TOXICOLOGICAL STUDIES OF PLANT NUTRIENT LIBREL™ ON TELEOST FISH

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CERTIFICATE

This is to certify that the thesis "TOXICOLOGICAL STUDIES OF PLANT NUTRIENT LIBREL™ ON TELEOST FISH" incorporate the results of investigation carried out by the candidate herself in the Department of Zoology, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara.

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CONTENTS

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Shalaka Sadekarpawar

LIST OF ABBREVIATIONS

µg/L	Microgram per liter
µmole	Microgram mole
^0C	Degree Celsius
A.W.W.A	American Water Works Association
AA	Ascorbic acid
ACP	Acid Phosphate
ALP	Alakaline phosphatase
ALT	Alanine transaminase
ANOVA	Analysis of Variance
APHA	American Public Health Association
APS	Ammonium Per sulphate
As	Arsenic
AST	Aspartate transaminase
B	Boron
BAF	Bioaccumulation factor
BCF	Bioconcentration factor
CAT	Catalase
Cd	Cadmium
Co	Cobalt
Cr	Chromium
Cu	Copper
DMRB	Design Manual for Roads and Bridge
DNPH	2,4-Dinitrophenylhydrazine
DPTA	Diethylene-triaminepenta acetate
DTNB	5,5'-DITHIO-BIS(2-NITROBENZOIC ACID)
EDTA	Ethylene diamine tetra acetic acid
EPA	Environmental Protection Agency
EU	European Commission
Fe	Iron
GSH	Reduced Glutathione
GSI	Gonado-Somatic Index
$H_2O_2^-$	Hydrogen Peroxide
Hb	Haemoglobin
Hct	Haematocrit
Hg	Mercury
HSI	Hepato-Somatic Index
Hsp	Heat shock proteins
K	Condition factor
KDa	Killo Dalton
K_n	Relative Condition factor
LC	Lethal concentrations
LCL	Lower Confidential limits
LP	Lipid Peroxides
LPO	Lipid Peroxidase
MANOVA	Multivariate Analysis of Variance
MCH	Mean Corpuscular Haemoglobin

MCHC	Mean Cell Haemoglobin Concentration
MCV	Mean Corpuscular Volume
MDA	Malondialdehyde
mg/L	Milligram per liter
mg/l	Milligram per litre
mmole	Milli mole
Mn	Manganese
Mo	Molybdenum
MPI	Metal Pollution index
MSDS	Material Sheet Datasheet Sheet
MT	Metallothionein
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitro Blue tertrazolium
Ni	Nickel
NOEL	NO Observable Effect Level
NPK	Nitrogen Phosphorus Potassium
OBF	Opercular beat frequency
OH^-	Hydroxyl radical
OUC	Ornithine Urea Cycle
Pb	Lead
PBS	Phosphate Buffer Saline
PCV	Packed Cell Volume
PCV	Packed cell volume
PMS	Phenazinemetho sulphate
PPM	Parts per million
RBC	Red Blood Corpuscles
Rm	Relative mobility
ROS	Reactive Oxygen Species
SASF	Stress activated serum factor
SD	Standard deviation
Se	Selenium
SOD	Superoxide dismutase
SPSS	Statistical Package for the Social Sciences
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid Reactive Substance
TBF	Tail beat frequency
TCA	Trichloracetic Acid
TEMED	N,N,N′,N′-Tetramethylethylenediamine
UCL	Upper Confidential limits
USEPA	United States Environmental Protection Agency
W.P.C.F	Water Pollution Control Federation.
WBC	White Blood Corpuscles
WHO	World Health Oraganization
Zn	Zinc

INTRODUCTION

All substances are poisons; there is none which is not a poison.
The right dose differentiates a poison from a remedy."

Paracelsus (1493-1541)

INTRODUCTION

Today more than 100 elements are known to man, out of whom less than 20 elements are essential for vigorous and healthy growth of plants. Essential elements are those which are important for plants to complete its life cycle by transforming photo energy to chemical energy synthesizing a whole variety of substances (White and Brown, 2010). When we talk about the nutrients required by the plants, then there are primary (nitrogen, phosphorus and potassium), secondary nutrients (calcium, magnesium and sulphur) and micronutrients (iron, copper, manganese, zinc and boron). There are 7 essential plant nutrient elements defined as micronutrients [boron (B), zinc (Zn), manganese (Mn), iron (Fe), copper (Cu), molybdenum (Mo), chlorine (Cl)] which are found in plant in relatively small amounts (Hochmuth *et al.*, 2010). A deficiency of any one of these obstructs its normal yields resulting in complete crop failure. Soils deficient in their ability to supply micronutrients to crops are alarmingly widespread across the globe (White and Zasoski, 1999; Kabata-Pendias, 2011). India which was dependent on external food supplies in the early 1960s has introduced high yielding cultivators in the form of fertilizers and micronutrients to meet the growing demands of food, fiber and fuel. This has lead to the exhaustion of the nutrients especially micronutrients in the form of trace elements (Gupta *et al.*, 2005).

Fertilizers containing trace elements (such as boron, copper, manganese, zinc, and cobalt) — in small quantities are called as micronutrient fertilizers. It is called *micronutrients* as they are needed only in minuscule amounts, these substances are the "magic wands" that enable the plants to produce enzymes, hormones and other substances essential for proper growth and development (Yoshida, 2008). They are distinguished according to trace element; there are also polymicronutrient fertilizers, which contain two or more trace elements. Other than these salts of trace elements, industrial wastes (slag or slurry), frits (alloys of salts with glass), and chelates (compounds of organic substances with metals, such as zinc and copper) are also used as

micronutrient fertilizers (Dar, 2004). The first experiments in Russia and abroad have shown beneficial effect of micronutrient fertilizers on the growth and development of plants in the 19th century. Among the countries that have made extensive use of micronutrient fertilizers (mainly after 1940) are the USA, Great Britain, France, Sweden, the Federal Republic of Germany, the German Democratic Republic, Poland, Bulgaria, Italy, and Japan (Sillanpaa, 1982).

Micronutrient fertilizers are specially formulated for delivering micronutrients with maximum bioavailability, tolerability, & safety. Micronutrients are available in two forms, chelated and nonchelated (Modaihsh, 1997). Most of the micronutrients are in chelated form, as they are absorbed quickly and easily by the crop thus providing effective organic nitrogen to overcome stress conditions and boosting up energy metabolism in the plant. Moreover it activates phytohormones and other growth substances in the crop increasing chlorophyll concentration and in turn boosting the photosynthetic activity maximizing growth and yield of crops like cotton, sugarcane, cereals, pulses, vegetables, fruit crops and floriculture. They are necessary building blocks that plants require needed in addition to NPK fertilizers (Ray *et al.*, 1993). The fertilizer effectively acts as a store from which the crops can extract nutrients as and when they need it. They mimic the natural form of nutrients in soils. Nutrient release is under the control of the plant itself (Ray *et al.*, 1997).

In the decade when attention is being directed towards improving the micronutrient density of staple foods, deficiencies in crops in the field continue to be reported and much remains to be done to ensure that micronutrient supply is not limiting crop yield. Katyal and Randhawa (1983) have studied the function, concentration and deficiency symptoms of the micronutrients on plants. Recently, B deficiency has been shown to be limiting for rice yield and grain quality on calcareous soils in the Punjab region of India (Rashid and Ryan, 2004). According to Stewart et al. (2005), at least 30- 50% of crop yield is attributable to commercial fertilizer (N, P, and K) inputs. Without micronutrient budget information such as those discussed by Yamada (2004) for soils, crops and fertilizers in Brazil, it is presently not possible to estimate the overall dependency of crop yield on micronutrients. Nevertheless, the extent of world soils that are low in micronutrients, especially Zn (Alloway, 2004), Fe and B (Shorrocks, 1987), suggests that micronutrients will continue to depress yield potentials in some areas unless they are managed for sustainable crop production. Alloway (2004) concludes that “it is highly probable that there

are several million hectares of paddy rice which could benefit from zinc fertilization". Moreover the loss of soil fertility remains a major concern in many parts of the world with losses ranging from 0.1 to 35 mm of topsoil per year. There is often a close relationship between DPTA extractable micronutrients (Cu, Mn and Zn) and organic carbon content of soils (Sharma *et al.*, 2004). Further, micronutrient cycling in soils is closely associated with organic matter turnover and crop residue management is important for maintaining the micronutrient balance of soils (Mythili *et al.*, 2003).

Micronutrient deficiencies are especially widespread in plants, animals, and human in many Asian countries due to the calcareous nature of soils, high pH, low organic matter, salt stress, continuous drought, high bicarbonates in irrigation water and imbalanced application of fertilizers. By producing more HCO_3^- and OH^- in the rhizosphere, the pH of the soil solution and, consequently, the pH of plant sap will increase enough to cause the precipitation of micronutrients, lowering their level of availability as a whole. Some of the adverse effects that will develop when plants are stressed with micronutrient deficiencies include lower crop yield and quality; imperfect plant morphological structure such as fewer xylem vessels with smaller size; increased bio and non-bio-stresses; widespread infestation of crops with various diseases and pests; low activation of phytosidrophores and lower fertilizer use efficiencies. The absence of micronutrient-fertilizers means inadequate absorption of trace elements by plants, which causes substantial yield losses in different crops, forages, and eventually results in poor health for domestic animals and humans. Hence, micronutrient deficiencies are common in our agricultural products (Malakouti, 2008).

The world population is expanding rapidly and will likely be 8 billion by the year 2025. Limited availability of additional arable land and water resources and the declining trend in crop yields globally makes food security a major challenge in the 21st century (Hinrichsen, 1998). According to the projections, food production on presently used land must be doubled in the next two decades to meet the food demand of the growing world population. To achieve the required huge increases in food production, greater emphasis in application of fertilizers and improvements of soil fertility are indispensable. Presently, in many developing countries, poor soil fertility, low levels of available mineral nutrients in soil, improper nutrient management, along with a lack of concern for plant genotypes having high tolerance of nutrient deficiencies or

toxicities are considered to be the major constraints contributing to food insecurity, malnutrition and ecosystem degradation (Malakouti and Balali, 2004).

India was dependent on external food supplies in the early 1960s. Micronutrient Fertility Mapping For Indian Soils have been well explored by Singh (2008) and have reported that the concentration of different micronutrients, variation in the fertility status of soils in different states of India varies from moderately low to very low. To meet the growing demand for food, fiber and fuel, high yielding cultivars were introduced. These high yielding crop cultivars were highly responsive to fertilizers. Thus, slowly the soils were exhausted of their nutrients. Application of major nutrients (nitrogen, phosphorus and potassium) became common, therefore the crops started responding to micronutrient fertilizers. Concerted efforts have been made through the All India Coordinated Research Project on Micronutrients to delineate the soils of India regarding the deficiency of micronutrients. At present about 48.1% of Indian soils are deficient in diethylene-triaminepentaacetate (DTPA) extractable zinc, 11.2% in iron, 7% in copper and 5.1% in manganese. Apart from the deficiency of these micronutrients, deficiencies of boron and molybdenum have also been reported in some areas. Areas with multi-micronutrient deficiencies are limited, thus simple fertilizers are sufficient to exploit the potential of crops and cropping systems. Based on the extent of deficiency, cultivated area, and crop removal, the micronutrient fertilizer demand in 2025 is projected using sufficiency and maintenance approaches (Gupta, 2005).

Trace elements mean elements present at low concentrations (mg/kg or less) in agroecosystems. Some trace elements, including copper (Cu), zinc (Zn), manganese (Mn), iron (Fe), molybdenum (Mo), and boron (B) are essential to plant growth and are called micronutrients. Except for B, these elements are also heavy metals, and are toxic to plants at high concentrations. Some trace elements, such as cobalt (Co) and selenium (Se), are not essential to plant growth but are required by animals and human beings. Other trace elements such as cadmium (Cd), lead (Pb), chromium (Cr), nickel (Ni), mercury (Hg), and arsenic (As) have toxic effects on living organisms and are often considered as contaminants. Trace elements in an agro-ecosystem are either inherited from soil parent materials or inputs through human activities. Soil contamination with heavy metals and toxic elements due to parent materials or point sources often occurs in a limited area and is easy to identify. Repeated use of metal-enriched chemicals, fertilizers, and

organic amendments such as sewage sludge as well as wastewater may cause contamination at a large scale. A good example is the increased concentration of Cu and Zn in soils under long-term production of citrus and other fruit crops. Many chemical processes are involved in the transformation of trace elements in soils, but precipitation-dissolution, adsorption-desorption, and complexation are the most important processes controlling bioavailability and mobility of trace elements in soils. Both deficiency and toxicity of trace elements occur in agro-ecosystems.

Indian agriculture is now in an era of multiple plant nutrient deficiencies. Nutrients like N, P, K, Zn, Mn, Mg, Mo, B, S and Cu are now of widespread practical importance from an application point of view. To meet this deficiency application of trace elements in the form of fertilizers or micronutrients have been used rampantly whereas remediation of soils contaminated with metals is not addressed (Zhenli *et al.*, 2005). Soil microorganisms are the first living organisms subjected to the impacts of metal contamination. Furthermore repeated use of such metal-enriched chemicals, fertilizers, and organic moieties cause contamination of aquatic ecosystem by surface runoff leading toxic effect to non target organisms especially freshwater fishes. A frequently overlooked agrochemical is the plant nutrients added for biofortification of the soil. These nutrient supplementations, though enhancing food production, can have disastrous effects on the aquatic ecosystem as they readily leach out in the surface run off. A classical example of this condition is the death of alligators in Lake Griffin, Florida in 2000. The sudden deaths of alligators were caused by blooms of algae *Cylindrospermopsis*. Such algal blooms result due to leaching of various plant nutrients used in nearby farms. After this incident it was realized that not only pesticides, but plant nutrients can have detrimental effects on the environment. Unlike pesticides, which directly kill the organism/s, these plant nutrients may boost the growth of one organism and cause imbalance in the ecosystem leading to extinction of one or more species.

Steady growth of crop yields during recent decades (in particular through the Green Revolution) compounded the problem by progressively depleting soil micronutrient pools. Farmers only apply micronutrients when crops show deficiency symptoms, while micronutrient deficiencies decrease yields even before symptoms appear. The resultant widespread and indiscriminate use of plant nutrients may have far reaching effects on organisms in the higher trophic levels of the food chain. Crops containing excess amounts of trace elements, when consumed by livestock and

humans can result in toxicity. The most toxic effects in livestock appear to be dependent upon different factors (Fraser *et al.,* 1986).

Zinc belongs to a class of microelements which are essential for organisms and plays a vital role in the physiology of living systems, higher concentrations can be toxic to organisms (Williams and Mount, 1965; Ambrose *et al.,* 1994) elevated levels of zinc in aquatic systems can be due to liquid effluent discharge, atmosphere deposition, the leaching of domestic sewage and metal bearing minerals insecticides (DWAF, 1996; Nussey, 1998). On a relative basis, surface drainage and atmospheric fallout are the most important inputs of zinc to aquatic environments (Spear, 1981). The toxicity of zinc to aquatic life is intensively investigated during the previous decades and a considerable amount of experimental data is compiled and reviewed (US EPA, 1980; Tuurala and Soivio, 1982; Somasundaram *et al.,* 1984; Larson and Hyland, 1987; Bagdonas and Vosyliene, 2006).

Iron is an objectionable constituent in water supplies for either domestic or industrial use. At certain concentrations, iron can also be toxic to aquatic life. The EPA red book (1976) recommended a criterion of 1.0 mg/l for freshwater aquatic life protection. The current water quality-based iron limits are derived from the implementation of general criterion (61.3(2) d"), which states that water, must be free from of any substance that is acutely toxic to human, animal or plant life. There are no EPA established acute or chronic criteria for iron. Furthermore, toxicity studies of iron on aquatic life are rare. Iron and other metals are known to get leached from the soil and rinsed into river systems (Ahtiainen 1992, Reynolds *et al.,* 1992, Vuori 1995) Compared to other metals, little is known about the toxicity of iron to fish. The effects of iron on fish have been studied primarily in connection with effluent discharges from industry and mining (Sykora *et al.,* 1972, Smith *et al.,* 1973, Lehtonen 1976, Smith and Sykora 1976, Vuorinen 1984, Grippo and Dunson 1996a, 1996b).

Manganese is a common constituent of point and nonpoint discharges from mining and smelting activities. Available data indicate that Mn is acutely toxic at relatively high aqueous concentrations, when compared with trace metals, and its toxicity is affected by water hardness with toxicity decreasing with increasing hardness. (Wepener *et al.,* 1992, Stubblefield *et al.,* 1997) examined the mechanism of toxicity of manganese to the banded tilapia (Tilapia

sparrmanii) in South Africa and found significant decreases in red blood cells, haemoglobin, mean cell volume, haematocrit, and white blood cells.

Copper

Copper is a micronutrient and toxin. It strongly adsorbs to organic matter, carbonates and clay, which reduces its bioavailability. Copper is a toxic metal commonly used in certain herbicides, especially copper sulfate (bluestone) which is frequently used to control algae. Copper will bioconcentrate in many different organs in fish and molluscs (Owen, 1981). Exposure of fishes to sublethal concentrations of Copper reduced food intake, gonadosomatic index and fertility while opercular beat and oxygen consumption registered an increase (James *et al.,* 2003). Its toxicity to fish varies with the species and the physical and chemical characteristics of the water. Even at recommended rates of application, this material may be poisonous to trout and other fish, especially in soft or acid waters. Its toxicity to fish generally decreases as water hardness increases (Pimental, 1971).

Boron

Boron is widely distributed in the environment, comprising about 0.001% of the earths' crust (Budavari *et al.,* 1996). Boron is an essential plant nutrient, but at relatively low concentrations it becomes toxic to plant growth (Reid and Davies, 1989). Throughout the world, boron has received broad attention. In some marine aquatic environments, mainly in India, China and in England, the boron level has been extensively studied by many investigators (Shirodkar *et al.,* 1982; Liddicoat *et al.,* 1983; Shirodkar and Sankaranarayanan, 1984; Satyanarayana *et al.,* 1989; Shirodkar and Singbal, 1992; Imai *et al.,* 1994; Nasnolkar *et al.,* 1997). A number of studies have considered boron as a tracer for anthropogenic pollutants (Blume *et al.,* 1980; Narvekar *et al.,* 1983; Tartari and Camusso, 1988; Neal *et al.,* 1998). Toxicological Data of Boron toxicity are available for Rainbow Trout and Gold fish (Birge and Black, 1977; Black *et al.,* 1993)

All the above studies suggest that the trace elements either alone or in various combinations do show the toxicity. Plant nutrients are known to be available in various trade names. One of them is the Librel which because of its stability, solubility and its compatibility with wide range of herbicides, fungicides, insecticides and other crop care products has been used extensively. **Librel™,** rapidly soluble chelated micronutrient mixture, manufactured by DuPont is used in

most of the agricultural, horticultural fruit crops to correct nutrient deficiency. It's stability and solubility in water leads to rapid crop absorption and optimum biological performance. Though its solubility is useful to the agricultural crops, it is easily carried to the natural water bodies with agricultural run offs ultimately impairing the non-targeted aquatic biota, including fishes. There are no toxicity reports available in fish or other aquatic organisms for Librel, a plant nutrient. According to EU regulations it is classified as irritant and known to irritate eyes and respiratory system when brought to contact in human beings. However, there are no ecological toxicity data available for the same.

Moreover, the studies conducted till date have been focused on the metal toxicity, but their role in minute quantities through the fertilizers by way of plant nutrients are not well documented. As fish production had always been associated with agriculture, it is mandatory to explore and understand the effects of plant nutrient supplementation on fish health so that one can take remedial actions to improve fish health, both in terms of ecology as well as economics. Hence, the aim of the present work is to look into the toxicity of the plant nutrient Librel TM on edible fishes.

Fish as a model of study

Fishes are major part of the human diet and it is therefore not surprising that numerous studies have been carried out on metal pollution in different species of edible fish. Predominantly, fish toxicological and environmental studies have prompted interest in the determination of toxicity elements in seafood (Begum *et al.,* 2009)Fish are ideal sentinels for behavioral assays of various stressors and toxic chemical exposure due to their: (1) constant, direct contact with the aquatic environment where chemical exposure occurs over the entire body surface; (2) ecological relevance in many natural systems (Little *et al.,* 1993); (3) ease of culture; (4) ability to come into reproductive readiness (Henry and Atchison, 1986), and (5) long history of use in behavioral toxicology. Alterations in fish behavior, particularly in non-migratory species, can also provide important indices for ecosystem assessment.

Ideally, test organisms should have the following characteristics: (1) high ecological relevance; (2) susceptibility to the stressor(s) in question, both in the field and in the laboratory; (3) have wide geographical distributions; (4) be easy to culture and maintain under laboratory conditions;

(5) have relatively high reproductive rates and, should have relatively early maturation and easy fertilization in order to produce sufficient numbers of organisms of the proper age and size for testing; (6) have environmental relevance to the potential exposure (have been exposed to the test contaminant in the wild); and (7) have the ability to yield reproducible data under controlled laboratory conditions (Kane, 2005).

Tilapia fish were selected as a research fish model because these fish were easily produced and economically important. Fish are known by their tendency to localize significant amounts of metals. They absorb metals from water through gills, skin and digestive tract. Bioconcentration and biomagnification for heavy metals were previously reported by many authors (Saeed, 2000). This study aims to describe the clinical signs, postmortem lesions and histopathological changes due to nickel toxicity beside estimation of alteration in hematological, biochemical parameters and bioaccumulation and distribution of nickel in Nile tilapia organs.

Since invertebrates are major food resource for fish, they constitute an important link in nickel transport chain to fish (Wong *et al.,* 1991). Also it induce decrease in body weight of *Oreochromis niloticus* fish (El-Saieed and Mekawy, 2001)

Objectives

- To evaluate the species specific acute response and observe the morphological and behavioural alterations on exposure to the toxicant to understand their mode of action on its health and survival.
- To study the effect of plant nutrient on hematological and biochemical parameters of teleost fish with particular reference to duration of the exposure of the plant nutrient.
- To characterize the effects of the plant nutrient on anti-oxidant and lipid peroxidation to test the hypothesis that the toxicity of plant nutrient may be mediated by oxidative stress.
- To evaluate the histological alteration in the kidney, liver and gills as a result of sub-chronic intoxication with the plant nutrient.
- To evaluate the trace element concentration in kidney, liver, muscle and gills due to sub-chronic exposure of plant nutrient in both the species.
- To evaluate proeotoxicity studies in muscle, liver and gills of both species.

Fish metal bioaccumulation is largely due to the differences in the uptake and depuration period (Tawari Fufeyin and Ekaye, 2007) along with the abiotic factors (Kargin, 1996). Various studies have been recorded portraying their toxic effect especially with reference to oxidative stress (Robert *et al.*, 2001; Farombi *et al.*, 2007) bioaccumulation (Vinodhini and Narayanan, 2008 and more references Hontela) and reproductive aspect (Sadekarpawar and Parikh, 2013) of trace metals on various fishes.

Trace elements are recognized as potent toxicants causing oxidative stress in the exposed animals through wide spectrum of mechanisms (Elbekai and El-Kadi, 2005; Benedetti *et al.*, 2007) by increasing formation of ROS which damage proteins, DNA and lipids (Frenzilli *et al.*, 2001 and Regoli *et al.,* 2004), enzyme inhibition, impairment of cell signalling and calcium homeostasis, changes in gene regulation and physiological alterations (Stohs and Bagchi, 1995; Elbekai and El-Kadi, 2005). The intracellular fate of metal ions in terms of is detoxification or the storage depends strongly on thiol-containing molecules, particularly reduced glutathione (GSH) and metallothioneins (MT) (Eaton *et al.,* 1980) which provide the platform for the binding of metals in the form of sulphydryl cysteine groups (Mason and Jenkins, 1995; Maracine and Segner, 1998). Tripeptide glutathione (γ-L-glutamyl-L-cysteinylglycine) is the most abundant low-molecular-weight thiol-containing molecule in the cell acting as a free radical scavenger in important non-enzymatic antioxidant processes (Belcastro *et al.,* 2009). Further studies by Fabrik *et al.,* (2008) have reported that various detoxification systems particularly stress induced molecules are present in the form of extra proteins called metallothioneins (MTs) are abundant throughout the whole animal kingdoms. Thus, MTs are a group of low molecular mass (6,000–7,000 Da) single-chain proteins, containing about 25–35% cysteine having have high binding capacity for metals (Ryan and Hightower, 1994). A difference in metal accumulation and MT levels varies at the species and organ level (De Boeck *et al.,* 2003). Its level is known to be high in gills, liver, kidney and intestine which are involved in the metal uptake, storage and excretion (Hogstrand and Haux, 1991; Roesijadi and Robinson, 1994; Viarengo *et al.,* 2007), moderate to negligible in muscle (Wang and Rainbow, 2010) and least in blood (Kito *et al.,* 1982b). MTs in its isoforms are present in trace amounts in cells for maintaining the homeostasis of copper and zinc and the protection of cells against oxidative damage (Adam *et al.,* 2008)

Librel, an EDTA chelated micronutrient mixture (Zn, Mn, Fe, Cu and B) is used extensively in Gujarat. Subchronic effect on the reproductive and hepatic indices as well as histological alterations have been documented on *O. mossambicus* (Sadekarpawar and Parikh, 2013) and *L. rohita* (unpublished data). However, studies regarding its metallotionien expression are lacking. Hence the present study is done with an objective to report the expression of the metallothionein in *Labeo rohita* exposed to a trace element mixture in three tissues i.e. liver, gills and muscle.

CHAPTER I

Acute Toxicity And Behavioral Responses Of Plant Nutrient To *Orechromis mossambicus* And *Labeo rohita*: A Comparative Study

INTRODUCTION

The widespread use of agrochemicals not only brought adverse influence on agro ecosystems but also caused alteration in physiological processes of non-target organisms. They through surface runoff reaches to the unrestricted areas like ponds and rivers which alters the physicochemical properties of water and is toxic to aquatic organism and cause deleterious effect or even death to the aquatic animals. In many countries, large scale mortality of fishes has been recorded due to pesticides in water bodies as pollutants. Insecticides, fungicides and herbicides constitute the major source of potential environmental hazards not only to birds, fish, and other animals but also to humans when they become part of food chains (Abd-Alla *et al*., 2002; Oruce and Usta, 2007). Long term exposure to these products causes countless abnormalities and reduces the life span of organisms (Hussain *et al*., 2011; Naz *et al*., 2011).

The toxicity study is essential to find out toxicants limit and safe concentration, so that there will be minimum harm to aquatic fauna in the near future. Among the several aspects of toxicity studies, the bioassay constitutes one of the most commonly used methods in aquatic environmental studies with suitable organisms. The necessity of determining the toxicity of substances to commercially aquatic forms at the lower level of the food chain has been useful and accepted for water quality management. Several studies have assessed the toxicity of various pesticide to the aquatic biota especially fishes (Vasait and Patil, 2005; Susan Anita *et al.*, 2010; Parikh *et al.*, 2010; Desai and Parikh, 2013; Sadekarpawar and Parikh, 2013). The wide use of fishes is probably due to their adaptability to the laboratory conditions as well as their availability and their varying degree of sensitivity to the toxic substance. Fish is usually affected by toxicants in aquatic environment. The moment effects on the exposed fish is well pronounced, abnormal behavior such as incessant gasping for air, backward swimming and secretion of mucus on the skin of fish would set in (Omitoyin *et al*., 2006). Furthermore, fish appear to possess the same biochemical pathways to deal with the toxic effects of endogenous and exogenous agents as do mammalian species (Lackner, 1998). It is important to examine

the toxic potential of pesticides on fish since they constitute an important link in food chain and their contamination by pesticides imbalances the aquatic system.

The acute toxicity of agrochemicals on fish has involved the determination of the LC50, which is the concentrations that kill 50% of group fish under specified conditions. Toxicity testing is an essential tool for assessing the effect and fate of toxicants in aquatic ecosystems (Shazili *et al.,* 2006; Adams and Rowland, 2003; Luoma and Rainbow, 2008). Acute toxicity tests are short-term tests designed to measure the effects of toxic agents on aquatic species during a short period of their life span (Ebramhimpour *et al.,* 2010).

Behaviour is usually a very complicated phenomenon through which the animal capable of adjusting it various functions to a constant or changing environment. In aquatic toxicology however, the nexus of behavioral sciences with the study of toxicants has only become prominent within the last 5 decades (Kane *et al.,* 2005). Physiologists have mainly approached this phenomenon by starting at the bottom with detailed studies of relatively simple behavioural components i.e. reflexes locomatory or vegetative automatisms and influence of definite parts of the nervous system or endocrine system (Kamble *et al.,* 2011). Behaviour is a highly structured and predictable sequence of activities designed to ensure maximal fitness and survival of the individual, behavioural endpoints serve as valuable tools to discern and evaluate effects of exposure to environmental stressors, and they integrate endogenous and exogenous factors linking biochemical and physiological processes, thus providing insights into individual- and community-level effects of environmental contamination (Brewer *et al.*, 2001; Vogl *et al.,* 1999).

Behavior allows an organism to adjust to external and internal stimuli in order to the best meet the challenge of surviving in a changing environment. Conversely, behaviour is also the result of adaptations to environmental variables. Thus, behaviour is a selective response that is constantly adapting through direct interaction with physical, chemical, social and physiological aspects of the environment. In the aquatic environment the concentration, transport, transformation and disposition (fate) of a toxicant are primarily controlled by (1) the physical and chemical properties of the compounds, (2) the physical, chemical and biological properties of the ecosystem, and (3) the sources and rate of input of the chemical into the environment. Various characteristics such as temperature, pH, hardness, alkalinity, free CO_2, chloride and dissolved oxygen of water may change the toxicity of water pollutants. Many researchers have studied the behavioural responses on the agrochemical exposure (Henry and Atchinson, 1986; Rao *et al.*, 2005; Prasanth *et al.,* 2005; Parikh *et al.,* 2010; Nagaraju *et al.,* 2011; Chawanrat *et al.,* 2007; Pestana *et al.,* 2007).

The purpose of this investigation was to evaluate the comparative acute toxicity of a chelated micronutrient Librel for the two fresh water teleosts fish in a static renewal bioassay. Furthermore, the behavioural responses to the micronutrient mixture exposure on fish was also taken into account along with physicochemical parameters so as to have an insight into species specific differences if any of two freshwater teleosts, *O.mossambicus* and *L.rohita*.

MATERIALS AND METHODS

Healthy specimens of freshwater fishes *O.mossambicus* and *L.rohita* were brought from the local pond of Baroda district. Fishes were acclimated for the period of 10 days in well aerated 40 L aquaria containing dechlorinated water. Fishes were daily fed with commercial fish food, *ad libitum* during the acclimation period. Temperature (27±2°C), dissolved oxygen (3.9 ± 0.02 mg/L) and pH (7.1±0.5) were monitored constantly during the experiments. In order to obtain information about the range of concentrations to be used in the main test, a series of range-finding tests were run on the micronutrient mixture. Acclimated fish were not fed 24 h before the start of the test to maintain their catabolic and anabolic physiological state as suggested by APHA (2005).

Healthy fishes having following weight and length, *O. mossambicus* (12 ± 2 cm, 25 ± 1.9 g) and *L. rohita* (20± 2 cm, 125 ± 5 g) were selected for the test (n=10) to determine the LC_{50} value of each fish. Based on the pilot experiments, the experiments were conducted to determine the toxicity in different concentrations. The concentrations used included 4600 mg/l, 4700 mg/l, 4800 mg/l, 4900 mg/l, 5000 mg/l, 5100 mg/l, 5200 mg/l, 5300 mg/l, 5400 mg/l and 5500 mg/l for O.mossambicus and 5600 mg/l, 5700 mg/l, 5800 mg/l, 5900 mg/l, 6000 mg/l, 6100 mg/l, 6200 mg/l, 6300 mg/l, 6400 mg/l and 6500 mg/l for *L.rohita* with three replicates each. Observations were made and recorded every 12 hours until the end of 48 hours. Test species were considered dead if they showed no movement at all. The experiments were carried out in static non-renewable systems. The results of the experiments were analyzed by linear regression probit analysis method (Finney, 1971) using SPSS software (version 21) computer program. Values were calculated using the regression line obtained by plotting the concentration against the death percentage on a probit scale, and the results were evaluated with probit analysis The 95% confidence levels for the LC50 values were obtained by Finney's method were calculated using the formula given by Mohapatra and Rengarajan (1995).

RESULTS

The physicochemical characteristics of the water maintained in the experiments are listed in Table I. Temperature ranged from 27 to 36°C during experimentation. The pH of the water ranged from 7.5 to 9, which was slightly higher than neutral. Dissolved oxygen ranged from 17 to 23 mg/L. The toxicant exposure has shown the changes in the physicochemical characteristics; pH showed an increase while dissolved oxygen, free CO_2 and chloride content was decreased. The main objective of this study was to compare the susceptibility of two freshwater fishes to plant nutrient. The observed percentage of mortality of *O. mossambicus* and *L.rohita* for plant nutrient in static tests continued for different hours and different concentrations are shown in Tables II-VII. The observed LC values and 95% confidence limits in static tests are shown in Table VI & VII The 48 hours median lethal concentration (LC_{50} values) reported were 5083.77 and 5997.82 $mg.l^{-1}$ with the line of best fit 26.9x – 99.7 and 42.63x – 1.61E2 respectively for *O.mossambicus* and *L.rohita* (Fig. I and II). The results indicated that *O.mossambicus* was more sensitive to plant nutrient compared to *L.rohita.*

The behavioral responses in the fish were species specific varied in accordance with the test concentrations. No mortality or morphological changes were observed in the control experiment for the 48 hr acute toxicity test. Fishes in the control experiment appeared active and healthy throughout the test period. Compared to control reduced activity was exhibited during early hours of exposure at all the concentrations in the both the species. Most of the fish which died during the experiment exhibited symptoms of poisoning such as change in colour as well as behaviour. Initially decreased swimming activity, restlessness, jerky movements, hyper secretion of mucus, opening of mouth for gasping, losing scales, decreased abnormal hyperkinetic activity, loss of equilibrium by swimming sideways, finally fishes collapsed and died at higher concentrations. This behavioural abnormality was more prominent in *O.mossambiccus* compared to *L.rohita.*

Table I: Physicochemical analysis of water used for the experiments

Sr. No.	Parameters	Range
1	Temperature (^{0}C)	27 – 35
2	pH	7.5 – 9
3	Alkalinity (mg/l)	150 – 186
4	Hardness (mg $CaCO_3$)	304 – 423
5	Free CO_2 (mg/l)	2.1 – 3.3
6	Chloride (mg/l)	6 – 12
7	Dissolved oxygen (mg/l)	17.5 – 22.3

Table II Parameter Estimates of Probit analysis for *O. mossambicus* and *L. rohita*

Fish Species	Parameter (Probit[a])	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
O. mossambicus	**Concentration (mg/l)**	25.692	5.827	4.409	.000	14.271	37.114
	Intercept	-95.221	21.584	-4.412	.000	-116.804	-73.637
L. rohita	**Concentration (mg/l)**	45.434	8.096	5.612	.000	29.567	61.302
	Intercept	-171.651	30.598	-5.610	.000	-202.249	-141.053

a. PROBIT model: PROBIT(p) = Intercept+BX (Covariates X are transformed using the base 10.000 logarithm.)

Table III Chi-Square Tests for 48hr LC_{50} value of *O.mossambicus* and *L.rohita*

Fish Species	Probit	Chi-Square	Df[b]	Sig.
O. mossambicus	Pearson Goodness-of-Fit Test	1.313	8	.995[a]
L. rohita	Pearson Goodness-of-Fit Test	2.072	8	.979[a]

a. Since the significant level is greater than .150, no heterogeneity factor is used in the calculation of confidence limits.

b. .Statistics based on individual cases differs from statistics based on aggregated cases.

Table IV Log concentration, observed responses in fish *O. mossambicus*

Number (Probit)	Concentration (mg/l)	Number of Subjects	Observed Responses	Expected Responses	Residual	Probability
1	3.663	10	1	1.323	-.323	.132
2	3.672	10	2	1.906	.094	.191
3	3.681	10	3	2.608	.392	.261
4	3.690	10	4	3.406	.594	.341
5	3.699	10	4	4.265	-.265	.426
6	3.708	10	5	5.142	-.142	.514
7	3.716	10	6	5.996	.004	.600
8	3.724	10	6	6.789	-.789	.679
9	3.732	10	7	7.496	-.496	.750
10	3.740	10	9	8.100	.900	.810

Table V Log concentration, observed responses in fish *L.rohita*

Number (Probit)	Concentration (mg/l)	Number of Subjects	Observed Responses	Expected Responses	Residual	Probability
1	3.748	10	1	.878	.122	.088
2	3.756	10	2	1.575	.425	.157
3	3.763	10	3	2.541	.459	.254
4	3.771	10	3	3.728	-.728	.373
5	3.778	10	5	5.029	-.029	.503
6	3.785	10	5	6.305	-1.305	.631
7	3.792	10	8	7.435	.565	.743
8	3.799	10	8	8.339	-.339	.834
9	3.806	10	9	8.998	.002	.900
10	3.813	10	10	9.437	.563	.944

Table VI Confidence Limits for fish *O.mossambicus* at different concentrations

Probit	95% Confidence Limits for Concentration (mg/l)			95% Confidence Limits for log(Concentration (mg/l))[a]		
	Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
LC_1	4127.053	3498.075	4406.250	3.616	3.544	3.644
LC_2	4229.122	3653.887	4483.221	3.626	3.563	3.652
LC_3	4295.186	3756.101	4533.023	3.633	3.575	3.656
LC_4	4345.563	3834.718	4571.034	3.638	3.584	3.660
LC_5	4386.976	3899.758	4602.333	3.642	3.591	3.663
LC_6	4422.536	3955.879	4629.265	3.646	3.597	3.666
LC_7	4453.952	4005.657	4653.118	3.649	3.603	3.668
LC_8	4482.271	4050.671	4674.681	3.651	3.608	3.670
LC_9	4508.182	4091.966	4694.473	3.654	3.612	3.672
LC_{10}	4532.165	4130.270	4712.856	3.656	3.616	3.673
LC_{15}	4632.829	4291.607	4790.973	3.666	3.633	3.680
LC_{20}	4714.426	4422.386	4856.097	3.673	3.646	3.686
LC_{25}	4785.572	4535.457	4915.161	3.680	3.657	3.692
LC_{30}	4850.379	4636.536	4971.948	3.686	3.666	3.697
LC_{35}	4911.215	4728.369	5029.238	3.691	3.675	3.702
LC_{40}	4969.648	4812.162	5089.524	3.696	3.682	3.707
LC_{45}	5026.845	4888.366	5155.153	3.701	3.689	3.712
LC_{50}	5083.777	4957.405	5228.081	3.706	3.695	3.718
LC_{55}	5141.354	5020.256	5309.606	3.711	3.701	3.725
LC_{60}	5200.526	5078.561	5400.518	3.716	3.706	3.732
LC_{65}	5262.401	5134.330	5501.690	3.721	3.710	3.740
LC_{70}	5328.406	5189.677	5614.782	3.727	3.715	3.749
LC_{75}	5400.563	5246.855	5742.957	3.732	3.720	3.759
LC_{80}	5482.065	5308.639	5892.048	3.739	3.725	3.770
LC_{85}	5578.619	5379.282	6073.311	3.747	3.731	3.783
LC_{90}	5702.525	5467.265	6311.908	3.756	3.738	3.800
LC_{91}	5732.863	5488.469	6371.232	3.758	3.739	3.804
LC_{92}	5766.003	5511.507	6436.417	3.761	3.741	3.809
LC_{93}	5802.664	5536.853	6508.976	3.764	3.743	3.814
LC_{94}	5843.884	5565.191	6591.106	3.767	3.745	3.819
LC_{95}	5891.253	5597.568	6686.185	3.770	3.748	3.825
LC_{96}	5947.397	5635.706	6799.816	3.774	3.751	3.832
LC_{97}	6017.152	5682.767	6942.381	3.779	3.755	3.842
LC_{98}	6111.148	5745.683	7136.849	3.786	3.759	3.854
LC_{99}	6262.286	5845.801	7455.062	3.797	3.767	3.872

a. Logarithm base = 10.

