The acute toxicity of Sodium fluoride and biochemical alterations in the tissues of fresh water Indian major carp *Labeo rohita.*

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Abstract

Fluoride is an element of high biological activity and shows adverse affects on fishes even in very low concentrations in the aquatic medium. Acute bioassay tests were conducted after exposure to Sodium fluoride for 24, 48 and 96 hrs on fry stage and also for 24 h bioassay on fingerling stage of *Labeo rohita* to determine the LC_{50} values. The 24 h LC_{50} value 427.56 mg/l, 48h LC_{50} value 411.90 mg/l and for 96 h 374.82 mg/l for the fry 24 h 334.29 mg/l respectively for fingerling stage of *Labeo rohita*. The fingerling stage was found to be more susceptible than fry stage. The toxicity of sodium fluoride is found to be increasing with increasing concentration. When the fingerlings were exposed to sublethal, lethal concentration and above lethal concentration of sodium fluoride and the biochemical changes that occur in different tissues i.e. gill, brain, muscle, liver and kidney were analysed. There is significant decrease of protein content in gill muscle, brain and kidney whereas significant increase in protein content of liver. When the fingerlings were exposed to sublethal concentrations of sodium fluoride and biochemical changes in different tissues viz. gill, brain, muscle, liver and kidney were assayed. A significant decrease and increase for proteins and glycogen. The food substrates of metabolism in the vital organs of the tissues of the heterotrophic fish are affected due to toxic stress where the enzyme inhibition and accumulation resulted in the ambient situation.

Key words : Biochemical change, LC_{50} value, significant, sodium fluoride, static renewal bioassay, total proteins and glycogen.

1. Introduction

Any impairment of water by adding contaminants either directly or indirectly is mainly anthropogenic render it unfit to support the biotic communities such as fish. Fluoride is one among them and comes from the element fluorine. The 17th most abundent element in the earth's crust and never occurs in free state in

nature. Fluorine exists as fluoride compounds which are constituents of materials in rock and soil (Dhar 2009). Naturally occuring fluorides make up approximately 0.06-0.9% of the earth's crust and form components of rock, clay and soil (*ATSDR 2003*). In India high concentration of fluoride i.e. 1.5 mg/l have been reported from Andhra Pradesh, Rajasthan and many other states (*Susheela, A.K. 2001*). At low concentrations fluoride deficiency can occur but at high concentrations of fluoride some deleterious effects can arise. In relation to drinking water too high (>1.5 mg/l) fluoride concentration can affect bone and teeth structure (*Edmunds and Smedley 1996 and 2003*). The first major natural source of inorganic fluorides is the weathering of flouride minerals (*CEPA 1995*). The most important inorganic fluoride minerals in the earth's crust are fluorapatite ($Ca_3(PO_4)_3F$), fluorite (CaF_2) and Cryolite (Na_3AlF_6). Volcanoes are the second major natural sources through the release of gases with hydrogen fluoride. The effluents from aluminium smelter lead into nearby surface waters can raise fluroide levels, according to *Roy et.al. (2009)* for an aluminium smelter on the Saguenay River (Que. Canada) other important human activities causing significant increase in flourode concentration of surface water are phosphate fertilizer plants (*Somasekhar* and *Rangaswamy, 1983*), plants producing fluoride chemicals (*Karunakaran & Subramanian 1992*) such as hydrogen fluoride, Calcium flouride, Sodium fluoride. Discharges of fluoridated municipal water also cause significant increase in fluoride concentration of recipient rivers (*Camargo et.al. 1992*). In addition, though there is low risk, aluminium smelter and other industries along the marine coast also increase fluoride concentration of sea waters (*Pankhurst et.al. 1980*).

The aquatic environment is severely affected by different types of chemicals which are toxic to the inhabiting organisms (*Kopeca et.al. 2006*). Fluoride must be considered as a serious pollutant since its concentration in many aquatic ecosystems is significantly increasing by anthropogenic activities. The toxic effects of elevated fluoride on various aquatic species, humans and live stock are well documented by *Dwivedi et.al. (1997), Camargo (2003).* In aquatic habitat fish are the most sensitive organism and get affected even upon a mild change in the surroundings. Static renewal bioassay are useful to study the toxic effect of Sodium fluoride on the fish. Andhra Pradesh with long coastal belt and aquaculture is of the paramount importance in agriculture hence it is desirable to study the toxic affect of fluoride on the capture and cultivable fish *Labeo rohita*. There are very few reports on the toxic effect of Sodium fluoride & industrial effluents on protein and glycogen of fishes (*Kumar & Gopal 2001*). The present study was undertaken to study the toxic affect and biochemical changes viz. total proteins and glycogen in some of the body tissues of *Labeo rohita*.

