of the fish was suspended 24 h prior to the experiments. Necessary permissions were sought and guidelines prescribed by the Animal Ethics Committee, Panjab University, Chandigarh (PU/IAEC/S/14/144) were =followed.

2.2 Chemicals
ACS reagent of potassium dichromate ($K_2Cr_2O_7$) was purchased from Merck. Other chemicals used were obtained from Hi-media.

2.3 Physicochemical characteristics of test water
The physicochemical characteristics of water were tested using the standard methods [6]. In the experimental water, the different physicochemical characters were: temperature 23±1˚C, pH 7.2±0.1, D.O. 8±1 mg/L, hardness 92.65±0.5 as $CaCO_3$ mg/L.

2.4 Toxicity tests
2.4.1 Acute toxicity
96 h LC_{50} of Cr (VI) for *C. idellus* was determined using semi-static acute toxicity tests, starting with the range finding tests to acute toxicity trials [6, 14]. Feeding to the fish was stopped 24 h before the experiment and were not fed during the acute toxicity tests, and the experiments were performed in triplicates. The preliminary test concentrations ranged from 42.41-70.69 mg/L. Based on the range, acute toxicity tests were conducted in a basic experimental setup of 'Syntax' plastic tanks of 60L capacity containing 40L of the test solution and 10 fish in each test concentration, with a control group maintained in dechlorinated tap water. Test water was changed daily to maintain the toxicant concentration. Mortality was recorded at 24 h interval and dead fish were immediately removed to avoid contamination. From these values, the threshold effect concentration was also determined.

2.4.2 Chronic toxicity
5.3 mg/L (one-tenth) and 10.63 mg/L (one-fifth) of 96 h LC_{50} were chosen as sublethal concentrations of Cr (VI) for chronic toxicity bioassay. The concentrations chosen in the present study correspond to the elevated and environmentally relevant level of Cr present in the water bodies [7, 15]. The experiment was performed in triplicate for 45 days and sampling was done on 15th, 30th and 45th day of the exposure period. The fish were evaluated for behavioural and morphological alterations every day for the entire length of the exposure duration.

2.4.2.1 Behaviour studies
The frequency of caudal and dorsal fin and opercular movements were observed on 15th, 30th and 45th day of the experiment. For this, all fish in the different groups were monitored for 30 min each and the frequency of the movements was calculated.

2.4.2.2 Acetylcholinesterase activity
For determining the activity of acetylcholinesterase in the serum of fish, blood was collected from caudal peduncle from benzocaine anaesthetized fish after 15th, 30th and 45th day. The serum was immediately processed for the AChE activity [16]. Serum was added to phosphate buffer and incubated at 37 ºC for 10 min followed by the addition of DTNB (Ellman's reagent). The reaction was initiated with the addition of acetylcholine iodide and absorbance was measured for 2 min at 412 nm using LABINDIA® UV 3000+ Analytical UV/VIS spectrophotometer. The concentration of protein was determined by a commercial diagnostic kit (Reckon Diagnostic Private Limited, Gujarat, India).

2.5 Statistical analysis
The data was analysed statistically using IBM SPSS version 21. Kolmogorov-Smirnov test was applied for determining the normal distribution of data. As the data was found to follow a normal distribution, the significance of difference among the different groups was tested using one-way ANOVA followed by Tukey's multiple comparison tests. Data are presented as mean ± SE. The values were considered significant at $p < 0.05$.

3. Results
3.1 Acute toxicity
96 h-LC_{50} of Cr (VI) to *Ctenopharyngodon idellus* was determined by Probit analysis [36] and was 53.08 mg/L (added as 151.65 mg/L of potassium dichromate) (Table 1, Fig 1). The fish in the lower concentrations (42.41 and 45.95 mg/L) displayed loss of equilibrium and convulsions after 24-48 h of exposure. The higher concentrations were able to induce rapid opercular movements and hyperactivity. In the highest concentration experimental set up, the fish reacted with erratic swimming and made air gulping actions, with more surfacing behaviour.

