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Research article

Sodium fluoride induced alterations in hematological parameters and oxygen consumption in Indian major carp *Labeo rohita (Hamilton)* as biomarkers of fluoride toxicity

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Key words: Exposure, fish, fluoride, hematological parameters, *Labeo rohita,* oxygen consumption.

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Abstract

Fish *Labeo rohita* was exposed to a concentration of fluoride 66.976mg/l for a period of 7 days and 15 days only to study the effects of fluoride on hematological parameters and the respiratory behaviour. Due to the manifestation of the toxic action the important blood parameters that were altered are erythrocytes, Haemoglobin, haematocrit, Packed Cell Volume and Mean Corpuscular Haemoglobin, and the Mean Corpuscular Haemoglobin concentration. When exposed to sodium fluoride for 7 days the values of RBC 2.552±0.38 MC/mm, Hb% 6.08 ± 1.140, PCV 15.06±3.00, MCV 58.65±5.685, MCH 23.802 ± 2.315 compared to control. After 15 days of exposure to sodium fluoride the decreased values are, erythrocytes 2.94±0.11, Hb 6.56 ± 1.26, PCV 15.6±2.85, MCV 51.82±5.26 and MCH 22.52±2.54 compared to control. MCHC values are increased as 40.504±2.82 for 7 days exposure and 42.05±2.79 for 15 days exposure compared to control. The changes in the poikilotherm blood of the fish having branchial heart have profound bearing on the oxygen consumption of the fish. The fish was exposed to less than LC_{50}, LC_{50} and more than LC_{50} concentrations of fluoride for 6 hours and 12 hours. The oxygen consumption of the fish was reduced when compared to control. After 12 hours there is significant reduction in the oxygen consumption of the fish *Labeo rohita*. The decrease in the hemoglobin levels, results in lowering the oxygen intake capacity of the fish.

Introduction

The contamination of fresh water with wide range of pollutants has become a matter of great concern not only because of threat to the public health but also with the damage caused to aquatic life. These pollutants cause deleterious effects to aquatic life and human beings [1, 2]. The aquatic environment is severely affected by different types of chemicals and effluents which are toxic to the inhabiting organism such as fish [3]. Fluoride is widely distributed in nature causes adverse effects at higher level. The most abundant element that occurs in earth's crust and is in the form of fluoride compounds which are constituents of material in rocks and soil and finally getting their way into water. Fluoride concentrations above the permissible level more than 1.5mg/l can affect bone, teeth structure [4] high concentrations of fluoride were reported in 14 states of India. The main sources of fluoride in ground water is fluoride bearing rocks such as cryolite, fluorite, fluorapatite and hydroxylapatite [5]. A review by Barbirer *et. al.* (2010) [6] has sketched a number of cellular processes in which fluoride can have negative effects.

Stress is a general and non specific response to any factor disturbing homeostasis. In the fish it may be induced by various abiotic environmental factors, biotic interaction and also by anthrapogenic activities. Any behavioural alterations can be considered as sensitive indicators of environmental stress. Fresh water invertebrates mainly cadisfly larvae and fishes that upstream migrating adults like solmons are to be more sensitive to fluoride [7]. In fishes the hematological parameters are reported to vary under stress [8]. The toxicant cause the mortality of it is legal whereas in sub-lethal concentrations render the animal not suitable to maintain life especially the fish the heterotropic cold blooded animals.

Stress involves various physiological changes causing affects which includes alteration in blood composition. In fishes the hematological parameters are reported to vary under stress. Stress includes alteration in blood cell count and activities such rapid changes in the characteristics of weight, growth in different seasons [9]. The blood is used as indices of pollution by the toxicants. They provide assessment factors of pollution and equip with necessary protective measures to take care for the fish population. Such alterations either increase or decrease in the blood parameters were studied and reported [10].

Blood parameters are considered as pathological indicators of whole body and therefore are important in diagnosing the structural and functional status of the fish exposed to toxicants [11]. A number of hematological indices such as HCT, Hb, Red blood cells and are used to assess the functional status of the oxygen carrying capacity of the blood stream and have been used as a indicator of pollution in the aquatic environment [12]. The present investigation was undertaken to find out the toxic effects of fluoride on the erythrocytes and related hematological parameters and the oxygen consumption capacity of the fresh water, edible fish *Labeo rohita* as the fishes are very important from nutritional point of view as well as the economic venture as it is one of the main component of aqua culture.