2. Materials and Methods

The fresh water fish *Labeo rohita* (Hamilton) fry of both sexes, length 1.5 to 2.5 cm and weight 45 to 70 mg, fingerlings 4-6 cm, weighing 2500-4500 mg have been used as the test organisms in the present investigation. Healthy and active fish were obtained at Nandivelugu fish form, Guntur district, Andhra Pradesh, India. The fish were acclimatized to the laboratory conditions in large plastic water tanks for 10 days at room temperature 28 $\pm$ 1^0C and 12-12 h dark and light cycle. Water was renewed every day during the period of acclimatization, the fish were fed (at libitum) with groundnut oil cake and ricebran. Feeding was stopped one day prior to acute toxicity test. All the precautions recommended by APHA tp toxicity test

of aquatic organisms (*APHA 1998, 2005 and 2012*) were followed. If mortality exceeds 5% in any batch of fish during acclimatization, the entire batch of that fish were discarded.

Physical and Chemical properties of water used for the present experiments are (in mg/l) : Turbidity - 8 silica units, Electrical conductivity at 28^0C – 816 micro ohms/em, p^H at 28^0C – 8.1, Alkalinity, Phenolpthalene – Nil, Methyle orange 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 ± 2^0C.

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. The water used for acclimatization of the fish and for conducting experiments was the same.

2.1. Acute toxicity test

Experiments were conducted to determine acute toxicity of fluoride to *Labeo rohita* fry and fingerlings for 24 h, 48 h and 96 hours in static renewal system. First pilot tests were conducted to choose the concentrations at which the mortality of fishes were observed. Five replicates were taken for each concentrations, along with control group. The concentrations of the test chemical used in short term definitive tests were in between lowest concentration at which there was no mortality and the highest concentration at which 100% mortality resulted. Ten fish were introduced in each test chamber having 10 litre of test solutions. The number of dead fish at each concentration are noted but precaution was taken to remove the dead fish immediately. The data observed from these tests were recorded from time to time. Toxicity experiments were conducted to choose the mortality rate from 10% to 90% for 24 h, 48 h and 96 hours in static renewal system.

Finney's probit analysis (*Finney 1971*) as recorded by *Roberts and Boyce (1976)* was followed to calculate LC_{50} values. The respective probit values were taken from *Fisher and Yates (1938)*. For the determination of the 95% confidential LC_{50} and a normal variate of 1.96 was taken into consideration.

Further the data is also analysed by probit analysis (excel method) and computer generated output is taken which has given 24 h LC_{50} lower and upper limits, regression equation, slope and R^2 values *(Finney 1971)* and is represented graphically.

2.2. Estimation of total proteins and glycogen

The fish *Labeo rohita* of size 6 to 8 cm in length and 2500-4500 mg in weight were brought from local fish form and acclimatized at 28 ± 2^0C in the laboratory for 10 days. Such acclimatized fish was exposed to 24 h to $<LC_{50}$ (33.429 mg/l), LC_{50} (334.29 mg/l) and $>LC_{50}$ (355.701 mg/l) sodium flouride concentrations. The surviving fish tissues were taken for estimation of total proteins and glycogen. The total proteins were estimated by the modified method of *Lowry et.al., (1951).* The animals were sacrificed and fresh tissue was collected from gill, brain, muscle, liver and kidney. 30 mg of each tissue was taken and homogenised in 5% trichloroacitic acid (TCA) and centrifuged at 3000 rpm for 10 minutes. The suspended protein residue was dissolved in 1 ml. of 1 N NAOH. 0.2 ml of the extract was taken into the test tube and the 5 ml of alkaline Copper solution (50 ml of 2% $NaCo_3$ in 0.1 N NAOH 1 ml of 9.5% $CuSo_4$. $5H_2O$ in 1%

Sodium or Potassium tartrate) was added. After 30 minutes, the optical density was measured spectro-photometrically at 540 nm.

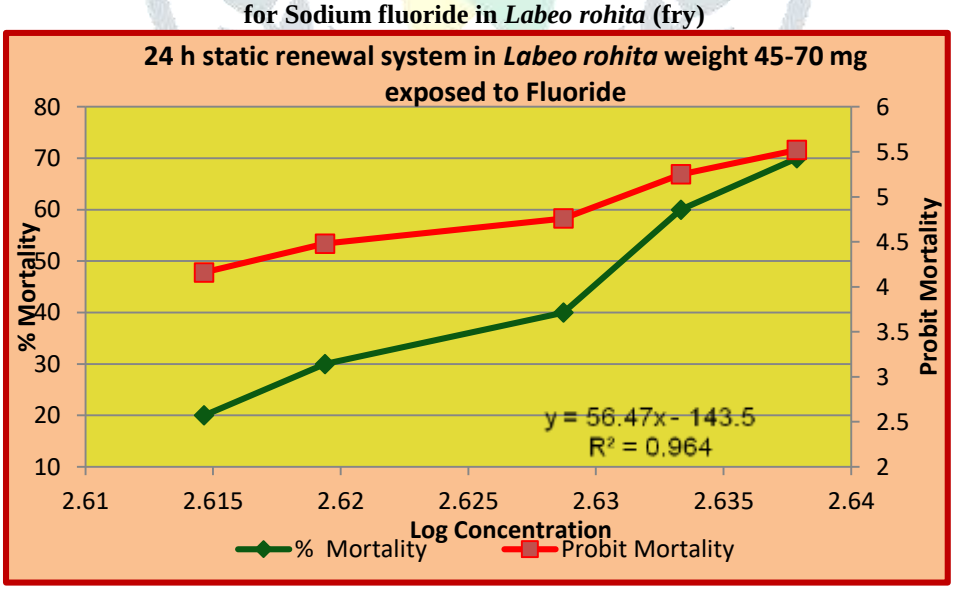
The standard graph was plotted by the method of *Lowry et.al, 1951* with Bovine serum albumin supplied by Singer Chemical Company (U.S.A.).