Table VII Confidence Limits for fish *L.rohita* at different concentrations

Probit	95% Confidence Limits for Concentration (mg/l)			95% Confidence Limits for log(Concentration (mg/l))[a]		
	Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
LC_1	5330.789	4983.009	5513.939	3.727	3.697	3.741
LC_2	5404.945	5088.446	5572.308	3.733	3.707	3.746
LC_3	5452.529	5156.299	5609.876	3.737	3.712	3.749
LC_4	5488.601	5207.807	5638.445	3.739	3.717	3.751
LC_5	5518.118	5249.985	5661.898	3.742	3.720	3.753
LC_6	5543.367	5286.071	5682.027	3.744	3.723	3.755
LC_7	5565.600	5317.843	5699.812	3.746	3.726	3.756
LC_8	5585.583	5346.388	5715.853	3.747	3.728	3.757
LC_9	5603.819	5372.421	5730.545	3.748	3.730	3.758
LC_{10}	5620.658	5396.440	5744.163	3.750	3.732	3.759
LC_{15}	5690.916	5496.308	5801.656	3.755	3.740	3.764
LC_{20}	5747.380	5575.849	5848.963	3.759	3.746	3.767
LC_{25}	5796.268	5643.810	5891.102	3.763	3.752	3.770
LC_{30}	5840.525	5704.224	5930.578	3.766	3.756	3.773
LC_{35}	5881.837	5759.274	5968.959	3.770	3.760	3.776
LC_{40}	5921.309	5810.255	6007.414	3.772	3.764	3.779
LC_{45}	5959.750	5858.003	6046.926	3.775	3.768	3.782
LC_{50}	5997.825	5903.125	6088.387	3.778	3.771	3.785
LC_{55}	6036.144	5946.158	6132.644	3.781	3.774	3.788
LC_{60}	6075.331	5987.689	6180.540	3.784	3.777	3.791
LC_{65}	6116.101	6028.437	6233.004	3.786	3.780	3.795
LC_{70}	6159.362	6069.319	6291.220	3.790	3.783	3.799
LC_{75}	6206.392	6111.551	6356.940	3.793	3.786	3.803
LC_{80}	6259.184	6156.872	6433.073	3.797	3.789	3.808
LC_{85}	6321.286	6208.143	6525.048	3.801	3.793	3.815
LC_{90}	6400.302	6271.183	6644.845	3.806	3.797	3.822
LC_{91}	6419.534	6286.251	6674.378	3.808	3.798	3.824
LC_{92}	6440.493	6302.572	6706.704	3.809	3.800	3.827
LC_{93}	6463.617	6320.470	6742.533	3.810	3.801	3.829
LC_{94}	6489.541	6340.413	6782.888	3.812	3.802	3.831
LC_{95}	6519.235	6363.114	6829.339	3.814	3.804	3.834
LC_{96}	6554.295	6389.745	6884.473	3.817	3.805	3.838
LC_{97}	6597.655	6422.455	6953.061	3.819	3.808	3.842
LC_{98}	6655.740	6465.941	7045.576	3.823	3.811	3.848
LC_{99}	6748.328	6534.610	7194.409	3.829	3.815	3.857

a. Logarithm base = 10.

Fig. I Linear relationship between probit mortality and logarithm concentration of plant nutrient concentrations to which *O. mossambicus* was exposed during the 48 hours exposure period.

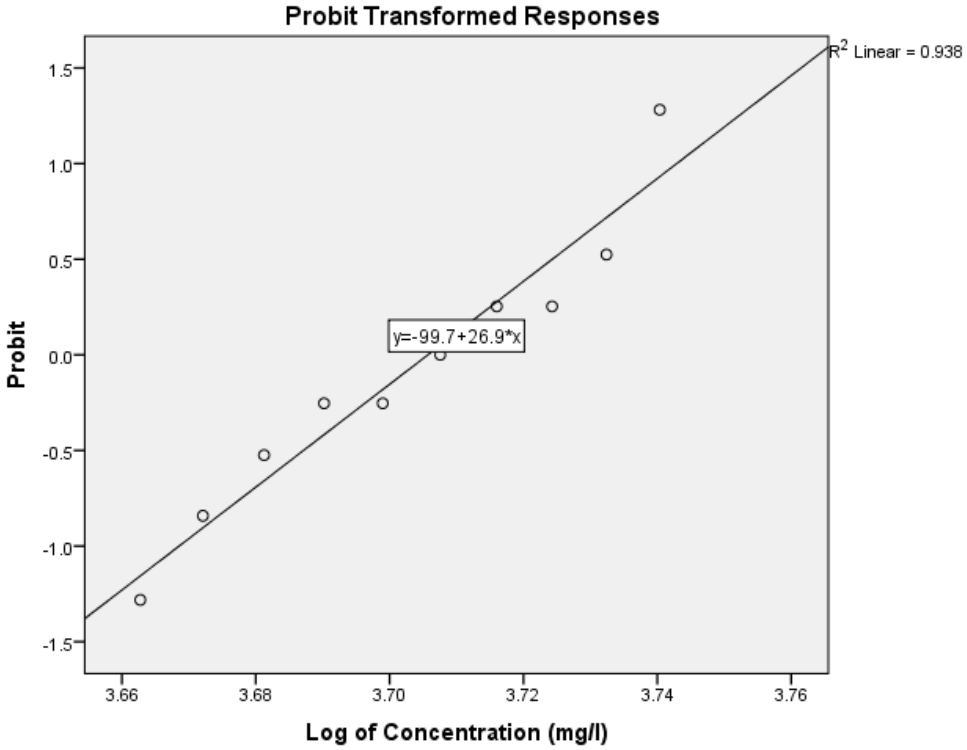
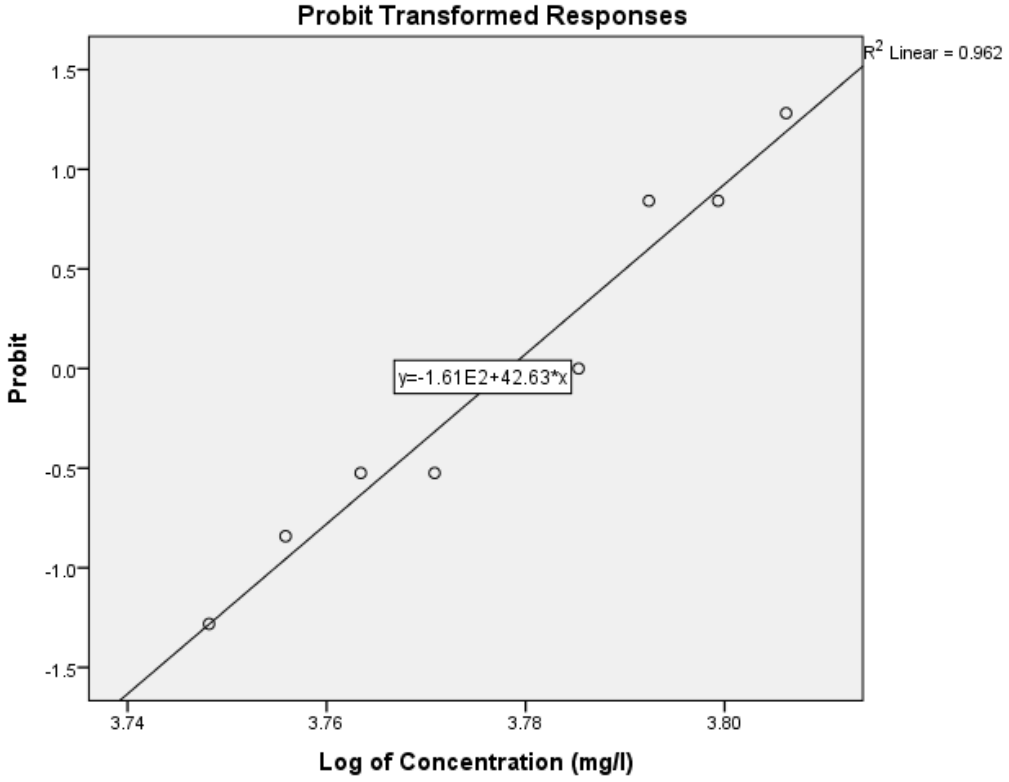


Fig. II Linear relationship between probit mortality and logarithm concentration of plant nutrient concentrations to which *L.rohita* was exposed during the 48 hours exposure period



DISCUSSION

Advancement in agriculture and industrial sectors have resulted into aquatic pollution due to discharges of untreated industrial wastes and runoff water into the water bodies causing devastating effects on the fish health (Javed, 2006). Toxicity testing has been widely used as a tool to identify suitable organisms as a bio-indicator and to derive water quality standards for chemicals. In the present study it was evident that exposure to plant nutrient lead to toxicity and it varied with the species and concentration. Fish mortality occurred when exposed to lethal concentration of plant nutrient. The 48hr LC_{50} value for *O.mossambicus* was lower than that of *L.rohita.* This infers that *O.mossambicus* is more sensitive to plant nutrient compared to *L.rohita*. The variation in the toxicity response has been reported to vary from species to species (Hassanein *et al.,* 2007; Adedeji *et al.,* 2008; Barbee *et al.,* 2010). This species specific difference could be due to the level of tolerance of animals to the toxicant (Inbamani and Sreenivansan, 1998; Ezeonyejiaku *et al.,* 2011) or is known to be dependent on various factors such as sensitivity to the toxicant, its concentrations and duration of exposure (Bantu and Vakita, 2013). Moreover the absorption of toxicant, its accumulation, biotransformation and excretion varies with the differences in the metabolic pathways among the species resulting into the different patterns of biotransformation leading to more or less toxic metabolites (Sheweita, 2000 and Harris *et al.,* 2002; CERI, 2007). Species length and weight, corporal surface to body weight ratio and breathing rate decides the scale of toxicity (Murthy, 1986 and Alkahem *et al.,* 1998; Ariole and Ezevununwo, 2013) In the present study we did not investigate the mode of accumulation of the toxicant. It is possible that accumulation must have occurred by absorption through the gastrointestinal and respiratory tracts as well as skin. Hence a comparative observation has proved that *L.rohita* is having more length and weight compared to *O.mossambicus* and proving the fact of having higher toxic response.

Statistical analysis of the data on toxicity brings out several important points. The chi square tests of goodness of fit (Heterogeneity) revealed that the mortality counts were not found to be significantly heterogeneous and were found to lie within the 95% confidence limits. The dose mortality graphs exhibit steep slope values which suggests that there is a large increase in the mortality of fish with relatively small increase in the concentration of the toxicant. The slope is, thus an index of the susceptibility of the target animal to the pesticides used (Yadav and Singh, 2013). A steep slope is also indicative of rapid absorption and onset of effects. Even though the slope alone is not a very reliable indicator of toxicological mechanism, yet it is a useful parameter, for such a study. Since the LC50 of both the fishes lay within the 95%

confidence limits, it is obvious that in replicate test of random samples, the concentration response lines would fall in the same range (Rand and Petrocelli, 1988). Our results are in agreement with the earlier reported work of Mushigeri and David, 2005 in freshwater fish *Cirrhinus mrigala* and Singh and coworkers (2010) for freshwater teleost *Colisa fasciatus* as well as Parikh and coworkers (2011) *in O.mossambicus.*

Behaviour links physiological functions with ecological pro-cesses (Scott and Sloman, 2004) and therefore it has been considered of relevance when studying the effects of pollution (Walsh and Bjordal, 2004; Leis, 2007), particularly in fish where several ecological relevant behavioural endpoints are easy observed and quantified (Scott and Sloman, 2004). Behaviour allows an organism to adjust to external and internal stimuli in order to best meet the challenge of surviving in a changing environment. Conversely, behaviour is also the result of adaptations to environmental variables. The effects in behavioural endpoints were well below LC_{50} values, indicating that these endpoints are more sensitive than mortality. The micronutrient mixture is a combination of various metals (Cu, Mn, Zn and Fe) which are known have varied behavioural responses (Hogstrand and Wood, 1996; Vieira *et al.,* 2009; Khunyakari *et al.,* 2001; Ezeonyejiaku *et al.,* 2011). Both the species, *O.mossambicus* and *L.rohita* have shown distinct behavioural changes at lethal concentrations. In the present study hyperactivity, agitated swimming, restlessness and loss of equilibrium was observed in *O.mossambicus* while *L.rohita* have shown the changes like slow and lethargic movement, decreased swimming activity, immobilisation and jerky movements. Scientists have noted that hyperactivity, lethargy, erratic movement and loss of equilibrium (Ayoola, 2002; Ladipi, 2011) could be due to the disruption of the functioning of nervous system of fish (Desai, 2013). The behavioural anomalies observed in both the fishes is probably due to the effect of one of major metal Cu whose neurological impairment have been proved in the *O.niloticus* and *C.gariepinus* (Ezeonyejiaku *et al.,* 2011) Moreover increased respiratory activity along with increased mucus secretion was seen in both the species. These responses increased with the increase in the toxicant concentration. Profused secretion of mucous on whole body parts, more pronounced in gill region is a protection device and it is well known that fish gills apart from the respiration are also the primary site of osmoregulation (Evans, 1987; Shephard, 1994; Pandey *et al.,* 2008) Lot have been stated for the mucous secretion in the fish, as in some components of mucous have a metal binding and ameliorative capacity (Arillo and Melodiaas, 1990; Coello and Khan, 1995; Lodhi *et al.,* 2006); mucous coating prevents the further metal entry by precipitation suggesting the adaptive mechanism against the environmental stress (Reddy and Reddy, 2013); Daoust *et al.,* (1984) have suggested that the

lamellar adhesion in gills as a result of contact stress causes erosion of mucous coating and epithelial lining. Increased respiratory behaviour can be attributed to an attempt made to extract more oxygen to meet energy demand (Reddy and Reddy, 2013) or a compensatory device to cope up oxygen deficiency as reported by Kasherwani (2009) in *Heteropneustes fossilis* due to cadmium toxicity.

The purpose of the acute toxicity test with the fish species under laboratory conditions is to help in the assessment of possible risks to similar species in natural environments. It is also aids the determination of possible water quality criteria for regulatory purposes and for use in correlation with acute testing of other species for comparative purposes. From the acute study investigation we conclude that plant nutrient Librel was toxic to both the fishes and based on the LC50 values *O.mossambicus* was more sensitive compared to *L.rohita*. It was established through this study that short-term exposure to plant nutrient resulted in negative alterations in behavior and mortality. Further the toxic effect in the fishes observed is the cumulative manifestation of the changes in the physicochemical factors and plant nutrient exposure. Hence, precautions must be taken when it is used in fish inhabiting areas since the excess application can affect the life of organisms living near the farming area and cause the high mortality among them.

CHAPTER II

Hematological And Biochemical Alterations In Oreochromis mossambicus And Labeo rohita Exposed To Plant Nutrient

INTRODUCTION

The aquatic environment is currently under threat due to an increase in heavy metal contamination by the human activities and causing high risk to non-target organisms (Kumar *et al.,* 2010; NEPC, 1980). The cry of pollution is heard from all the nooks and corners on global level. It has become a major challenge and threat to the very existence of mankind on the earth. Moreover, anincrease in agricultural practices inorder to overcome the needs of increasing population the degradation of aquatic system is a worldwide phenomenon. Heavy metals are major cause of concern for aquaticenvironment because of their toxicity, persistent, and tendency to accumulate in the organisms. Such an aquatic contamination cause ecological (biological integrity, biodiversity, ecological processes) as well as economic (aquaculture, production of potable water, fishing, bathing, recreation) effects.Fresh water environments compared to seas and oceans, are more vulnerable to pollution stress inasmuch as they are smaller systems and have more limited numbers and kinds of organisms. Pollution of lakes and rivers pose alarming dangers to aquatic life;especially fishes (Caring, 1992). Biomarkers in fishes have been used within environmental monitoring programs to estimate the degradation of aquatic ecosystems (Seriani*et al.,* 2009).

Fish serves as bio-indicator of water quality and the impact of the toxicant can be well understood by analyzing either blood or serum of the fish, because blood is a pathophysiological reflector of whole body (Sharma and Singh, 2004; 2006).Hematological study is important in toxicological research because a hematological alteration is a good method for rapid evaluation of the chronic toxicities of a compound (Kori-Siakpere*et al.,* 2006). Fish blood is known to exhibitpathological changes before the onset of any externalsymptoms of toxicity thus reflecting the physical andchemical changes occurring due to heavy metalaccumulation in body of fish.Hematological changes in fish may be used for assessing the effects of contaminants, because blood parameters respond to low doses of pollutants (Ranzani-Paiva*et al.,* 2000; Affonso*et al.,* 2002; Adhikari*et al.,* 2004; França *et al.,* 2007; Seriani*et al*., 2009; Seriani *et al.,*

2010). Thus, fish blood is being studied increasingly in toxicological research and environmental monitoring investigations (Mulcahy, 1975; Bansal*et al.*,1980).

Fishes exposed to metals, pesticides and effluents exhibit hematological changes, not only after laboratory exposure, but also when the exposure occurs in the field (Ranzani-Paiva*et al.,* 1997; Oliveira-Ribeiro*et al.,* 2006; Adhokari, 2004; Shah, 2006; França*et al.,* 2007, Serian*iet al.,* 2010). A thin epithelial membrane separates fish blood from the water and any unfavorable change in the water body is reflected in the blood (Kori-Siakpere*et al.*,2008). It is a pathophysiological indicator of the whole body function and therefore blood parameters are important in diagnosing the structural and functional status of fish exposed to a toxicant.A number of haematological indices such as haemoglobin (Hb), haematocrit (Hct), red blood cells (RBCs), white blood cells (WBCs) and so on, have been used as an indicator of metal pollution in the aquatic environment. Furthermore, it should be noted that haematological indices are of different sensitivity to various environmental factors and chemicals (Dordevic*et al.,* 2000; Gagnon *et al.,* 2006; Akinrotimi *et al.,* 2012). Previous haematological studies of pollutants brought to the knowledge that erythrocytes are the major and reliable indicators of various sources of stress (O' neal and Weirich, 2001).

Blood parameters are considered good physiological indicators of the whole body conditions and therefore can be used in diagnosing the structural and functional status of fish exposed to toxicants (Adhikari*et al.,* 2004; França*et al.,* 2007; Serian*iet al.,* 2009). They have been increasingly employed in environmental monitoring programs toindicate physiological changes due to toxicants (Françaet*et al.,* 2007;Serian*iet al.,* 2009; Zutshi*et al.,* 2009). However, the knowledge on the fish hematology stillneeds to be expanded, to provide data for differentspecies (Affonso*et al.,* 2002, Françaet *et al.,* 2007; Ranzani-Paiva *et al.,* 2000; 2008; Zutshi *et al.,* 2009; Serian*iet al.,* 2010) and its exposure to the different toxicants.Such a study would be helpful as the exposure of fish to several different types of chemical agents may induce differential changes in hematological variables (Shahi and Singh, 2011). The values of hematocrit, hemoglobin, and number of erythrocytes are indicators of toxicity with a wide potential for application in environmental monitoring and toxicity studies in aquatic animals (Barcellos *et al.,* 2003; Ahmad *et al.,* 2004; Miron *et al.,* 2005; Ferrari *et al.,* 2007). In addition the determination ofthe packed cell volume (PCV), and obtaining total erythrocyte counts and red blood cell indices, such as mean cell volume, mean corpuscular hemoglobin concentration, and mean corpuscular

hemoglobin, all can be useful in diagnosing disease.PCV varies within and between species and seems to correlate with thenormal activity level of the fish.Hematological abnormalities have also been studied in various toxicants exposed fish: *Channapunctatus*to lead (Hymavathi and Rao, 2000); *C. punctatus* to cadmium (Karuppasamy*et al.*, 2005); and *O.mossambicus* to fungicide curzate (Desai and Parikh, 2012).

Along with alterations in the haematological profile the fishes also exhibit alterations in the metabolism and biochemical processes (Luskova *et al.*, 2002). For example, several studies indicate that after exposure to a toxicant, fishmay exhibit an increase or decrease in levels of plasma glucose, serum protein, creatinine and urea. However the exact changes can vary depending on thetoxicant type, species of fish, water quality and length of exposure (Monteiro *et al.*, 2005; Jee *et al.*, 2005). Microelements such as Zn, Mn, Fe, Cu and Mn play key roles in living processes and either an excess or deficit can disturb biochemical functions in both humans and animals (Przybyl and Koligot, 1997) ln this study, the effect of the micronutrient mixture on the haematological and biochemical profile of freashwaterteleosts, *Oreochromismossambicus* and *Labeorohita* were studied. Till now there is lack of knowledge about the toxic potential of a micronutrient mixture having EDTA chelated chemistry in trace amounts. Such a study is vital as it not only assesses the health of fish subjected to changing environmental conditions but also for the deteriorating water quality.

MATERIALS AND METHODS

Experimental design:

The specimens of freshwater fishes, *O. mossambicus* (12 ± 2 cm, 25 ± 1.9 g)and *L. rohita* (20± 2 cm, 125 ± 5 g)of similar size in length and weight were brought to the laboratory from a local pond of Baroda district, stocked in well aerated tanks containing chlorine free water and acclimated for 10 days. Temperature, pH, and dissolved oxygen of the water were maintained at 27 ± 2° C, 7.1 ± 0.5, and 3.9 ± 0.02 mg/L, respectively. If in any batch, mortality exceeded 5%during acclimatization, that entire batch of fish was discarded. They were fed with commercial fish pallets. 30% Water was renewed every alternate day to provide freshwater, rich in oxygen. Ten well-acclimatized fish were transferred from the stock to each experimental tank containing 40 L of water exposed to the concentration of 500mg/L in *O.mossambicus* and 600 mg/L in *L.rohita*for the period of 45 days. A control group was also maintained in the same condition for the basic test. After the study period the fishes were sacrificed. The LC_{50} values in the respective time intervals were calculated using software by transforming mortalities (percentage values) into a probit scale (Finney, 1971).

Experimental Procedure

On basis of LC_{50} value sub acute study dose $LC_{50}/20$ was chosen for hematological and biochemical studies. The experimental regime wasmaintained in the laboratory for 45days.A control group was alsomaintained. The experiment was performed semi statically with a group of 10 fishes in experimental aquaria. Hematological and biochemical examinations of the experimental as well as the control fish were carried out at 15^{th}, 30^{th} and45^{th}days of exposure. All the groups were kept under continuous observation during the experimental period. Commercially food pallets were given to fishes once in day during the experimental period *ad libitum*. After the completion of the exposure fish were caught very gently using a small dip net, one at a time with least disturbance. They were slowly released in the tough containing 1% clove oil to make it immobile, blotted dry and blood was collected by tail ablation.

***Haematological and biochemical estimation of fish**:*

The tail ablation was done using a single stroke from a heavy, sharp seizure. The caudal peduncle of the fish wasseveredand first drop of blood was discarded. Afterwards freely oozing blood was collected using separate heparinized disposable syringe. The blood was then transferred to the eppendrof containing anticoagulant, thoroughly mixed using a thin, blunt glass rod, during the process ofcollection itself. The blood was stored in -4°C prior to hematological and biochemical estimations. An alterations in the hematology and biochemical profile was recorded usingNIHON KOHDEN Automated Hematology Analyzer (Celtics α, Japan).The difference between the control and the Librel exposed fishes was determined by One-Way ANOVA. If there was significant difference, Dunnett t-tests were employed to recognize difference in the alterations found in between the control and the exposed groups. The significant level of the tests was set at 5% ($p<0.05$).

RESULTS

The alterations in the haematological profiles of the exposed and control fishes of both the fishes are shown in the graphs (Table I and Fig. I-XII). There was a species specific change in the parameters. There was significant ($p<.05$) increase in the HB, RBCs ad PCV in *O.mossambicus* in contrast to *L.rohita* which have shown a significant increase in the exposed fishes compared to control. The level of significance varied with time, showing most significant ($p<.001$) alteration at the 30th day compared to the 15th and the 45th day in both the fishes. MCH, MCV and MCHC values significantly ($p<.05$) increased at 15th day with a sudden insignificant decrease at 30th day and at increase at 45th day in both fishes.

There was time dependent decrease in the values of protein of both the fishes, in case of *L. rohita* the decrease was significant at 45th day ($p<.05$) while in case of the *O.mossambicus* the values decrease significantly at 30th ($p<.05$) and 45th ($p<.001$) day. Albumin showed a contrasting results, in case of *O.mossambicus* it decreased significantly ($p<.05$) at 30th and 45th day while in *L.rohita* it increased significantly ($p<.001$) at 30th and 45th day.Globulin showed a significant ($p<.001$) time dependent decrease in its levels in both fishes. WBC count have shown a differential results, in *L.rohita* it decreased significantly ($p<.001$) at 15th and 45th day and increased on 30th day in contrast of *O.mossambicus* where an insignificant increase was reported at 15th day and significant ($p<.001$) decrease in 30th and 45th day. Platelet count have shown the following results, in *L.rohita* it decreased but the decrease was significant ($p<.001$) at 30th day while in *O.mossambicus* and increase was seen which was significant ($p<.001$) at 15th day. Urea showed a time dependent significant ($p<0.05$) increase in both species.

Table I: Time dependent alterations in blood parameters in both species

Parameters	*O.mossambicus*				*L.rohita*			
	Control	**15 days**	**30 days**	**45 days**	**Control**	**15 days**	**30 days**	**45 days**
Hb	8.24±0.29	8.30±0.05*	7.41±0.06**	7.92±0.05*	3.47±0.24	4.30±0.29*	7.02±0.32**	4.86±0.48*
RBCs	1.84±0.14	1.51±0.08*	1.48±0.02**	1.52±0.01*	0.69±0.03	0.77±0.09*	1.38±0.17	1.03±0.07*
PCV	27.66±0.66	25.62±0.86*	18.71±0.24**	21.5±0.5*	6.75±4.78	10.02±0.08	19.49±0.79**	12.28±1.13*
MCV	142.95±3.09	166.44±3.27	125.5±2.58	138.75±2.81*	140.56±1.84	133.49±2.32*	144.22±0.95	123.28±0.86*
MCH	41.23±1.11	53.68±1.55*	49.42±0.99	51.82±0.95*	49.85±0.79	61.55±3.27	52.47±2.28*	48.19±1.04
MCHC	30.99±0.55	32.44±1.07	39.73±1.55*	36.83±1.30*	35.33±0.90	46.24±0.89	36.45±0.69*	38.35±0.66*
WBC	110±5	114.1±16.55	59.26±5.25	77.26±1.65	62.66±2.75	36.66±1.15	118±6.24	47.1±1.56*
Platelet	90.66±17.00	227±23.64	97±15.13	58±9.84**	22±1*	20±10	10.16±0.76	26.83±2.75*
Glucose	65.22±0.95	47.44±0.50	73.77±1.34*	90.85±1.45*	44.94±1.41	69.14±1.00*	48.11±1.34**	71.15±1.11**
Protein	7.22±0.67	6.18±0.16	5.29±0.11	5.06±0.16	4.95±0.35**	4.70±0.42	4.23±0.25	3.86±0.22*
Albumin	4.42±0.05	4.11±0.10	3.88±0.10*	3.81±0.16*	1.49±0.05	1.54±0.01	1.75±0.04*	1.92±0.05**
Globulin	2.73±0.23	1.92±0.24	1.73±.23	1.40±0.08*	3.60±0.08	3.12±0.08	2.91±0.03*	2.16±0.06*
Urea	32.3±0.60	34.3±0.85	36.76±0.92	38.36±1.47*	21.6±0.65	23.18±0.41	24.4±0.45	36.03±0.25*

***The mean difference is significant at the 0.05 level.*
****The mean difference is significant at the 0.01 level.*

Fig. I: Alterations in the Hemoglobin (Hb) in *O.mossambicus* and *L.rohita* exposed to Plant nutrient Librel.

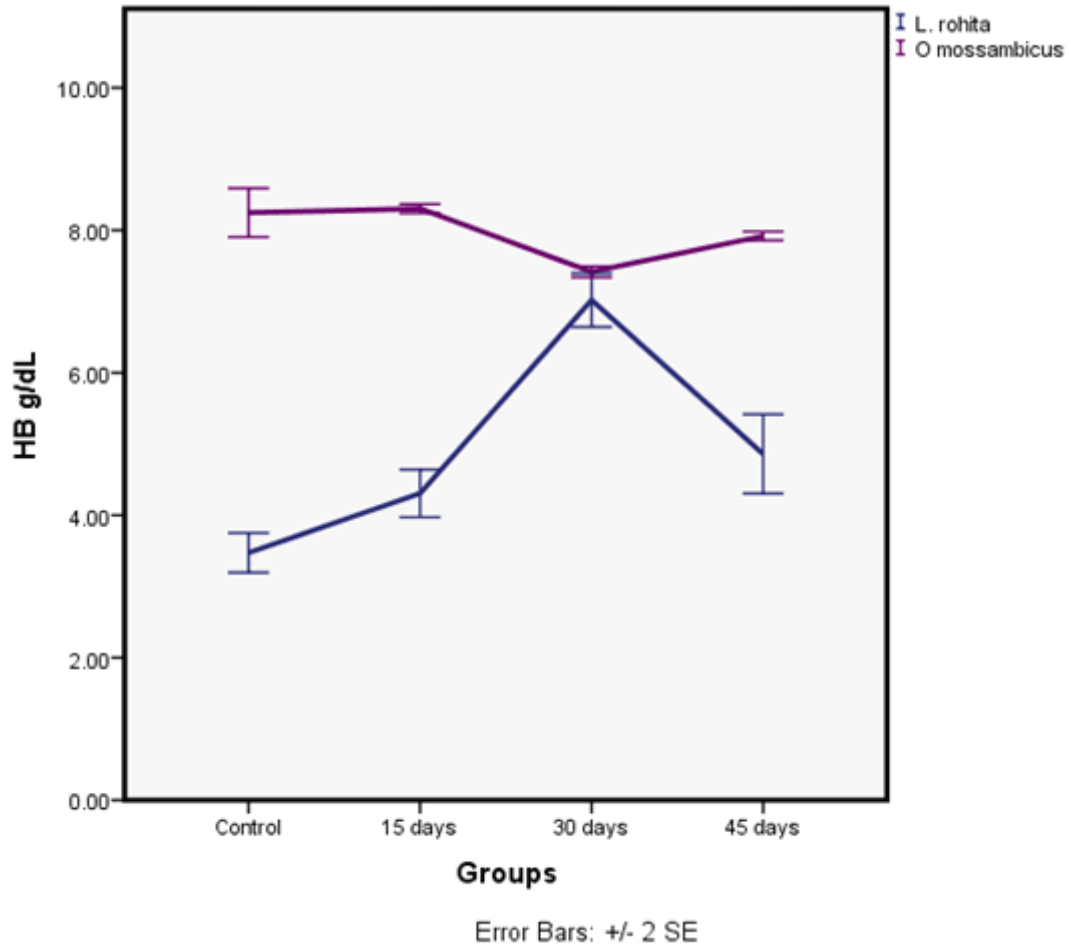


Fig. II: Alterations in the Red blood cells (RBCs) in *O.mossambicus* and *L.rohita* exposed to Plant nutrient Librel.

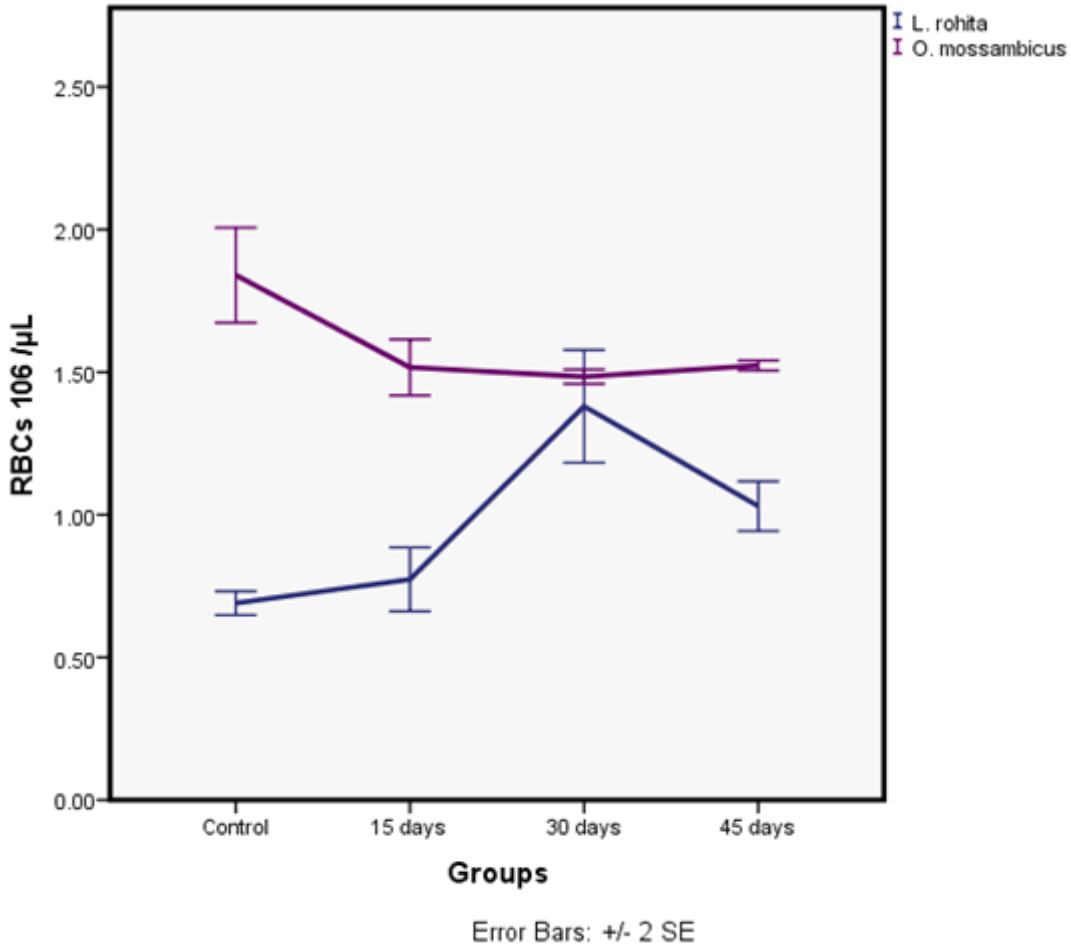


Fig. III: Alterations in the Packed Cell Volume (PCV) in *O.mossambicus* and *L.rohita* exposed to Plant nutrient Librel.

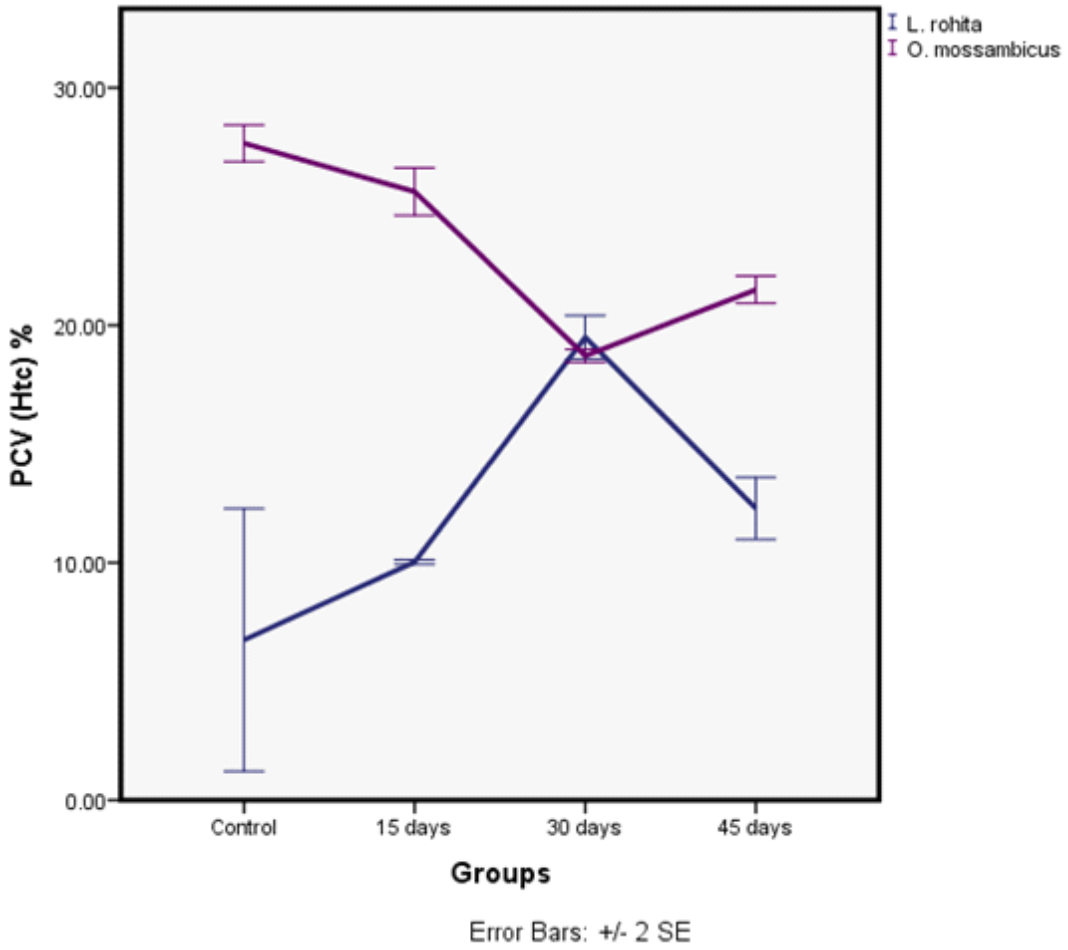


Fig. IV: Alterations in the Mean Corpuscular volume (MCV) in *O.mossambicus* and *L.rohita* exposed to Plant nutrient Librel.