Materials and methods

Collection and maintenance of the test organism

The fresh water fish *Labeo rohita* (Hamilton) 6 to 7 cm in length and 10 to 15g in weight irrespective of their sex have been chosen for the present investigation of blood parameters and the fish measuring 4 to 6cm in length and 2 to 3g in weight in case of oxygen experiment. The healthy and active fish were obtained from Rao's hatcheries, Nandhivelugu, Andhra Pradesh, India. The fish were acclimatized to the laboratory conditions in large cement tanks for 10 days at room temperature of 28 $\pm$ 1^{0}C. The water used for acclimatization and conducting experiments were clean and unchlorinated ground water, during the period of acclimatization, the fish were fed with the ground nut cake and rice bran. Feeding was stopped one day prior to the acute toxicity test and such acclimatized fish were only used for experimentation.

Physical and chemical properties of water used for the present experiment are in mg/lit. Turbidity-8 silica units, electrical conductivity at 28^{0}C – 816 MHOS/Cm, pH at 28^{0}C – 8.1, Alkalinity (Phenolphthalein) – Nil, Alkalinity (Methulorange)-472, Total hardness as $CaCO_3$ – 232, Non carbonate hardness as $MgCO_3$ – Nil, Nitrate nitrogen as (N) – Nil, Sulphate (as SO_4) – Trace, Chloride (as Cl) – 40, Fluoride (as F^-) – 1.8, Iron (as Fe) – Nil, Dissolved oxygen 8-10 ppm, Temperature 28 ± 2^{0}C. Sodium fluoride reagent grade was used as a toxicant supplied by LOBA Chemical Company, Bombay. The test solution of Sodium Fluoride was prepared by using water as solvent.

Lethal values

Bioassay tests were conducted to determine acute toxicity of fluoride to Labeo *rohita*. 96h LC_{50} values were obtained by conducting experiments in static renewal system. All the precautions recommended by APHA toxicity test to aquatic organisms, 1998, 2005 and 2012 were followed [13, 14]. Finney probit analysis (1971) [15] as recommended by Roberts and Boyce (1972) [16] was followed to calculate LC_{50} values. The 96h LC_{50} values were obtained were 334.88mg/lit (95% confidential limits 325.761 – 352.289). The fish were exposed to 10% of LC_{50} value 33.488mg/lit, LC_{50} value 334.88mg/lit and more than LC_{50} value to study oxygen consumption.

Blood sampling

In natural conditions the fish will be usually exposed to sub-lethal concentrations that's why 20% of 96h LC_{50} values were chosen to conduct the chronic experiments to study the hematological alterations in the fish. 10 fish were introduced in each test chamber have 10lit of test solution. Multiples of 5 chambers were taken.

The blood samples were taken from the heart with help of hypodermic syringe (clean and dry). From the heart blood was drawn by inserting or injecting the needle to the body wall. The insertion was exactly in the middle line 0.05 to 1cm cranially from the posterior margin of the opercular cover and directed dorsally and caudally at an angle of 45^{0}C, care was taken to see that the blood was not haemolized.

Estimation red blood corpuscle (RBC Count)

RBC count was made with Neubauer crystalline counting chamber as described by Davidson and Henry, 1969 [17]. The blood was drawn into the RBC pipette upto 0.5mark and immediately the diluting fluid is drawn upto 101 mark (thus the dilution is 1:200). The solution was mixed thoroughly by shaking gently. It was allowed to remain stable for 2 to 3 minutes. The counting chamber and cover glass were cleaned and the cover was placed over the ruled area. Again the solution was mixed thoroughly and the stemfull of solution was expelled and a drop of fluid was allowed to flow under the cover slip holding the pipette at an angle of 40^0 it was allowed to stand still (2 to 3 min) till the RBC settled. Afterwards the ruled counting area was focused under the microscope and the number of RBCs were counted in fine small square of the RBC column under high power and the number of RBCs for sq. mm were calculated accordingly.

$$\frac{\text{No. of cells} \times \text{Dilution Factor} \times \text{Depth factor}}{\text{Areas counted}}$$

Estimation of haemoglobin concentration (Hb)

The haemoglobin concentration was estimated by acid haematin method [18]. N/10 Hydrochloric acid was taken upto 10 mark in the graduated tube blood was sucked into the haemoglobin pipe and then transferred into the graduated tube containing N/10 hydochloric acid. The pipette was rinsed 2 to 3 times with dilute hydrochloric acid. It was allowed to remain in dark for 10 to 20 mins after through mixing of N/10 HCl were added drop by drop mixing by each dilution until the blood colour matched with the standard colour. Then the results were

read from the scale on the graduated tube and Hb concentration was expressed in gram %.