The total glycogen was estimated, employing the method of *Kemp et.al (1954)* 30 mg of each tissue was taken and homogenized in 80% methanol and centrifuged at 3000 rpm for 10 minutes. The tissue residue was suspended in 5 ml of TCA and boiled for 15 minutes at 100^0C and then cooled in running water. The solution was made upto 5 ml with TCA to compensate for the evaporation and then centrifuged. From this 2 ml of supernatant was taken into the test tube, 6 ml of concentrated H_2So_4 was added and the mixture was boiled for 10 minutes. The mixture was cooled and the optical density was measured at 520 nm. The standard graph was plotted with D-glucose (Analar supplied by B.D.H. Bombay) by the above method. The glucose obtained was converted into glycogen by the multiplication factor 0.98 *(Hawks 1951).*

3. Results & discussion

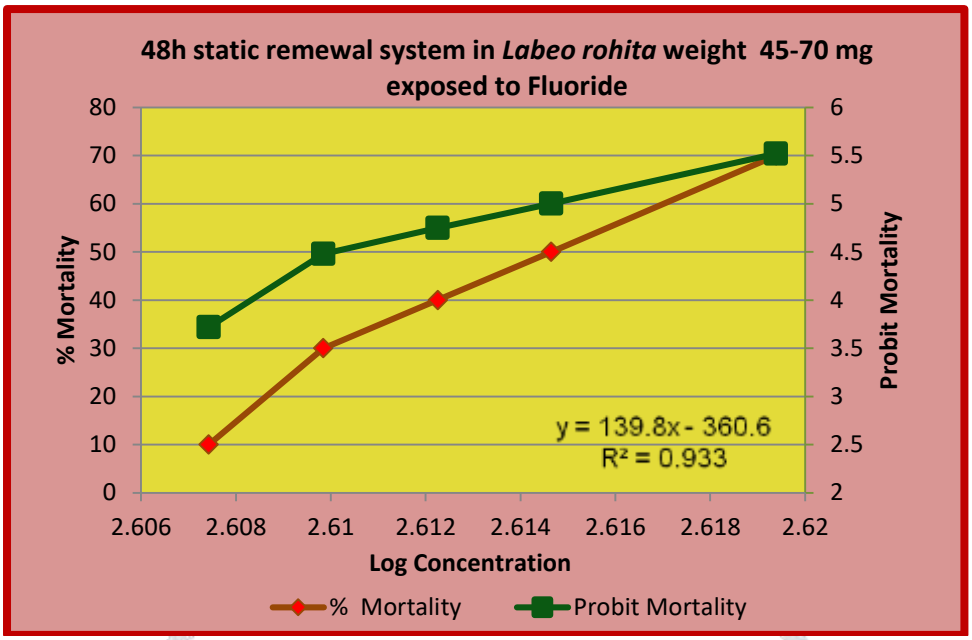
Fishes are considered as index organism for assessment of toxicological studies. Bioassay studies of any toxicant will give the idea of intensity of pollution of a given component. The results of the bioassay studies of *Labeo rohita* are as follows. The 24 h LC_{50} value 427.56 mg/l, and the 95% confidential limits 425.98 - 428.94, 48 h LC_{50} value 411.90 mg/l and 95% confidential limits 410.35 mg/l 412.64, 96 h LC_{50} value 374.82 mg/l and 95% confidential limits are 371.42 - 377.18 for fry stage of *Labeo rohita* respectively. For fingerling stage 24 h LC_{50} value is 334.29 mg/l and 95% confidential limits are 331.76 - 336.48. The regression equation and R^2 values were recorded graphically and represented as figures 24, 48, 96 hrs – fry graphs 1, 2, 3 and respectively and for 24 h fingerlings as graph 4.