Table 1: Mortality of *C. idellus* exposed to different concentrations of Cr (VI) for 96 h and empirical Probit values

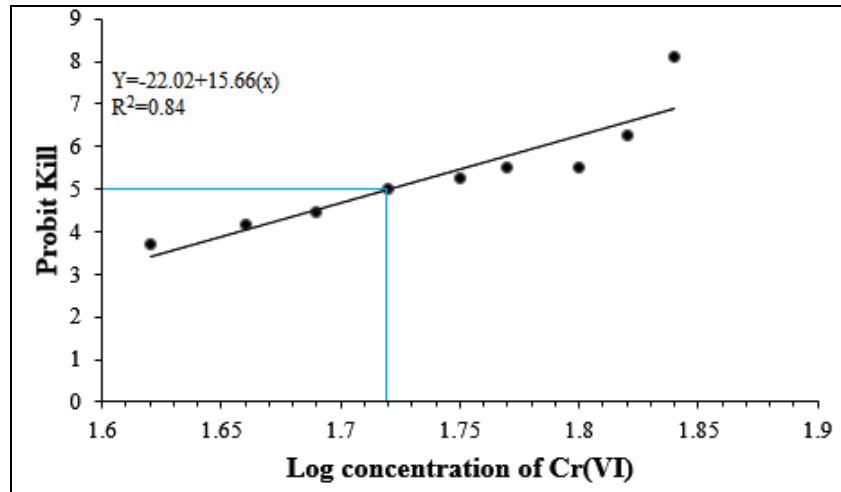| Cr(VI) concentration (mg/L)	Total number of fish	Number of dead fish	Mortality (%)	Log concentration	Empirical Probit value
42.41	10	1.00	10	1.62	3.72
45.95	10	2.00	20	1.66	4.16
49.48	10	3.00	30	1.69	4.48
53.02	10	5.00	50	1.72	5
56.55	10	6.00	60	1.75	5.25
60.09	10	7.00	70	1.77	5.52
63.62	10	7.00	70	1.80	5.52
67.16	10	9.00	90	1.82	6.28
70.69	10	10.00	100	1.84	8.09

Fig 1: Empirical Probit values of the death of *C. idellus* against log concentrations of Cr (VI).

3.2 Chronic toxicity
3.2.1 Morphology
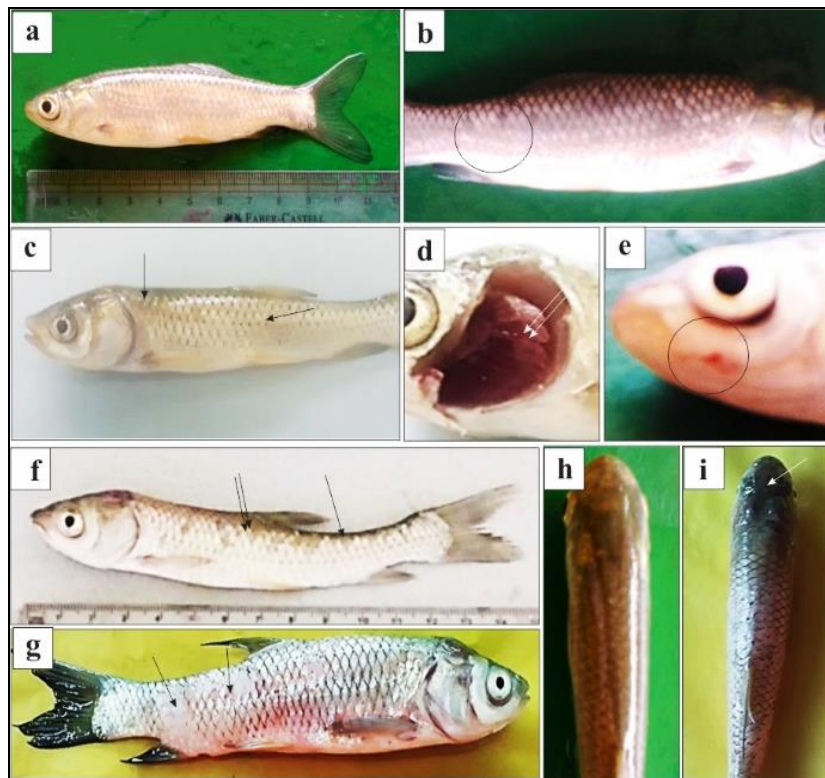The fish displayed cryptic changes, shedding of scales, haemorrhages and thick coating of mucus on the body (Fig 2).