Estimation of packed cell volume (PCV)

PCV was estimated by using Wintrobe's tube [19]. The blood samples were collected into a vial which contained an anti coagulant. The 2% EDTA blood was mixed thoroughly in a vial by repeated inversion and then filled Wintrobes tube upto 100 mark. The Wintrobe's tubes were placed in the centrifuge and centrifuged at 2,500 rpm for 30mins. The original column of blood in the tube being 100mm. The volume of packed cell can be read directly as percentage. The line that crossed the top of the packed erythrocyte column represented the PCV present.

Main Corpuscular Volume (MCV)

MCV expresses the average volume of red cells. For obtaining the MCV the Packed Cell Volume is provided by red cell count and the result is multiplied by 10. MCV is thus expressed as cubic micro.

$$\text{MCV} = \frac{\text{Haematocrit \%}}{\text{RBC in millions/mm}^3} \times 10$$

Mean corpuscular haemoglobin (MCH)

MCH represents the average weight of Hb containing in each cell. MCH is influenced by the size of the cell and concentration of Hb. For getting MCH the Hb concentration is usually divided by red blood cells and the result is multiplied by 10 and is expressed as micro gram (µg)

$$\text{MCH} = \frac{\text{Haemoglobin (g/100ml)}}{\text{RBC in millions/mm}^3} \times 10$$

Mean corpuscular haemoglobin concentration (MCHC)

MCHC refers to the average concentration of the Hb in the red cells. In contrast to MCH, MCHC is not influenced by size of the cell. For getting MCHC the Hb is divided by the packed cell volume expressed in terms of grams percent (g %).

$$\text{MCHC} = \frac{\text{Haemoglobin (g/100ml)}}{\text{Haematocrit}} \times 100$$

The effect of fluoride on Haemogram of *Labeo rohita* (Hamilton) exposed to sub-lethal concentration of 66.976mg/l of fluoride for 7 days and 15 days as shown in graph 1.

Estimation of oxygen

The fish was exposed to 110th of 96h LC_{50} value i.e., 33.488 mg/lit LC_{50} concentration of 334.88 mg/lit and more than LC_{50} i.e., 355 mg/lit. The aim is to study the oxygen consumption of *Labeo rohita* when exposed to different concentrations of fluoride.

The experiments on the oxygen consumption of the fish *Labeo rohita* was carried out using respiratory apparatus developed by Job (1955) [20]. In each experiment, the respiratory chambers one with fish, the other without fish i.e., control were taken. At the end of each hour samples were collected and the amount of oxygen present was estimated by Winklers method as in Golterman [21]. The amount of oxygen consumed by control and test fish was calculated by using the given formula.

$$\begin{aligned} O_2 \text{ consumed by fish per gm body weight per hour} \\ = [\alpha - \beta \times \text{N. of hypo } 8 \times 1000] \\ /[\text{Vol. of sample taken} \\ \times \text{Correction factor} \\ \times \text{Weight of the fish} \\ \times \text{Time interval for the sample}] \end{aligned}$$

Results and discussion

The alteration in erythrocyte count and related hematological parameters of the fish *Labeo rohita* exposed to sub-lethal concentration to (66.976 mg/l) of fluoride for 7 days and 15 days are represented in Graph-1. There is significant decrease in RBC number, haemoglobin (Hb), Packed Cell Volume (PCV), Mean Corpuscular Volume (MCV), Mean Corpuscular haemoglobin (MCH) but there is significant increase in MCHC % after 7 days and 15 days of exposure to the toxicant fluoride.

Sanguineous values are influenced by various factors such as size, nutritional state [22]. It was reported by Sunil Kumar *et. al.*, (2014) [23], reduction in RBC count and Hb, PCV, MCV, MCH and MCHC in the *Channa punctata* exposed to different concentrations of fluoride (10, 20, 30 and 40 ppm) for 15 days. Prominent clumping of erythrocytes was also reported. There was significant decrease in RBC count in fish *Cyprinus carpio* when exposed to toxic heavy metal pollutants. It indicates that the pollutants like heavy metals have strong influence on hematological parameters of fish [24]. Kamble and Velhal, (2010) [25] reported that the dose of fluoride concentration 100ppm, 200ppm and 300ppm caused the time dependent and dose dependent transient effect on RBC, WBC count and Hb which indicates immunological suppression.