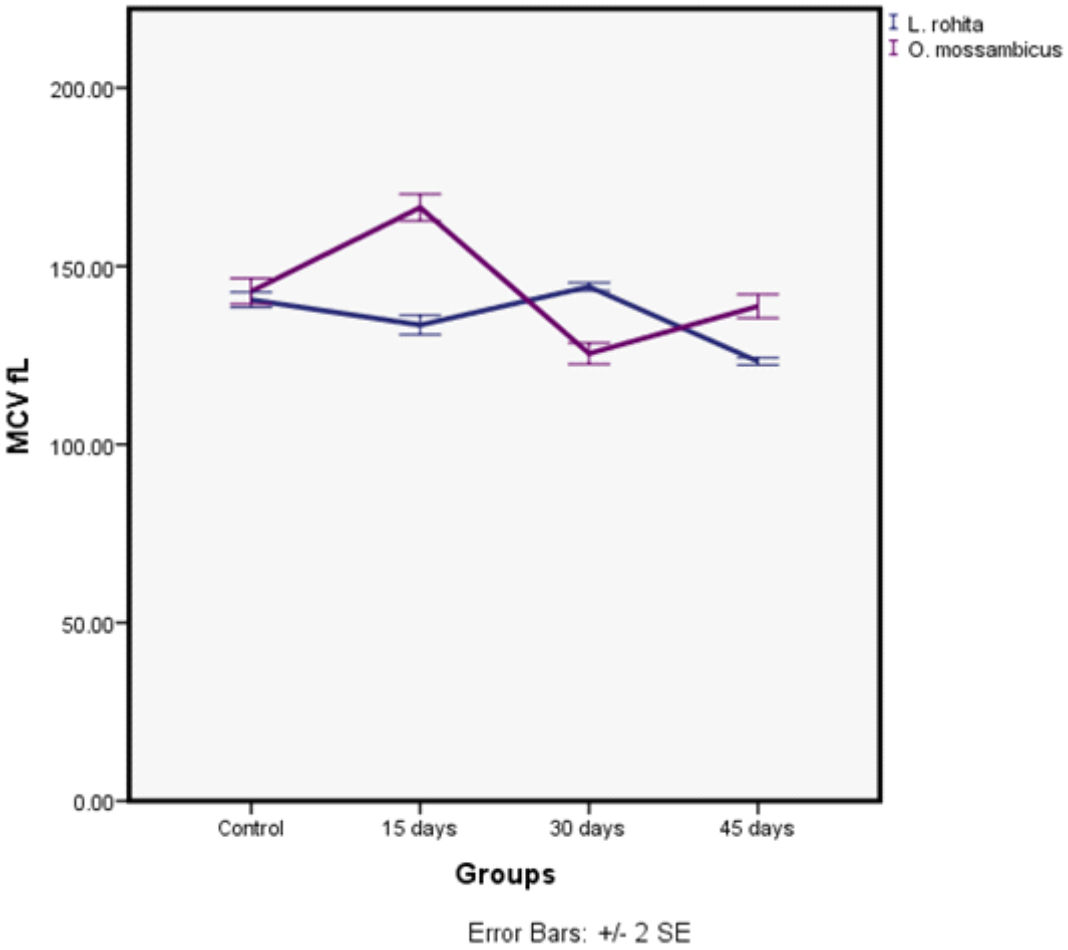


Fig. V: Alterations in the Mean corpuscular hemoglobin (MCH) in *O.mossambicus* and *L.rohita* exposed to Plant nutrient Librel.

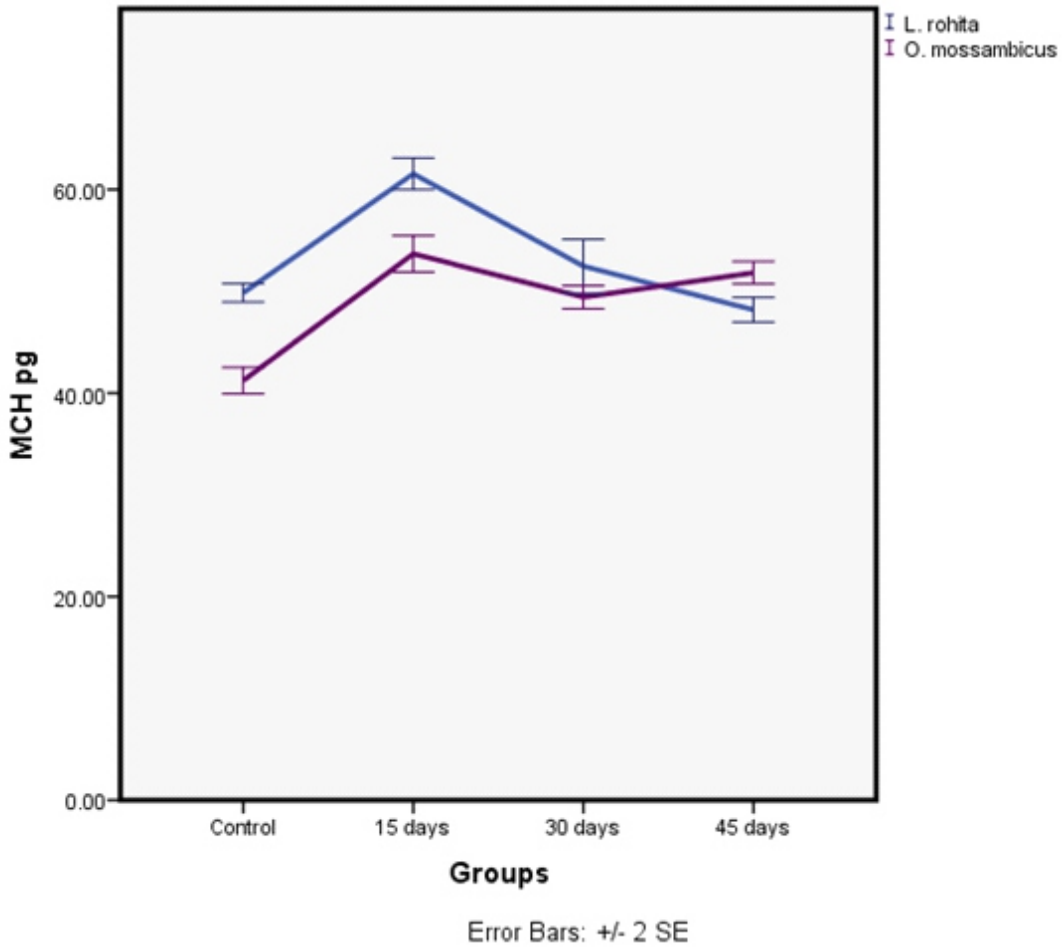


Fig. VI: Alterations in the Mean corpuscular hemoglobin concentration (MCHC) in *O.mossambicus* and *L.rohita* exposed to Plant nutrient Librel.

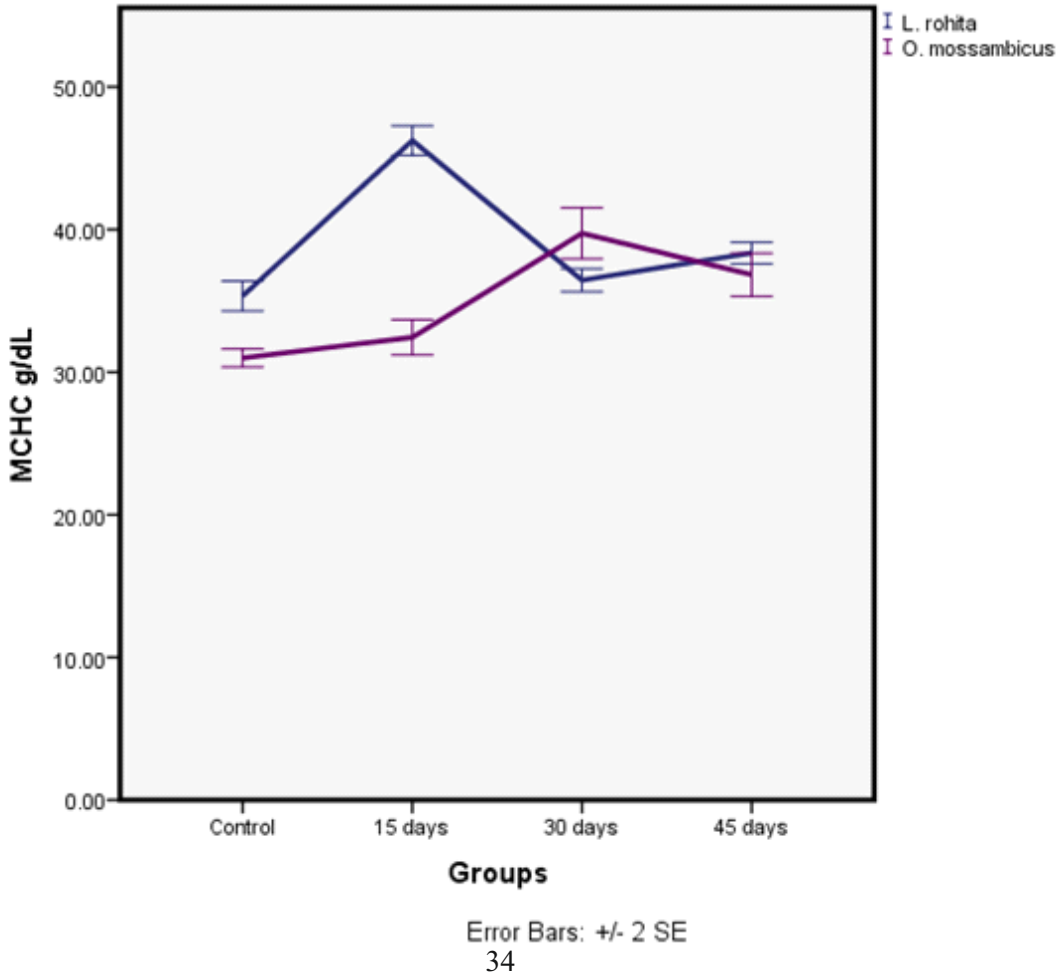


Fig. VII: Alterations in the Glucose in *O.mossambicus* and *L.rohita* exposed to Plant nutrient Librel.

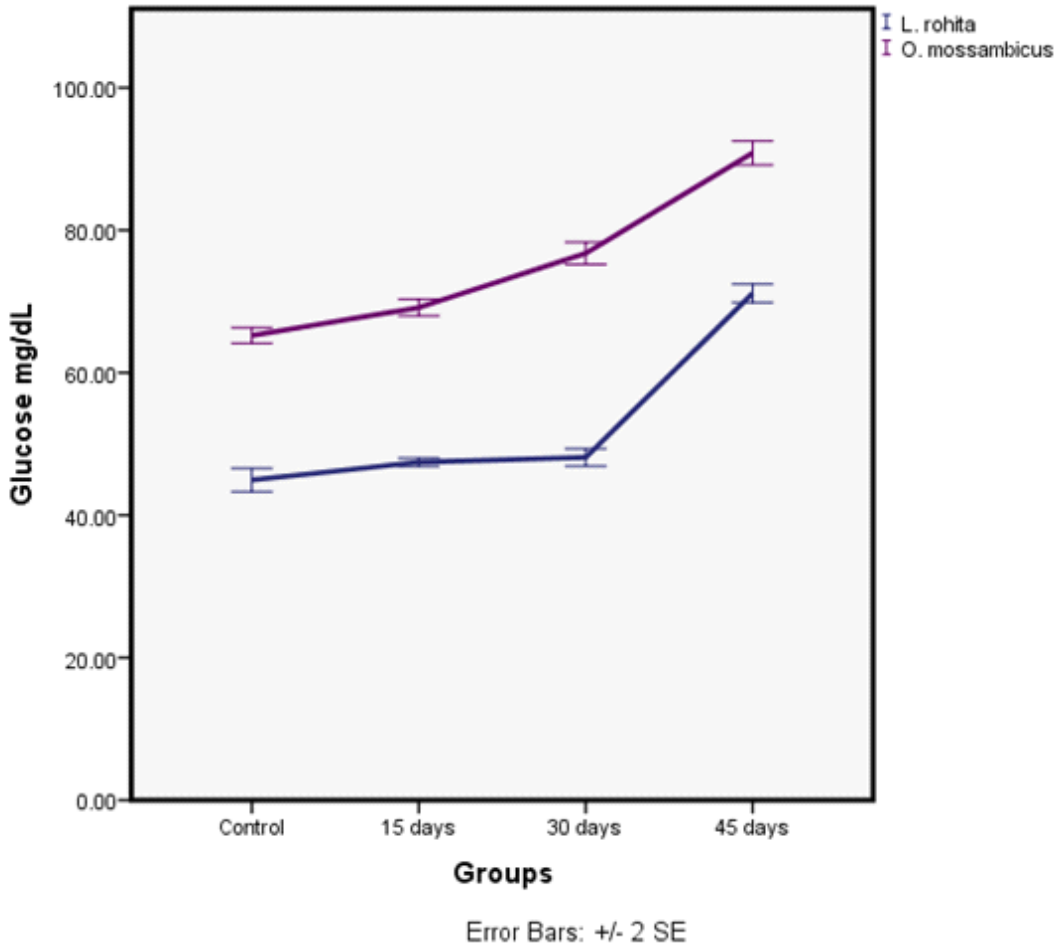


Fig. VIII: Alterations in the Protein in *O.mossambicus* and *L.rohita* exposed to Plant nutrient Librel.

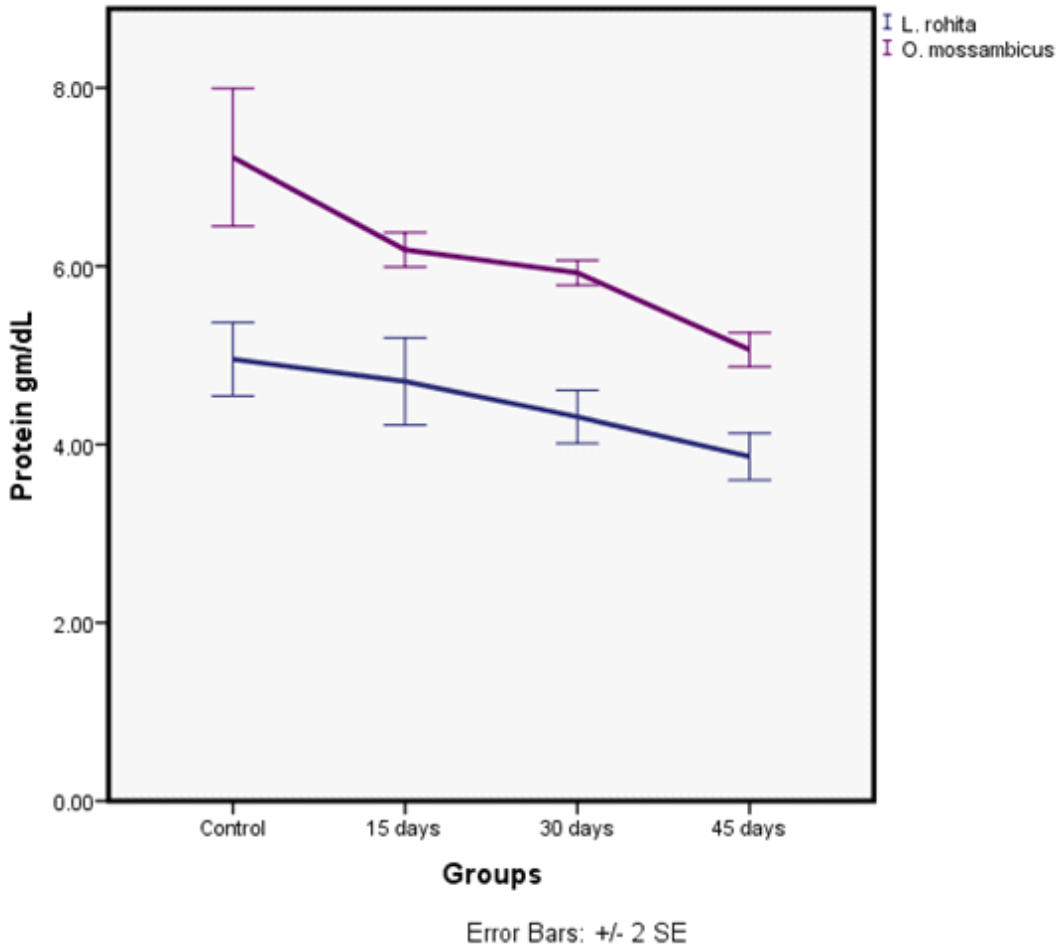


Fig. XI: Alterations in the Albumin in *O.mossambicus* and *L.rohita* exposed to Plant nutrient Librel.

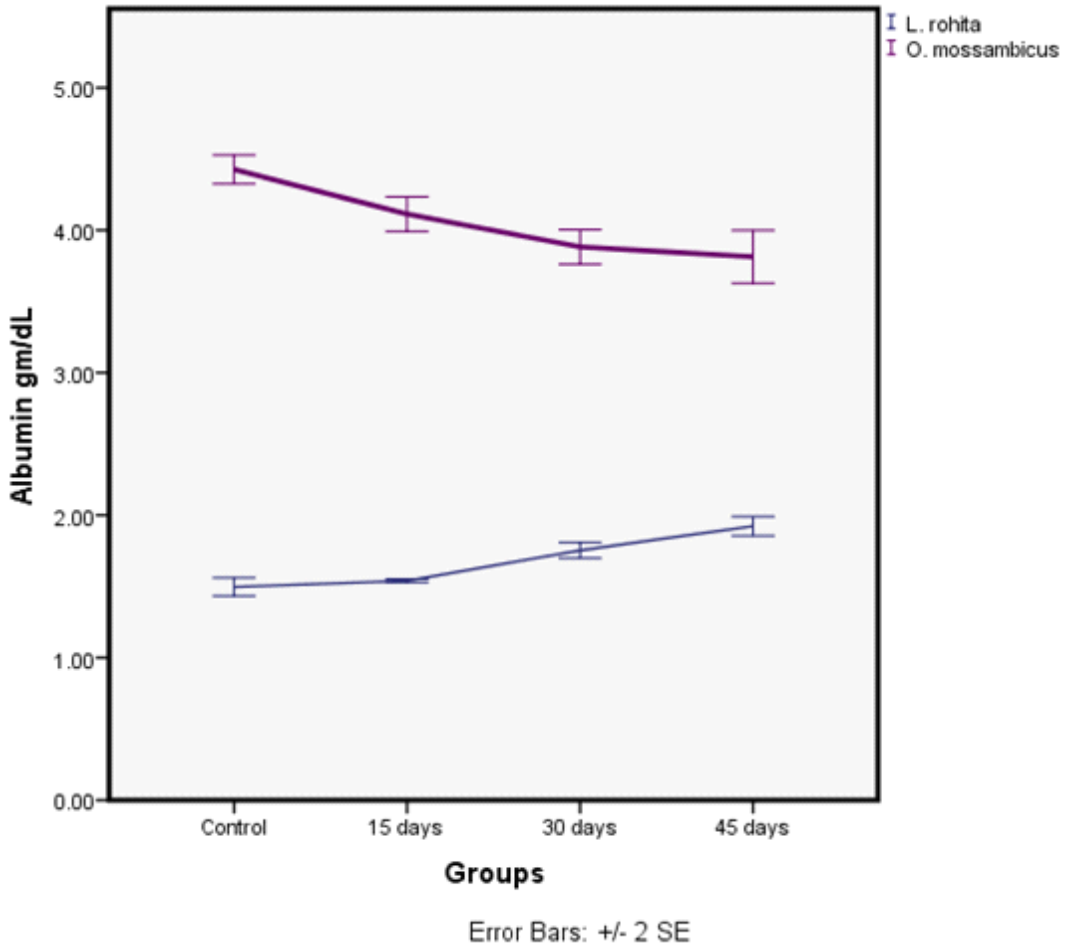


Fig. X: Alterations in the Globulin in *O.mossambicus* and *L.rohita* exposed to Plant nutrient Librel.

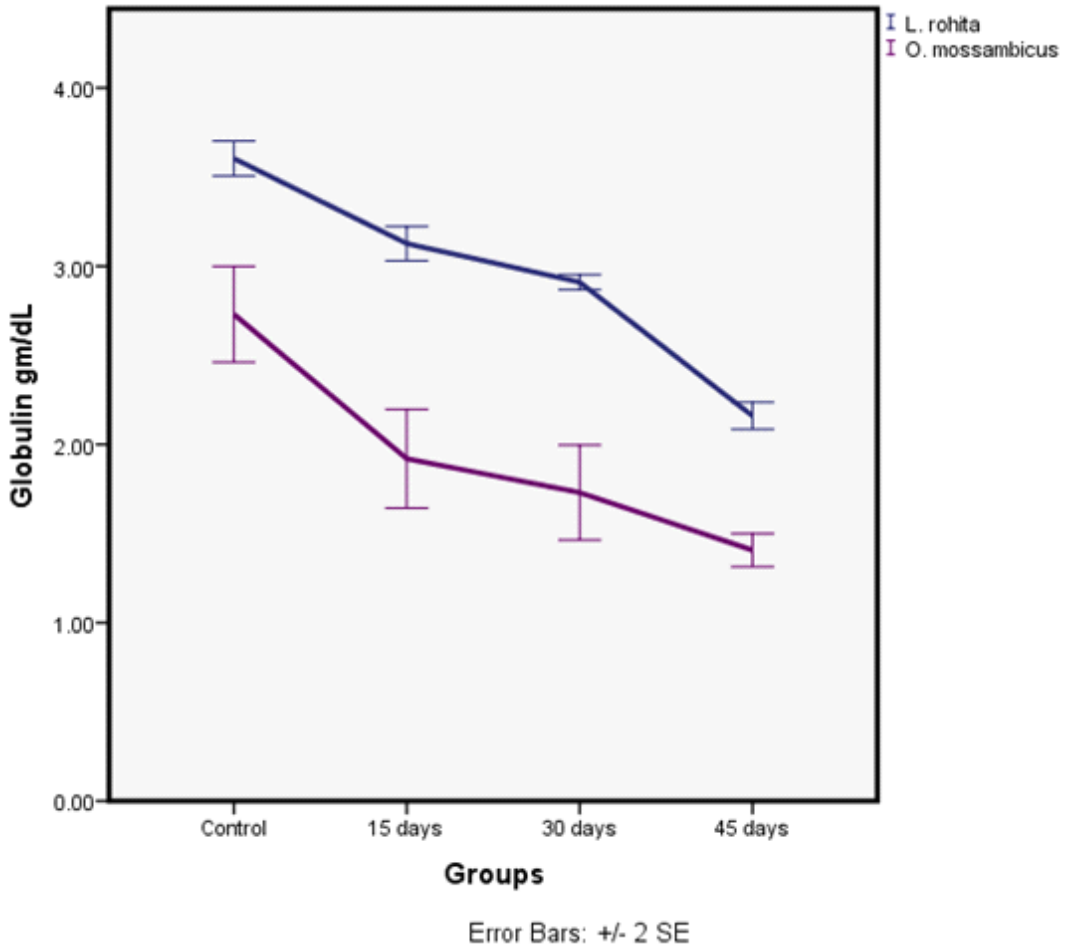


Fig. XI: Alterations in the Hemoglobin (Hb) in *O.mossambicus* and *L.rohita* exposed to Plant nutrient Librel.

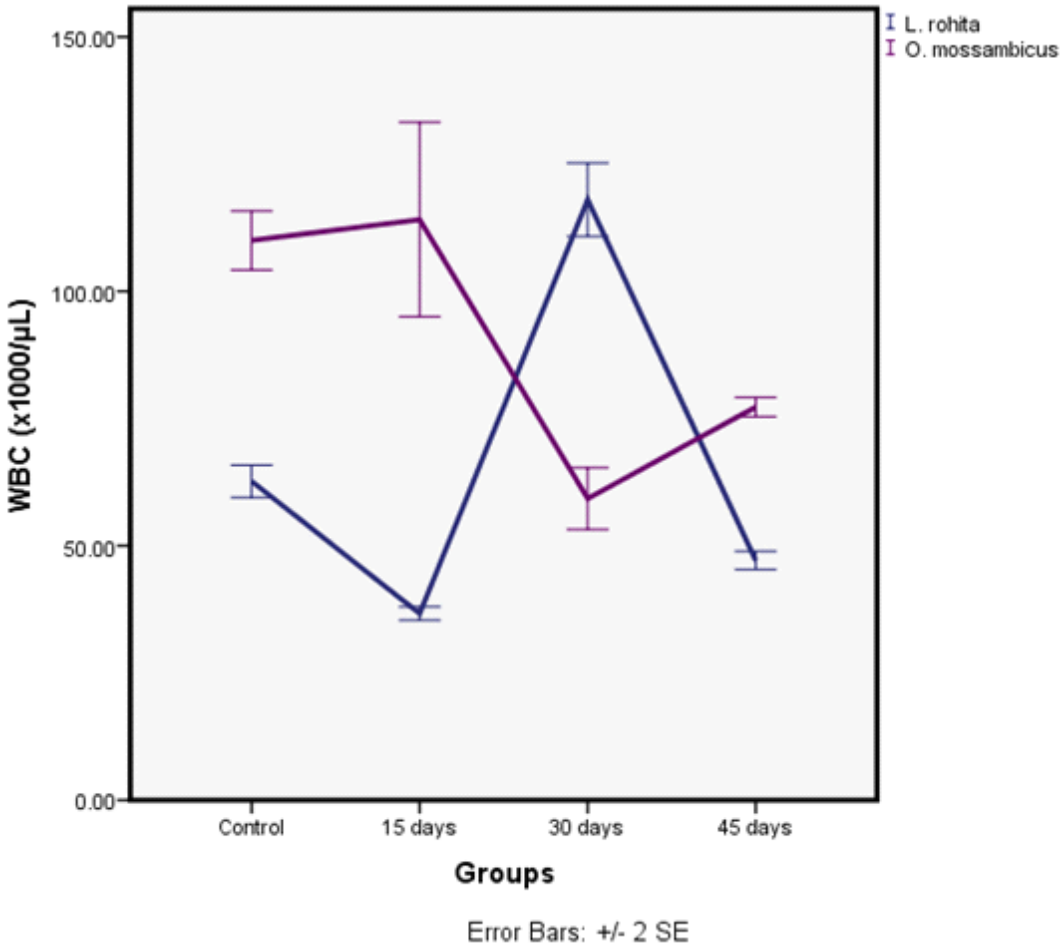


Fig. XII: Alterations in the Hemoglobin (Hb) in *O.mossambicus* and *L.rohita* exposed to Plant nutrient Librel.

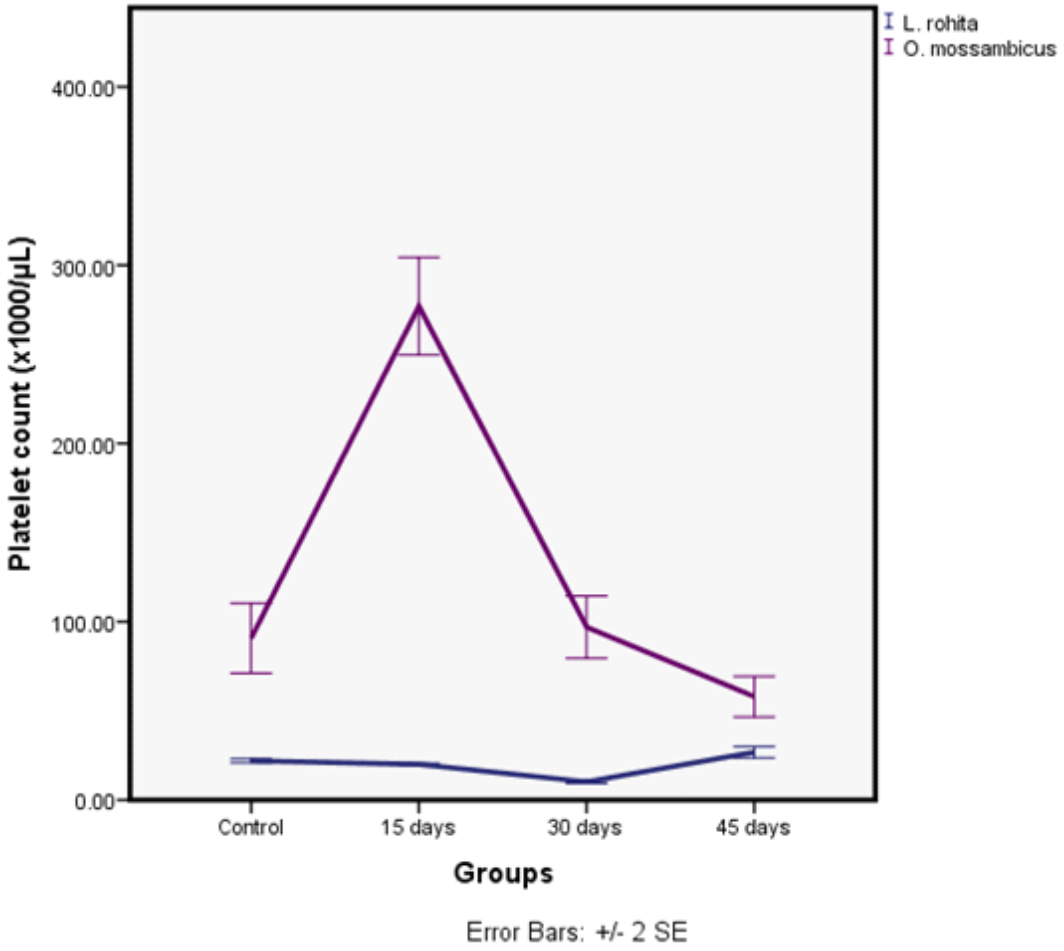
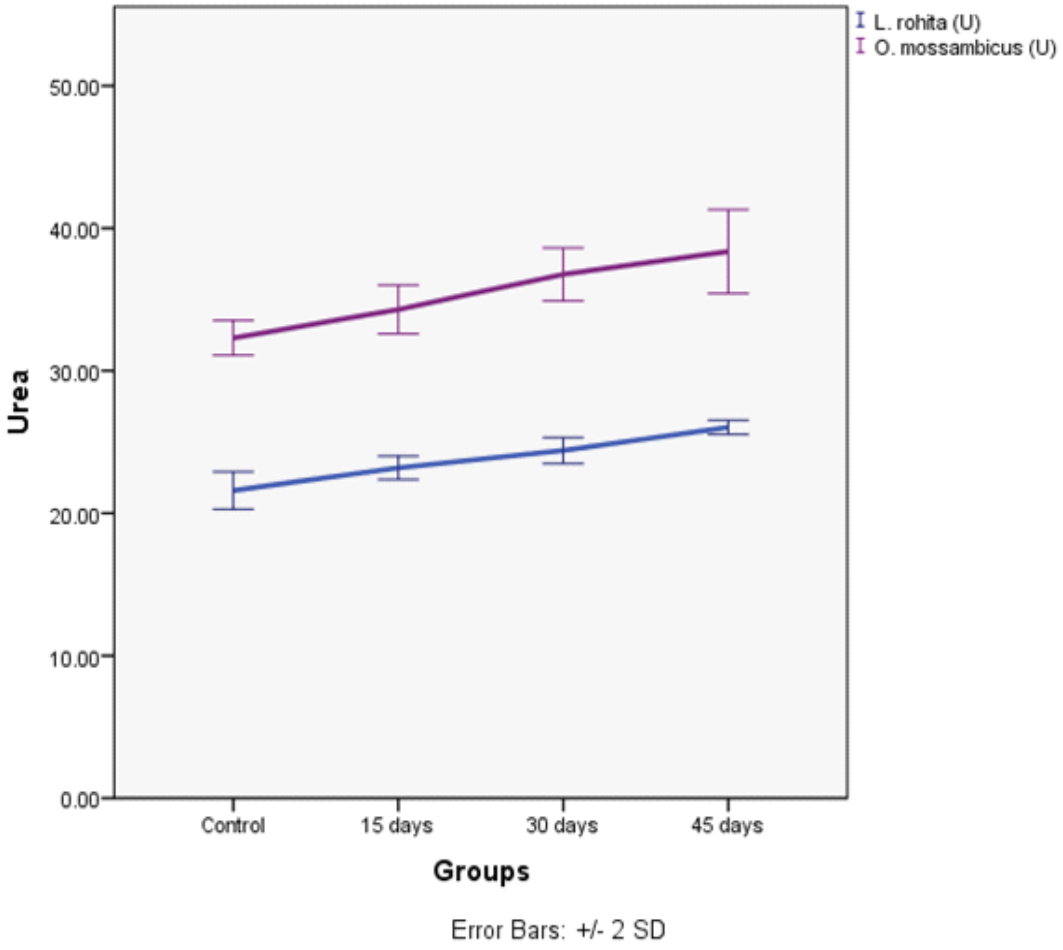


Fig. XII: Alterations in the Urea in *O.mossambicus* and *L.rohita* exposed to Plant nutrient Librel.



DISCUSSIONS

Hematological indices are very important indicators of changes in the internal and/or external environment of animals. In fish, exposure to chemical pollutants can induce either increases or decreases in hematological levels (Kori-Siakpere, 2006; Oboh, 2011).The impacts of Librel on the hematological profile of tilapia have been assessed in the present investigation.

Hematological system is the most important vital system which reflects the total health status in any organism as it provides movement for useful land unuseful components. It is the only transport medium for gases during respiration along with the transport of nutritive materials and other important biomolecules to various parts of body. Any alteration in animal's body like in liver, kidney, brain, digestive system or any infection is reflected in the haematological system. Blood being a liquid connective tissue connects all tissues to each other. It is through this system that compounds are transported in the other parts of the body. These compounds in turn gradually harm the haematopoietic system to damage RBCs, WBCs and other cells resulting into the disturebed haematological parameters (Pathak*et al.,* 2013). In the present study, reduction in hemoglobin was accompanied by lowest PCV value in *O.mossambicus*. Moreover an increase in WBC count; reflect the occurrence of leucocytosis in the treated fish samples. This was perhaps, a typical defensive response of the fish against a toxic invasion or probability may be of leukemia (Sudha, 2012).This decrease in the erythrocyte count or in the percent of PCV indicates the worsening of an organism stateand developing anemia as they are positively correlated. The anemia could be due to the destruction of RBC triggered by the influx of micronutrient into the erythrocytesand may also be of hemolytic type of RBC. Similar observations were also reported by Tilak *et al.,* (2007), Saravanan *et al.,* (2010) and Saeed *et al.,* (2012). In contrast *L.rohita* has shown increased levels of RBCs, Hb and PCV values which could be due to an increased loss of scales and haemorrhage. Moreover here the duration of exposure also plays an important role. With the increase in the exposure period *L.rohita* showed, erratic swimming, air gulping, loss of reflex, loss of scale, haemorrhage and molting. They finally settled at the bottom motionless with slow opercular movement. Such results were also reported by Ayotunde and his coworkers (2004) in *O.niloticus* when exposed to drumstick.

MCV, MCH and MCHC increased considerably with time compared to control. However the increase in these indices can be attributed to direct or feedback responses of structural damage to RBC membranes resulting in hemolysis and impairment in hemoglobin synthesis, stress related

release of RBCs from hemopoietic organ and hypoxia, induced by micronutrient exposure. Our results are in agreement with earlier reported alterations in these indices of Clarian gariepinus exposed to lead nitrited (Adeyemo,2007; Shah, 2006)

In the present investigation, total leucocyte count was increased aftertreatment of parathion and malathion in all the three fishes *Catla catla*, *Cirrhinus mrigala* and *Labeo rohita* as compared to control fishes of the same group (Table-3).The increase is more in *Catla catla* following Cirrhinusmrigala and Labeorohita and dose dependent i.e. effect is increasing with increased sublethal dose. Both thepesticides were harmful but malathion was more reactive than parathion. The increasewas gradually non-significant to significant with increasing dose. The present result isin accordance to the findings of Agarwal and Srivastava (1980) in fresh water fish C*olisa fasciatus* after manganese poisoning; Goel and Kalpana (1985) in *Heteropneustes fossilis* after zinc treatment; Aruna and Gopal (1987) in fish aftersublethal exposure of mercury; Saravanan and Harikrishnan (1999).

Glucose is one of the most important sources of energy for the animals and has been studied as an indicator of stress caused by physical factors in particular pollutants (Manush*et al.,* 2005).An increase in the levels of glucose in fishes in response to micronutrient mixture exposure indicates quantum of stress imposed on fish during subchronic toxicity and its physiological attemptsto overcome it. Glucose is synthesized from hepatic tissue proteins and amino acids due to the intensive glycogenolysis induced due to stress (Almeida *et al.,* 2001). Nakno and Tomlinson *et al.,* observed that all types of stress elevated the secretion of catecholamine which in turn increased the break downof glycogen and elevated blood glucose level. Stressors induce the changes that alter the homeostasis of the animals (Luebke *et al.,* 1997; Bols *et al.,* 2001; Rehulka, 2002). The stressors first activate the chromaffin cell present in the wall of the cardinal veins and in some cases the heart and the kidneys of the teleosts (Mazeaund and Mazeaund, 1981), which in turn releases the adrenalin and small amount of nor-adrenalin that stimulates the conversion of liver glycogen into blood glucose and the utilization of the glucose by muscle. Umingel, (1977) reported that blood sugar has a direct co-relation to metabolism. On the other hand the increase in blood sugar noticed could also be attributed to the differences in the respiration and the activity (Ghosh, 1987). The progressive accumulation of the blood glucose reported in this investigation reveal that both the fishes become hyperglyceamic. Same results were observed by Omoregie *et al.,* (1990) in tilapia exposed to stressed environmental condition

as a result of a incomplete metabolism of the blood glucose due to impaired osmoregulation. According to Coles (1980) increased blood glucose concentration results from an imbalance between the hepatic output of glucose and the peripheral uptake of sugar. Though there are no reports on diabetes in fishes, however stress imposed upon the fish during the toxicity trial might be the possible reason for hyperglycemia. In the present study, exposure of librel at different time period caused an increase in the blood glucose level leading to lethargy.

The proteins are most diverse bio-molecule which are ofprime importance in biochemical reactions and cellularstructures.Serum proteins have immunological properties in fishes and other animals as well as in human(Kumar and Dahiya, 2013).Proteins are indispensable constituents of the body and their metabolism is almost confined to the liver. Fall in serum protein level may be due to impaired function of kidney or due to reduced protein synthesis owing to liver cirrhosis (Garg*et al.,* 1989; Ravichandran *et al.,* 1994;Kumari and Kumar, 1995). There are reports on the changesinduced by pollutants on protein contentof serum (Abidi, 1990; Das and Mukherjee, 2001).Serum proteins were found to decrease due to Librel exposure in the present study. This could be attributed torenal excretion or impaired protein synthesis or due toliver disorder (Kori-Siakpere, 1995; 2008). Moreover this could alsoresult from the breakdown of protein into amino acids first and possibly into nitrogen and other elementary molecules.Verma*et al.,* (1979) and Abdel-Tawwab and Wafeek (2008) have observed the same results in fishes. Moreover byAbdel-Tawwab1 and Wafeek (2008) have opined thatsuch alterations result in the depletion oftotal protein in the plasma of fish. It is obvious that prolonged exposure offish to most toxicants, interferes with protein metabolism and the present work also supports the observations. Moreover the histopathological damages caused to the kidney offish by these toxicants (Sastry and Sharma, 1981) can lead to significant loss of blood proteins by renal excretion, further augmenting its depletion in the blood (Verma*et al.,* 1979).