Fig 2: Morphological alterations induced in *C. idellus* with acute and chronic chromium exposure. (a) Unexposed fish showing normal morphology, (b) fish with loss of scales, (c) cryptic variation (arrow), (d) whitening of gill filaments, (e) haemorrhage near the operculum. With increased exposure of Cr (VI), fish showed (f) bending of the body (arrow), with cryptic changes and (g) loosening of scales making patches of skin without scales. The fish also developed (i) haemorrhage on the head (arrow) in comparison to the normal fish (h).

3.2.2 Behaviour changes
The alterations observed in fin and opercular movements of *C. idellus* on exposure to Cr have been given in Table 2 and Fig 3. The frequency of opercular and fin (dorsal and caudal) movements in the control group remained almost the same throughout the experimental duration. The opercular movement frequency (Fig 3a) on the 15th day of exposure displayed an increase of 14.5 and 19.6% with 5.3 and 10.63 mg/L of Cr respectively. The incessant exposure on the 30th day with 10.63 mg/L Cr led to a non-significant decline in the frequency, which become significant (19.04%) on the 45th day of exposure. The lower concentration of Cr was able to decrease the opercular frequency by 28.57% by the 45th day. The dorsal fin movement (Fig 3b), on the 15th day of experiment with 5.3 mg/L Cr witnessed an elevated frequency by 19.59%, which decreased by 36.48% till the 45th day. The higher concentration of Cr, 10.63 mg/L was also able to reduce the dorsal fin frequency by 54.05% by the 45th day. The caudal fin movement (Fig 3c) also flaunted modified frequency with Cr exposure. The treatment of fish with higher Cr concentration (10.63 mg/L) diminished the frequency by 19.79 and 37.5% by 30th and 45th day of exposure; while the lower concentration (5.3 mg/L) lessened it by18.84% on the 45th day.

Table 2: The frequency of fin and opercular movement shown by *C. idellus* exposed to 5.30 and 10.63 mg/L of Cr(VI).

Behaviour	Exposure Period	Experimental Groups		
		Control	Cr (5.30 mg/L)	Cr (10.63 mg/L)
Opercular frequency	15d	67.2±3.023	77.0±4.472[a]	80.4±2.839[a]
	30d	67.7±3.120	62.4±3.931	53.8±7.07
	45d	67.9±3.091	48.0±2.846[a]	54.4±4.319[a]
Caudal fin frequency	15d	19.2±0.374	20.2±0.734	22±0.707
	30d	19.6±0.327	17.0±0.894	15.4±0.748[a]
	45d	19.5±0.241	13.8±0.583[a]	12±0.71[a]
Dorsal fin frequency	15d	29.6±2.541	35.4±1.749	35.4±1.326
	30d	28.9±2.362	24.8±2.395	22.8±1.772
	45d	28.5±3.263	18.8±0.800[a]	13.6±1.691[a]