Fig 1: Graphical representation of 24 h LC50 value in static renewal system for Sodium fluoride in *Labeo rohita* (fry)



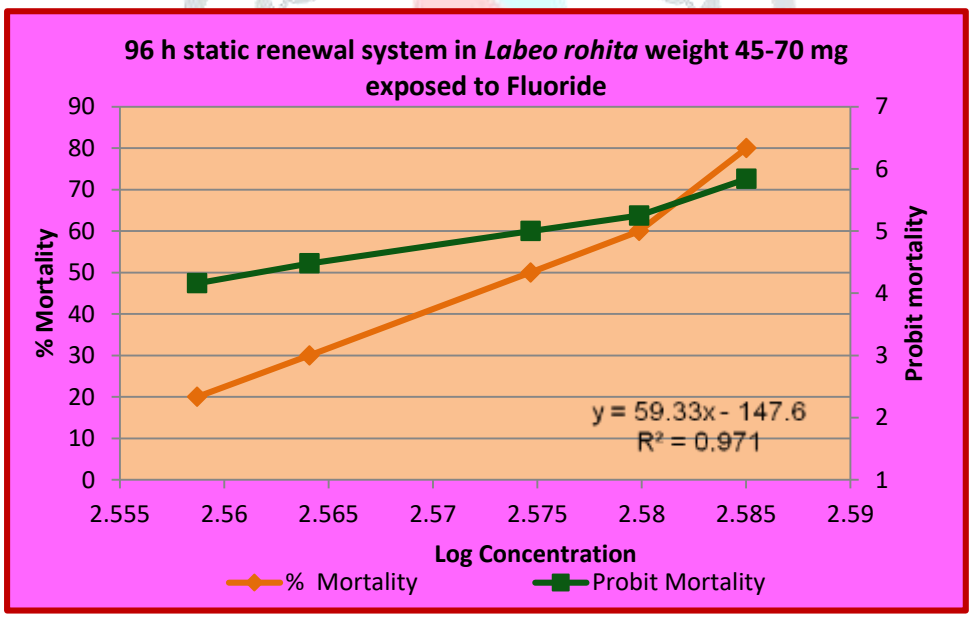
LC50=427.56 mg/L; 95% confidential limits: 425.48 – 428.94

Fig 2: Graphical representation of 48 h LC50 value in static renewal system for Sodium fluoride in *Labeo rohita* (fry)



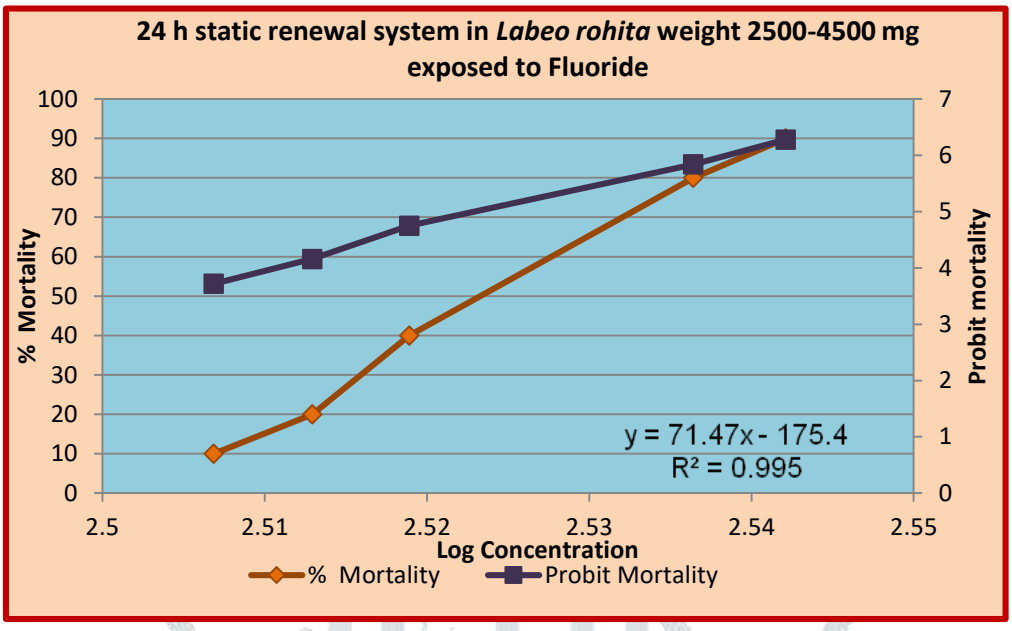
LC50=411.90 mg/L; 95% confidential limits: 410.35 – 412.64

Fig 3: Graphical representation of 96 h LC50 value in static renewal system for Sodium fluoride in *Labeo rohita* (fry)



LC50=374.82 mg/L; 95% confidential limits: 371.42 – 377.18

Fig 4: Graphical representation of 24 h LC50 value in static renewal system for Sodium fluoride in *Labeo rohita*(Fingerling)