Proteins are mainly involved in the architecture of the cell. During chronic period of stress they are also a source of energy. During stress conditions fish need more energy to detoxify the toxicant and to overcome stress. Since fish have fewer amounts of carbohydrates so next alternative source of energy is protein to meet the increased energy demand. The depletion of protein fraction in liver may have been due to their degradation and possible utilization of degraded products for metabolic purposes (Singh *et al.,* 2010).Chronic study in *Channa punctatus* exposed to endosulfan have also shown the decrease in the total serum protein levels

due to low assimilation of food (Joseph and Raj, 2010).The decrease in serum protein level in fish exposed for longer duration in the present study may be due to the low assimilation of food. Das *et al.,* (2004) stated that reduction of protein content in serum occurs due to shrinkage and lysis of RBCs causing plasma dilution and/or protein catabolism where structural protein converts to energy. According to Radha *et al.,* (2005) the reduction of protein content may be due to increased proteolytic activity and decreased anabolic activity of protein as observed by Jenkins *et al.,* (2003).Further, due to this degradation of protein in liver, the serum protein level has been increased which was released. Reddy *et al.,* (1995) and Singh and Sharma (1998) have also reported decline in protein constituent in different fish tissues exposed to sub-lethal concentration of insecticides in liver and increase in serum. Serum or organ proteins of fish are occasionally studied to estimate the toxic potential of many substances including metals. Results of such investigations are valuable in observing the proteins involved in the metabolism of the toxic substance. The decrease in total protein may be due to the inhibition of RNA synthesis disturbing the protein metabolism. Micronutrient exposure has led to serum total protein depletion probably because of excessive renal excretion or due to the diver disorder (Singh *et al.,* 2011). Decrease in the serum proteins also supports the view of (Abdel-Tawwab, M., and Wafeek, 2010), who reported that the decrease in amino acid incorporation and desegregation of polysomes lead to decrease in protein synthesis. In the present study reduction in the serum oral protein may also be attributes to intensive proteolysis which contributes to the increase in the free amino acids to be fed into TCA cycle as ketoacids. It is, therefore, evident that in case of continous esposure of the micronutrient mixture the deleterious effects of these substances on protein synthesis and kidney function accounts for the progressive reduction in the concentration of total serum protein.

Albumin, globulin and A/G ratio have found to be decrease with the increase duration of micronutrient exposure. Measurement of albumin, globulin, and total protein in serum or plasma is of considerable diagnostic value in fish, as it relates to general nutritional status (Schaperclaus *et al.,* 1992).The decline in albumin, globulin and A/G ratio correlates with decrease in protein content as these are the integral content of protein itself (Singh *et al.,* 2011). Serum protein, albumin, and globulin were significantly lower in Tilapia and Rohu. These results may be due to the disturbances in the liver protein metabolism due to micronutrient toxicity, as was found to be the case with other contaminants (Dange and Masurekar 1984; Abdel-Tawwab *et al.,* 2007a; b).

On the other hand, Nguyen (1999) reported that a low albumin may result from impaired synthesis, loss through urine or feces, or increased catabolism. Serum albumins are synthesized in liver, and changes in serum proteins are inevitable during the pesticide/metal toxicity. Further, Singh and Agarwal (2006) have reported changes in albumin, globulin and A/G ratio after the exposure of pesticides in *Channa striatus.*

Urea is synthesized from NH_4^+ and HCO_3 in the liver via the ornithine-urea cycle (OUC). Urea may also formed by the degration of uric acid or arginine. Elasmobranchs utilize the ornithine-urea cycle whereas teleost synthesize urea by uricolysis or arginolysis. Few teleostean species synthesize significant amounts of urea in response to environmental conditions that limit ammonia excretion (*O.a. grahami,* Randall *et al.,* 1989*; Opasanus beta,* Walch *et al.,* 1990;*Heteropneustesfossilis,* Saha and Ratha, 1989). These species are unique in expressing the full complement of OUC enzymes but in other adult teleosts some of the genes for OUC enzymes appear to be repressed (Wright, 1993). The OUC is used as a 'safeguard' mechanism to prevent ammonia toxicity during particularly sensitive stage of neural development (Wright, 1995) Teleost fishes are primarily aminotelic but their blood contains significant amount of urea and indeed in some teleost it may account for 20% or more of total nitrogen excreted (Joshi, 2002). Renal disorders also elevate serum urea release. Creatinine is another nitrogenous waste product that is eliminated by kidneys when excretion is suppressed in renal insufficiency. The high levels of blood urea and creatinine result either from increase breakdown of tissue or dietary or impaired excretion or increased synthesis or decreased urinary clearance by the kidney or decrease degradation of these compounds (Adham *et al.,* 2002) the present suggest that micronutrient exposed fish adapt glomerular dysfunction rather than tubular insufficiency as blood levels of urea and creatinine depends largely on glomerular function. In consistent with this explanation of decreased total protein level with micronutrient exposure urea is the end product of protein catabolism in mammals but in fish ammonia is the end product of protein, so the marked increase in blood urea nitrogen could be attributed to impaired excretion of urea through kidney which is supported by increase in blood creatinine level, a more sensitive and specific indicator of impaired kidney function (Amin and Hashem, 2012).

Thus from the present study it can be concluded that evaluation of haematological parameters along with biochemical changes of fish exposed to toxicants provide valuable information in the assessment of fish health and in monitoring stress responses. The exposure of *O.mossambicus*

and *L.rohita* to sublethal concentrations of Librel caused marked variations in the blood indices of both the fishes an indication that this micronutrient mixture is highly toxic to fish and its use should be controlled so as to prevent its entrance into water bodies to minimize aquatic pollution consequent of agrochemicals. Furthermore, the results of the present investigation reveal that under experimental condition, blood parameters of tilapia and rohu weresensitive to Librel exposure. These findings permit us to conclude that Librel is highly toxic to fish. Hence, the presence of micronutrient in waterways surrounding the agriculture fields could have adverse impact on the survival of the fish. Therefore it is necessary to monitor, the level of micronutrient content in the surrounding aquatic environments.

CHAPTER III

Plant Nutrient Induced Alterations In The Condition Factor, Hepatosomatic Index And Gonadosomatic Index Of *Oreochromis mossambicus* and *Labeo rohita*.

INTRODUCTION

Aquatics environment gets contaminated with a variety of pollutants generated from diverse sources (industries, agricultural and domestic). Amongst the pollutants, pesticides, heavy metals and detergents are the major cause of concern for aquatic environment because of their toxicity, persistency and tendency to accumulate in organisms. A sustainable agriculture is a system of agriculture that is committed to maintain and preserve the agricultural base of soil, water and atmosphere ensuring future generations the capacity to feed themselves with an adequate supply of safe and wholesome food (Khitoliya, 2004). To satisfy human hungers with the increasing population growth and changing dietary patterns have resulted in more and more land moving from forest or grasslands into agricultural production. Initially the crop productivity could be maintained with nitrogen fertilizer alone as the other major nutrients needed by the plants were provided by the soil. However, due to gradual usage of the nutrients the same is no longer possible by applying NPK alone. Tiwari(2002) in his study has reported the growing emergence of plant nutrient deficiencies in the farmfields and is of the view that the Indian agriculture is in an era of multiple nutrient deficiencies. Hence to sustain the high yields farmers have to apply the six main nutrients (N, P, K, S, and Zn). Most of the soils of Gujarat have been reported to be deficient in Zn and Fe (Patel et. al 1998). The supplementation of micronutrients through multi-micronutrients mixture under such situations becomes more important to provide balanced nutrition to the crops (Patel, and Singh, 2010).

India has come a long way since independence, in respect of production and consumption of fertilizers. From a mere 69.8 thousand tons of fertilizers (1950-1951) to 22.57 million tons (2007-2008), the fertilizer consumption increased from a mere 0.7 Mt in 1951 to 28.3 in the year 2011. This resulted in the food grain production from 640 kg/ha to 1921 kg/ha in (Rao2011). This clearly states that as earth can only supply limited amount of nutrition for every additional ton increase in the food production external application is needed. This increased use of fertilizer with the motive of soil nutrient enrichment has led to eutrophication and deterioration of surface

water quality due to transpiration of nutrients applied through fertilizers via leaching and /or runoff. Further eutrophication may lead to excessive growth of phytoplankton and filamentous algae, increase in aquatic plant life, increase in turbidity (cloudiness) of water, increase in rate of sedimentation, development of anoxic conditions (low oxygen levels), decrease in species diversity, and an increase in the frequency of algae blooms causing a dearth of oxygen and a change in fish species composition (Khan, and Ansari, 2005).

Fish around the world are found occupying almost any aquatic habitat. In particular, freshwater fish are severely threatened as the freshwater ecosystems are considered the most endangered of the world (Dudgeon , 2006). The ultimate destination of most contaminants is water; rivers, lakes, aquifers, or sea, are receptors of wastewaters with a complex mixture of xenobiotics. Fish in their natural environments are typically exposed to numerous stressors including unfavorable or fluctuating temperatures, high water velocities and sediment loads, low dissolved oxygen concentrations, limited food availability, and among other types of natural episodic variables. In addition, anthropogenic stressors such as contaminant loading can add to the insults that fish may already experience in many systems. All these factors, individually or together, can impose considerable stress on physiological systems of fish and impair their health (Sedeño-DíazLópez-López, 2012).

Analyses of HSI and GSI of fish offers information on the general health condition of the organisms. Pollution may damage organisms directly by increasing their mortality, or interfering with the processes of food acquisition and uptake, and reducing their growth and reproduction rates. Growth represents the integration of feeding, assimilation and energy expenditure over a period of time which is commonly assessed by condition factor. Poor growth means less energy is available for reproduction, which will in turn reduce the species fitness and lead to a decline inwell being of the fish and a reduction in overall population of the species. Growth and reproduction therefore can serve as a time-integrated indicator of the general well being of the organism. Hepatosomatic Index (HSI) is defined as the ratio of liver weight to body weight. It provides an indication on status of energy reserve in an animal. Gonado-Somatic Index (GSI) is the ratio of gonad weight to body weight used to estimate reproductive condition.GSI is generally indicative of reproductive success. It has been also reported that HSI, GSI and condition factor (K) are not only responsive to pollution but can be affected by other factors such as temperature and food availability (Pelissero et al., 1991; Liao et al., 2006, Davis et al.,

2009).K has been used as an indicator of health in fishing biology studies since the beginning of the 20 century, such as growth and feeding intensity (Sutton et al., 2000).Le Cren (1951) proposed relative condition factor (Kn) in preference to 'K' as the former considers all the variations like those associated with food and feeding, sexual maturity, etc., while the latter does so only if the exponent value is equal to 3. Thus 'K' factor measures the variations from an ideal fish, which holds the cube law while 'Kn' measures the individual deviations from the expected weight derived from the length-weight relationship. Any stresses in the natural environment can have an effect on fish overall health and condition; therefore, Kn can be employed as an integrative biomarker.Both anthropogenic and natural stressors are incorporated into Kn; however, there are natural fluctuations or differences in condition factor of fish due to species, sex, and season (e.g. temperature, spawning, photoperiod, prey quantity/quality) (Le Cren, 1951; Sutton et al., 2000; Björnsson et al., 1989).

Alterations in the HIS, GSI, K and Kn was further validated by histological examination. As reported by Vitale and coworkers (2005) histological examinations accurately represent the altered status of individual fish. Hence keeping this in mind in the present study an attempt is made to look into the histological alterations in gonads as well as liver of freshwater teleost fish, *Oreochromis mossambicus* and *Labeo rohita*.

MATERIALS AND METHODS

Oreochromis mossambicus,commonly known as Tilapia, having an average weight (25±2 g) and size (12±3 cm) and *Labeorohita* commonly known as Rohu, having an average weight (125±5 g) and size (25±3 cm) was brought from the local pond of Baroda and acclimatized to the laboratory conditions in a well aerated dechlorinated tap water for the period of 10 days. Commercial fish food was supplied to the fishes during whole experimental period. Test animals were categorized into treated and control groups (20 animals in each group). After establishing the LC_{50} values (5000 mg/l) acclimatized fishes were exposed to a sublethal dose of 250 mg/l and 300 mg/l ($LC_{50}/20$) for *O.mossambicus* and *L.rohita* respectively. The exposure period for the treated group was 15, 30 and 45 days. Control group was kept in dechlorinated water without any treatment. 30% water was changed after every 24 hours and physicochemical properties of water were measuredtwice in a week.

After the period of exposure fishes were removed and washed with freshwater. Control as well as treated groups were euthanized by decapitation and weighed; blood was allowed to drain and dissected to take out organs. The prime goal of the study was to know the reproductive and the metabolic health of the fish for the EDTA chelated micronutrient mixture (LIBREL™). Hence the total organ weight (liver and gonads) was taken for the Hepatosomatic (HSI) and Gonadosomatic (GSI) indices. The indices were calculated according to the following formula:

HSI = (Liver weight (g) / Fish weight (g)) × 100,

GSI = (Gonad weight (g) / Fish weight (g)) × 100.

K = (Fish weight (g)/Length^3) × 100.

Means ± standard deviation (SD) were calculated for both group. The homogeneity of variance were checked by Levene's test. Multivariate analysis of variance and Dunnett's multiple comparisons tests were used to determine significant differences between control and exposure groups using SPSS software version 21. The significant level for all statistical analyses was set at $p< 0.05$.

Further the tissues were fixed in 10% formalin for 24 hours, hydrated, embedded in paraffin wax, sectioned at 7-8 µm, stained with hematoxylin and eosin, observed microscopically to know the histological alterations. The photomicrography was done on system uses current Leica Delta technology and includes the modular DMRB research microscope frame with digital camera.

The results of the experimental groups were compared with the control group to quantify the effect of the EDTA chelated micronutrient mixture (LIBREL™) on the test animal.

Results

Plant nutrient exposure resulted into increase in HSI of and significant decrease in GSI and condition factor (Tables I & II and Figures I-IV).The P-values and level of significance found from the Dunnetts' multiple comparison tests are presented in the table (III-IV). Time dependent and gender specific changes were clearly observed. Females were found to be more sensitive compared to males ensuing insignificant changes in the GSI in *O.mossambicus*. In contrast *L.rohita* showed significant ($p<0.001$) changes in both sexes throughout the exposure period. As far as HSI is concerned insignificant changes were obtained at 15th day of exposure in both the species, whereas, at 30th day and 45th day of exposure resulted into moderately significant ($p<0.05$) and highly significant ($p<0.001$) change respectively. K index resulted into an insignificant decrease in *O.mossambicus* and highly significant decrease ($p<0.001$) in *L.rohita* . Along with the GSI, HSI and K the histological alterations in gonads and liver were also observed in the present study. Time dependent and species specific moderate to severe histological alterations were seen in all the organs (fig.).

Table I: Time dependent changes in gonadosomatic index (GSI) and hepatosomaticindex (HSI) of fish males*(n=5)*exposed to plant nutrient

Fish Species	Groups	HSI			GSI		
		Min.	Max.	Mean ± SD	Min.	Max.	Mean ± SD
O.mossambicus	**Control**	0.51	0.93	0.7210±0.207	0.27	3.11	2.0699±1.566
	15 Days	0.58	1.08	0.7769±0.267	0.10	0.46	0.2332±0.195
	30 Days	0.63	0.91	0.7532±0.142	0.09	0.21	0.1456±0.0605
	45 Days	1.00	1.72	1.4506±0.396	0.25	0.34	0.2969±0.045
L.rohita	**Control**	0.69	0.98	0.8500±0.147	1.21	1.42	1.3200±0.105
	15 Days	1.08	1.33	1.2232±0.129	0.99	1.23	1.1233±0.122
	30 Days	1.67	1.98	1.8067±0.158	0.77	1.11	0.9467±0.170
	45 Days	1.55	1.99	1.8400±0.251	0.45	0.69	0.5667±0.120

Table II: Time dependent changes in gonadosomatic index (GSI) and hepatosomatic index (HSI) of fish females*(n=5)* exposed to plant nutrient

Fish Species	Groups	HSI			GSI		
		Min.	Max.	Mean ± SD	Min.	Max.	Mean ± SD
O.mossambicus	Control	0.24	0.50	0.4010±0.140	0.70	4.14	2.7688±1.824
	15 Days	0.66	1.12	0.8722±0.230	1.17	3.73	2.8780±1.478
	30 Days	0.92	1.09	0.9936±0.087	1.58	1.80	1.6775±0.113
	45 Days	1.06	1.53	1.2161±0.270	0.42	0.93	0.7092±0.265
L.rohita	Control	1.23	1.66	1.4467±0.215	2.56	3.11	2.8767±0.284
	15 Days	1.87	2.01	1.9567±0.075	2.03	2.42	2.1867±0.205
	30 Days	2.09	2.23	2.1433±0.057	1.22	1.89	1.5567±0.335
	45 Days	2.03	2.56	2.3467±0.279	0.99	1.54	1.2500±0.276

Table III: Dunnetts' multiplecomparison tests showing the P values ofGSIin fish

GSI	15 days		30 days		45 days	
	Males	Females	Males	Females	Males	Females
O.mossambicus	0.052	0.999	0.043*	0.563	0.061	0.151
L.rohita	0.234	0.040*	0.021*	0.001**	0.000***	0.000***

Table IV: Dunnetts' multiple comparison tests showing the P values of HSI in fish.

HSI	15 days	30 days	45 days
O.mossambicus	0.190	0.102	0.000
L.rohita	0.099	0.002	0.000

Table V: Dunnetts' multiple comparison tests showing the P values of K in fish.

Condition factor	15 days	30 days	45 days
O.mossambicus	0.966	0.744	0.944
L.rohita	0.018	0.000	0.000

Fig. I: Alterations in the Gonadosomatic index (GSI) in *O.mossambicus* exposed to Plant nutrient Librel.

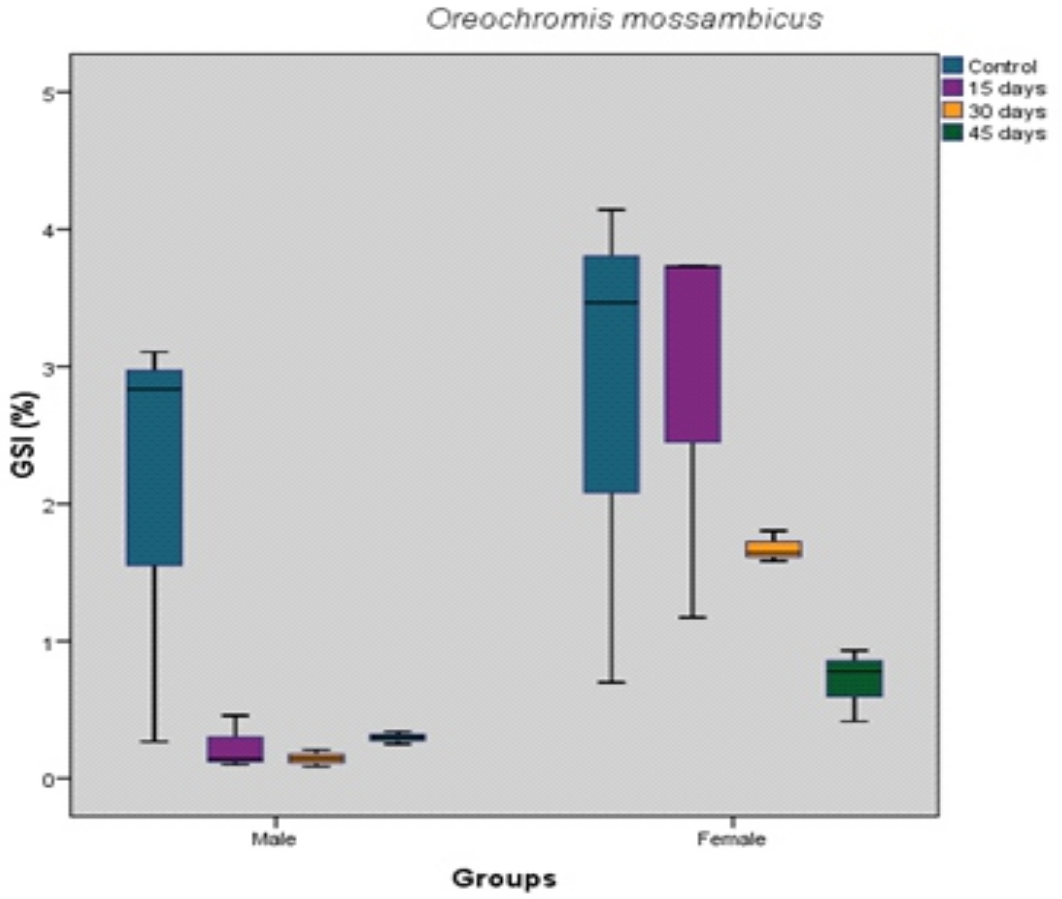


Fig. II: Alterations in the Gonadosomatic index (GSI) in *L.rohita* exposed to Plant nutrient Librel.

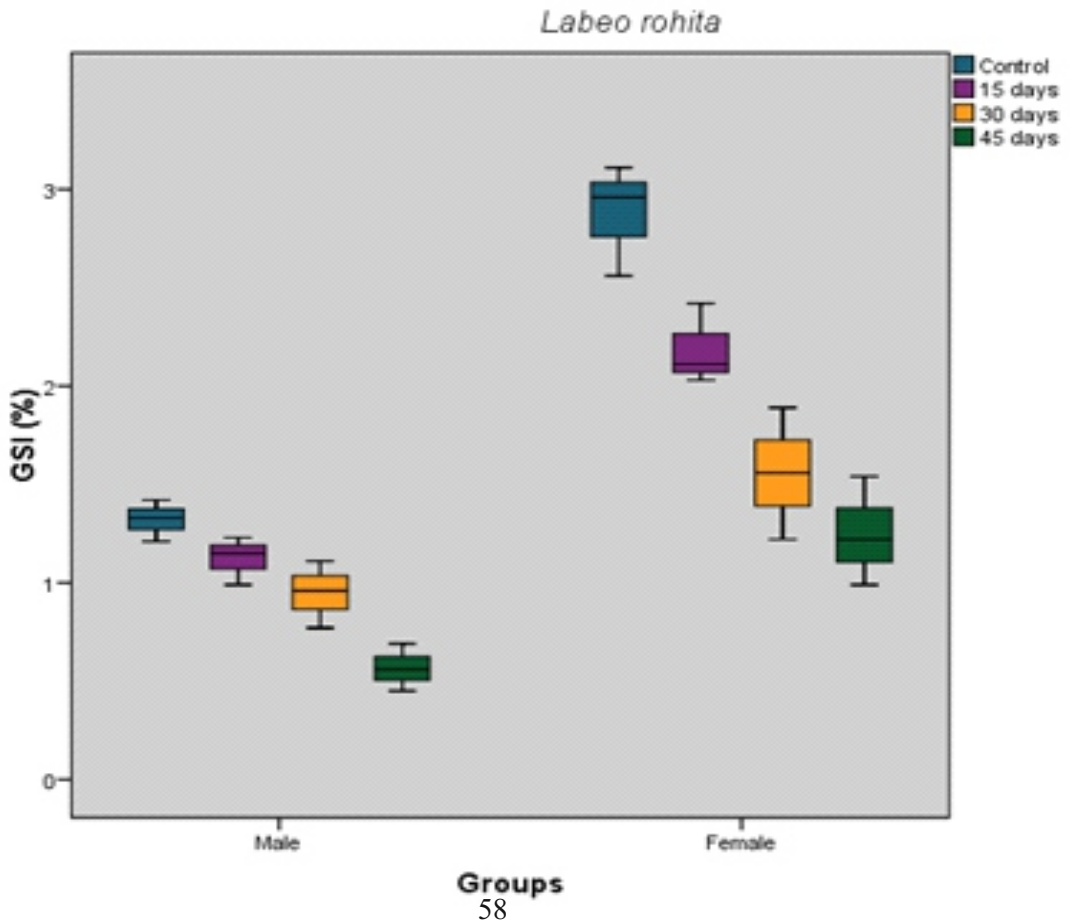


Fig. III: Alterations in the Hepatosomatic index(HSI) in *O.mossambicus* and *L.rohita* exposed to Plant nutrient Librel.

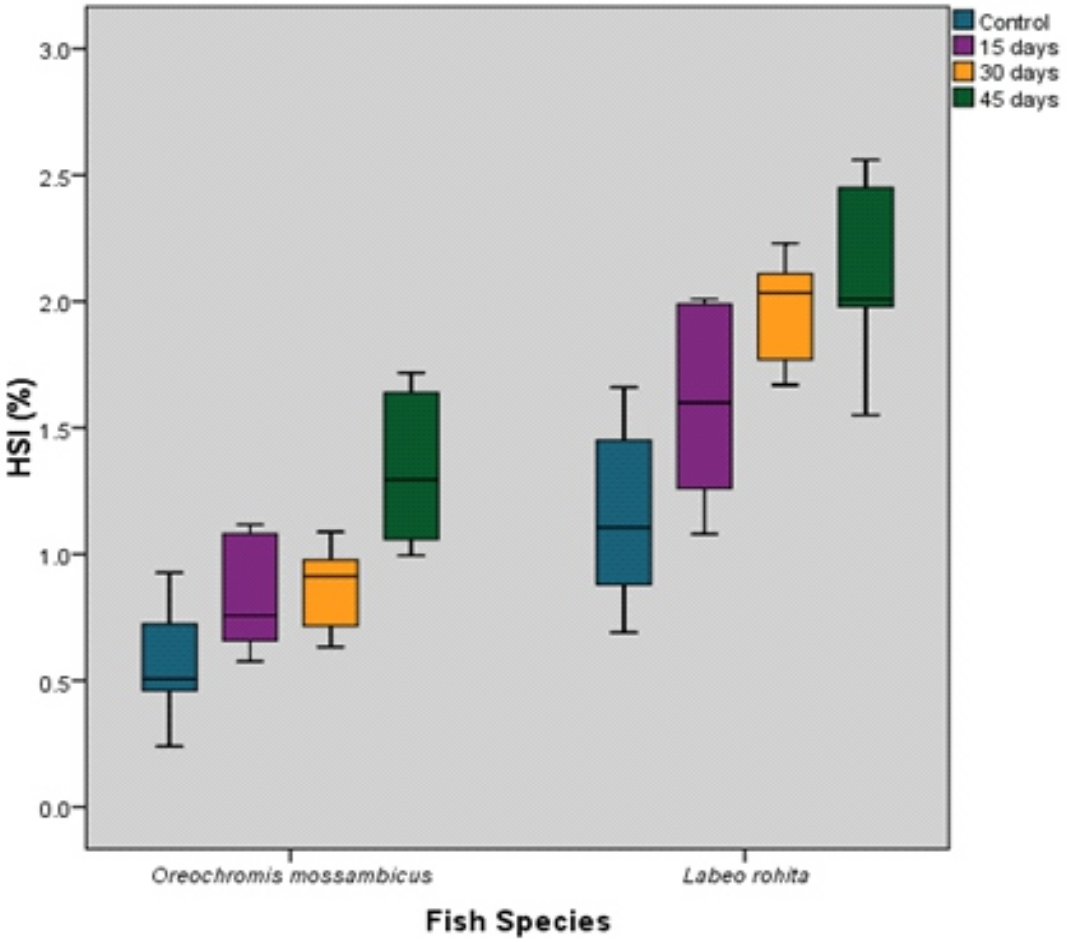
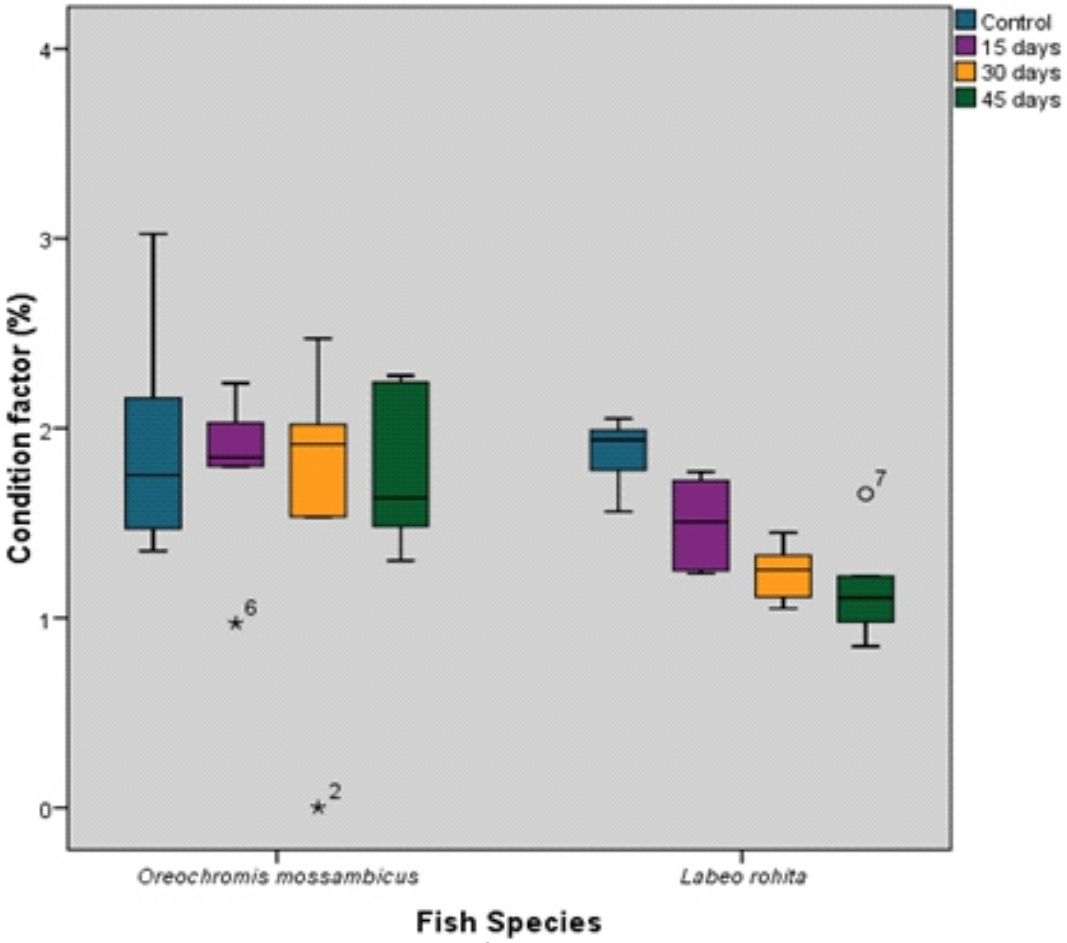
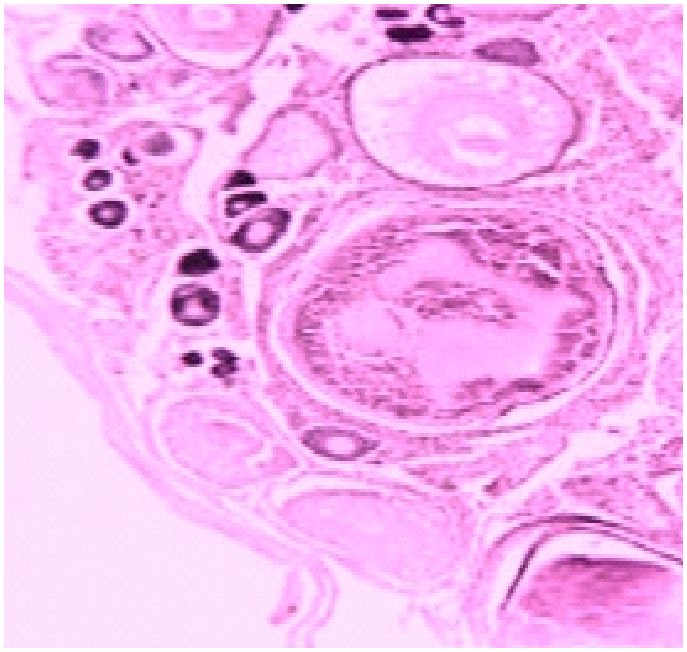
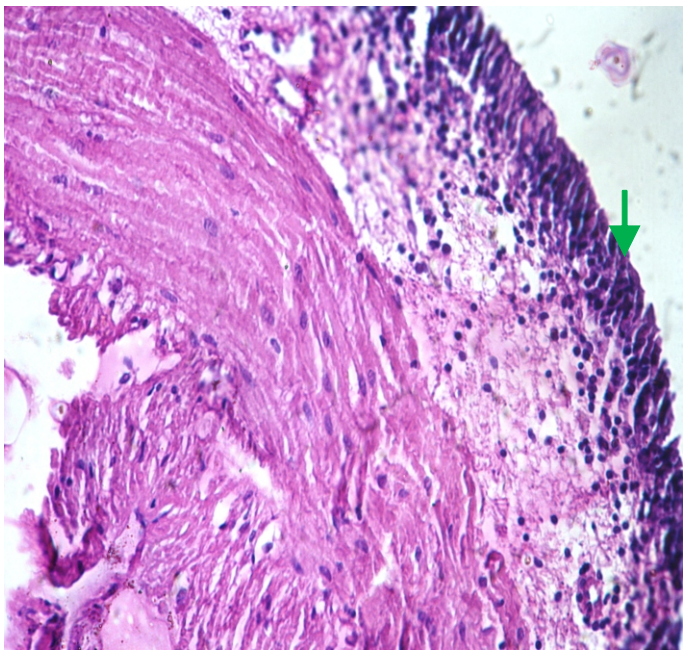


Fig. IV: Alterations in the Condition factor (K) in *O.mossambicus* and *L.rohita* exposed to Plant nutrient Librel.