According to Kumar *et. al.*, (2010) [26] fluoride causes significant decrement in count of RBC, Hb content and Packed Cell Volume in *Clarias batrachus*. Guru *et. al.*, (2014) [27] reported the total erythrocyte count Hb, PCV, MCV, MCH and MCHC progressively decrease if an increase in the concentration of fluoride in *Channa punctata*. Similar results were reported by Srinivasa Rao *et. al.*, (2018) [28] in the fish *Ctenopharyngodon idella* after exposure to the toxicants

deltamethrin technical grid and 11% EC among the blood parameters reported RBC, Hb and HCT decreased whereas WBC is increased. The hematological parameters of the fish altered due to exposure to different pesticide toxicants i.e., synthetic pyrethroids reported by Gopal Rao *et. al.*, (2017) [29], and Neelima *et. al.*, (2015) [30]. Hematological alterations by different toxicants to the fish *Clarias batrachus* by the toxicant monocozeb, by Swarnakumari *et. al.*, (2018) [31] to the fish ctenopharyngon idella [valencies], to the fish *Silver carp* by the toxicant diazinon by George *et. al.*, (2017) [32], monocrotophos on the fish *Labeo rohita* by Anusiya (2015) [33] were reported. *Cyprinus carpio* exposed to permethin 25% EC by N. Gopala Rao *et. al.* (2017) [29].

Hematological alterations of the different pesticide toxicant to the fish *Cyprinus carpio* by Vaiyanan (2015) [34], endosulphan as pesticide toxicant to *Ctenopharygodon idella* by Renu and Bala Singh (2016) [35] was reported. Tough the fluoride is present in sub-lethal concentrations the ambient water defilement definitely pose a threat in which blood serve as a bio marker as opined by Kaviraj and Gupta (2014) [36]. This results in the disturbance of the equilibrium in homeostasis.

It is indicated that the Hematological alterations of the toxicant exposed fish might be due to physiological changes of haemopoietic system which is considered to be the most sensitive point [37]. The present study reports are in cognizant with the earlier reports i.e., when *Channa punctata* was exposed to 10ppm of sodium fluoride there was decrease in total leucocyte count, lymphocytes, haematocrit values and an increase in erythrocyte count [38]. The development of anemia in the fish exposed to toxicants may be at the interference of toxicants with haemopoiecis and / are alteration of cell membrane by hydrolysis of acetyl choline in the body fluids of cholinesterases of the erythrocytes. In the fish stress suppresses the intake of food which leads to hematological changes and oxygen carrying capacity of blood in *Clarias batrachus* [39]. It was reported that *Oreochromis mossambicus* on exposure to textile mill affluent as a function of feeding / starvation resulted increase in concentration produced dose dependent increase in RBC, WBC, Hb and HCT and decrease in MCV, MCH and MCHC. This anaemia is due to disturbance of erythropoisis, fatty haemosynthesis and osmaregulatory misfunctioning due to an increase in the rate of erythrocyte destruction in haemopoietic organs [40]. Stress induced by altering the homeostasis of the environment leading to decrease in values of RBC, Hb, HCT, MCH, MCB in *Rita rita* when exposed to sodium fluoride [41]. Earlier reported the diminishing levels of the MCH value might be due to reduction in the Haemoglobin content [42].

It was reported that the fish when exposed to pesticides / chemicals, changes were induced in respiratory mechanism and metabolic rate [43]. Since the respiratory distress is recognized as one of the symptoms of pesticide toxicity [44] the observed decrease in the rate of oxygen consumption should be due to the respiratory distress as a consequence of the impairment of oxidative metabolism or it may be due to reduction in the uptake by gills, probably due to the tissue damage in the said organ [45]. The reduction in the oxygen consumption of the fish exposed to sub-lethal concentration of the toxicants is probably due to continuous exposure which leads to tissue damage of gill by degenerative changes [46]. The results of the present study during the oxygen consumption as per the Graphs 2(a) and 2(b) indicate the toxic stress.