LC50=334.29 mg/L; 95% confidential limits: 331.76 – 336.48

The resulted LC_{50} values showed that the toxicity increased to the fish when it is exposed to long duration. When exposed to 96 h the mortality resulted even in low concentration. In fingerling stage for 24 h the values were compared to fry stage, it was found the mortality resulted even in less concentration than 24 h LC_{50} value of fry. Hence the fingerling stage is found to be more susceptible than fry. It was found that the higher concentrations of fluoride inhibits the growth of fishes such as weight, length and of fingerlings of *Heteropneustis fossilis (Tripathi, et.al., 2005).* Effect was more pronounced in fingerlings in comparison to young and mature fish. (*Ellis et.al., 1948).* They reported that the fish egg exposed to 1.5 ppm of fluoride had delayed hatching time. *Shi et.al 2009* have reported the significant increase in fluoride concentration in bone and gill, cartilage of skin, of Siberian sturgeon when exposed to lethal dose. It was stressed that factors like temperature and hardness greatly effect fluoride toxicity (*John* and *Singler 2011)* and (*Pimental 1983*). Herbert and Shurben (1964) recorded the mortality in yearling rainbow trout at 8.5 mg/l fluoride. Acute toxicity of fluoride effluent in *Catla catla* fry was reported by Pillai and Mane (1985). *Catla* fry died whthin one hour at different concentrations of effluent i.e. 100%, 90%, 80%. They reported that 16 h LC_{50} value was 28.7 ppm. Beside high fluoride content the low p^H of the effluent also played major role in killing the fry. *Chitra et.al (1979)* reported LC_{50} of NaF for *Channa punctatus* bloch as 10 ppm. *Chitra et.al 1983* also reported LC_{50} value using mixtures of NaF and $HgCl_2$ at different concentrations was found to range from 3 ppm of $HgCl_2$ + 10 ppm of NaF to 5 ppm of $HgCl_2$ + 20 ppm of NaF. *Smith et.al (1985)* reported acute toxicity of fluoride to stickle back (*G. aculeatus*) fat headminnow (*P. promelas*) and juvenile rainbow trout. The results indicate that the medium lethal concentration varied with species and initial water hardness. *Pimental et.al (1983)* reported water hardness influence the toxicity of fluoride to rainbow trout *(S. gairdnerii)* and 96 h LC_{50} values increases from 51 to 193 mg/l as water hardness level rose from 17 to 385 mg/l $CaCo_3$.

Suvetha et.al (2015) reported in *Labeo rohita* the LC_{50} values for 24 h & 96 h are 0.438 and 0.38 mg/l respectively is static bioassay when exposed to pesticide toxicant deltamethrin. They opined that toxicant exerted toxic effect due to the high rate of gill absorption. It was reported by Vijayalakshmi (2018) in case of *Catla catla* the toxicity increases with increase in concentration. In case of *Labeo rohita* the fluoride is accumulated on exposure to sublethal concentrations for 15 days.

Fluoride induced changes in the behaviour of fresh water fishes *Tilapia mossambica* as reported from different experiments (*Aziz et.al 2014* and *Manna et.al 2007)* who observed the adverse effect due to fluoride toxicity induces enzyme inhibition, gastric damage and disruption of immune system. The physico-chemical factors such as temperature, p^H alkalinity and hardness also influence the toxicity *(John M. Neuhold et.al 2011).*

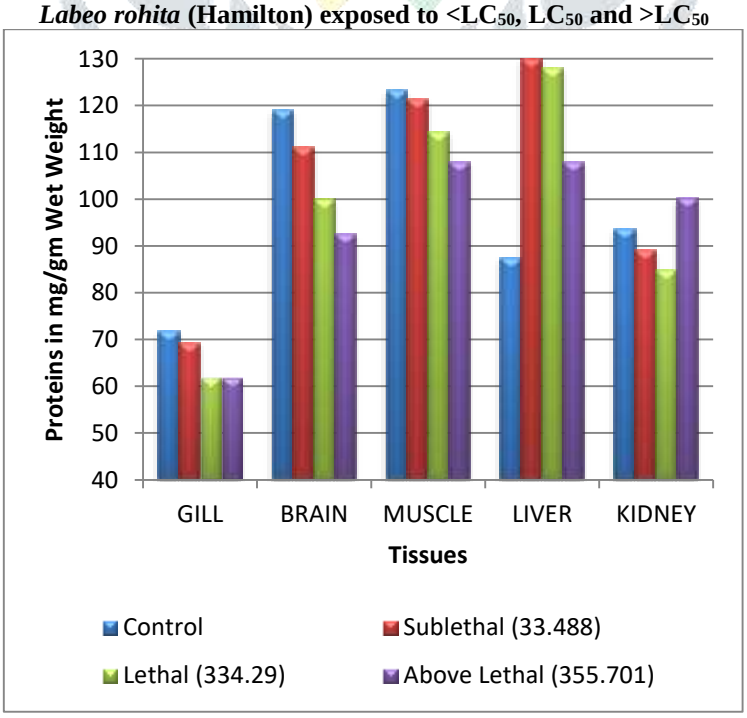
The above studies support the present study of fluoride toxicity on *Labeo rohita*. The fingerlings that is 6 to 8 cm size fish is found to be more sensitive than fry of size 2.5 to 3.5 cm.