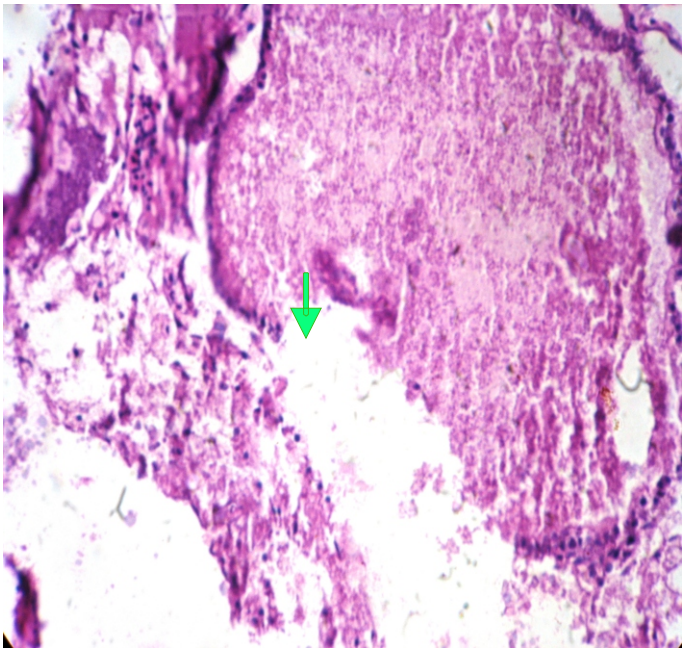




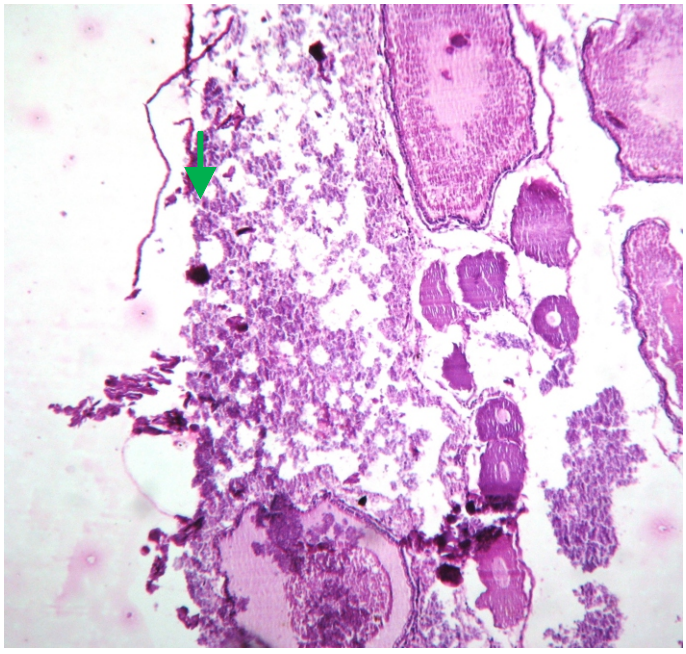
a. Control Group



b. 15 days group

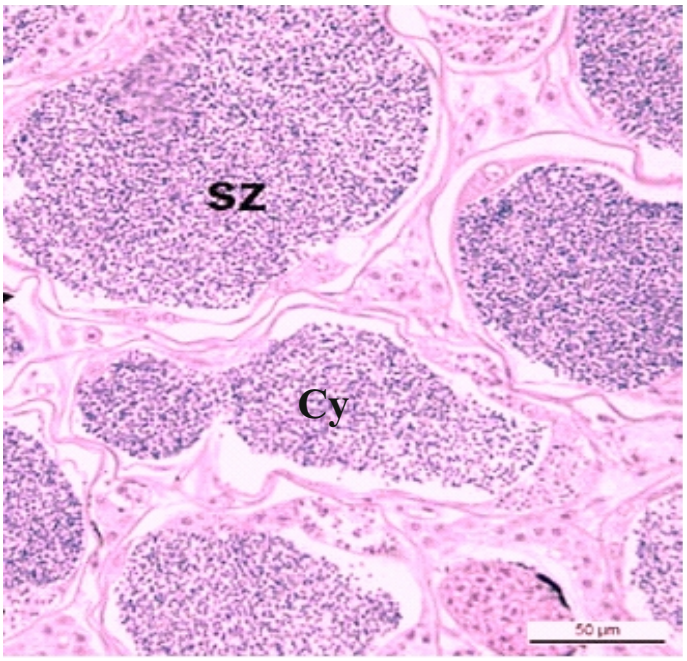


c. 30 days Group

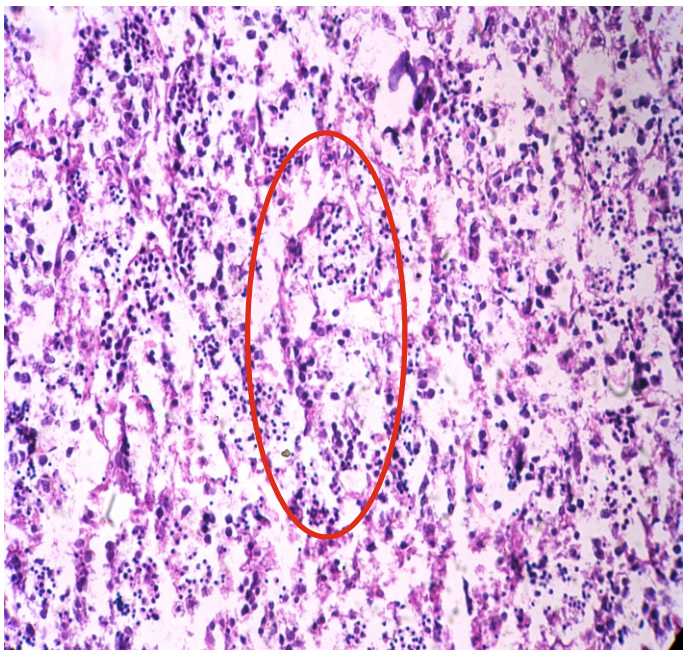


d. 45 days Group

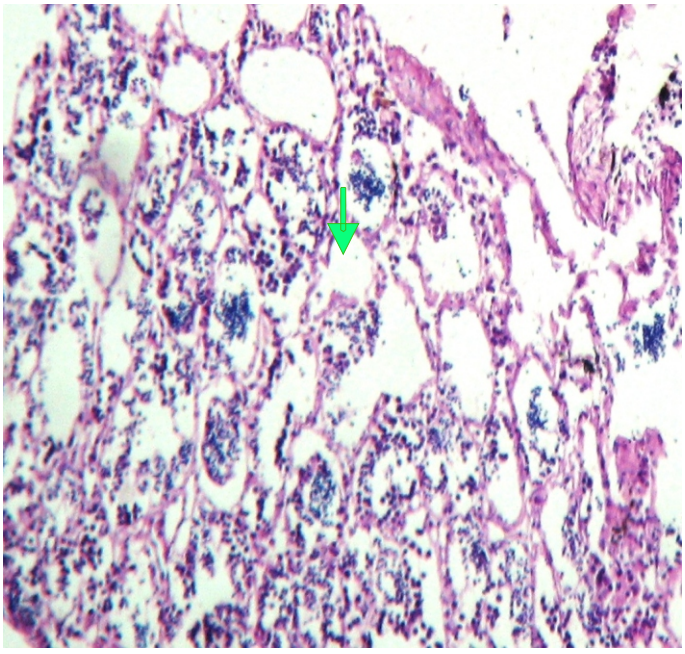
Photomicrographs I: Cross sections of Ovaries of *O.mossambicus* fish stained with H.E. (a) Ovary at control fish showing normal ovary tissue (x200); (b) Ovary at 15 day exposure fish to Librel (x200); (c) Ovary at 30 day exposure fish to Librel (x200); (d) Ovary at 45 day exposure fish to Librel (x200)
Control group showing ovarian stages and nucleus; Exposed groups showing degenerative changes in the cellular elements of oocytes, Loss of circularity of nucleus degeneration in wall of oocytes, Large vacuoles
Thinning and degeneration of Ovarian wall (green arrow); Oocytic stages are not intact
Degeneration of germinal epithelial cell of oocytes (red arrow), vacuoles (blue arrow)



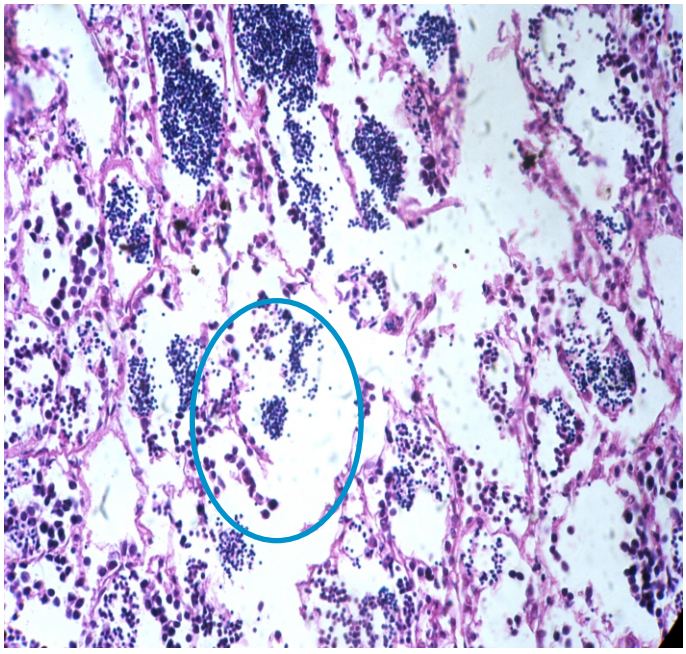
a. Control Group



b. 15 days group



c. 30 days Group

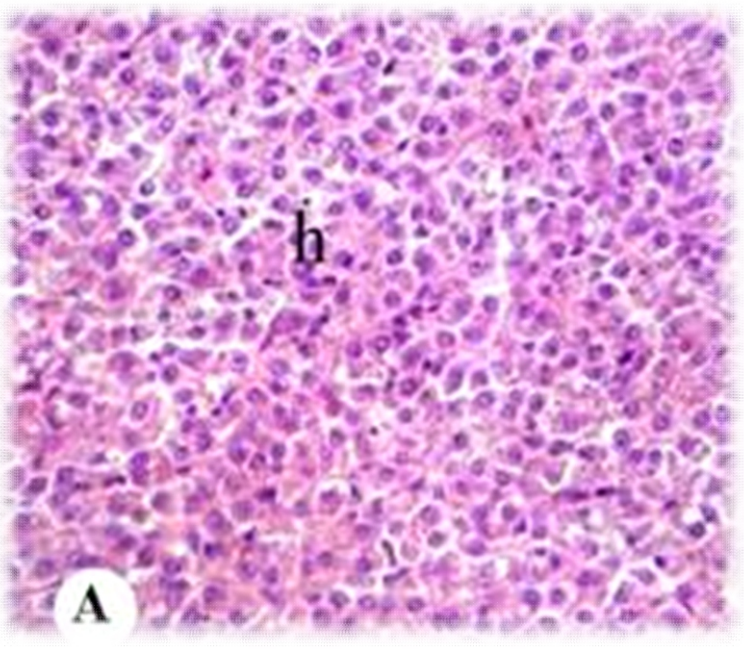


d. 45 days Group

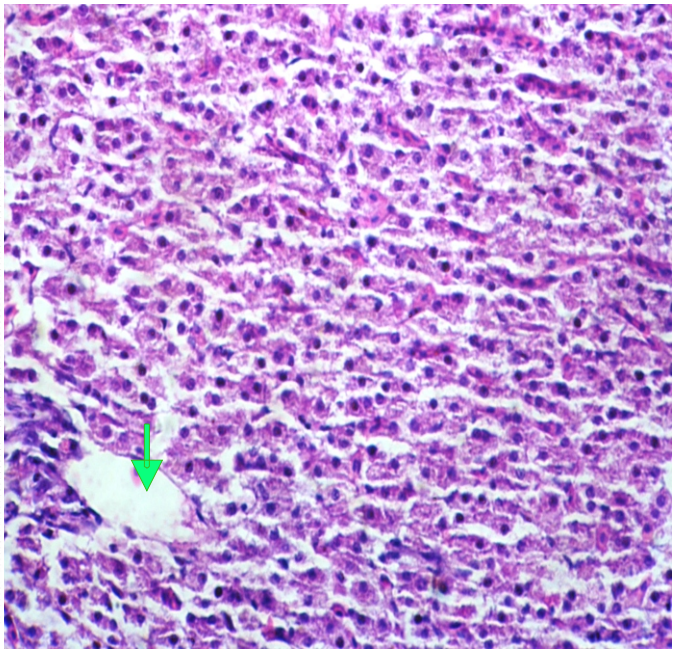
Photomicrographs II: Cross sections of Testis of *O.mossambicus* fish stained with H.E. **(a)** Testis of control fish showing normal testis tissue (x200) **(b)** Testis of 15 day exposure fish to Librel (x200); (c) Testis of 30 day exposure fish to Librel (x200); **(d)** Testis of 45 day exposure fish to Librel (x200).

Control group showing undisturbed cysts and spermatozoa; Exposed groups showing degenerative changes in the cellular elements of spermatozoa; Disorganization of intertubular tissue.

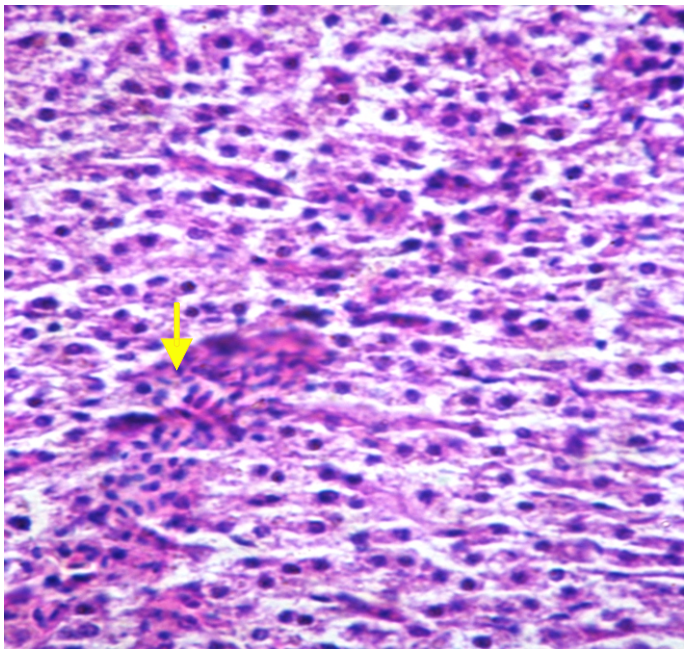
Cy-undisturbed cyst; sz-spermatozoa; Disorganization (red circle) and degeneration (blue circle) of tubular cysts associated with Intertubular vacuolization (green arrow).



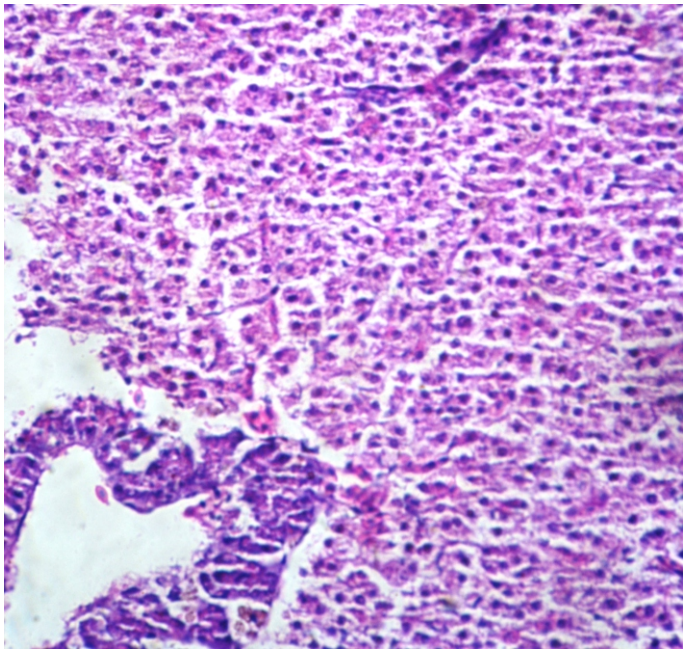
a. Control Group



b. 15 days group



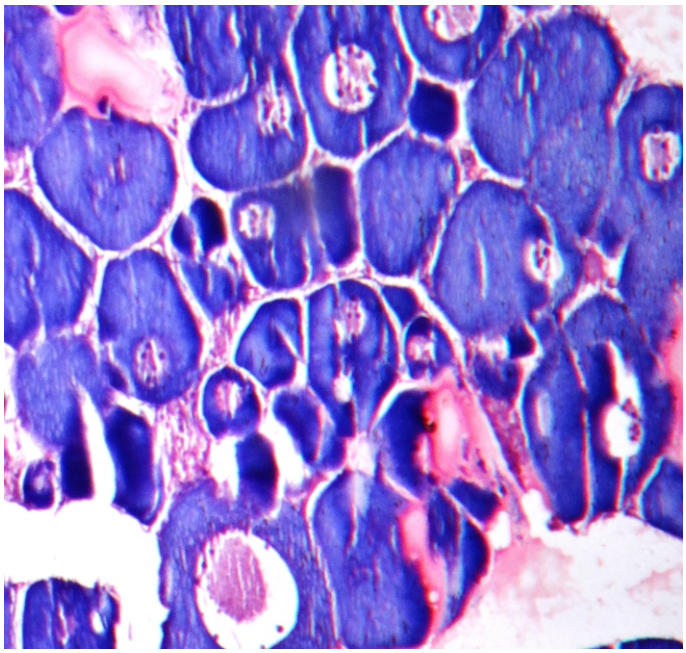
c. 30 days Group



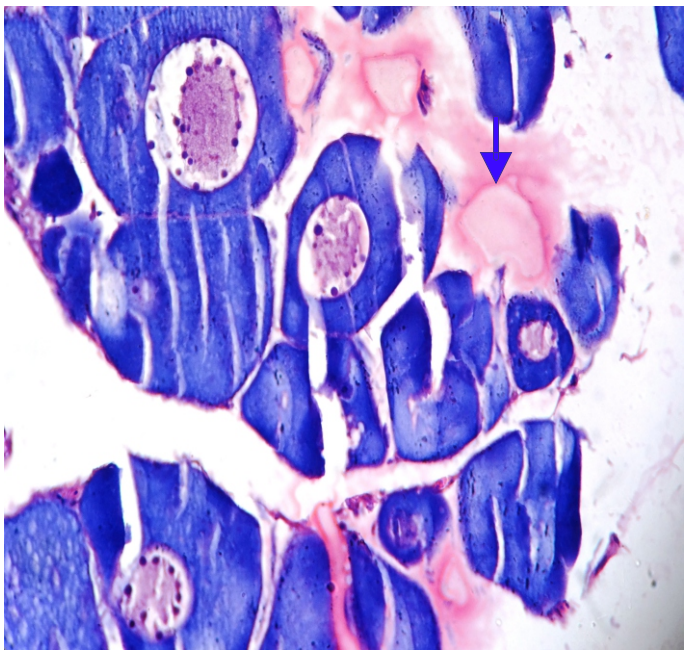
d. 45 days Group

Photomicrographs III: Cross sections of Liver of *O.mossambicus* fish stained with H.E. (a) Liver of control fish showing normal ovary tissue (x200); (b) Liver of 15 day exposure fish to Librel (x200); (c) Liver of 30 day exposure fish to Librel (x200); (d) Liver of 45 day exposure fish to Librel (x200)
Control group showing intact hepatocytes with a distinguished cell out line; Exposed groups showing vacuolation, hemorrhage of blood cells, indistinguished cell outline of hepatocytes.

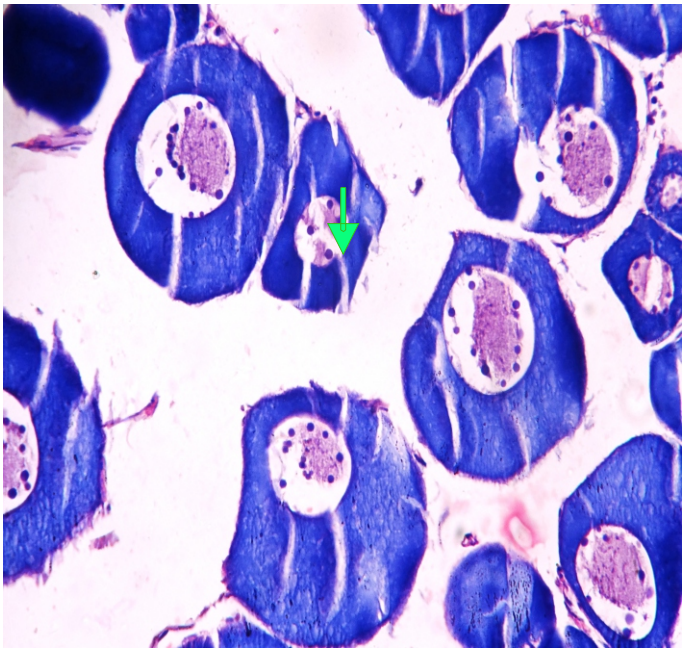
H: hepatocytes; Cell outline of hepatocytes became indistinguishable. Dilation and hemorrhage of blood vessels in hepatic sinusoids (yellow arrow) resulting into its blockage. Mild vacualation (green arrow).



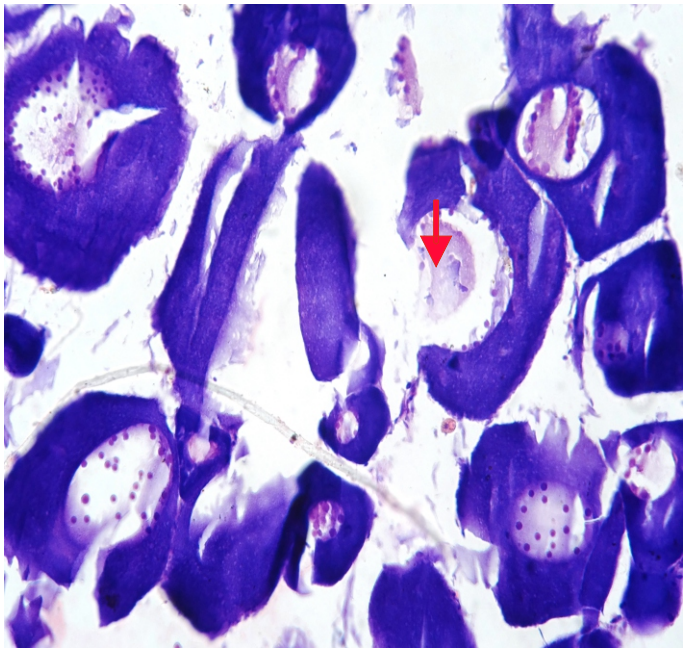
a. Control Group



b. 15 days group

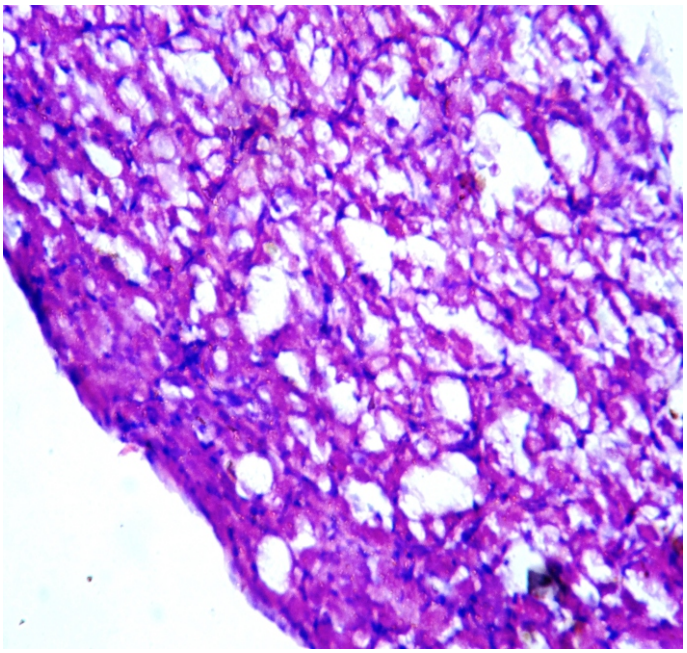


c. 30 days Group

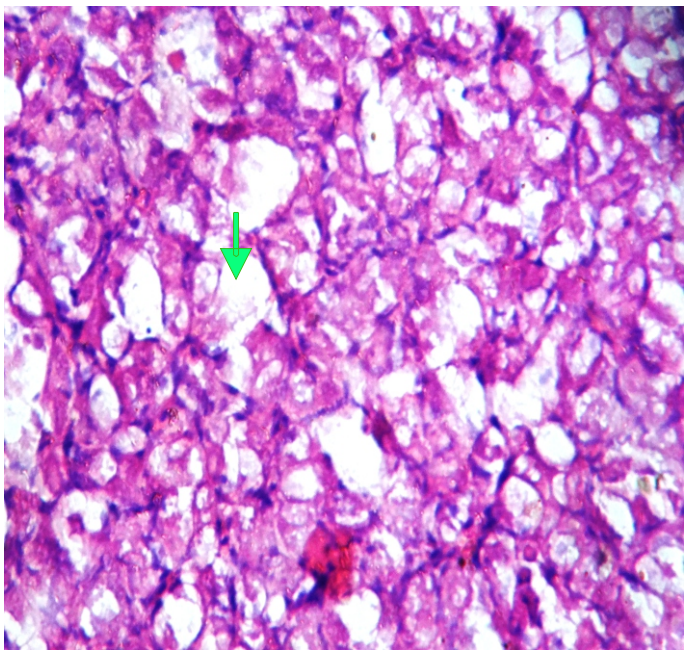


d. 45 days Group

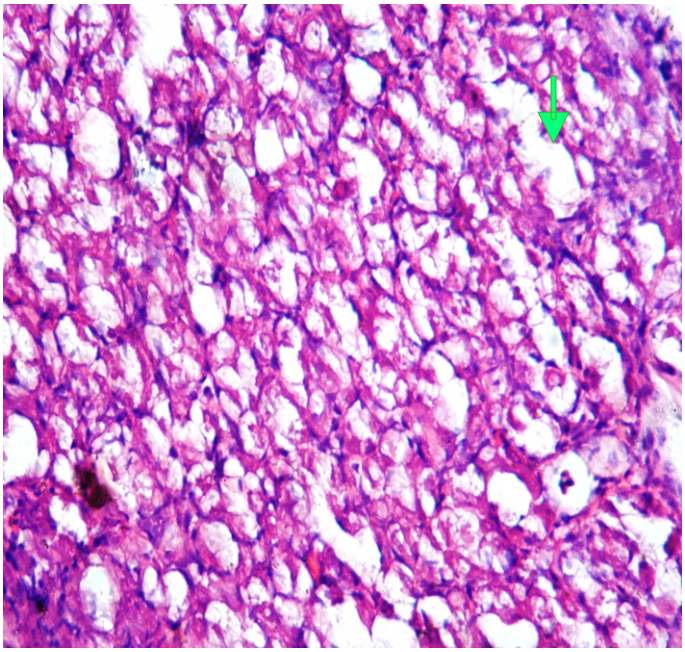
Photomicrographs IV: Cross sections of Ovaries of *L.rohita* fish stained with H.E. (a) Ovary at control fish showing normal ovary tissue (x200); (b) Ovary at 15 day exposure fish to Librel (x200); (c) Ovary at 30 day exposure fish to Librel (x200); (d) Ovary at 45 day exposure fish to Librel (x200)
Control group showing ovarian stages and homogenours cytoplasm, Nuclei having distinct nucleoli and normal distribution of chromatic network ; Exposed groups showing degenerative changes in the cellular elements of oocytes, liquefied cytoplasm, deformed nucleoli and crumpled chromatin network
Thinning and degeneration of Ovarian wall (green arrow); liquified cytoplasm (blue arrow), crumpled chromatin network (red arrow)



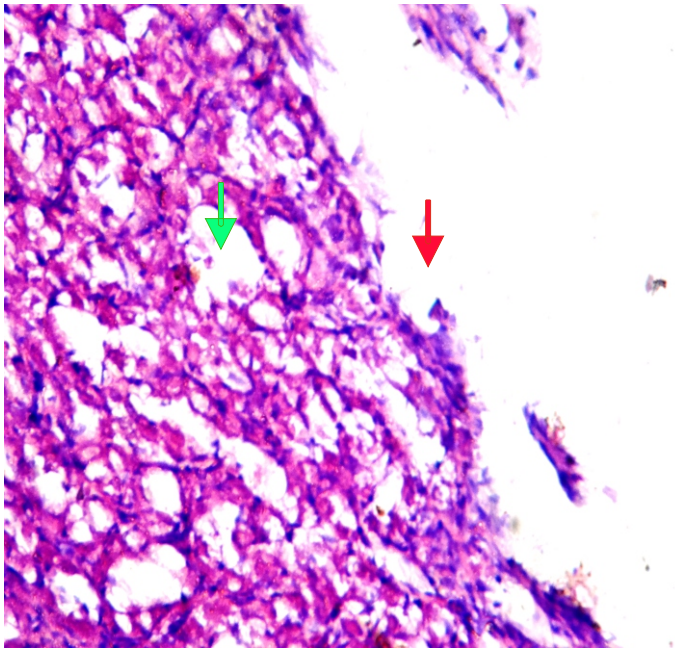
a. Control Group



b. 15 days group



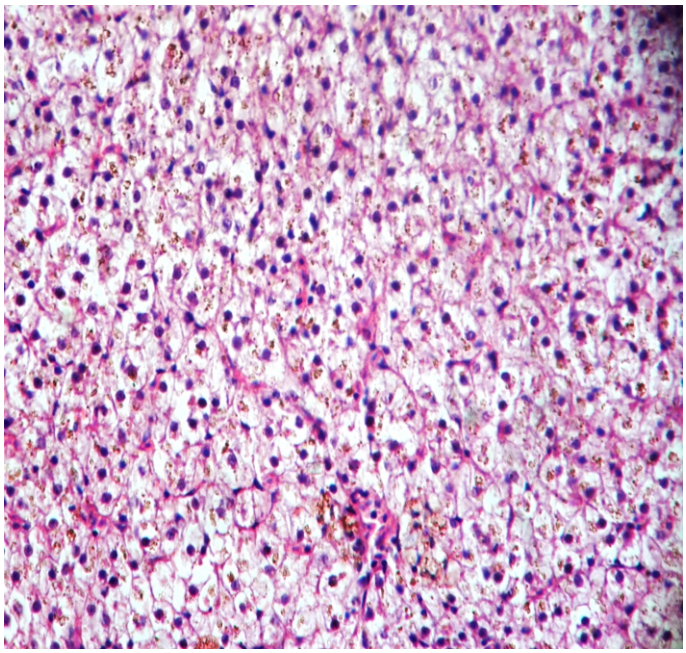
c. 30 days Group



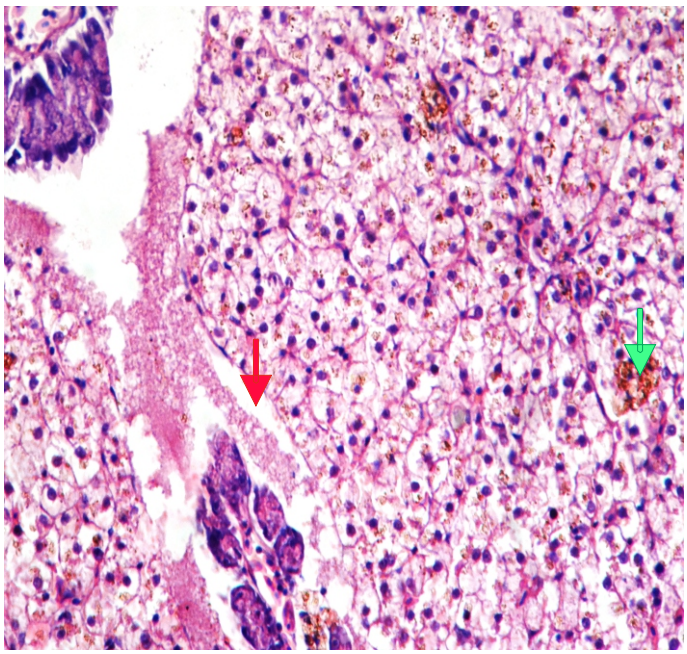
d. 45 days Group

Photomicrographs V: Cross sections of Testis of *L.rohita* fish stained with H.E. **(a)** Testis of control fish showing normal testis tissue (x200) **(b)** Testis of 15 day exposure fish to Librel (x200); (c) Testis of 30 day exposure fish to Librel (x200); **(d)** Testis of 45 day exposure fish to Librel (x200).

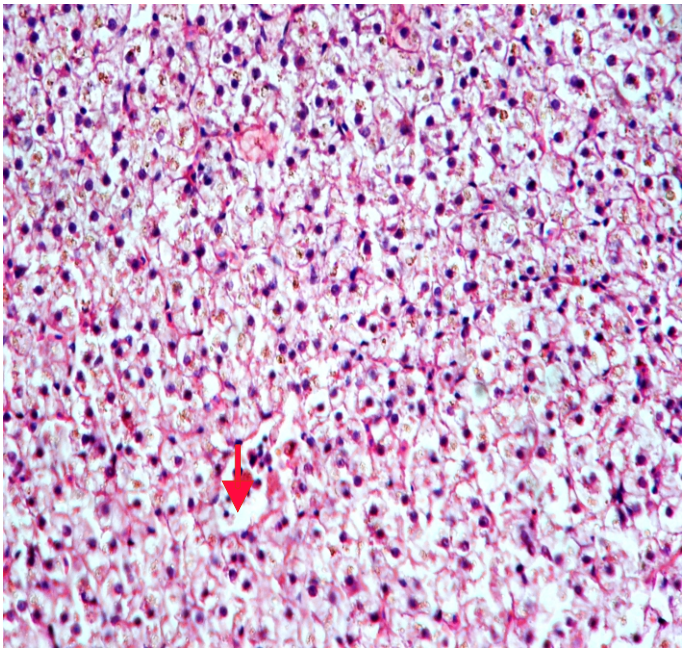
Control group seminiferous tubules have definite interlobular membrane; Exposed groups showing damaged membrane and spermatogenic cell layer; Disorganization of intertubular tissue; Complete disintegration of interlobular membrane
Damaged membrane (red arrow) (blue circle) of tubular cysts associated with Intertubular vacuolization (green arrow).



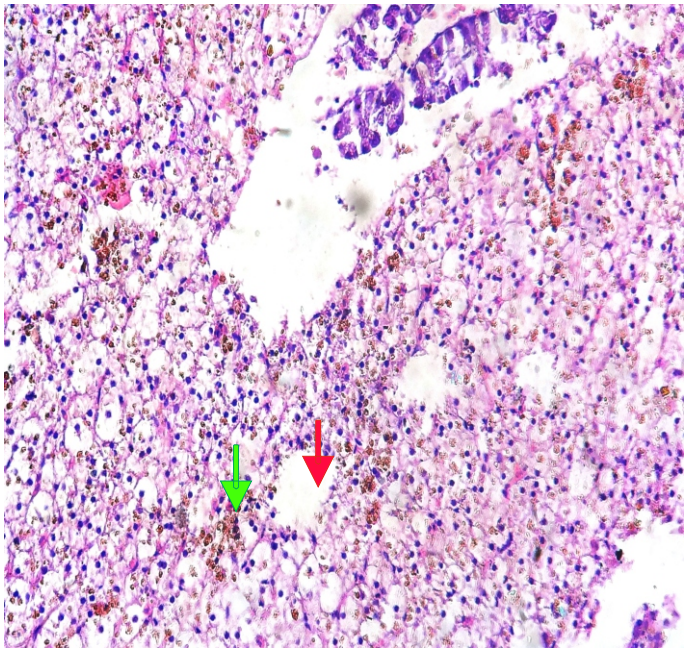
a. Control Group



b. 15 days group



c. 30 days Group



d. 45 days Group

Photomicrographs VI: Cross sections of Liver of *L.rohita* fish stained with H.E. (a) Liver of control fish showing normal ovary tissue (x200); (b) Liver of 15 day exposure fish to Librel (x200); (c) Liver of 30 day exposure fish to Librel (x200); (d) Liver of 45 day exposure fish to Librel (x200)

Control group showing intact normal hepatic acini arrangement in cord of one layer thickening surrounding the bile duct; Exposed groups showing vacuolation, obliterated liver structure, melanomacrophage aggregated, cytoplasmic vacuoles and pycnotic nuclei were observed.

Cell outline of hepatocytes became indistinguishable. cytoplasmic vacuoles (red arrow) resulting into its blockage. melanomacrophage (green arrow).

Discussion

Gonadosomatic Index (GSI) is the percentage ratio of the gonad weight and body weight used to determine fecundity among fish (Janz et al, 1997). It is generally used as an indicator of fish sexual condition. In the present studies Plant nutrient exposure resulted into a time-dependent decrease in the GSI, suggesting that the effects were expressed at long time exposure. Furthermore, in both the species it was females which showed significant changes compared to males, implying that the females are more sensitive. Our results are in agreement with Hanson *et al.* (2007) who have also documented that female GSI decreased significantly than males as compared to control in 3 fishes *O. niloticus*, *C. gariepinus* and *C. nigrodigitatus.*To authenticate the GSI results the histological observations were also performed. Normal histology of the ovary of *O. mossambicus*reveals that it is surrounded by an ovarian wall that is differentiated into an outer thin peritoneum, a thicker tunica albuginea made up of connective tissues, muscle fibers and blood capillaries. The innermost layer is the germinal epithelium which joins with the tunica albuginea at several places and projects into the central lumen, the ovocoel, in the form of finger like projections called ovigerous lamella. Fish exposed to the plant nutrient showed progressive thinning and degeneration of ovarian wall which was apparent at the 30th and 45th day. Oocytic stages were not intact. Degeneration of germinal epithelial cells of oocytes caused vacuolation. At the 30^{th} and 45^{th} day more vacuolated follicular epithelium and degenerative cytoplasm was observed. The cytoplasm showed vacuolization at the periphery of oocyte which gradually extended towards the centre. Analogous histopathological findings were reported by Hossain*et al.* (2002) in the ovaries of *Anabas testudineus* and *C. punctatus* on the exposure of pesticide, dimecron 100SCW and by Giri*et al.*(2000) on exposure of insecticide basathrin in catfish, *H. fossilis*. They reported marked damage in germinal epithelium, atresia of ocyte, stromal hemorrhage, vacuolization of oocytes and general inflammation. Hilderbrand*et al*.(1973).

Normal histology of the ovary of *O. mossambicus* revealed an intact ovarian wall with an outer thin peritoneum and a thicker tunica albuginea made up of connective tissues, muscle fibers and blood capillaries. The innermost layer is the germinal epithelium which joins with the tunica albuginea at several places and projects into the central lumen, the ovocoel, in the form of finger like projections called ovigerous lamella. The cytoplasm showed vacuolization at the periphery of oocyte which gradually extended towards the centre. Thus the histopathological alterations were seen to get adversed with time. Moreover the study have shown insignificant changes in the

testicular GSI of fishes. Though the changes were insignificant but the histological makeup of testes was time dependent. Progressively there was an increase in the vacuolization, disorganization and distortion of seminiferous tubules (Fig. 4). At the 45th day of exposure condensation of spermatocytes besides inflammation and inter-tubular vacuolation was very much prominent. As reported by some investigators (Sokal*et al*. 1985, Ruby *et al*.1986, 1987, Gaber*et al*.2013) testicular inflammation has been documented as one of the common responses on the aquatic animals exposed to environmental toxicants. Testis in fish represents the most dynamic organ having a high cell turnover during the reproductive period which makes it vulnerable to a wide variety of chemical toxicants. Scientists such as Katti and Sathyanesan, (1985)observed exposure time dependent and concentration-mediated changes in testis of *Clariasbatrachus* treated with lead (Kinnberg et al.2000) in *Xiphophorus maculates* (Zutshi2005) in *Glosso gobiusgiuris* (Zutshi, and Murthy, 2001) in *Glossogobiusgiuris* and (Kumar et al. 2007) in *H. fossilis* have reported various cytotoxic effects on testis due to the exposure of the different toxicants. These changes may culminate in a partial or total arrest of spermatogenesis. Thus the present study suggests that the extent of damage in the fish gonads depends on the time of exposure of the toxicant. These changes in the histological make clearly states that reduced GSI in the present study may be due to lowered gonadal activity under plant nutrient stress and impairment of the production of steroid hormones which might have arrested the formation of germ cells and cause degeneration or necrosis (Sharma et al. 2012).

Liver is the metabolic organ. It is a target for the metabolism in the fish body, the liver index (HSI) is a useful biomarker to detect the hazardous effects of the environmental stressors (Pait, and Nelson, 2003). In the present study a significant increase in the HSI was documented on exposure of the plant nutrient. Pesticides are metabolized in the liver through cytocrhrome P450 system through hepatotoxic intermediates (Das and Gupta, 2013) Several field and laboratory studies have recorde an increase in HSI in fishes exposed to organochlorine (Kurutap and Doran, 2001; Roche et al., 2000), while Salvo et al 2008 and Bhattacharya and Kaviraj, 2009 have reported reduction in HSI. These differences reflect the nature of response of the liver to different toxicants. In the present study increased HSI is thus reflecting the response of the liver to the plant nutrient. As reported by Authman (2011) increased HSI may be attributed to the accumulation of lipid in liver tissue of fish on plant nutrient exposure, furthermore Munshi and Dutta (1996) have stated that the HSI of Osteichthyes is normally between 1% and 2%. The HSI

values of the exposed fishes exceeded this range. Although hepatosomatic indices can vary with nutrition, season, fish condition and disease, a relationship between hepatosomatic index and levels of contamination was reported by Sloof et al. (1983).The possible interpretation of the variation of hepatosomatic index may coincide with that suggested by Fabacher and Baumann (1985), that the enlarged livers could result in increased activity of hepatic mixed-function oxidase enzymes and thus develops an increased ability to metabolize xenobiotics. The teleost liver is one of the most sensitive organs with regard to showing alterations in histoarchitecture, biochemistry, and physiology following exposure to various types of environmental pollutants (Roy and Bhattacharya, 2006). Bruslé and González I Anadon (1996) stated that, fish liver histology could serve as a model for studying the interactions between environmental factors and hepatic structures and functions. The harmful effect of metal pollution on fish liver histology may, however, depend on the duration of the exposure and the concentration level of the specific metal (van Dyk et al., 2007). The present study documents pathologic changes in fish treated with Plant nutrient for three different time intervals. The degree of pathology gradually increased during the entire days of experiment which exhibited time-dependent changes.

Liver histology of control and exposed fish is briefly illustrated in Fig. 5. In the control group, the liver exhibited a normal architecture and there were no pathological abnormalities, with hepatocytes presenting a homogenous cytoplasm, and a large central or subcentral spherical nucleus, whereas in the tissues of exposed fish cell outline of hepatocytes was indistinguishable and it is evident that this effect increased with the increase in the exposure time. The effect on the cell outline resulted in the mild vacuolation in the cytoplasm which were much more prominent at the 15th day. Hemorrhage of blood vessels in hepatic sinusoids was seen which eventually resulted to its blockage and affecting the metabolic activity. Researchers (Montaser*et al*.2010,Biuki*et al*.2012, Pugazhvendan*et al*.2009) in their work have also identified the same liver damage due to the toxicity of heavy metals.Furthermore, Agius and Robert, (2003), Stentiford et al. (2003) and Authman et al., (2008). have got similar histological alterations in liver of flounders and have correlated the altered pathological condition with the expenditure of energy in the detoxification process. Thus our results are in agreement with the results of thosementioned by Liao et al. (2006) when exposed medaka (Oryziaslatipes) to sublethal exposure of methylmercury chloride, Roy and Bhattacharya (2006) when exposed *Channapunctatus* to arsenic,andvan Dyk et al. (2007)when exposed *Oreochromismossambicus* to

cadmium and zinc. The study suggests that in situ long-term exposure could be responsible for integrated biological effects related to essential physiological functions, like metabolism and development or reproduction.