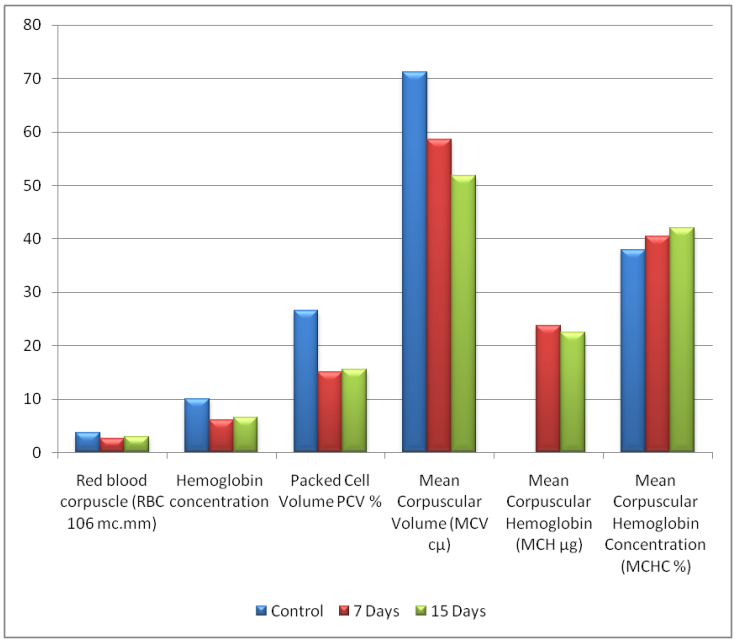
Similar observations in decline of oxygen uptake due to gill damage were reported [28]. The decrease may be due to the absorption of the fluoride across the gills from there into the blood streams results in high toxicity to the fish. Oxygen uptake of fishes is said to be intimately connected at the damage of the gill, changes in the architecture of the gill would lower the diffusing capacity of the gill with consequent hypoxic / anoxic conditions and thus respiration may become problematic task for the fish. Laboured breathing was observed in the fish with concomitant gill damage respiring through mouth as an indication of respiratory distress and / or hypoxic condition in and around the fish [47].

The decrease in oxygen uptake may also be due to the interference of the toxicant with Hb lowering its transport efficiency. The reduction in Hb may also be responsible for decreased oxygen transport as reported by Kumar *et. al.*, (2010) [25] in Clarias batracus. In the present study the decrease in uptake capacity of fluoride exposed fish *Labeo rohita* was supported by previous studies. The *Labeo rohita* when exposed 3 different concentrations of NaF i.e., LC_{50}, < LC_{50}, and > LC_{50} oxygen consumption was reduced drastically in the high concentration at 6 hours duration. Even after 12 hours exposure significant reduction in the oxygen consumption toxicant exposed fish was observed. The decrease was less in sub-lethal concentration but not significant. Fluoride ion acts as enzymatic poison inhibiting the enzyme activity and ultimately interrupting the metabolic processes such as glycolosis and synthesis of proteins [48]. This decrease in protein synthesis leads to decrease synthesis of Hb. [49], Bajpai *et. al.*, (2012) [45] reported histological alterations in gills, kidneys and intestine of fresh water fish *Heteroneustes fossilis* (Bloch).

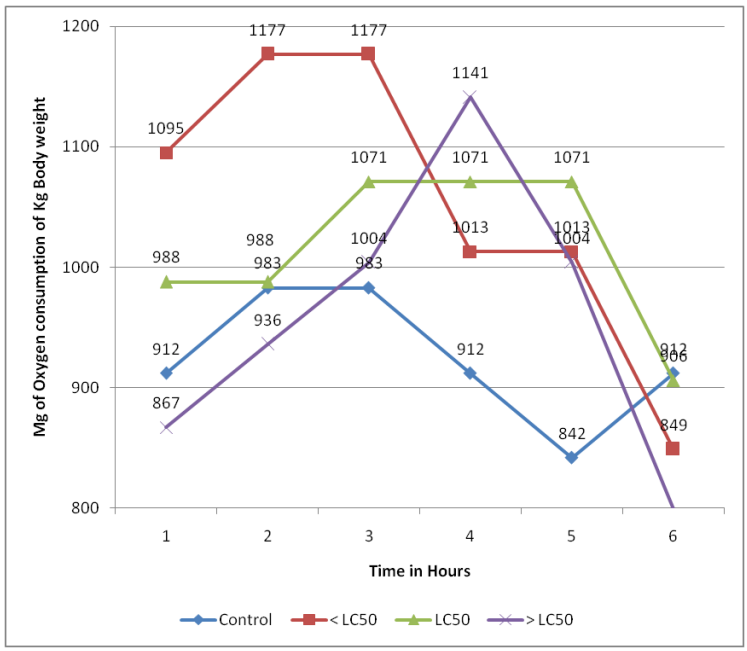
As the fish was exposed to toxicant it inhibited the cell proliferation i.e., in the haemopoietic tissue leading to reduce growth and interrupted the mechanism. The same was reported by Jha (2004) [50]. The DNA and cytogenetic alteration in aquatic organisms due to impaired enzymatic function in turn metabolism, cyto toxicity and reduced growth in fluoride. Chromosomal aberrations increased with increase in fluoride dose in

Clarias batrachus [51]. Molecular studies in fluoride toxicity influences the metabolic pathways which are involved in cell proliferation and apoptosis [6]. The cytotoxicity and genotoxicity changes induced by F^- resulted in reduced growth and abnormal development. The cytotoxicity changes alters gill filament structure and thereby leads to hypoxic conditions thus leading to laboured breathing and high ventilation.