Data are presented as mean ± SE (n=5). Significance ($p < 0.05$) is determined by one-way ANOVA followed by Tukey's post hoc test.
'a': indicates statistically significant difference with respect to control.
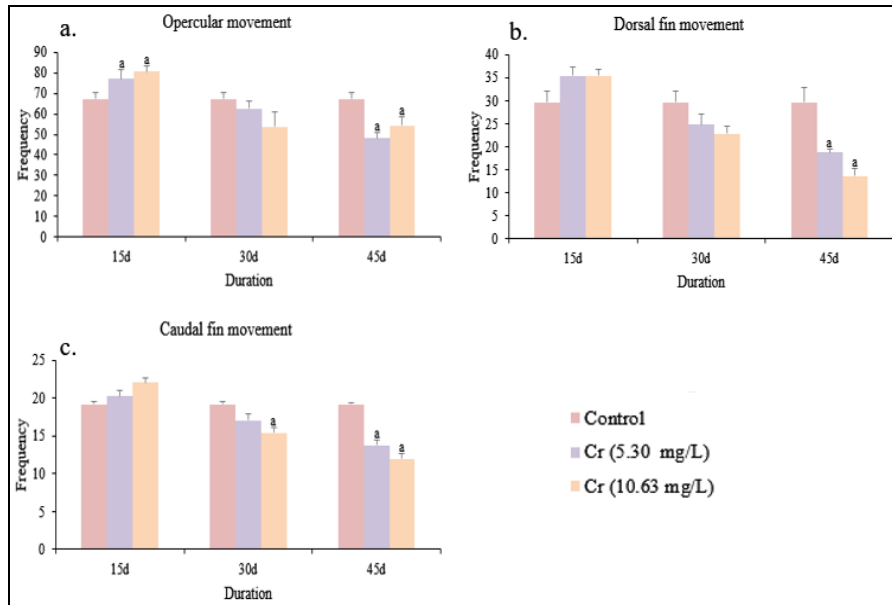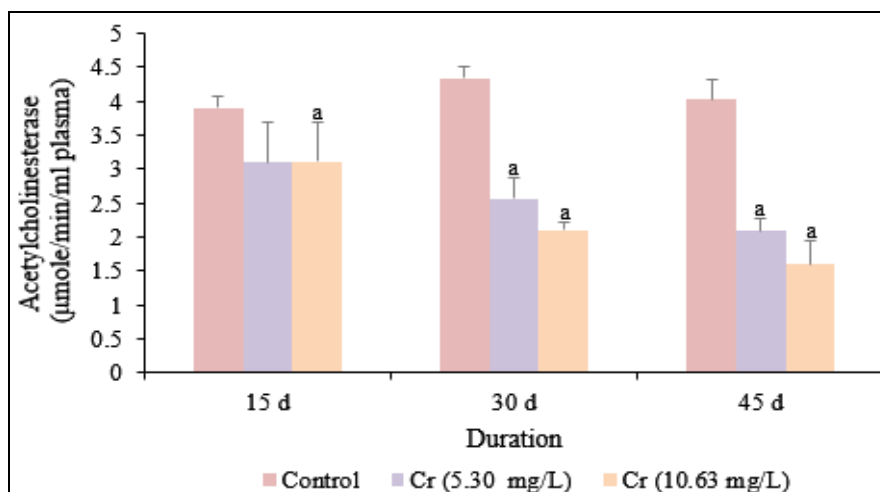

Fig 3: Alteration in (a) Opercular, (b) dorsal and (c) caudal fin movement (per min.) of *C. idellus* exposed to Cr (VI) at different exposure periods. Data are presented as mean ± SE (n=5). Significance ($p < 0.05$) is determined by one-way ANOVA followed by Tukey's post hoc test. 'a': indicates statistically significant difference with respect to control.

3.2.3 Acetylcholinesterase activity

The activity of AChE (Fig 4) showed a decreasing trend with increasing duration and concentration in the Cr treated fish. In fish treated with 5.3 mg/L Cr led to decline in the activity of enzyme by 40.87 and 48% on 30th and 45th day of exposure with values of 5.55±0.320 and 5.95±0.18 respectively, while the exposure with 10.63 mg/L diminished the activity of acetylcholinesterase by 20.47, 51.64 and 60.3% in comparison to control on the three exposure endpoints i.e., 15th, 30th and 45th day with values of 6.11±0.588, 7.10±0.128 and 7.60±0.341, respectively.