But *Hemens et.al. (1972)* reported that larger fish are more tolerent in high concentration. In present study the LC_{50} values are high because the hardness of water is low and the p^H is also not normal. Sensitivity to fluoride is dependent on several factors such as size of the fish, temperature, calcium and chloride concentrations in the environmental medium. Further it was reported any increase and decrease of bio availability of micronutrient metals adversely affect the physiological activities of various organs *(Azmat et.al 2011)* and that leads to mortality of fish.

When the fish *Labeo rohita* was exposed to sublethal concentration i.e. (33.429 mg/l), 334.29 mg/l and above lethal concentration (355.701) for 24 hours some biochemical changes in the tissues were observed. The observed values for biochemical constituents of proteins and glycogen of different tissues and standard deviation along with the percentage change over the control are represented in graphs 5 and 6.

Graph – 5

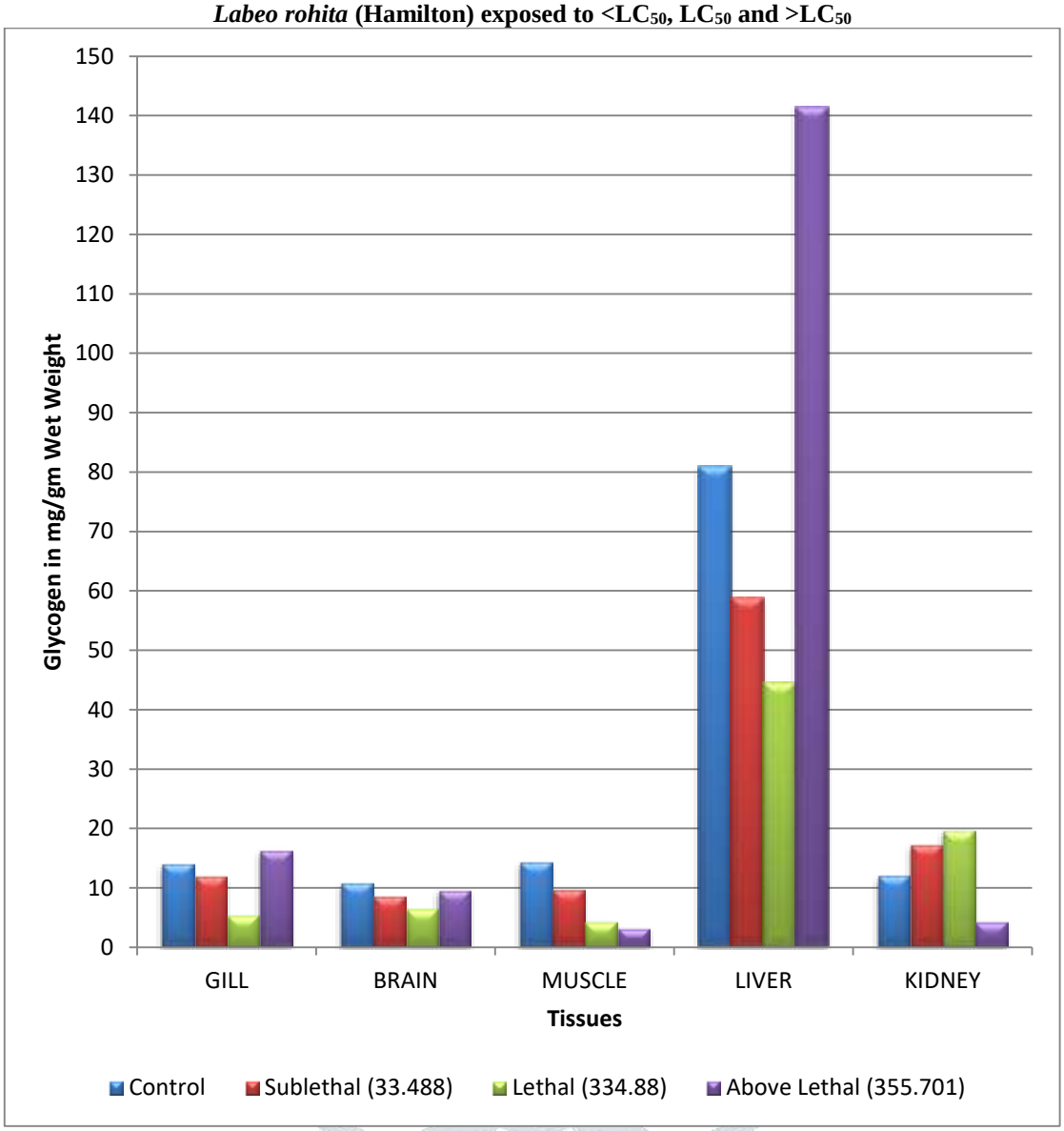
The Amount of Total Proteins mg/gm Wet Weight in Tissues of Fish *Labeo rohita* (Hamilton) exposed to <LC_{50}, LC_{50} and >LC_{50}