Condition factor is one of most important parameters, which throws light on the physiological state of fish in relation to indication of the onset of sexual maturity (Salam and Davies, 1994). It has been used as an indicator of health in fishing biology studies since the beginning of the 20 century, such as growth and feeding intensity (Honeyfield *et al*.2008).Condition factor decrease with increase in length (Badawy,1998, Barakat, 2004). The condition factor provides information on the variation of fish physiological status and may be used for comparing populations living in certain feeding, climate and other conditions (El-Kabbany *et al*.2000, Gupta *et al*.2002, El Nameki, and Badawy, 2005). Therefore, condition factor can be used to determine the feeding activity of a species to determine whether it is making good use of its feeding source (Gupta *et al*.2002, Radwan, and Atalla, 2005, El Nameki, and Badawy, 2005). Fisheries biologists routinely employ the body condition as a straightforward measure of fish condition because it is an inexpensive, non-lethal alternative to proximate analysis of tissues (Sutton and others, 2000), though there are assumptions that may be violated and limitations to its application. Condition factors, when low or having declined, may be interpreted as a depletion of energy reserves, such as stored liver glycogen and body fat. Prudent interpretation of fish condition factor data considers that condition factors can vary seasonally because of changes in feeding activity and nutrient availability, sexual maturational stages when energy is shifted from somatic to gonadal processes, geographically or spatially because of subpopulation differences, and sex. In fisheries science, the condition factor is used in order to compare the “condition”, “fatness” or wellbeing of fish. It is based on the hypothesis that heavier fish of a particular length are in a better physiological condition (Bagenal and Tesch, 1978). Condition factor is also a useful index for monitoring of feeding intensity, age, and growth rates in fish (Bakare, 1970; Fagade, 1979) . It is strongly influenced by both biotic and a biotic environmental conditions and can be used as an index to assess the status of the aquatic ecosystem in which fish live (Khan and Ansari, 2005). In the present studies the condition factor revealed species specific alterations. A time dependent decrease was a prominent feature expressed by *L.rohita*, whereas *O.mossambicus* showed significant decrease only on 45th day of exposure possibly due to its sturdy physiology. Such a contrasting results has also been observed by Arslan et al., (2004) in *Salmotrutta* and by Naeem

et al., 2010 and 2011 in *O. mossambicus* and *L.rohita*.According to Barton, et al 2003; Barton 2002 a fish that is heavier for a given length is generally considered to be a healthier fish. Thus a fish with higher condition index is generally considered to be a healthier fish with extra weight and extra energy reserves. Lighter fish lack energy reserves and tend to be more susceptible to environmental stressors. A low body condition may also suggest muscle wasting (proteolysis) indicating a starvation response (Barton, 2002). It has also been suggested that females with a lower body condition reduce reproductive investment and have an increased risk of mortality (Courtney et al., 2012). Condition factors, when low or having declined, may be interpreted as a depletion of energy reserves, such as stored liver glycogen and body fat.

Putting down all the changes together obtained in the HSI, GSI as well as K it can be concluded that the fishes are more susceptible to the plant nutrient which has resulted into the metabolic and reproductive damage/fecundity which is apparent in sequential changes observed in histomorphological structures of liver as well gonads of the test organism. However the effects whether reversible or irreversible can be deduced only after the conduction of the recovery studies.

CHAPTER IV

Biochemical And Oxidative Stress Response of Plant Nutrient In *Oreochromis mossambicus* and *Labeo rohita*

INTRODUCTION

In agriculture, soils'inability to supply micronutrients to crops are alarmingly widespread issue of interest and concern across the globe due to the deficiency of trace elements such as zinc, iron, copper, manganese and boron (White and Zasoski, 1999). Several researchers have addressed this issue of the micronutrient deficiency (Katyal and Vlek, 1985;Rashid and Ryan, 2004;Welch and Graham, 2005;Gupta, 2005)in India and have deduced that the least requirement of human existence needed to meet metabolic demands during all seasons is not met. In contrast the dramatic increase in the agricultural production has resulted into the increased absorption of the nutrients from the soil leading to the increased use of traditonal fertilizer practices, NPK, trace metals and micronutrients in the soil. These chemicals though helpful to the plants, in excess enters the nearby aquatic sytems through runoff affecting the non targeted aquatic species especially fishes (Jezierska and Witeska, 2006). Inadvertently the ingestion of such metal contaminated fishes represents a human health risk (Jomova and Valko, 2011; Mozaffarian and Rimm, 2006;Wang*et al.*, 2005).

Free radicals are atoms,molecules, or ions with unpaired electrons on an otherwise open shell configuration. Oxygen and nitrogen free radicals are essential in thephysiological control of cell function in biologicalsystems and are continuously produced in living cells(Halliwell and Gutteridge, 1989; 1999) during basic cellular metabolismin aerobic organisms. These being highly reactive are known to take part in chemical reactions. Such oxygen free radicals and nonradical reactive species (superoxide anion radical, O_2^-; hydrogen peroxide, H_2O_2; peroxyl radicals, ROO; nitrogen oxide, NO),referred as reactive oxygen species(ROS) are known to be used by organisms for advantageous biological effects (Droge, 2003).

ROS concentration is a dynamic parameter, i.e. they are continuously generated and eliminated, in other words the amount of ROS produced is virtuallyequal to the eliminated one. However, due to some reasons ROSconcentration may be changed leading to disturbance of redox statuscalled as oxidative or reductive stress."Oxidativestress" is a situation when steady-state ROS concentration is transientlyor chronically enhanced, disturbing cellular metabolism and its regulationand damaging cellular constituents(Lushchak, 2011b; Nishida, 2011). On the other hand "Reductive stress" may

bedefined in with the only difference that steady-stateROS concentration is decreased (Lushchak, 2011). The maintainence of "redox homeostasis" is essential in the higher animals for the physiological health of the organisms. (Ames*et al.,* 1993). But, during metabolicprocesses, a small proportion (2–3%) of free radicalsmay escape from the protective shield of antioxidantmechanisms, causing oxidative damage to cellularcomponents. These have propensity todamage crucial cellular components, such as lipids, carbohydrates,proteins, and DNA (Kelly *et al.,* 1998). Exposureto numerous xenobiotics can cause oxidative stress throughvarious mechanisms, such as redox cycling, uncoupling ofelectron transport chains, different oxidases, autooxidation of certain cellular components and xenobiotics and enzymaticoxidation of NADPH by NADPH-oxidaseand depletion of radical scavengers and antioxidant enzymes (Papa and Skulachev, 1997; Bonnefont-Rousselot, 2002; McAnulty *et al.,* 2003; Agnisola, 2005).Xenobiotics such as transition metals, azo dyes and quinones are known to exert oxidative stress through redox cycling (Halliwell and Gutteridge, 1999).This leads to an imbalance between the generation and the neutralization of the ROS byantioxidant mechanisms within an organism thus, causing oxidative stress (Davies, 1995).

Oxidative stress has become an important subject for aquatic toxicology (Livingstone, 2001).Biological systems have developed an adequate enzymatic and non-enzymatic antioxidant mechanisms to protect their cellular componentsfrom oxidative damage duringtheir evolution. The damage caused by the ROS can be measured through cellular markers which includes various adaptive cellular responses such as increased concentrations of non-enzymatic and enzymatic antioxidants as well as by the measurements of cellular damage, such as perturbed redox balances, lipid peroxidation and DNA oxidation (Di Giulio, 1991).The antioxidant defense system includes enzymes suchas superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) (Droge,2002) and the cellular damage caused by malondialdehyde (MDA) content, which is known as an end-product of lipid peroxidation. Enzymes are biochemical macro-molecules controlling the metabolic processes of organisms (Roy, 2002) whose analysis is widely used for rapid detection to predict early warning of toxicant toxicity (Dutta and Areids, 2003). Analysis of biochemical parameters could help to identify target organs of toxicity as well as the general health status of animals. It may also provide an early warning signal in stressed organism (Folmar, 1993). The source of these parameters is the indicators responding to the environmental effects and can also serve as markers for toxicant exposure in fish. The plasma transaminase ALT, AST, as well as acid

and alkaline phosphatases can be used to establish the tissue damage of the liver and kidney (Nemcsok *et al.,* 1981; Nemcsok and Boross, 1982).Cellular damage releases the Alanine transaminase (ALT) and Aspartate transaminase (AST) into blood stream and the levels of these enzymes have the potential to indicate hepato-toxicity and histopathological changes(Kumari *et al.,* 2011).They participate in transamination reactions found predominantly in liver, cardiac cells and striated muscle tissues. Moreover they are sensitive measure of histopathological changes within a shorter time (Balint *et al.,* 1997) whose release indicate the tissue damages in liver, kidney, and gill (Rajyasree and Neeraja, 1989; Oluah, 1999).Alterations in alkaline phosphatase (ALP) and acid phosphatase (ACP) activities in tissues and serum have been reported in fish (Jyothi and Narayan, 2000).

Alkaline phosphatase (ALP) is present in almost all tissue of an organism especially in cell membrane catalysing the hydrolysis of monophosphate esters.The functional activity of this enzyme increases during exposure to heavy metal toxicity. Increased activity of ALP has been found in such pathological process of liver impairment, kidney disfunction and bone diseases.Alterations in alkaline phosphatase (ALP) activities in tissues, organs and serum have been reported in fish exposed to toxicants of varying concentrations (Jyothi and Narayan, 2000).

Trace elements though essential gets bioaccumulated when in excess. Fish are the final trophic link of hydro ecosystems, metals in trace amounts are taken up through different organs of fish and gets concentrated at various levels in fish bodies which are not metabilized and carry-overed from feed to food of animal origin (Papagiannis, *et al.*, 2004). Responses to toxicants by aquatic organisms are broad ranged depending on the compound, exposure time, water quality, and the species. They attack non-target organisms simultaneously, and till now the effect of the single metal on the fish species are many but the combination of the metals in the trace amounts is sparce. Various bioaccumulation studies have been carried out earlier on freshwater teleost fish (Malik, *et al.*, 2010; Rauf, *et al.*, 2009; Flora, *et al.*, 2008) being economically important but the knowledge of its subchronic effect on the combined action of these trace elements having a EDTA chelation chemistry have not studied yet. Hence, in the present study,two freshwater teleost fish *O.mossambicus* and*L.rohita,*are taken as a experimental models to have an understanding of biocehmical changes and oxidative stress response of plant nutrient element mixture at subchronic level. In addition, species andtissue-specific (liver, kidney, muscle and gill) defences were also studied.

MATERIALS AND METHODS

Animal acclimation and study procedure

Labeo rohita were obtained from the local ponds of Vadodara district and transferred to the laboratory. They wereacclimated for 10 days at 27 ± 4^0C, pH 7.4 ± 0.05, dissolved oxygen 8 ± 0.3 mg/L, total hardness 188 mg/L $CaCO_3$ with a 12:12 light:dark photoperiod. Fish were supplied daily with commercialfish food during acclimation.Animal maintenance and experimental procedures werein accordance with the guideline of A.P.H.A., A.W.W.A. and W.P.C.F. (1998).

The Trace element mixture used was a commercial formulation of Librel TMX, Chelated Micronutrient mixture(Nutrient % by Wt.Min., Zn-4.0, Mn-0.5, Cu-0.3, Fe-2.0 and B-0.5).

Adult fishes were exposed to sub-lethal concentrations of micronutrient mixture of 300 mg/L ($1/20^{th}$of LC_{50} value) for 15,30 and 45 days after acclimatization period. Experimental and controlwater was refreshed every third day to minimize loss of mixture concentration. No mortality occurred underany of these conditions. After each experimental periods, the fishes from the control and experimental groups were euthenised by decapitation and the tissue samples (gills, muscles, liver and kidney) were dissected out blot free and were store at -80ºC for furtheer biochemical analysis.

Antioxidant enzymes and biochemical parameters

All enzyme activities were measured spectrophotometrically.Frozen tissue were quickly weighed and then homogenized in a homogenizer. The buffers for homogenization of tissues were: 0.1 M sodium phosphate buffer pH 7.4at a ratio of 1:10 w/v for CAT, LPO, GSH; in 6% TCA for AA assay and in 0.89% KCl keeping the proportion 1:4 for SOD activity assay. ALP Alkaline Phosphatase (ALP), Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) were done using standard diagnostic kits purchased from Reckon Diagnostics.

Antioxidant enzymesestimation:

Estimation of ascorbic acid: (Roe and Oesterling, 1944)

Principle:

Ascorbic acid is converted to dehydro ascorbic acid by shaking it with norit.It is then coupled with 2,4-DNPH in presence of thiourea as mild reducing agent then converted into a red coloured compound which is assayed colorimetrically.

Reagents:

a.Standard ascorbic acid

b. 2,4- DNPH solution

c. 85% H_2SO_4, 6% TCA.

Procedure:

Homogenize the weighed tissue in 6% TCA. Add norit to it. shake well allow it to stand for 15 minutes. Filter with whatmann paper 42. The mixture containing 4ml homogenate followed by 1 ml and after addition of 4 drops of 2,4 DNPH and put it into water bath for 15 minutes. Lastly 5 ml of 85% H2SO4 added. Wait for 30 min and then read the absorbance at 540 nm against a blank containing all the reagents. A series of standards were run along with blank treated in a similar manner to determine the ascorbic acid content. Values were expressed as mg/ g wet tissue.

Assay of Redused Glutathione (GSH):

Total redused glutathione was estimated by the method ofEllman and Fiches (1959).

Principle:

The Glutathione assay is a modification of the method first described by Tietze. The general thiol reagent, 5-5'dithiolbis(2-nitrobenzoic acid) (DTNB or ellman's reagent) react with GSH to form the 5 thionitrobenzoic acid (TNB) and Gs-TNB.

Reagents:

a. DTNB (0.6 Mm) in 0.2 M phosphate buffer (pH – 8.0)

b. TCA 5%

c. standard glutathione.

Procedure:

Precipitated protein in the homogenates of gills, liver, kidney and muscle with 0.1 ml 5% TCA and 0.4 ml distilled water. Mixed the contents well for complete precipitation of proteins and centrifuged. To 0.5 ml clear supernatant, added 2.5 ml of 0.2 M phosphate buffer and 50 µl of DTNB. Read the absorbance at 412 nm against a blank containing all the reagents. A series of standards were run along with blank treated in a similar manner to determine the glutathione content. Values were expressed as nmoles/100 g wet tissue.

Assay of Superoxide Dismutase (SOD)

Superoxide dismutase in different tissues was determined using the method of Kakkar*et al.*, (1984).

Principle:

The assay of SOD is based on the inhibition of the formation of NADH-phenazine methosulphate-nitrobluetetrazoliumformazon. The colour formed at the end of the reaction can be extracted into butanol and measured at 560nm.

Reagents:

a. 0.1 M PBS, n-butanol

b. 0.052 M sodium pyrophosphate buffer (pH 8.3)

c. 0.0025 M Tris-HCl buffer(pH 7.4)

d. 186 µM phenazinemethosulpahte (PMS)

e. 300 µM Nitro blue tetrazolium (NBT)

f. 780 µM NADH and Glacial acetic acid.

Procedure:

Weighed samples of tissues were homogrnised in 0.1 M PBS and subjected to differential centrifugation under cold condition. The supernatant was used as the enzyme source. Assay mixture contained 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of PMS, 0.3 ml of NBT,1.3 ml of distilled water and 0.1 ml of the enzyme source. The tubes were kept at 30°C for 90 seconds and the reaction was stopped by the addition of 1 ml of glacial acetic acid. Reaction mixture was shaken vigorously with 4.0ml of n-butanol. The mixture was allowed to stand for 10 minutes and centrifuged. The upper butanol layer was removed. Absorbance of the chromogen in butenol was measured at 560 nm against n-butanol blank. A system devoid of enzyme served as control, one unit of enzyme activity is defined as the enzyme concentration required to inhibitchromogen production by 50% in one minute under the assay conditions and specific activity is expressed as unit/ mg protein.

Assay of Catalase (CAT)

Catalyse level in different tissues was determined using the method of Maehly and Chance (1955).

Principal:This method is based on the fact that dichromate in acetic acid is reduced to the chromatic acetate .when heated in presence of hydrogen peroxide with formation of perchromic acid as unstable intermediate.the chromium acetate is measured colourimetically at 610 nm. The catalase preparation is allowed to spilt hydrogen peroxide at regular time interval and the reaction is stopped by addition of dichromatic acid .mixture of hydrogen peroxide liberated is determined colourimetrically.

Reagents:

a. 0.01M phosphate buffer (pH 7.0)

b.30mM H_2O_2.

Procedure:

The estimation was done spectrophotometrically following the decrease in absorbance at 230 nm. The reaction mixture contained 0.01 M phosphate buffer. 30 mM hydrogen peroxide and the enzyme extract prepared by homogenizing the tissue in phosphate buffer and centrifuging at 5000 rpm. Specific activity was expressed as international Units / mg protein. 1 IU = change in absorbance / min / extinction coefficient (0.021).

Assay of Lipid peroxidase (LPO)

LPO was estimated by the method of Niehaus and Samuelson, 1968

Principal: Lipid peroxide leads to formation of an endoperoxide that is malondialdehyde (MDA), which reacts with thiobarbituric acid (TBA) and gives thiobarbituric acid reactive substance (TBARS). TBARS gives a characteristic pink colour that can be measured caloriemetrically at 532nm.

Reagents:

TCA-TBA-HCl reagent: 15% (W/V) Trichloro acetic acid, 0.375% (W/V) Thiobarbituric acid (TBA) in 0.25 N HCl. 0.1 M Tris-HCl buffer (pH 7.5).

Procedure:

The tissue homogenate of different tissues were prepared in Tris-HCl buffer and was combined with thiobarbituric acid reagent and mixed thoroughly and heated for 15 minutes in a boiling water bath. It was then cooled and centrifuged for 10 minutes at 600 g. The absorbance of the sample was read spectrophotometrically at 535 nm against a reagent blank that contained no tissue extract. The extinction coefficient for malondialdehyde is 1.56×10^5/ M/ cm. The values are exptresses as millimoles / 100g wet wt of tissue.

Statistical analysis

The significance of Wilk's Lambda test, $p < .001$ and the insignificance of Levene's test Equality of Error Variances, $p > .05$ were considered for testing MANOVA.Multivariate analysis of variance (MANOVA) wasperformed to test for significant differences between the control and exposed groups by SPSS software. Means were compared and statistical significance was established by Dunnett's test ($p<0.05$).

RESULTS

The results of this investigation are as presented in TableIa, b (Wilk's lambda test), IIa, b (Levene's test of Equality of Error variances) and IIIa, b (Dunnett's test)respectively,the positive values in the Post hoc tests indicates an increase in the activity of the parameters studied and vice versa. Significant increase in GSH and LPO ($p<0.001$) was obtained in all the tissues. An increase in AA ($p<0.05$) was also observed in liver, kidney and gills but an insignificant increase in muscle was obtained.Significant increase in the ALP ($p<0.001$) and ALT ($p<0.05$) was obtained in muscle, kidney as well as gills with a significant decrease in liver. ASTshowed an significant increase ($p<0.001$) in Gills and Liver, whereas kidney and muscle showed an significant decrease. In contrast to the differential trend in all the parmeters,CAT expressed significant ($p<0.001$) decrease in all the tissues except in gill. Further, there was an insignificant decrease in the SOD activity for all the exposed tissues compared to the control. (Table III).

Table Ia: Wilks' lambda test for *O.mossambicus*

Tissues	Wilks' Lambda	F	Hypothesis df	Error df
Gills	.003	51197.02	12.000	13.520
Muscles	.014	2894.22	12.000	13.520
Liver	.011	5352.10	12.000	13.520
Kidney	.002	125851.43	15.000	11.444

Table Ib: Wilks' lambda test for *L.rohiat*

Tissues	Wilks' Lambda	F	Hypothesis df	Error df
Gills	.000	524219.544	12.000	13.520
Muscles	.000	746.659	12.000	13.520
Liver	.000	17290.6	12.000	13.520
Kidney	.000	166396	15.000	11.444

Table IIa: Levene's Test of Equality of Error Variances for *O.mossambicus*

Parameters	Gills		Muscles		Liver		Kidney	
	F	Sig.	F	Sig.	F	Sig.	F	Sig.
SOD	6.265	.017	6.435	.016	.639	.611	1.084	.410
CAT	0.768	.543	.418	.745	3.630	.064	.601	.632
GSH	4.427	.041	.473	.709	6.134	.018	.623	.620
AA	2.583	.126	.556	.658	2.076	.182	.779	.538
LPO	10.929	.470	13.849	.002	.354	.788	.498	.694
ALP	1.488	.290	.982	.448	.143	.931	1.115	.399
ALT	0.645	.608	1.564	.272	1.280	.345	2.928	.100
AST	1.588	.267	.765	.545	2.349	.149	.885	.489

Table IIb: Levene's Test of Equality of Error Variances for *L.rohita*

Parameters	Gills		Muscles		Liver		Kidney	
	F	Sig.	F	Sig.	F	Sig.	F	Sig.
SOD	2.281	.156	2.281	.156	3.108	.089	2.281	.156
CAT	1.544	.277	1.544	.277	3.108	.089	1.544	.277
GSH	2.281	.156	2.281	.156	1.461	.296	2.281	.156
AA	2.281	.156	1.461	.296	2.281	.156	2.281	.156
LPO	1.461	.296	1.461	.296	1.461	.296	2.281	.156
ALP	3.108	.089	3.108	.089	2.281	.156	3.108	.089
ALT	1.461	.296	1.461	.296	2.281	.156	2.333	.150
AST	1.461	.296	1.461	.296	2.281	.156	1.461	.296

Table IIIa: Post Hoc tests (Dunnetts' t test) showing comparison between the exposed groups and control in tissues of *O.mossambicus*

Multiple Comparisons		SOD	CAT	GSH	AA	LPO	ALP	ALT	AST
		MD ± SE	MD ± SE	MD ± SE	MD ± SE	MD ± SE	MD ± SE	MD ± SE	MD ± SE
GILLS	15d vs C	12.29±23.73	713.0* ± 95.03	161.84* ± 26.57	39.84* ± 16.57	-19422.66*± 362.33	-34.50± 19.78	24.0* ± 6.13	178.0* ± 23.31
	30d vs C	57.32 ± 23.73	1300.00* ± 95.03	441.57* ± 46.57	125.83* ± 16.57	21414.3*± 216.57	394.00* 15.60	-3.33± 2.31	-54.66 ± 8.31
	45d vs C	75.00 ± 23.73*	1912.87* ± 96.33	265.20* ± 47.57	83.92* ± 16.57	10951.3* ± 16.57	326.00* 15.60	80.33* ± 8.31	192.6* ± 31.55
MUSCLE	15d vs C	4.76± 16.57	-139.9*± 8.28	91.26*± 16.57	22.27*± 8.31	-3768.6 ± 8.31	17.00± 15.60	195.66* ± 8.31	-88.66*± 8.31
	30d vs C	18.57± 16.57	-201.79* ± 8.28	29.10* ± 16.57	39.63*± 8.31	2695.33± 8.31	53.66* ± 8.31	-1.66± 8.31	-159.33* ± 8.31
	45d vs C	44.42± 16.57	175.40± 8.28	-137.90* ± 16.57	66.42* ± 8.31	-5637.66 ± 8.31	77.00* ± 8.31	257.00* ± 8.31	117.66*± 8.31
LIVER	15d vs C	4.76±16.57	-139.95*± 8.31	-91.26*±16.57	22.27*± 16.57	-3768± 16.57	17.00± 15.60	195.66± 15.60	-88.66*± 8.31
	30d vs C	18.57±16.57	-201.79*± 8.31	29.10*±16.57	39.63*± 16.57	2695± 16.57	53.66± 15.60	-1.66 ±15.60	-159.33* ± 8.31
	45d vs C	44.42*± 16.57	-201.79*± 8.31	-137.90*± 16.57	66.42*± 16.57	-5637± 16.57	77.00 ±15.60	257.00 ±15.60	117.66* ± 8.31
KIDNEY	15d vs C	49.76*± 16.57	-248.47 ± 8.29	40.62*± 16.57	39.45*± 16.57	-16361.66*± 16.57	188.00 ± 15.60	4.00± 9.68	-218* ± 8.31
	30d vs C	31.05*± 16.57	-367.97* ± 8.29	126.57*± 16.57	80.00*± 16.57	6199.33*± 16.57	112.00 ± 15.60	63.00** ± 9.68	-525.00*** ± 8.31
	45d vs C	31.96*± 16.57	205.99*± 8.29	-226.23*± 16.57	144.20* ± 16.57	27227.66* ± 16.57	607.33* ± 15.60	19.33 ± 9.68	-564.66 ± 8.31

Table IIIb: Post Hoc tests (Dunnetts' t test) showing comparison between the exposed groups and control in tissues of *L.rohita*

Multiple Comparisons		SOD	CAT	GSH	AA	LPO	ALP	ALT	AST
		MD ± SE	MD ± SE	MD ± SE	MD ± SE	MD ± SE	MD ± SE	MD ± SE	MD ± SE
GILLS	15d vs C	8.62±16.57	1738.47*±8.28	271.84* ± 16.57	70.74 *± 16.57	-16500.3*± 8.31	-119.6***± 15.6	30.66* ± 8.31	198.66* ± 8.31
	30d vs C	16.96 ± 16.57	-3138.89* ± 8.28	391.57* ± 16.57	141.32* ± 16.57	25000.0 *± 16.57	394.00* 15.60	10.00 ± 8.31	-91.00* ± 8.31
	45d vs C	16.00 ± 16.57	-1315.24* ± 8.28	345.20* ± 16.57	251.49* ± 16.57	14300 ± 16.57	326.00* 15.60	120.33* ± 8.31	83.33* ± 8.31
MUSCLE	15d vs C	33.100 ± 16.57	-52.68 *± 8.28	1385.13*± 16.57	3.66 ± 8.31	-6240.33* ± 8.31	18.33 ± 15.60	171.66*± 8.31	-74.33* ± 8.31
	30d vs C	31.91 ± 16.57	-271.66* ± 8.28	996.43** ± 16.57	7.000 ± 8.31	1160.00 *± 8.31	53.00* ± 8.31	8.000 ± 8.31	-199.0* 8.31
	45d vs C	39.76 ± 16.57	-327.71* ± 8.28	43.15 ± 16.57	34.33** ± 8.31	-3539.66* ± 8.31	33.00 ± 8.31	199.33* ± 8.31	86.33* ± 8.31
LIVER	15d vs C	40.72 ±16.57	-514.33*± .31	957.95 *±16.57	64.63*±16.57	-870.00 *±16.57	-56.3*±15.60	-43.66 ±15.60	101.66* ± 8.31
	30d vs C	53.10* ±16.57	-1021.00 *± 8.31	919.11 *±16.57	108.51*±16.57	3965.00 *±16.57	308.66* ±15.60	404.00* ±15.6	243.00* ± 8.31
	45d vs C	51.67 ±16.57	-1373.66***± 8.31	78.26 **±16.57	151.74**± 16.57	9200.0 *±16.57	-59.33* ±15.60	-39.00 ±15.60	174.33* ± 8.31
KIDNEY	15d vs C	31.43 ± 16.57	-421.40* ± 8.29	2321.50* ± 16.57	57.14* ± 16.57	-16060.00 ± 16.57	261.00***± 15.60	13.66 ± 9.68	-147.3* ± 8.31
	30d vs C	35.72 ± 16.57	3371.77* ± 8.29	1488.70*± 16.57	69.72** ± 16.57	5925.00* ± 16.57	70.00* ± 15.60	58.33* ±9.6	-62.0* ± 8.3
	45d vs C	-9.04 ± 16.57	-593.19* ± 8.29	124.59*± 16.57	175.18* ± 16.57	28900.0*± 16.57	644.00* ± 15.60	16.33 ± 9.68	-37.66* ± 8.31

MD ± SE – Mean difference ± Standard error
The mean difference in significant at the .05 level.
** indicates mean difference is significant at 0.05 level*

Fig. I: Alterations in the Superoxide dismutase (SOD) in *O.mossambicus* tissues exposed to Plant nutrient Librel

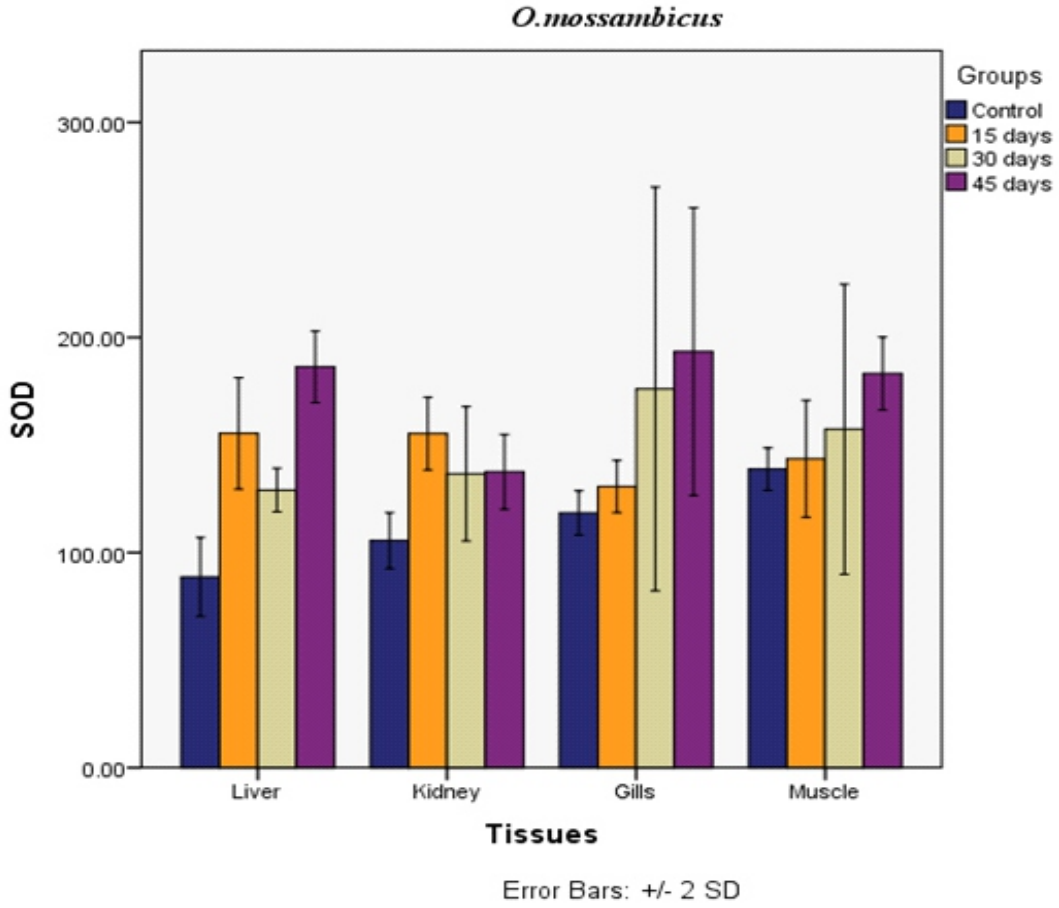


Fig. II: Alterations in the Superoxide dismutase (SOD) in *L.rohita* tissues *e*xposed to Plant nutrient Librel

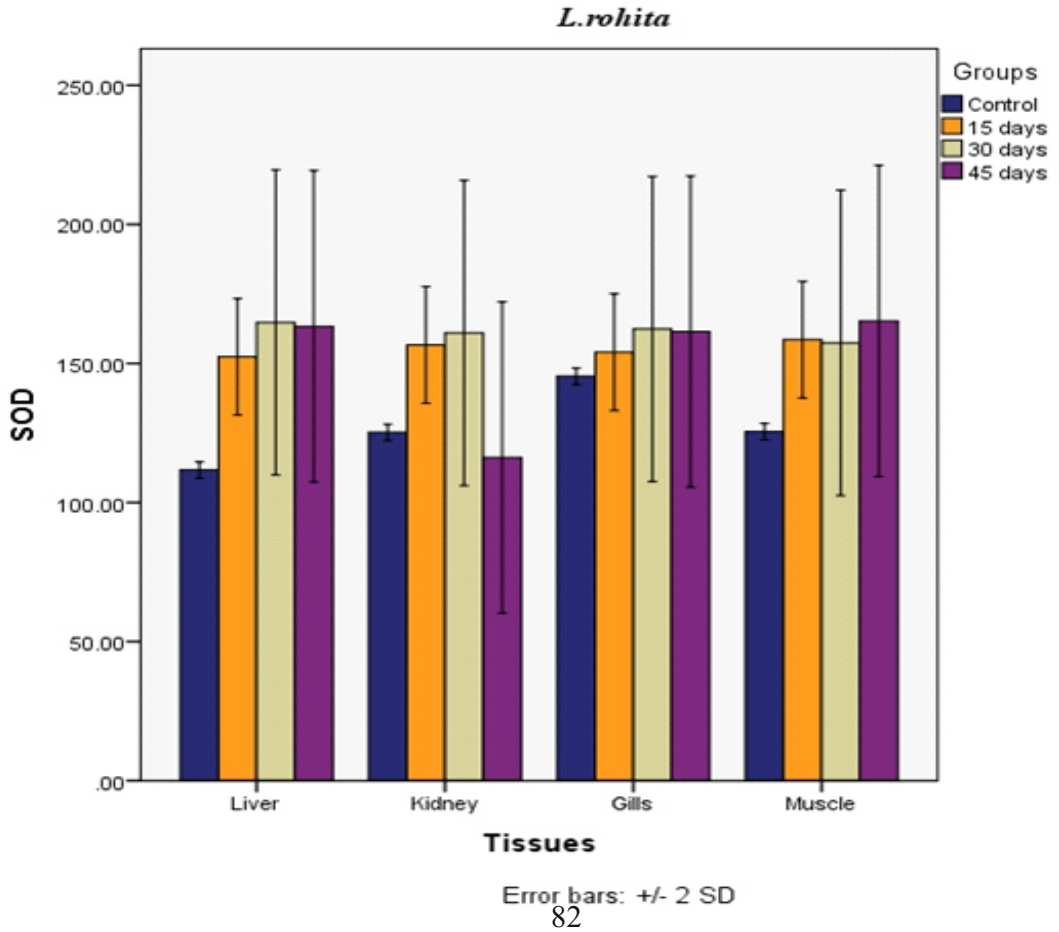


Fig. III: Alterations in the Reduced glutathione (GSH) in *O.mossambicus* tissues exposed to Plant nutrient Librel

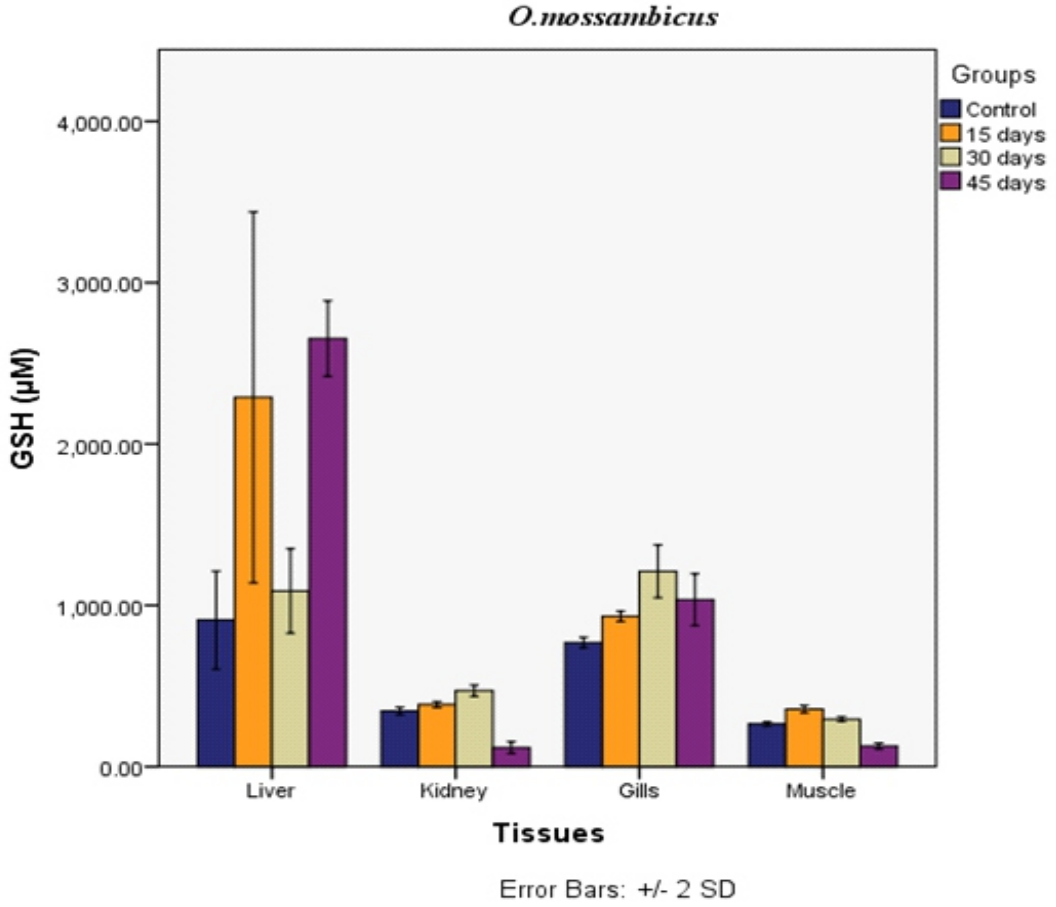


Fig. IV: Alterations in the Reduced glutathione (GSH) in *L.rohita* tissues *e*xposed to Plant nutrient Librel