Fig 4: Acetylcholinesterase activity in serum of *C. idellus* exposed to 5.3 and 10.63 mg/L of Cr(VI) at different exposure periods. Data are presented as mean±SE (n=5). Significance ($p < 0.05$) is determined by one-way ANOVA followed by Tukey's post hoc test. 'a': indicates statistically significant difference with respect to control.

4. Discussion

The present investigation was taken up with an aim to study the acute and chronic effects of chromium on the morphology and behaviour of *Ctenopharyngodon idellus*. The 96h LC_{50} value of chromium was determined to be 53.08 mg/L for *C. idellus* with threshold effective concentration (TEC) of 41.35 mg/L. Species mean acute value (SMAV) of chromium (VI) for different fish species has been documented to be in the range of 30.0 to 139.9 mg/L[17,18]. LC_{50} of chromium for *Carassius auratus* to be 85.7 mg/L [11]; while for *Cyprinus carpio,* it is 87.96 mg/L [19]. In the case of endemic species, it has been reported to be 34 mg/L and 39.4 mg/L for *Cirrhinus mrigala* and *Labeo rohita* [20, 21]. The acute toxicity values of chromium are organism specific [22] and age-specific [23]. Thus, the present LC_{50} displays the SMAV defined by US EPA[17]; and exhibited less sensitivity of *C. idellus* the towards metal than endemic fish.

The morphology displayed alterations in the acute as well as chronic tests with cryptic colouration, loss of scales and haemorrhages being prominent among others. Darkening of body colour is associated with the reversible response of melanophores [24] and is controlled by the sympathetic nervous system [25]. Heavy mucus secretion produced by goblet and other cell types is associated with making the epithelium impermeable in stressed conditions [26]. In teleosts, the skin, gills and urinogenital system are the principal mucosal surfaces and is the first line of defence [27]. Chromium is also known to affect skin by producing allergic reactions [28].

During the present investigation, chromium also induced behavioural alterations with erratic swimming, loss of balance and hyperactivity. Present observations are in concurrence with the findings of other workers [18, 29, 30, 40]. The behavioural changes point towards systemic toxicity, and has been connected to the oxidative stress-induced neurotoxicity [18]. The lowered respiratory efficiency with Cr (VI), evident by decreased opercular frequency, might be a result of low ATP levels in the blood. Similarly, the decreased frequency of caudal fin resulting in a decreased force of propulsion for swimming [31] followed by cessation of swimming has been noticed. The cessation of movement has been linked to the altered carbohydrate metabolism and induced neurotoxicity by the metal [18, 30].

To assess the neurotoxicity induced by chromium, acetylcholinesterase activity was studied which decreased in a concentration and time-dependent manner. Chromium (VI) reduced the activity of AChE [32] either due to blockage of the active site, altering in enzyme structure, or different amino acid sequences [33]. These modifications inhibit the formation of enzyme-substrate complex, which is either reversible or irreversible [34, 35]. The altered acetylcholinesterase activity produces neurotoxicity and alters the normal behaviour of animals, as evident from the present study.

5. Conclusion

Chromium at the environmentally relevant sublethal concentrations was able to induce morphological and behavioural modification with a decrease in acetylcholinesterase activity. The results suggest the potential toxic effects of chromium on *C. idellus* and prove it to affect the normal survival of fish in chromium contaminated waters.