In the present study the sub-lethal concentrations were selected to study the toxicant effect on fish. The toxicant concentration in majority of cases will be sub-lethal i.e., real concentration in natural environment. When the fish was exposed to NaF for 7 days and 15 days there is significant decrease in Hb and number of RBC, this decrease is purely due to the decrease of Hb quantity and reduction in the RBC count. Hb the oxygen transport pigment, production is decreased due to the stress of the toxicant on the fish. Hence the values of hematological parameters can be considered as biomarkers of stress.



Graph 1. Haemogram of *Labeo rohita* (Hamilton) exposed to sub-lethal concentration 66.976mg/l of fluoride after 7 days and 15 days of exposure.



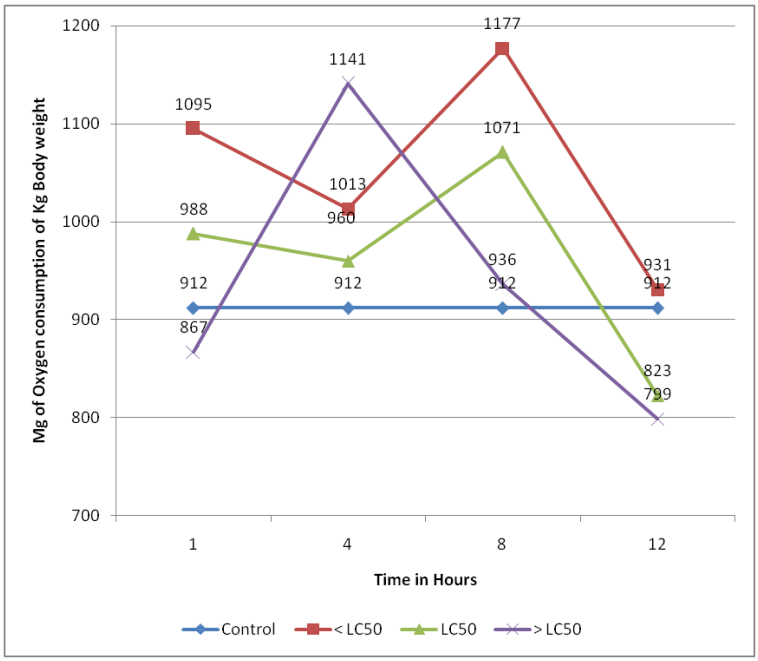
Graph 2(a). The amount of oxygen consumed in mg/kg body weight of the fish *Labeo rohita* (Hamilton) exposed to fluoride.

$< LC_{50}$ = 33.488mg Fluoride / *l*

LC_{50} = 334.88mg Fluoride / *l*

$> LC_{50}$ = 355.701mg Fluoride / *l*

Determination of five values and the average statistically significant.



Graph 2(b). The amount of oxygen consumed in mg/kg body weight of the fish *Labeo rohita* (Hamilton) exposed to fluoride.

< LC_{50} = 33.488mg Fluoride / *l*
LC_{50} = 334.88mg Fluoride / *l*
> LC_{50} = 355.701mg Fluoride / *l*
Determination of five values and the average statistically significant.

Conclusion

From the above results it can be concluded that the sodium fluoride is found to be toxic to the edible fish *Labeo rohita* and it has induced variations in hematological parameters. The decrease in levels of RBC, Hb, PCV and increase in MCHC revealed the haematotoxic effects of the chemical pollutants. The evaluation of hematological parameters will help in early detection of clinical pathology as well as the contamination of the environment. It can also be stated that the aquatic fauna have to be protected from contamination of media or by preventing leaching of chemicals into the aquatic environment. Further investigations are recommended to understand histo-chemical variations, oxygen consumption and its related enzymatic studies due to toxic stress of sodium fluoride on major carps as the major carps are main components of aquaculture for which the attention has to be paid.

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