Results are the mean values of five determinations and the standard deviation, significant at p.0.05 level; "T" test

Graph – 6

The Amount of Total Glycogen mg/gm Wet Weight in Tissues of Fish *Labeo rohita* (Hamilton) exposed to <LC_{50}, LC_{50} and >LC_{50}



Results are the mean values of five determinations and the standard deviation, significant at p.0.05 level; "T" test

Total proteins

It was observed there is variation in the distribution of proteins, suggests the difference in metabolic calibers of various tissues. In liver the protein quantity is high as it is the seat for the synthesis of proteins and the controlling centre of metabolism. In the lyotropic series the decrement of protein exposed to sublethal concentration of fluoride is in the order of brain > kidney > gill > muscle. In the lethal concentration the decrement is brain > gill > kidney > muscle and in above LC_{50} concentration brain > gill > muscle. However there is increment of protein in liver in all concentrations. The decrease is significant at 0.05 level of 'T' test. The decrease is due to protein ketabolism as an alternative source of energy (Kabeer et.al., 1981). Due to rapid swimming activity in toxicant exposed fish there is more energy utilization from protein as it is the source of energy and lead to decrease of protein in muscle, gluconeogenesis.

The increase in protein is more in sublethal and lethal concentration and increase is not significant in above lethal concentration. The fish is trying to synthesize more protein as a source of energy to fight against the toxicant stress and it might also be due to alteration in enzyme activity. The increased levels in the kidney at above lethal concentrations is as the fish is not able to cope up with the toxicant stress and due to loss of ammonotelic nature, as a result of histopathological changes and tissue damage.

The decrement of protein in brain leads to impairment of brain function. The brain can not have the complete control on the other organs like gill, muscle, liver and kidney by which the failure in their physiological activity happens. The gills are important organs that fulfill the multiple functions including gas exchange, osmotic pressure regulation, acid base balance, ion transport. All these are related to gill chloride cells (Malatt and Stinson 1990). Once the gills are damaged the osmatic regulation functions could be affected resulting in physiological and histological changes in fish (Haque et.al., 2012). As a result of physiological changes the enzyme activity in muscles is also altered leading to decrement of protein. The increment in the liver is due to structural damage of liver that leads to supressed proteolytic enzyme activity (Aziz et.al., 2013) and also disturbance in metabolism (glucogenesis, glycogenolysis, gluconeogenesis).

The decreased trend of the protein content in gill, brain, muscle and kidney may be due to metabolic utilization of the keto acids for the alternative path way of synthesis of glucose, directing the free amino acids for the synthesis of proteins / for the maintenance of osmotic and ionic regulation *Schmidt (1975)*.

The fish *Labeo* is under toxicant stress. Under toxicant stress many organisms will mobilize proteins as a source of energy via the oxidation of amino acids. Decreased protein level may be attributed to stress mediated immobilization of these compounds to fulfil an increased element for energy by the fish to cope with the environmental condition created by toxicant. *(Jenkins et.al 2003)*. The depletion in the total protein content may be due to augumented proteolysis and possible utilization of their product for metabolic purposes as reported by *Ravinder et.al (1988)*. The decline in protein may also be related to impaired food intake, increased energy cost of homeostatis, tissue repain and detoxification mechanism during stress *Neff 1985)*.

The present reports are in congnisize with the previous reports. The decrease in protein content of muscle, testis, liver of fluoride exposed fish *Channa punctata* (bloch) was reported by *Gupta 2003* and in *Clarias batrachus* by *Kumar et.al (2007)*.

Among various tissues of the control animals higher glycogen level was observed in liver as liver is the organ of glycogenesis. Liver glycogen is largely concerned with storage. In *Labeo rohita* when exposed to sublethal concentration glycogen decrement was in the order of muscle > liver > brain > gill. When exposed to lethal concentration the decrement was in the order of muscle > gill > liver > brain. When the fish was exposed to above lethal concentration the decrement was in muscle > gill > brain. Whereas in kidney there is increment in sublethal and lethal concentration and decreased in above lethal concentration. In the present study there is decrease in muscle glycogen due to more activity of muscle which utilises more energy for rapid swimming activity as a result of toxicant stress which curtails feeding.