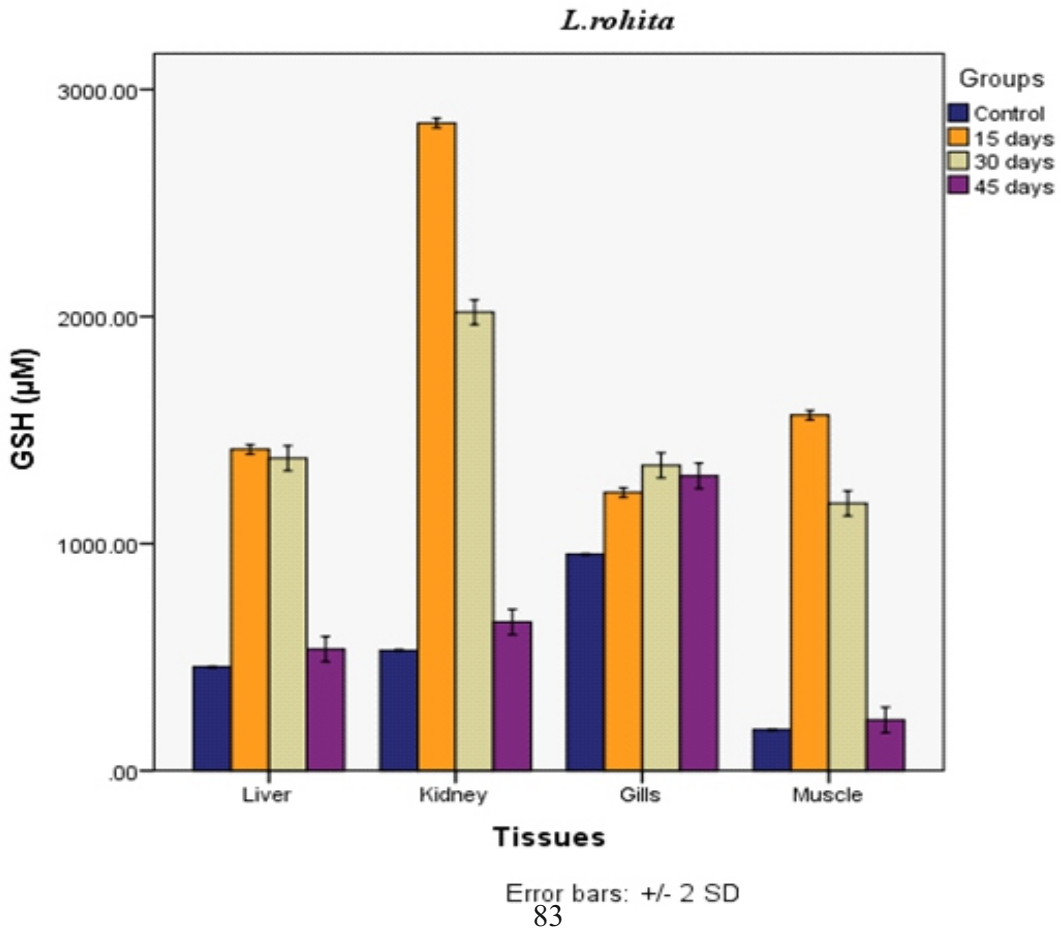


Fig. V: Alterations in the Ascorbic acid (AA) in *O.mossambicus* tissues exposed to Plant nutrient Librel

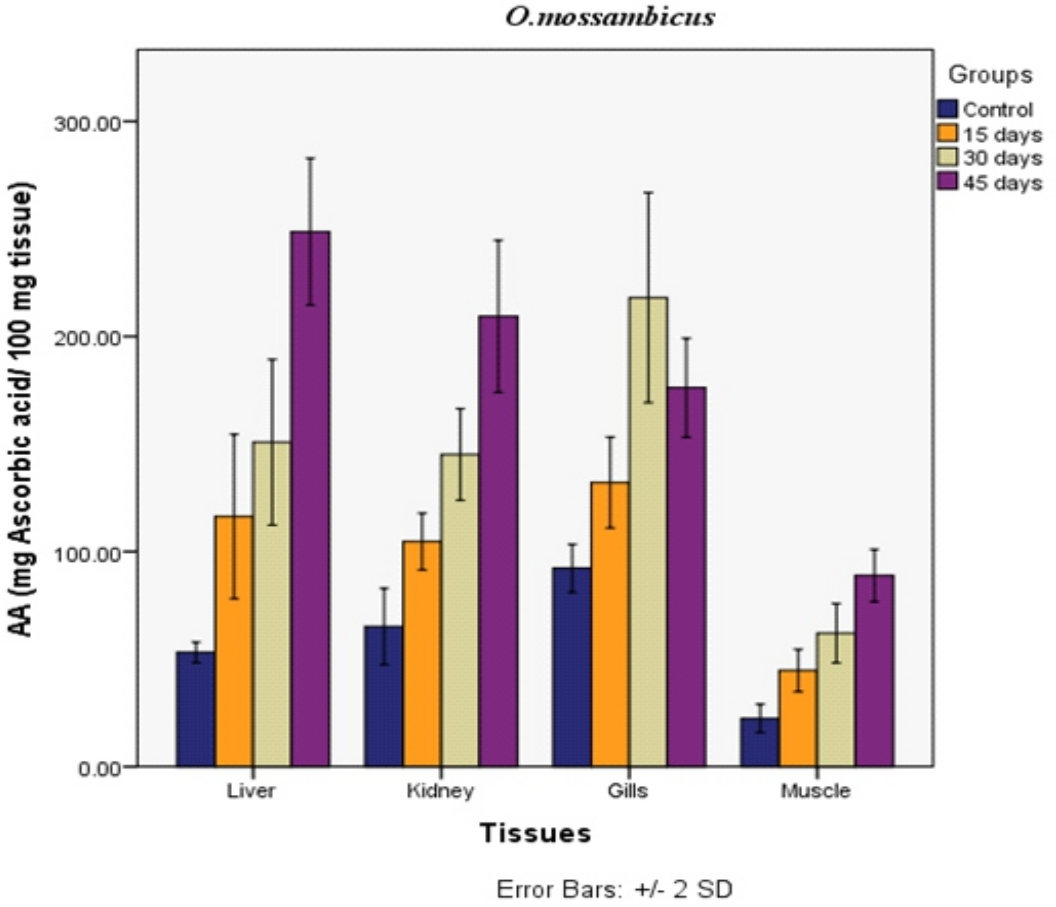


Fig. VI: Alterations in the Ascorbic acid (AA) in *L.rohita* tissues *e*xposed to Plant nutrient Librel

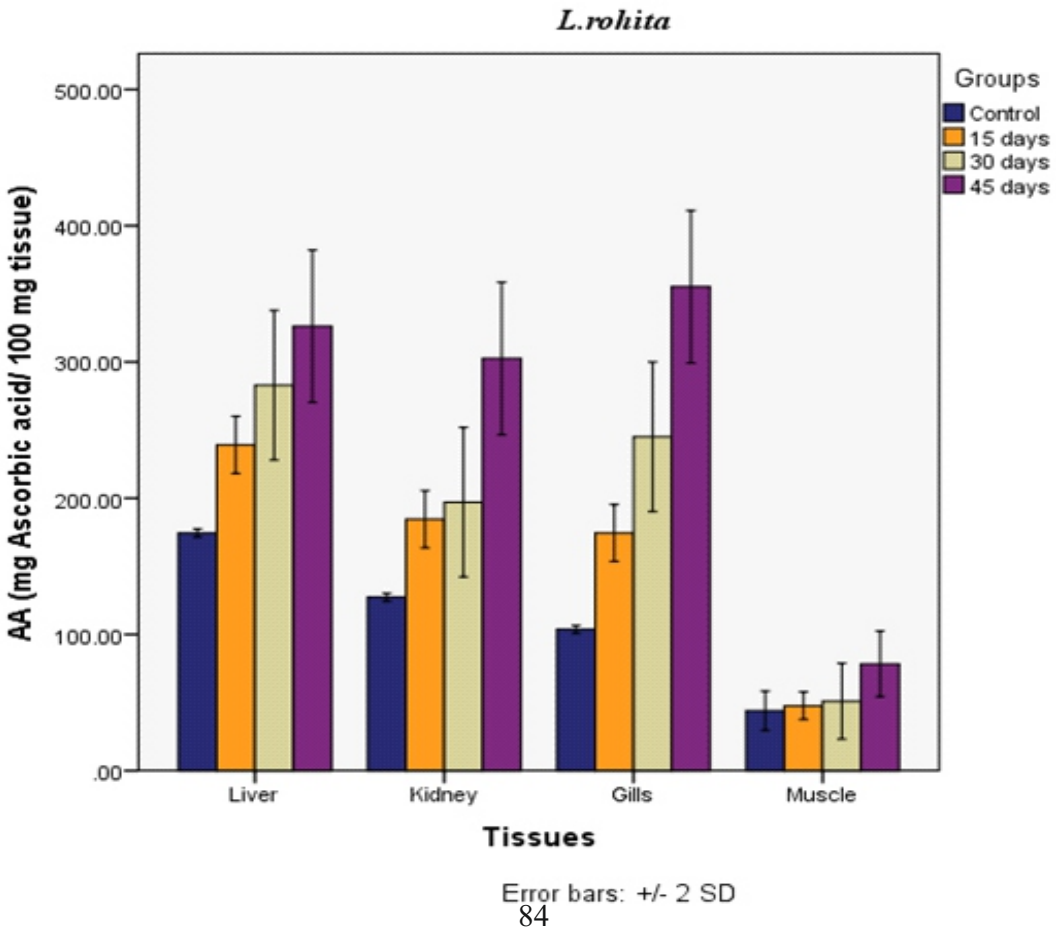


Fig. VII: Alterations in the Malonaldehyde (MDA) in *O.mossambicus* tissues exposed to Plant nutrient Librel

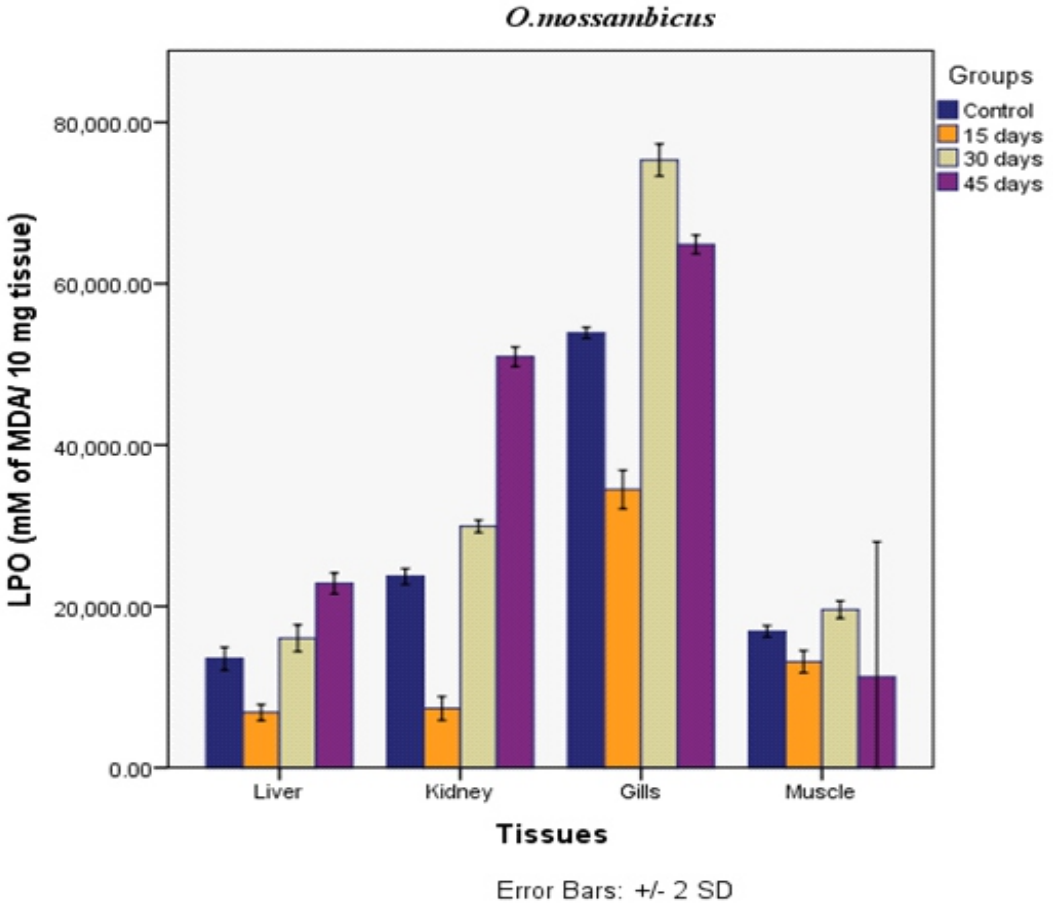


Fig. VIII: Alterations in the Malonaldehyde (MDA) in *L.rohita* tissues *e*xposed to Plant nutrient Librel

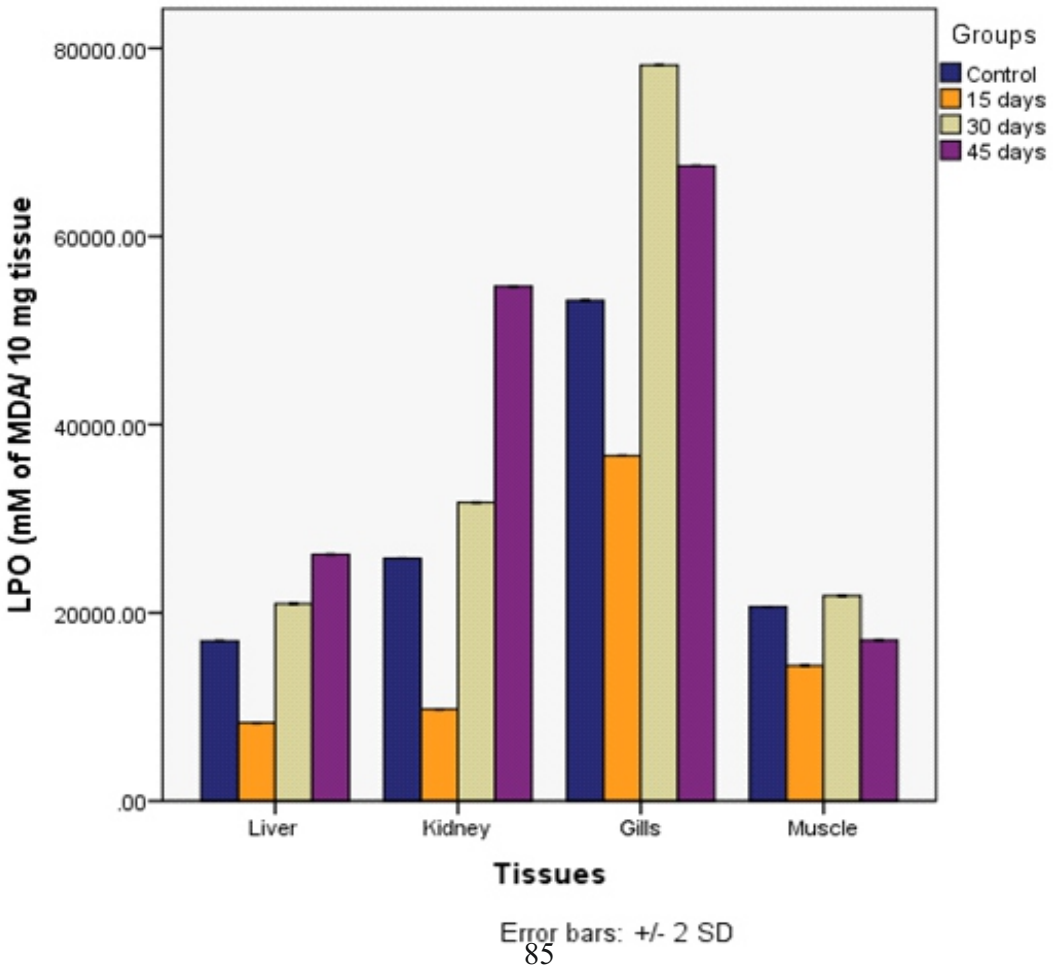


Fig. IX: Alterations in the Catalase (CAT) in *O.mossambicus* tissues exposed to Plant nutrient Librel

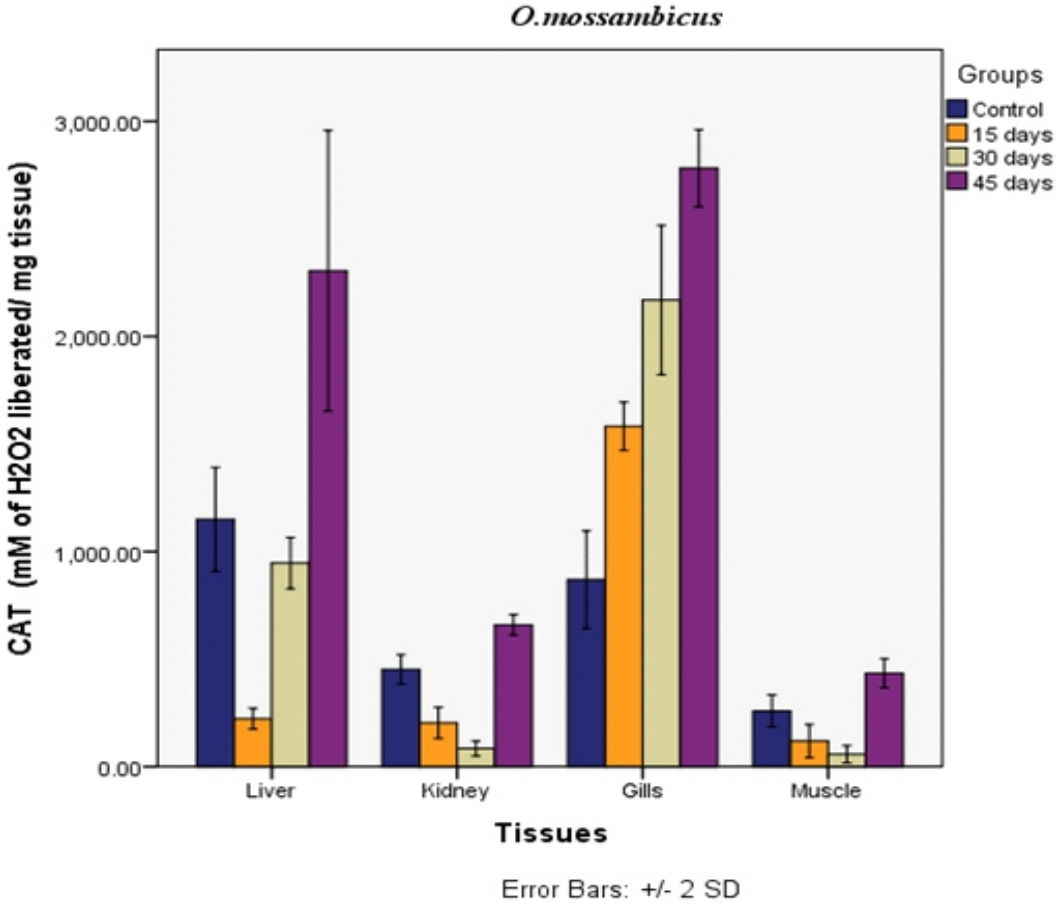


Fig. X: Alterations in the Catalase (CAT) in *L.rohita* tissues *e*xposed to Plant nutrient Librel

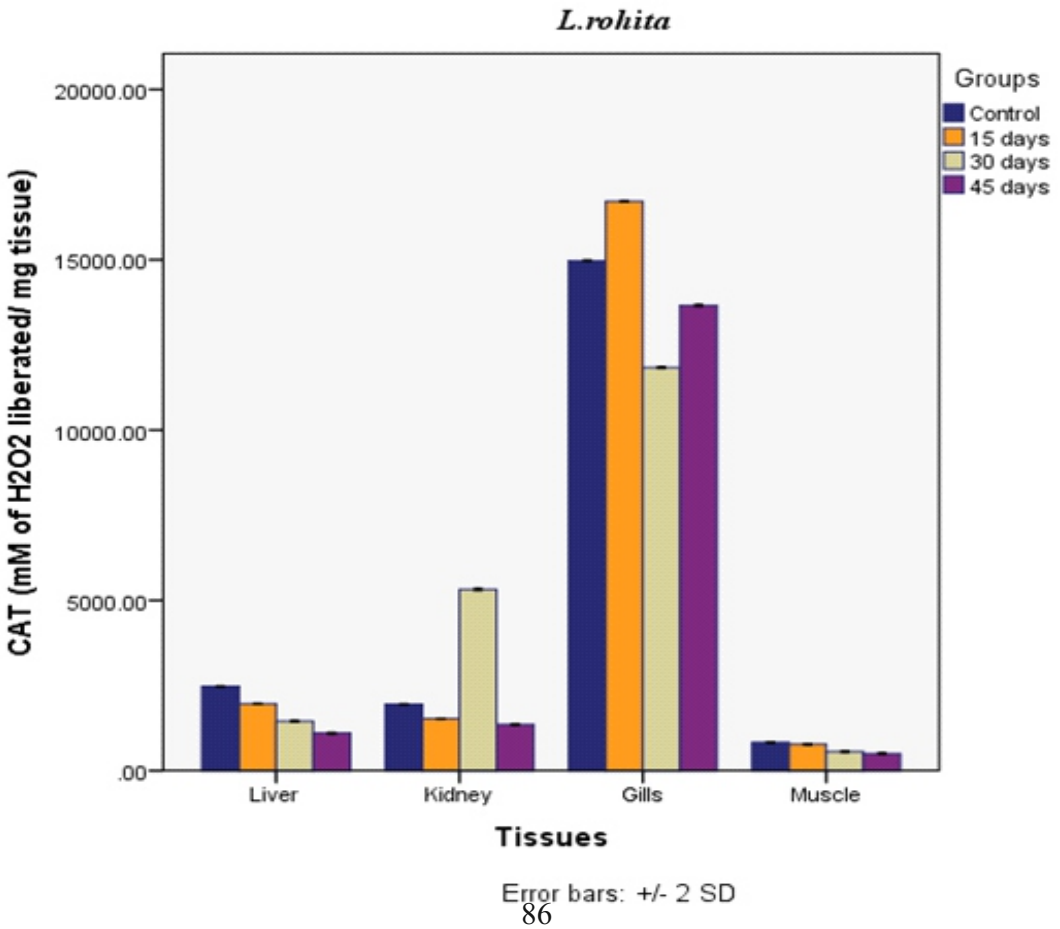


Fig. XI: Alterations in the Alkaline phosphatase (ALP) in *O.mossambicus* tissues exposed to Plant nutrient Librel

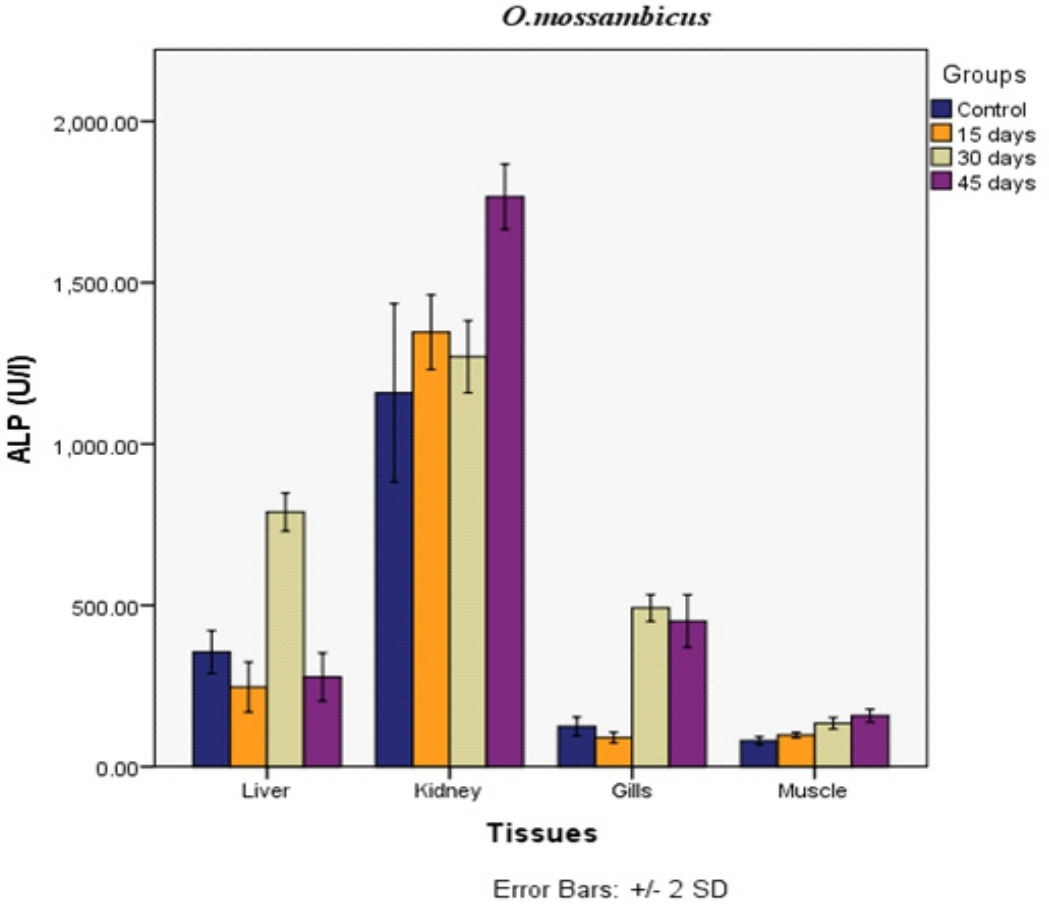


Fig. XII: Alterations in the Alkaline phosphatase (ALP) in *L.rohita* tissues *e*xposed to Plant nutrient Librel

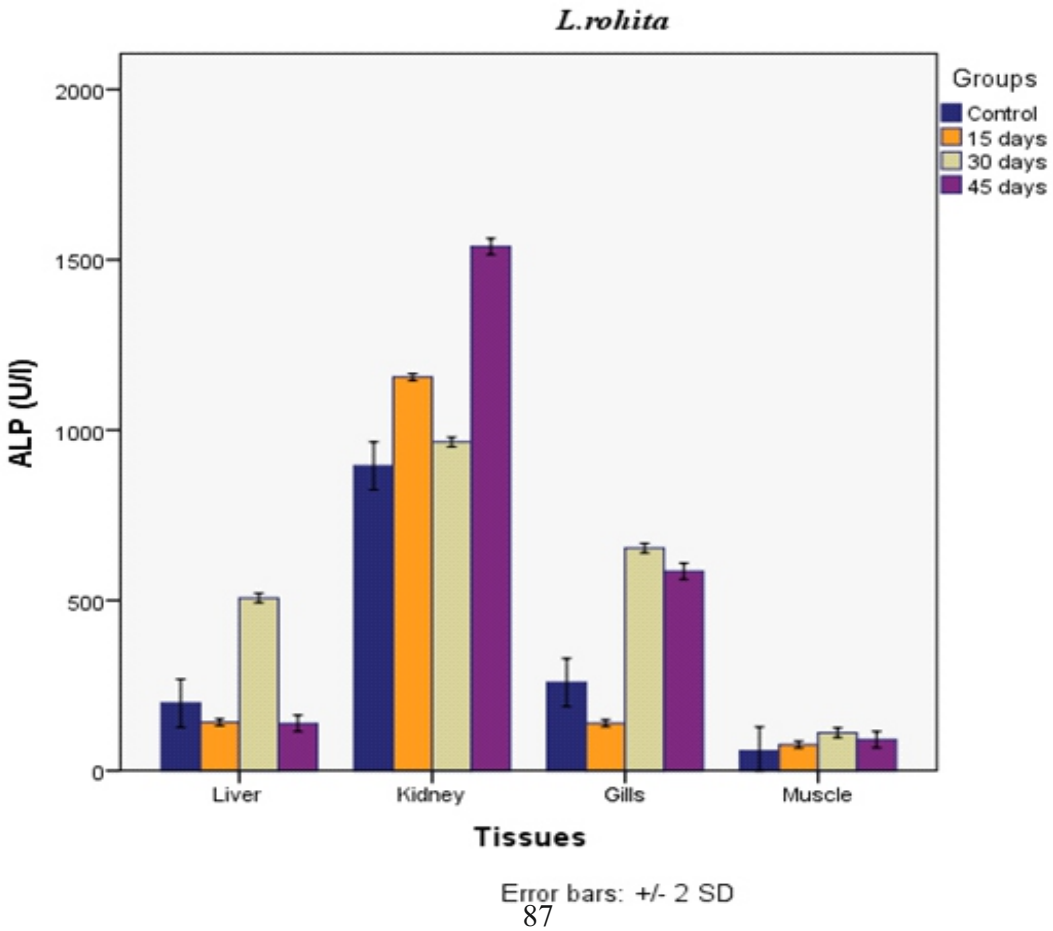


Fig. XIII: Alterations in the Alanine transaminase (ALT) in *O.mossambicus* tissues exposed to Plant nutrient Librel

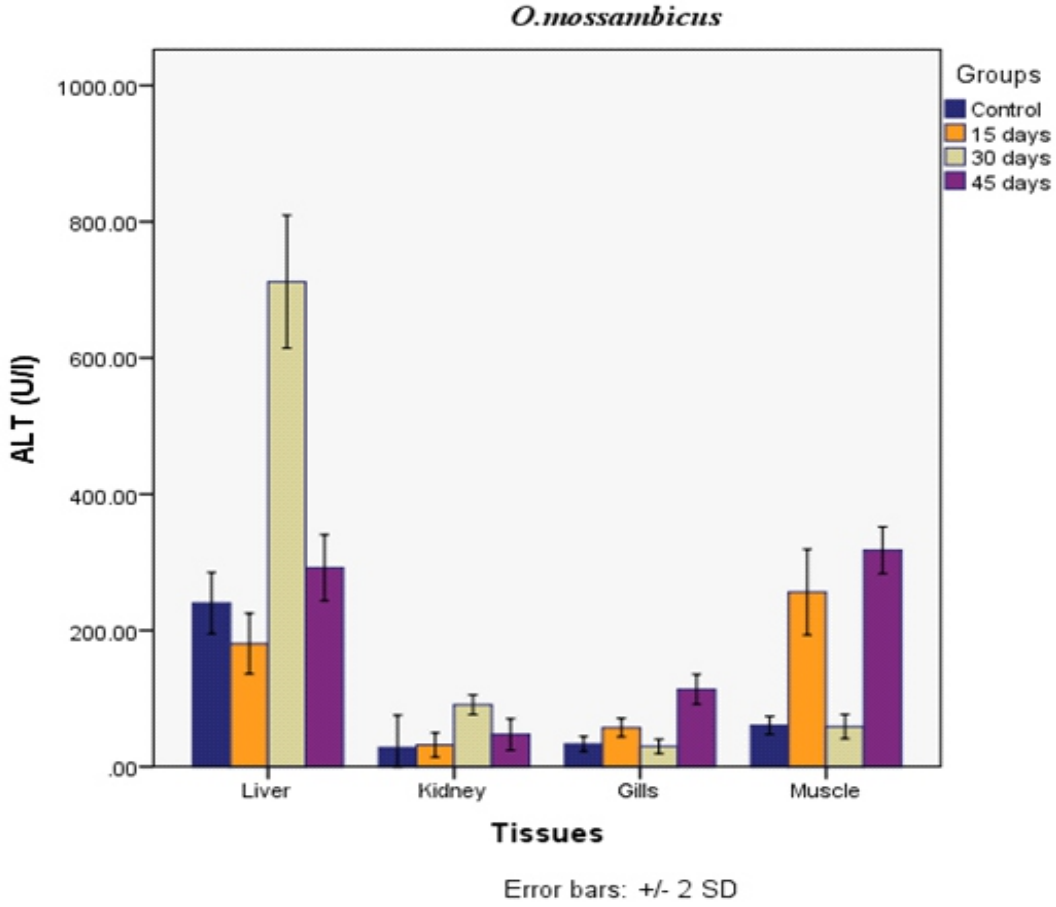


Fig. XIV: Alterations in the Alanine transaminase (ALT) in *L.rohita* tissues *e*xposed to Plant nutrient Librel

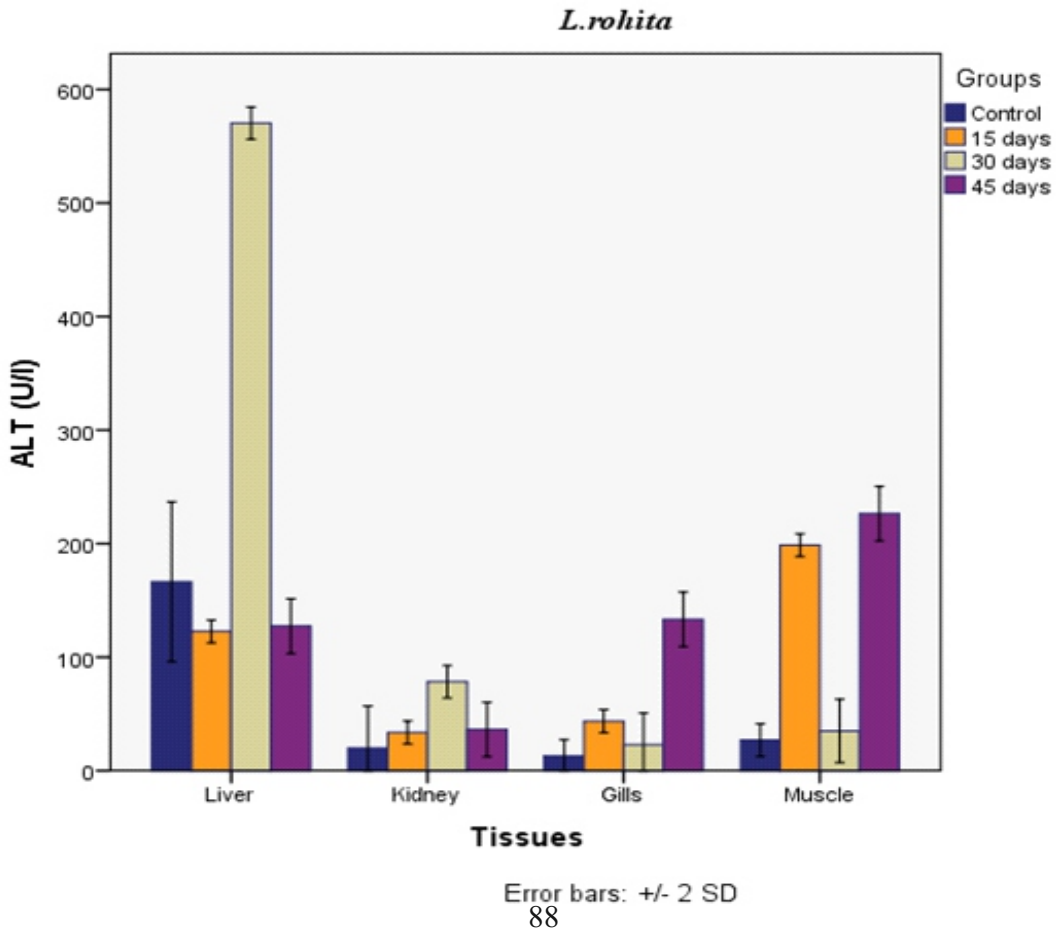


Fig. XV: Alterations in the Aspartate transaminase (AST) in *O.mossambicus* tissues exposed to Plant nutrient Librel

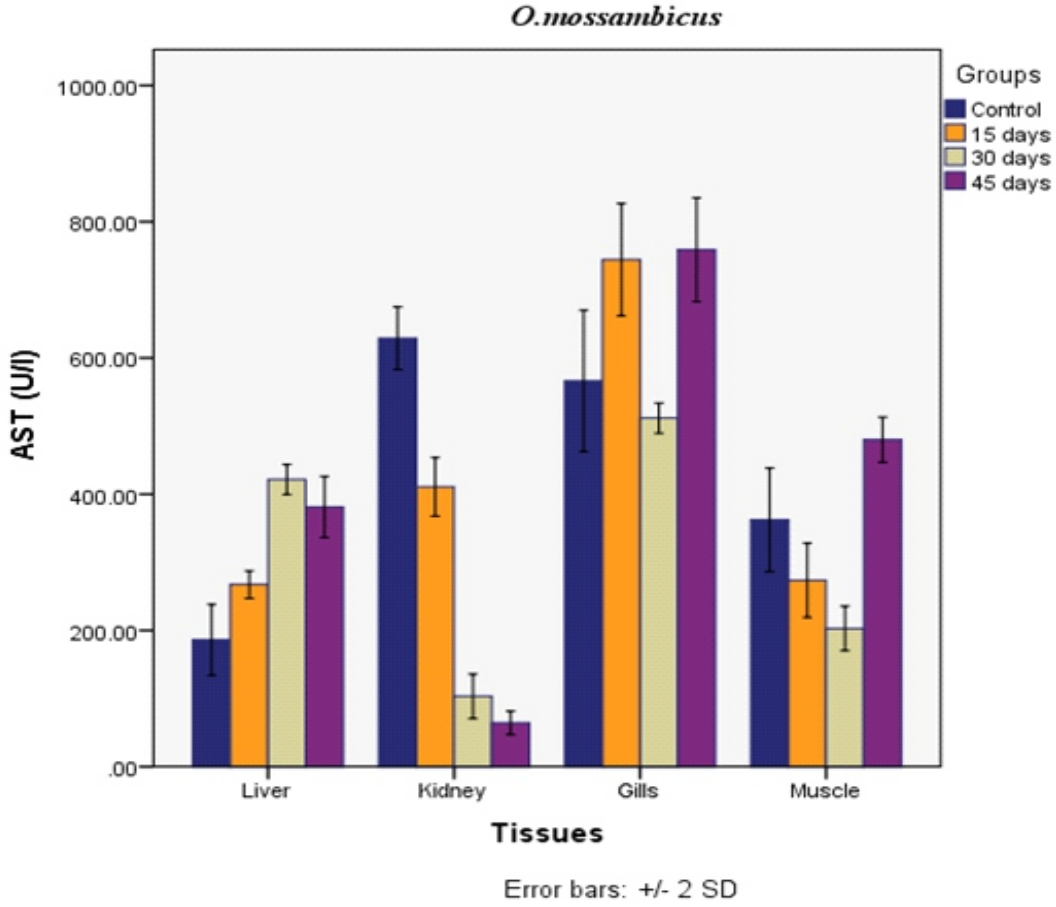
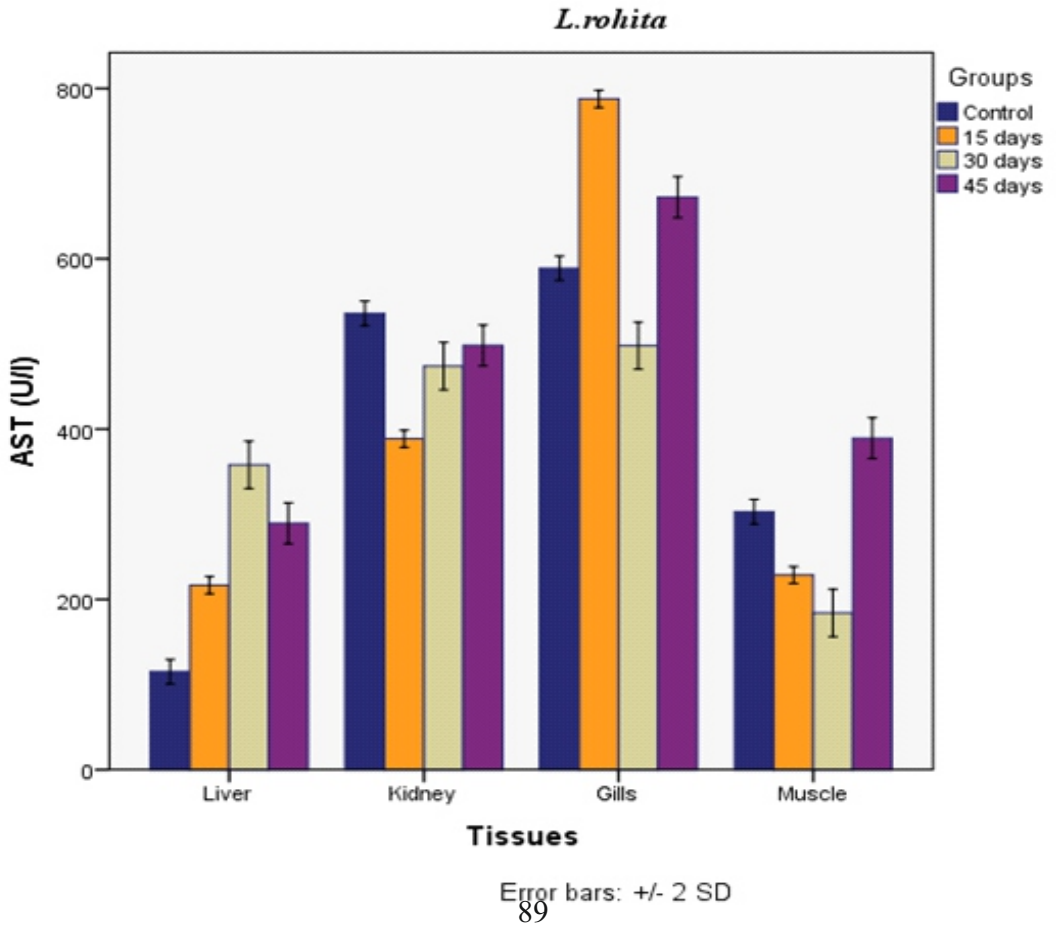


Fig. XVI: Alterations in Aspartate transaminase (AST) in *L.rohita* tissues *e*xposed to Plant nutrient Librel



DISCUSSION

Stress response in biological systems is an innate mechanism of survival in the oddest circumstances. In the present day situation the stress factors have multiplied in the exponential mannner with the advent of modern agricultural and industrial practices. Thus an improved agricultural practice has now become one of the major defaulters in creating a situation of pesticide/agrochemical overload.The studies showing the adverse effects of the pesticides of different group of chemicals are numerous (Arufe *et al.,* 2004;Atamanalp *et al.,* 2002;Atamanalp *et al.,* 2003;Belmonte *et al.,* 2005;Crestani *et al.,* 2007; Dobsikova *et al.,* 2006;Dong *et al.,* 2009; Velisek *et al.,* 2010; Parikh *et al.,* 2010; 2011;2012;2013; Desai and Parikh, 2014). The overall objective of the study was to test the stress response and perceive the toxicity threshold of trace element mixture librel on fresh water teleost fish *O. mossambicus* and *L. rohita.* Exposure of the micronutrient resulted in alterations in the activity of reactive oxygen species upregulating the antioxidant defence system.Several of soluble enzymes have been considered as a relevant stress indicator. Therefore, activities of enzymes have been commonly used in the diagnosis of tissue damage caused by environmental pollution. An increase of these enzyme activities in the extracellular fluid or serum is a sensitive indicator of even minor cellular damage (Palanivelu *et al.,* 2005) and indicates stress-based tissue impairment.