6. Acknowledgement

7. References

1. Cieslak-Golonka M. Toxic and mutagenic effects of chromium (VI) -- a review. Polyhedron. 1996; 15(21):3667-3689.
2. Zahoor A, Rehman A. Isolation of Cr(VI) reducing bacteria from industrial effluents and their potential use in bioremediation of chromium containing water. Journal of Environmental Sciences (China). 2009; 21(6):814-820.
3. Oliveira H. Chromium as an environmental pollutant: insights on induced plant toxicity. Journal of Botany, 2012, 375843.
4. Salnikow K, Zhitkovich A. Genetic and epigenetic mechanisms in metal carcinogenesis and carcinogenesis: nickel, arsenic, and chromium. Chemical Research in Toxicology. 2008; 21:28-44.
5. Ashraf A, Bibi I, Niazi NK, Ok YS, Murtaza G, Shahid M *et al*. Chromium (VI) sorption efficiency of acid-activated banana peel over Organo-Montmorillonite in aqueous solutions. International Journal of Phytoremediation. 2017; 19(7):605-613.
6. APHA. Standard Methods for Examination of Water and Waste Water. Edn. 23rd, American Public Health Association. Washington, 2015.
7. Jaishankar M, Tseten T, Anbakagan N, Mathew BB, Beeregowda KN. Toxicity, mechanism and health effects of some heavy metals. Interdisciplinary Toxicology 2014; 7:60-72.
8. Awasthi Y, Ratn A, Prasad R, Kumar M, Trivedi SP. An *in vivo* analysis of Cr^{6+} induced biochemical, genotoxicological and transcriptional profiling of genes related to oxidative stress, DNA damage and apoptosis in liver of fish, *Channa punctatus* (Bloch, 1793). Aquatic Toxicology. 2018; 200:158-167.
9. IARC. Lista de classificações da IARC. http://monographs.iarc.fr/ENG/classification/latest_classif.php, 2018.
10. Abdel-Baki AS, Dkhil MA, Al-Quraishy S. Bioaccumulation of some heavy metals in tilapia fish relevant to their concentration in water and sediment of Wadi Hanifa, Saudi Arabia. African Journal of Biotechnology. 2011; 10:2541-2547.
11. Velma V, Vutukuru SS, Tchounwou PB. Ecotoxicology of hexavalent chromium in freshwater fish: a critical review. Reviews on Environmental Health. 2009; 24(2):129-145.
12. Mendil D, Uluozlu OD, Hasdemir E, Tuzen M, Sari H, Suicmez M. Determination of trace metal levels in seven fish species in lakes in Tokat, Turkey. Food Chemistry. 2005; 90:175-179.
13. Singh P, Chowdhuri DK. Environmental presence of hexavalent but not trivalent chromium causes neurotoxicity in exposed *Drosophila melanogaster*. Molecular Neurobiology. 2017; 54(5):3368-3387.
14. OECD Test No. 203: Fish, acute toxicity. OECD guidelines for the testing of chemicals, Section 2, OECD Publishing, Paris, 1992.
15. CPCB. Report on groundwater quality in Kanpur, status, sources and control measures: GWQS/8/1996–97. Central Pollution Control Board, India. 1997; 1:4-5.

16. Ellman GLE, Courtney KD, Robert VA, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology. 1961; 7(2):88-90.

17. US EPA. Ambient water quality criteria for chromium-1984. EPA-440/5-84-029. Office of Water Regulations and Standards, Washington, 1985.

18. Mishra AK, Mohanty B. Acute toxicity impacts of hexavalent chromium on behavior and histopathology of gill, kidney and liver of the fresh water fish, *Channa punctatus* (Bloch). Environmental Toxicology and Pharmacology. 2008; 26:136-141.

19. Shaukat T, Javed M. Acute toxicity of chromium for *Ctenopharyngodon idella, Cyprinus carpio* and *Tilapia nilotica*. Journal of Agriculture and Biology. 2013; 15(3):590-594.

20. Virk S, Sharma A. Alterations in the biochemical constituents of muscles of *Cirrhinus mrigala* following exposure to and withdrawal from metals. Bulletin of Environmental Contamination and Toxicology. 2003; 70:106-111.

21. Vutukuru SS. Acute effects of hexavalent chromium on survival, oxygen consumption, hematological parameters and some biochemical profiles of the Indian major carp, *Labeo rohita*. International Journal of Environmental Research and Public Health. 2005; 2:456-462.

22. Bakshi A, Panigrahi AK. A comprehensive review on chromium induced alterations in fresh water fish. Toxicology Reports. 2018; 5:440-447.

23. Sanyal T, Kaviraj A, Saha S. Toxicity and bioaccumulation of chromium in some freshwater fish. Human and Ecological Risk Assessment. 2017; 23(7):1655-1667.