The liver is the main metabolic organ and plays an important role in the uptake, accumulation, biotransformation and excretion of toxic elements (Pedlar et.al., 2002). Histopathological changes are reported in liver by fluoride toxicity in *Cyprinus carpio* and *Channa punctatus* (bloch) by Haque et.al., 2012 include vacuolar degeneration and focal necrosis and nuclear pyknosis. Cao et. al., 2013. The liver glycogen is used as a source of energy by gluconeogenesis and leads to decreased levels of glycogen in liver. Aquatic animals generally depend on glycogen source for energy due to intoxication of trace metal fluoride for the maximum utility of their reserve food to combat adverse condition. Similar depletion of liver glycogen levels (glycolysis) was observed after methyl alcohol administration by Eletskii (1965), in fluoride treated rats. Depletion in the liver glycogen levels in the fresh water fish *Rasbora daniconius* exposed to paper mill effluent by Panthan et.al. (2009). Similar report of glycogen depletion in liver of *Tilapia mossambica* due to exposure to sublethal concentration of sodium fluoride was reported by Bagle et.al., (2015) and in fish *Labeo rohita* by Kale M.D. and Muley (2015). Increase in the kidney is due to disturbance in the excretory organs to combat with the toxicant stress and to supply more energy resources to fish. In the above lethal concentration the fish might be under severe toxicant stress leading to depletion of all energy resources and finally leading to kidney failure. Increased glycogen level in liver in above lethal concentration is due to disturbance of carbohydrate metabolism as it was observed to effect the enzymes of glycolytic pathway and krebs cycle leading to depletion and disturbance in cell membrane potential and ATP depletion. Schuliga et.al., (2012). The function of muscle glycogen is to act as readily available source of energy due to glycolysis *(Harper 1985)*. The depletion of glycogen content in liver and muscle of *C.mrigala* was reported by *Anitha (2010)*. The depletion was observed in fish exposed to both lethal and sublethal concentration of fenvalerate. In *Labeo rohita* the glycogen content of muscle remained unchanged with the control fish when exposed to fenvalerate.

Earlier reports that fluoride can induce many biochemical changes in mammals including rats, rabbits, goats and humanbeings *Chinoy et.al (1994), Chitra et.al (1983)* and *Kumar et.al (2007)* observed that fluoride affects the certain biomolecules and enzymes in different tissues of fresh water fish - *Channa punctata* and *Clarius batrachus* (Linn). A significant reduction of glycogen content was found in the muscle and testis at the lower concentration (35 mgF/l) but it increased in all three tissues at the higher concentration (75 mgF/l) when exposed to sodium fluoride *Kumar et.al (2007).* Fluoride induces changes in the behaviour of fresh water fishes reported from *Aziz et.al (2014)*. The decline in the liver and muscle suggests enhanced conversion of glycogen to glucose to meet an increased energy requirement under toxicant stress. Increased swimming activity of the fish during experiment supports this change due to toxic action.

Reported by *Helimeyer et.al (1970)* on exposure to technical grade fenvalerate caused changes in the glycogen content resulting in the disruption of enzymes associated with carbohydrate metabolism. *Aziz et.al (2013)* reported that fluoride increased the ALP, ALT and AST levels of enzyme activity in the gills of fresh water fish *Tilapia mossambica.* Increased level of these three biomarker enzymes are due to disturbances of carbohydrate and protein metabolism. All the earlier studies are in cognisize with the present study that is under toxicant stress there is disturbance in metabolism of proteins and glycogen the two important biomolecules.

3.1. Conclusion

Fresh water fish *Labeo rohita* fry and fingerlings were exposed to sodium fluoride and the LC_{50} values were determined. The fingerling stage was found to be more sensitive and the toxicity of fluoride is influenced by hardness of water i.e. in the medium. When the fish *Labeo rohita* was exposed to sublethal and lethal concentrations of fluoride the normal physiological function was effected. Biochemical changes of total proteins and glycogen were observed in different tissues of fish. There is significant decrease in glycogen content after acute exposure to NaF. Decrease in glycogen was more in muscle and gills in lethal concentrations, the gills are the first organ to come in contact with toxicant. Liver glycogen levels were affected as it is prime detoxifying organ with enzyme activity. Increased stress demands more energy which inturn leads to glycogen lysis to release glucose into blood.

In the present investigation, depletion of protein in muscle was found which curtails the growth of heterotrophic fish. The decrease may be due to proteolytic activity, rapid utilization of protein under stress condition as the sodium fluoride interefers in metabolic process i.e. protein synthesis in fish. The decrease in these biomolecules in heterotrophic fish, growth is curtailed and the venture of aquaculture is affected. Further research is required to examine the enzymatic analysis of fluoride exposed fish for chronic periods and also cellular level changes in fresh water fish.

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