24. Mills MG, Patterson LB. Not just black and white: pigment pattern development and evolution in vertebrates. Seminars in Cell Development and Biology. 2009; 20:72-81.

25. Burton D. A physiological interpretation of pattern changes in a flatfish. Journal of Fish Biology. 2008; 73:639-649.

26. Shephard KL. Mucus on the epidermis of fish and its influence on drug delivery. Advanced Drug Delivery Reviews. 1993; 11(3):403-417.

27. Gomez D, Sunyer JO, Salinas I. The mucosal immune system of fish: the evolution of tolerating commensals while fighting pathogens. Fish and Shellfish Immunology. 2013; 35:1729-1739.

28. Jomova K, Valko M. Advances in metal-induced oxidative stress and human disease. Toxicology. 2011; 283(2-3):65-87.

29. Nath K, Kumar N, Srivastav AK. Chromium induced histological alterations in the gills of a freshwater teleost, *Colisa fasciatus*. The Fish Biology Journal Medaka. 1997; 9:37-40.

30. Begum G, Rao JV, Srikanth K. Oxidative stress and changes in locomotor behavior and gill morphology of *Gambusia affinis* exposed to chromium. Toxicology and Environmental Chemistry. 2006; 88(2):355-365.

31. Cui Z, Yang Z, Shen L, Jiang HZ. Complex modal analysis of the movements of swimming fish propelled by body and/or caudal fin. Wave motion. 2018; 78:83-97.

32. Moshtaghie AA, Afrang M, Manuchehr M. Changes in catecholamines and acetylcholinesterase levels of cerebellum, mid-brain and brain cortex in chromium treated rats. Iranian Journal of Pharmaceutical Research. 2004; 3:149-153.

33. Sabullah MK, Sulaiman MR, Shukor MYA, Syed MA, Shamaan NA, Khalid A *et al.* The assessment of cholinesterase from the liver of *Puntius javanicus* as detection of metal ions. The Scientific World Journal. 2014; 571094.

34. Loewenstein-Lichtenstein Y, Schwarz M, Glick Nørgaard-Pedersen DB, Zakut H, Soreq H. Genetic predisposition to adverse consequences of anti-cholinesterases in "atypical" BCHE carriers. Nature Medicine. 1995; 1(10):1082-1085.

35. Podoly E, Shalev DE, Shenhar-Tsarfaty S, Bennett ER, Assayag EB, Wilgus H *et al*. The butyrylcholinesterase K variant confers structurally derived risks for Alzheimer pathology. The Journal of Biological Chemistry. 2009; 284(25):17170-17179.

36. Finney DJ. Probit Analysis. Cambridge University Press, Cambridge, England, 1980.

37. Clemedson C, Barile FA, Chesne C, Cottin M, Curren R, Eckwall B *et al.* MEIC evaluation of acute systemic toxicity. Part VII. Prediction of human toxicity by results from testing of the first 30 reference chemicals with 27 further *in vitro* assays. Alternatives to Laboratory Animals. 2000; 28:159-200.

38. Maheshwari DG, Shaikh NK. An overview on toxicity testing method. International Journal of Pharmacy and Technology. 2016; 8(2):3834-3849.

39. Richetti SK, Rosemberg DB, Ventura Lima J, Monserrat JM, Bogo MR, Bonan CD. Acetylcholinesterase activity and antioxidant capacity of zebrafish brain is altered by heavy metal exposure. Neurotoxic ology. 2011; 32(1):116-122.

40. Jindal R, Kaur M. Acetylcholinesterase inhibition and assessment of its recovery response in some organs of *Ctenopharyngodon idellus* induced by chloropyrifos. International Journal of Science, Environment and Technology. 2014; 3(2):473-480.

41. Jindal R, Verma S. *In vivo* genotoxicity and cytotoxicity assessment of cadmium chloride in peripheral erythrocytes of *Labeo rohita* (Hamilton). Ecotoxicology and Environmental Safety. 2015; 118:1-10.