In the present study the alterations in the ROS parameters, LPO, transaminases and phosphatases were evident. The oxidative stress is known to be caused due to an increase in ROS, or impairment of antioxidant defence systems, or a default to repair oxidative damage (Buege and Aust, 1978; Ahmad *et al.,* 2000; Gravato*et al.,* 2006) threatening the integrity of cellular macromolecules such as membrane lipids, proteins and DNA. An increase in the ROS such as superoxide anion radical (O^{2-}), hydroxyl radical (OH^{-}), and hydrogen peroxide ($H_2O_2^{-}$)are known to be removed by the enzymes SOD, GSH and CAT respectively (Cao, *et al.,* 2010). The activities of the enzymes usually increases as an adaptive response to free radical overload during moderate oxidative stress by means of an increased synthesis. However, a severe oxidative stress suppresses glutathione levels due to the mutilation of adaptive mechanism (Sevcikova*et al.,* 2011; Zhang *et al.,* 2004). GSH depletion may reduce the cellular ability to scavenge free radicals which affects the general oxidative potential of the tissue. Our results are in agreement with the earlier reported work(Sevcikova*et al.,* 2011) where an increase in GSH and SOD was clearly evident on day 15^{th} and 30^{th} in all the tissues and decrease on day 45^{th}. The increase in GSH and SOD in the study suggests their role in

combating the buildup in the ROS content. At the same time the decrease in the CAT activity is probably due to the cell damage and its inability to counter effect the agrochemical toxicity (Sivaperumal and Sankar, 2008). Charge of AA is well established as a scavenger of free radical and is regarded as aresourceful antioxidant (Stegeman, 1992). GSH plays crucial role in the cellular antioxidant protection by direct interaction of the SH group with ROS or involvement in the enzymatic detoxification reactions of ROS as a cofactor or a coenzyme (Cheeseman and Slater, 1993). The enzymatic reactions of GSH, CAT and SOD have been featured to be significant but they are not 100% effective in eliminating the free radicals as hydroxyl free radicals are not removed by these mechanisms. To compensate this, the body's non-enzymatic protective mechanisms is taken over byAA which has a well defined role as a scavenger whose inherent traitof performing redox reactions contributes in filling of an electron in the outer shell of R and thereby neutralising it to a nonreactive species. AA content in the present study was found to be increased significantly ($p<0.001$) compared to control in liver and kidney. As AA has a central position in curing the impairedcondition occurred during the agrochemical exposure, the increased AA content thereby is a self explanatory mechanism adopted by the teleost fish. Furthermore, as stated by Mahajan and Zambare (2001) Sometimes vitamin C and vitamin E acts incombination for detoxification, hence, increased AA content is probably performing the detoxicification task.

The direct consequence of failure of antioxidant system is the accumulation of ROS in the system leading to the higher rate of formation of lipid peroxides. Lipid peroxides (LP) are products of oxidatively damaged lipids resulting from lipid peroxidation reactions induced by ROS whose quantification by thiobarbituric acid represents the extent of oxidative damage (Meister and Anderson, 1983). The significant increase in the levels of LPO at 30^{th} and 45^{th} day suggests malfunctioning of tissue. In addition, LPO is considered animportant indicator of oxidative damage of cellular components due to excess generation of ROS which can lead to several biological effects ranging from alterations in signal transduction to gene expression and apoptosis and oxidative stress development (Kannak and Jain, 2000; Babusyte*et al.,* 2009; Cayir*et al.,* 2009). Increase on LPO is always known to be parallel with SOD increase, which is again due to an enhanced production of superoxide anion radical. These alterations in the antioxidant enzymes and scavengers clearly depicts that oxidative stress was obvious in the exposed fishes which in turn damaged lipids which is proved by the alterations in the LPO levels. Our results are in agreement with the earlier reported work of Ahmad *et al.,* 2000; Favari*et al.,* 2002; Sayeed*et al.,* 2003; Abdollahi*et al.,* 2004;Roberts and Oris,

2004;Bagnyukova*et al.,* 2006;Wang *et al.,* 2006;Abbas and Ali, 2007;Farombi*et al.,* 2007;Gabriel *et al.,* 2007;Xing *et al.,* 2012)

Transaminases play an important role at the junctionbetween the carbohydrate and protein metabolism by interconverting the strategic compounds viz; ketoglutarate, pyruvate and oxaloacetate on one hand and alanine, aspartate and glutamate on the other hand. Alterations in transaminases has been reported exhibiting an important role in carbohydrate and amino acid metabolism in various tissues of fish [Lushchak, *et al.,* 2001;Jomova and Marian, 2011; Khalaf, *et al.,* 1985; Dhanapakiam *et al.*,2006]. Any change in the transaminases thereby suggest that the agrochemicals have a component which will have an impact on carbohydrate metabolism.The increased transaminase activity in the gills, muscles and kidney might be due to increase in transamination reaction i.e. transferring of NH_2 group from amino acid to a ketoacid.Documented evidences showed that transamination and transdeamination reactions are prominent under stress condition (Rajender *et al.,* 1986; Dhanapakiam *et al.,* 2006).In most of the cases, it has been observed that different enzymes behave differently and even the same enzyme behave in different ways in different species (Kalele and Dhande, 2005; Devlin, 2006; Gupta and Kumar, 2006;Kumar *et al.,* 2011;Salahuddin and Khola, 2013). The differential expression of ALT and AST in the tissues as well as the species reported in the present study thus is supported by the work of these scientists.

Alkaline phosphatase is an ubiquitous transport enzyme present in almost all tissue of an organism especially in cell membrane. It catalyses the hydrolysis of monophosphate esters and also has wide substrate specificity. Firat *et al.,* (2011) have reported that ALP may increase due to the cellular damage in the liver and that high levels of these enzymes usually in an indicative of necrosis in the liver of animals. Exposure to the heavy metals resulted in increases in and ALP activity of plasma/serum of fish *Sparus aurata* (Vaglio and Landriscina 1999) and *Cyprinus carpio* (Karan *et al.,* 1998). Time dependent increase in the ALP activity is in agreement with the earlier reported work of Das and Mukherjee 2003 in *Labeo rohita*, Jee *et al.,* (2005) in Korean rockfish (*Sebastes schlegeli*), Borges *et al.,* (2007) in fish Bagre(*Rhamdia quelen*)andEl-Sayed and Saad, (2008) in Nile tilapia(*Oreochromis niloticus*). The researchers concluded that necrosis of liver and subsequent leakage of this enzyme into blood stream might be responsible for increase of this enzyme in blood.

Thus from the present studies it can be concluded that the plant nutrient exposure has led to the alterations in the antioxidants, lipid peroxidation, transaminases and phosphatase, and that the alterations in the parameters in the target tissue (i.e., liver, gill, kidney and muscles) is due to the damage leading to dysfunction. The results of the present studies also indicate that

the activities of certain biomarkers in *O.mossambicus*are more sensitive to the plant nutrient than those in *L. rohita* suggesting the differences in the defense capacity of the teleost fish and that the uptake and elimination pathways differ substantially among tissues.

CHAPTER V

Determination of Trace Metalconcentration In Liver, Muscle, Kidney and Gills Of *Oreochromis mossambicus* and *Labeo rohita* Exposed to Plant Nutrient

INTRODUCTION

The pollution of the aquatic environment with trace heavy metals has become a worldwide problem during recent years because they are indestructible and most of them have toxic effects on organisms (MacFarlane and Burchett, 2000). Among environmental pollutants, metals are of particular concern due to their potential toxic effect and ability to bioaccumulate in aquatic ecosystems (Censi*etet. al*., 2006). The presence of heavy metals in aquatic ecosystems is the result of two main sources of contamination; natural processes and anthropogenic activities (Goullé*et al*., 2012; Moore and Attar, 2011). Various anthropogenic activities, domestic and industrial effluents lead to degradation of the quality of both natural and man-made water bodies (Kamaludeen*et al.,* 2003; Gupta and Mahapatra, 2003; Strong and Burgess, 2008).Metal pollution from multifarious sources like effluents from industries, agricultural runoff anduntreated sewage system has adverse effects on aquatic ecosystem. Intensification of agriculture practices to meet the demand hasresulted in increased release of a wide range of agrochemicalcompounds to the environment(Desai and Parikh, 2014). In agricultural systems that integrate livestock and crop production, rigorous agricultural practices are recognized as significant sources of metal accumulation. Fertilizers and agricultural lime contain metal impurities, and it is widely reported that application of these agrochemicals, especially phosphate fertilizers, results in accumulation of metals in agricultural soils. Metallo-pesticides, including insecticides, fungicides, and herbicides are also known to contain various metals that can increase metal accumulation. (Senesi*et al.,* 1999; Nicholson *et al.,* 2003; Yabe *et al.,* 2012).Metal accumulation is not only threat to the water bodies as reported by Szolnoki*et al.,* (2013) there occurs cumulative impacts of human activities on urban garden soils leading to accumulation of metals therein.

Metal concentration in aquatic ecosystems are usually monitored by measuring their concentrations in water, sediments and biota (Ergul*et al.,* 2008) which generally exist in trace levels in water and attain considerable concentration in sediments and biota (Unlu*et al.,* 2008). Trace metals including both essential and non-essential elements have a particular

significance in ecotoxicology, since they are highly persistent and all have the potential to be toxic to living organisms (Storelli*et al*., 2005). These pollutants when compared with other types of aquatic pollution are less visible but its effects on the ecosystem and humans are intensive and very extensive due to their toxicity and their ability to accumulate in the aquatic organisms (Edem*et al*., 2008).Some metals are known to be toxic even at low concentrations, including chromium, lead, cadmium, arsine and mercury (Nguyen *et al.,* 2005). While others, such as copper, iron, zinc, manganese and cobalt, are known to be essential elements and play important roles in biological metabolism at very low concentrations but at the same time if excess or deficit can disturb biochemical functions in both humans and animals (Yilden, 2003). As metals, unlike organic pollutants, are nonbiodegradable their content has steadily increased in water and subsequently accumulated in sediments, plants, fishes, and even in humans (Che*et. al.,* 2006).Studies on trace metals in rivers, lakes, fish and sediments (Tüzen, 2003; Canli and Atli, 2003; Karadede *et al.,* 2004; Ozmen *et al.,* 2004; Begum et al., 2005; Ansari *et al.,* 2005; Tuzen and Soylak, 2007; Fernandes *et al.,* 2008; Ozturk*et al.,* 2008; Pote*et al*., 2008; Praveena *et al.,* 2008 and Turkmen *et al.,* 2009) have been a major environmental focus especially during the last decade. Sediments have been reported to form the major repository of metals in aquatic system while both allochthonous and autochthonous influences could make a concentration of trace metals in the water high enough to be of ecological significance (Oyewo and Don-Pedro, 2003).

The presence of metal pollutant in fresh water is known to disturb the delicate balance of the aquatic systems. Fish are often at the top of the aquatic food chain and may concentrate large amounts of some metals from the water (Mansour and Sidky, 2002).They are notorious for their ability to accumulate the metals in their muscles. Any of these metals can destroy life when they concentrate in the body above acceptable levels. Such a contaminated fish can cause health hazards when they enter into the human body through consumption (Ozuni et al., 2010). Hence, there is a need to carefully screen to ensure the unnecessary high level of some toxic trace metals that are being transferred to humans through fish consumption (Adeniyi and Yusuf, 2007; Akan *et al.,* 2012). So it is essential to determine the bioaccumulation capacity of heavy metals in organisms especially the edible ones in order to assess the potential risk to human health. Such an assessment can serve as a bio-indicator of their impacts on these organisms as well as give an insight to the degree of pollution of the water body in particular (Farkas*et al.,* 2003).In environmental assessment, BioconcentrationFactor (BCF) is considered to be an important parameter. It is a measure of the tendency for a

substance in water to accumulate in fish tissue or in tissues of other organisms. According to USEPA, 1986 this parameter is an important determinant for human intake of aquatic organisms.

Various studies have dealt with effective concentration of heavy metals in water and its inverse relationship towards BCF(Mason *et al.,* 2000; Van der Oost*et al.,* 2003; Papagiannis*et al*., 2004; McGeer*et al.,* 2009).This work examines the theoretical and experimental basis for the use of BCF in the hazard assessment of Zn, Fe, CuandMn.It is an attempt to have an insight into the tissue specific and species specific concentration of metals into the fish after establishing its acute and subchronic toxicity as discussed in the previous chapters.***Hence this study is geared towards determining the accumulation of the trace metals in the fish tissues as well as in the water with the view to establish the comprehensive evaluation of metals in various tissues i.e. liver, kidney, gills and muscles.***

Materials and Methods

Healthy *O. mossambicus* and *L.rohita*,were collected from local fresh water bodies of Baroda district and acclimatized at laboratory conditions for 10 days. Fishes were maintained in 25 ± 2 C, pH 7.4 ± 0.05, dissolved oxygen 8 ± 0.3 mg/L, total hardness 188 mg/L CaCO3 with a 12:12 light:dark photoperiod accordance with the Guidelines of A.P.H.A., A.W.W.A., W.P.C.F (Jomova and Valko, 2011). Fishes were daily supplied with commerecial food during acclimation and experimental period. Acclimatized fishes were exposed to water containing the test chemical at the concentration of 300 mg/L ($1/20^{th}$ of LC_{50} value) for 45 days at the semi static system. The Trace element mixture used was a commercial formulation of Librel TMX, Chelated Micronutrient mixture (Nutrient % by Wt.Min., Zn-4.0, Mn-0.5, Cu-0.3, Fe-2.0 and B-0.5). No test chemical was put into the aquarium containing the control fish.The water in the control and metal containing aquarium was renewed everyday in order to maintain the concentration of the test mixture. At each interval of 15, 30 and 45 days of long-term exposure, fish were sampled from each group for determination of metals (Zn, Fe, Cu and Mn) in different organs (gills, liver, muscle and kidney).

Water samples at every intervals were prepared using the method of APHA (1995) and different fish tissues were digested after drying according to the method described in APHA 3111B (Direct Acetylene Flame Method). The levels of Fe, Cu, Zn and Mn in digests as water were determined using atomic absorption spectrophotometer.

Bioaccumulation factor was calculated according to using the following equation:

$$\textbf{Bio-concentration factor (BCF)} = \frac{\textbf{Concentration of M in dry fish tissue (mg/kg)}}{\textbf{Concentration of M in water (mg/L)}}$$

The calculation of BAF and BCF are usually same, the interpretations are slightly different, with accumulation in organisms arising from water only for BCF and from water and dietary sources for BAF (Environment Canada, 2000). Therefore, in general, BAF is derived from measurements in natural environments and BCF is more readily measured under laboratory conditions. The metal pollution index wascalculated using the equation:

$$\textbf{MPI} = (CF_1 \times CF_2 \times \text{----- } CF_n)^{1/n}$$

Where, *Cfn* is the contents for the metal n in the sample(Usero*et al.,* 1997).

Average concentrations and standard deviations were calculated for each element, tissues and fish species. The significance levels of the differences between element concentrations in the

studied fish organs and between experimental groups were determined using the Mann-Whitney (Sokal and Rohlf, 1987) test. Heavy metal contents determined in water and fish tissue samples were evaluated statistically using analysis of correlation by SPSS (version number-21) statistical package. The statistical analyses were determined as 0.05.

Results

The result of metals determined in the water at different exposure periods are presented in Table I along with the standard values. They were in the order Fe (18.83 mgL-1) >Zn(6.34 mgL-1) >Mn (4.29 mgL-1) >Cu (0.7 mgL-1).The alterations in the trace metal concentrations in water and tissue of *O.mossambicus* and *L. rohita* were determined and their means and S.D. are presented in Table (II-V). Time dependent increase in the metal content of the tissues as well as water was observed (Table II – V). Amongst two fish species, *L.rohita* exhibited significantlyhigher ability to amass metals than *O.mossambicus*. The order of pattern of accumulation of metals in the tissues was liver>gills>kidney>muscle.Fe exhibited highest concentration in liver of both the fishes. Individual metal concentration assessment exhibited higher values of Fein *L.rohita* (2108.20±771.67 mg/Kg) than *O.mossambicus* (1589.20±45.30 mg/Kg). Organ wise concentration that tracked the order for Fe was: L>G>K>M. The second highest trace metal in order was Zn in both the fishes, where*L.rohita (*184±4.35 mg/Kg) revealed higher concentration compared to *O.mossambicus*(155.3±2.33 mg/Kg). Organ wise accumulation that followed the order for Znconcentrationwas K>G>M>L. Next in the order was Cu, and it was *L.rohita* (75.04±10.60 mg/Kg) which exhibited higher concentration compared to *O.mossambicus* (60.00±3.17 mg/Kg). Organ wise accumulation that followed the order for Cu accumulation was: L>G>M>K. Mne xhibited the least concentration, where*L. rohita* (131±3.60 mg/Kg) accumulated higher Mn compared to *O.mossambicus* (43.50±11.77 mg/Kg). Organ wise accumulation that followed the order for Mn accumulation was: M>L>K>G. To have an insight for the interspecific differences Mann-whitney test was performed (Table VI). Concentration of trace metals (mean and standard deviations) in the tissues of both the teleost fish over the period of time are presented in (Fig. I) where all trace element showed altered affinities in the studied fish tissues and did not differ significantly between the tissues except kidney.

The overall relationship among the various elements was calculated by Pearson correlation co-efficient and data is presented in the form of a matrix (Table VII&VIII). In water, all metals showed positive correlation with the other metals.Metal concentration for inter tissue correlation for *O.mossambicus*gills, Zn showed positive correlation with other three metals; while other three metals showednegative correlation with each other.In the Liverof showed negative correlation with all the other three metal ions,while Zn, Fe and Mn showed positive correlation with each other. In muscle Cu and Mn showed positive with each other as well as with Fe and Zn, on the other hand Zn and Fe showed anegative correlation with each other.In

kidney, a positive correlation amongst Zn, Fe and Cu and a negative correlation of MN towards all the three was reported. Whereas, in*L.rohita*: Zn showed negative correlation with other three metals; while other three metals showed positive correlation with each other. In liver muscle and kidbey Zn, Fe and Mn showed positive correlation with eachother while negative correlation of these three metals was seen with Cu.

In order to estimate the toxicity of trace metals accumulated in the experimental set up the BCF for each trace metal was calculated (Fig. II and III). The order of BCF for trace metals was Cu>Fe>Mn>Zn for both the fishes. However, when tissue BCF was compared the order was for *O.mossambicus*and*L.rohita*

- Cu: liver>gills>muscle>kidney
- Fe: liver>gills>kidney>muscle
- Zn:liver>kidney>gills>muscle

except for Mn which differed in both the fishes. For *O.mossambicus* it was liver>muscle>kidney>gills and *L.rohita*muscle>liver>kidney>gills.

Table I: Quality Guidelines and Standards by International Organization or country

Heavy metals	WHO (guidelines)	Metal content in present study
Cu	2	0.7
Fe	-	18.83
Mn	0.5	4.29
Zn	5.0	6.34

Table II: Concentration of Zn in water and selected tissues of fish exposed to sublethal concentration of plant nutrient (*n = 6*).

Tissues	*O.mossambicus*				*L.rohita*			
	Days of Exposure				Days of Exposure			
	Control	15 days	30 days	45 days	Control	15 days	30 days	45 days
Gills	10.62 ± 1.05	50.00 ± 7.37	66.1 ± 11.90	80.2 ±10.09	11.33 ± 0.99	40.81± 7.41	40.72 ± 4.71	41.43 ±1.83
Liver	1.33 ±0.09	15.99 ±2.69	60.54 ±9.60	126.88 ±5.94	5.22 ± 2.81	19.93 ± 3.66	57.60 ± 2.46	142.32 ± 3.08
Muscle	0.65 ±0.81	6.52 ±1.014	7.80 ±2.091	28.85 ±2.15	0.36 ± 0.54	2.64 ± 1.85	22.69 ± 2.51	61.91 ±3.75
Kidney	10.66 ± 0.99	55.33 ± 2.8	82.33 ±1.00	155.3 ± 2.33	12.33 ±1.36	36.15 ±3.19	99.32 ±5.22	184 ±4.35

Values are presented in Means ± S.D.

Table III:Concentration of Fe in water and selected tissues of fish exposed to sublethal concentration of plant nutrient (*n = 6*).

Tissues	*O.mossambicus*				*L.rohita*			
	Days of Exposure				Days of Exposure			
	Control	15 days	30 days	45 days	Control	15 days	30 days	45 days
Gills	4.13 ± 0.19	3.47 ±0.13	385.5 ±0.10	850.25 ±111.54	2 ± 1	5 ± 5	151.41 ±26.06	330.50 ±28.30
Liver	16.49 ±20.92	93.16 ±8.34	642.86 ±140.95	1589.2 ±45.30	41.44 ± 41.44	178.40 ±9.66	1142.073 ±99.77	2108.20 ±771.67
Muscle	1.55 ±1.18	7.28 ±1.27	24.25 ±3.45	121.7 ±23.50	0.14 ± 0.16	0.87 ± 0.42	10.53 ± 0.72	51.77 ± 2.93
Kidney	14.33 ±2.98	47.33 ±2.87	88.95 ±3.22	149.22 ±5.98	12.33 ±1.22	53.21 ±2.33	99.23 ±5.3	168.83 ±7.06

Values are presented in Means ± S.D.

Table IV: Concentration of Cu in water and selected tissues of fish exposed to sublethal concentration of plant nutrient (*n = 6*).

Tissues	*O.mossambicus*				*L.rohita*			
	Days of Exposure				Days of Exposure			
	Control	15 days	30 days	45 days	Control	15 days	30 days	45 days
Gills	0.77 ±0.69	3.58 ±0.92	27.11 ±8.18	61.4 ±7.09	0.21 ± 0.11	0.97 ± 1.22	33.04 ± 20.24	46.01 ±6.10
Liver	2.58 ±0.15	6.26 ±0.20	59.25 ± 9.04	60.04 ±10.60	0.11 ± 0.18	1.48 ±0.76	15.75 ± 4.15	75.00 ± 3.17
Muscle	1.06 ±1.17	5.11 ±1.31	19.93 ±2.46	41.04 ±4.89	0.14 ± 0.16	0.92 ± 0.66	2.53 ± 0.64	27.94 ± 2.35
Kidney	1.22 ± 0.82	9.22 ±0.98	19.23 ±2.33	25.33 ± 1.98	0.99 ± 0.19	15.32 ±0.33	20.32 ±1.22	27.52 ±2.03

Values are presented in Means ± S.D.

Table V: Concentration of Mn in water and selected tissues of fish exposed to sublethal concentration of plant nutrient (*n = 6*).

Metals	*O.mossambicus*				*L.rohita*			
	Days of Exposure				Days of Exposure			
	Control	15 days	30 days	45 days	Control	15 days	30 days	45 days
Gills	0.95 ±0.61	6.99 ±2.08	10.37 ± 14.61	29.58 ±0.89	0.02 ± 0.02	0.34 ± 0.22	5.93 ± 1.39	15.52 ± 3.68
Liver	1.55 ±0.69	30.17 ±15.53	67.07 ±6.60	115.55 ±5.011	5.22 ± 1.15	15.96 ± 4.10	32.83 ± 6.21	64.86 ± 3.64
Muscle	2.25 ±0.99	9.7 ±1.50	23.50 ±11.77	43.50 ±1.52	37.06 ± 17.69	62.70 ± 3.60	99.00 ± 3.50	131 ± 2.51
Kidney	5.32 ±0.87	15.33 ±2.39	25.38 ± 4.23	35.39 ±5.65	3.23 ±0.99	9.33 ±1.33	29.33 ±2.33	38.93 ±3.98

Values are presented in Means ± S.D.

Table VI: Time dependent significance of differences in the concentrations of metals in the organs of fish species exposed to plant nutrient

Samples	*O.mossambicus*			*L.rohita*		
	Days of Exposure			Days of Exposure		
	15 days	30 days	45 days	15 days	30 days	45 days
Zn						
Gills	0.050	0.050	0.050	0.050	0.050	0.050
Liver	ns	0.050	0.046	0.050	0.050	0.050
Muscle	0.050	0.050	0.050	ns	0.050	0.050
Kidney	0.01	0.01	0.01	0.01	0.050	0.01
Fe						
Gills	0.050	0.050	0.050	ns	0.050	0.050
Liver	0.050	0.050	0.050	ns	0.050	0.050
Muscle	0.050	0.050	0.050	0.050	0.050	0.050
Kidney	0.01	0.01	0.01	0.01	0.050	0.01
Cu						
Gills	0.050	0.050	0.050	ns	0.050	0.050
Liver	0.050	0.050	0.050	0.050	0.050	0.050
Muscle	0.050	0.050	0.050	ns	0.050	0.050
Kidney	0.050	0.050	0.050	0.050	0.050	0.01
Mn						
Gills	0.050	0.050	0.050	0.050	0.050	0.050
Liver	0.050	0.050	0.050	0.050	0.050	0.050
Muscle	0.050	0.050	0.050	0.050	0.050	0.050
Kidney	0.050	0.050	0.050	0.050	0.050	0.01

Values presented are the significance levels obtained using Mann-Whitney U test

Table VII: Interwater and inter tissue Pearson Correlation of *O.mossambicus*

		W-Zn	W-Cu	W-Fe	W-Mn	G-Zn	G-Fe	G-Cu	G-Mn	L-Zn	L-Fe	L-Cu	L-Mn	M-Zn	M-Fe	M-Cu	M-Mn	K-Zn	K-Fe	K-Cu	K-Mn
W-Zn	PC	1	.713	.977	.989																
	Sig.		.495	.136	.095																
W-Cu	PC	.713	1	.846	.601																
	Sig.	.495		.359	.589																
W-Fe	PC	.977	.846	1	.935																
	Sig.	.136	.359		.231																
W-Mn	PC	.989	.601	.935	1																
	Sig.	.095	.589	.231																	
G-Zn	PC					1	-.905	.827	.557												
	Sig.						.279	.380	.624												
G-Fe	PC					-.905	1	-.511	-.151												
	Sig.					.279		.659	.903												
G-Cu	PC					.827	-.511	1	.927												
	Sig.					.380	.659		.244												
G-Mn	PC					.557	-.151	.927	1												
	Sig.					.624	.903	.244													
L-Zn	PC									1	1.000**	-.704	.827								
	Sig.										.002	.502	.380								
L-Fe	PC									1.000**	1	-.702	.826								
	Sig.									.002		.504	.382								
L-Cu	PC									-.704	-.702	1	-.981								
	Sig.									.502	.504		.123								
L-Mn	PC									.827	.826	-.981	1								
	Sig.									.380	.382	.123									
M-Zn	PC													1	-.828	.089	.464				
	Sig.														.379	.943	.693				
M-Fe	PC													-.828	1	.485	.112				
	Sig.													.379		.678	.928				
M-Cu	PC													.089	.485	1	.923				
	Sig.													.943	.678		.251				
M-Mn	PC													.464	.112	.923	1				
	Sig.													.693	.928	.251					
K-Zn	PC																	1	1.000**	1.000**	1.000**
	Sig.																		.000	.000	.000
K-Fe	PC																	1.000**	1	1.000**	1.000**
	Sig.																	.000		.000	.000
K-Cu	PC																	1.000**	1.000**	1	1.000**
	Sig.																	.000	.000		.000
K-Mn	PC																	1.000**	1.000**	1.000**	1
	Sig.																	.000	.000	.000	

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table VII: Interwater and inter tissue Pearson Correlation of *L.rohita*

		W-Zn	W-Fe	W-Cu	W-Mn	G-Zn	G-Fe	G-Cu	G-Mn	L-Zn	L-Fe	L-Cu	L-Mn	M-Zn	M-Fe	M-Cu	M-Mn	K-Zn	K-Fe	K-Cu	K-Mn
W-Zn	PC	1	.989	.977	.933																
	Sig.		.095	.136	.234																
W-Fe	PC	.989	1	.935	.976																
	Sig.	.095		.231	.140																
W-Cu	PC	.977	.935	1	.835																
	Sig.	.136	.231		.370																
W-Mn	PC	.933	.976	.835	1																
	Sig.	.234	.140	.370																	
G-Zn	PC					1	-.986	-.999*	-.985												
	Sig.						.108	.033	.110												
G-Fe	PC					-.986	1	.975	.942												
	Sig.					.108		.141	.218												
G-Cu	PC					-.999*	.975	1	.993												
	Sig.					.033	.141		.077												
G-Mn	PC					-.985	.942	.993	1												
	Sig.					.110	.218	.077													
L-Zn	PC									1	.933	-.874	.998*								
	Sig.										.234	.323	.045								
L-Fe	PC									.933	1	-.990	.956								
	Sig.									.234		.089	.189								
L-Cu	PC									-.874	-.990	1	-.906								
	Sig.									.323	.089		.278								
L-Mn	PC									.998*	.956	-.906	1								
	Sig.									.045	.189	.278									
M-Zn	PC													1	.672	-.992	.990				
	Sig.														.531	.080	.090				
M-Fe	PC													.672	1	-.574	.770				
	Sig.													.531		.611	.441				
M-Cu	PC													-.992	-.574	1	-.965				
	Sig.													.080	.611		.170				
M-Mn	PC													.990	.770	-.965	1				
	Sig.													.090	.441	.170					
K-Zn	PC																	1	1.000**	1.000**	1.000**
	Sig.																		.000	.000	.000
K-Fe	PC																	1.000**	1	1.000**	1.000**
	Sig.																	.000		.000	.000
K-Cu	PC																	1.000**	1.000**	1	1.000**
	Sig.																	.000	.000		.000
K-Mn	PC																	1.000**	1.000**	1.000**	1
	Sig.																	.000	.000	.000	

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Fig. I: Average Zn concentration (mg/kg) in the tissues of *O.mossambicus* exposed to Plant Nutrient

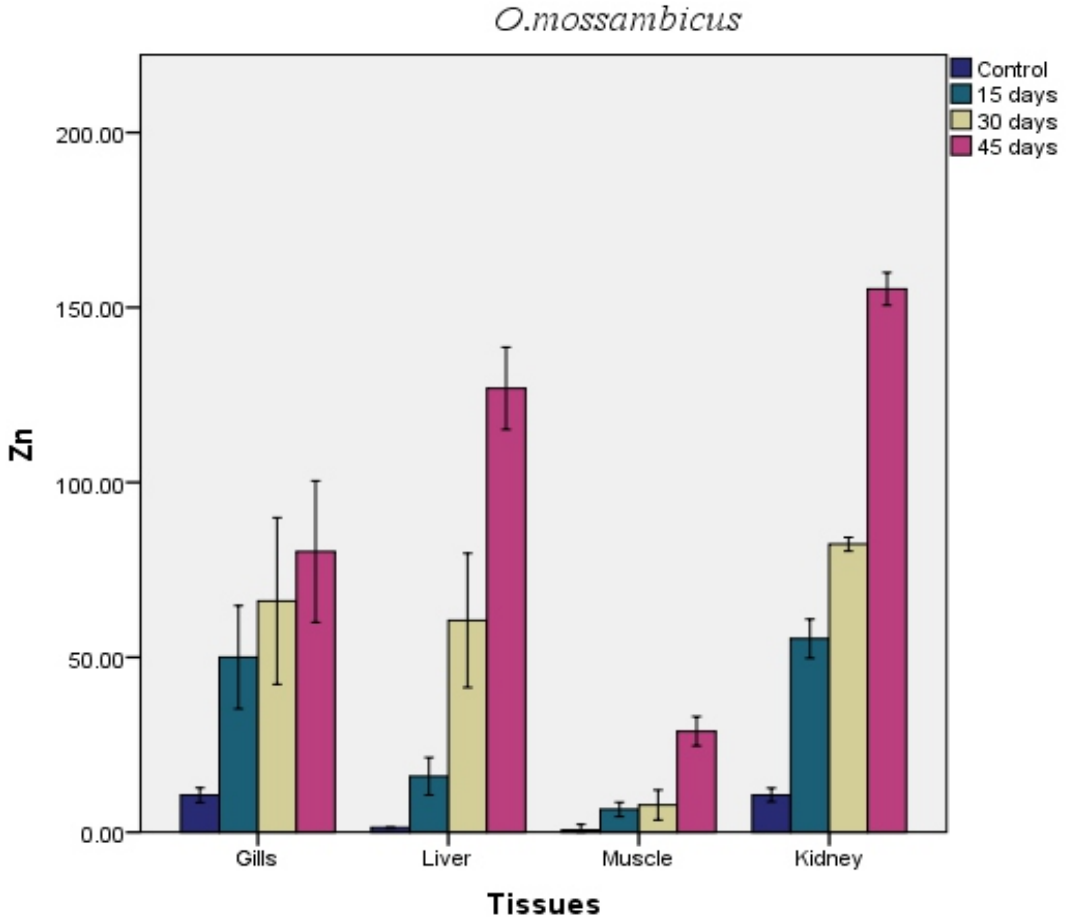


Fig. II: Average Zn concentration (mg/kg) in the tissues of *L.rohita* exposed to Plant Nutrient

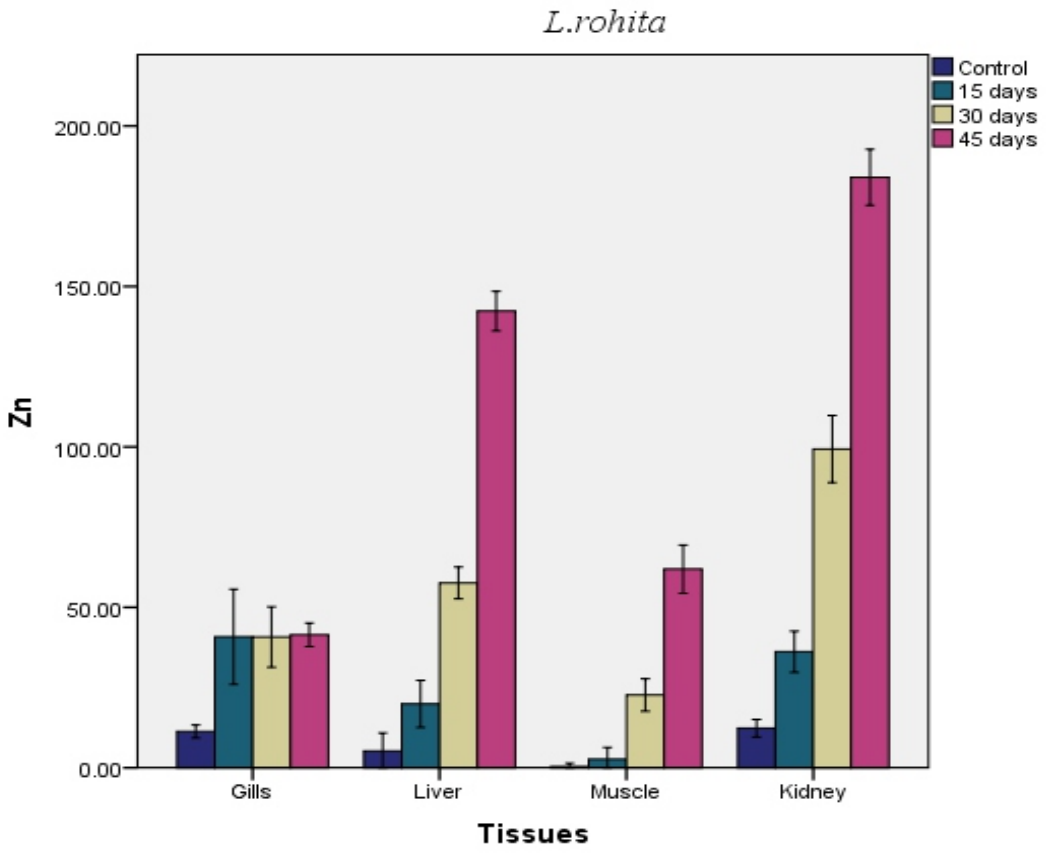


Fig. III: Average Fe concentration (mg/kg) in the tissues of *O.mossambicus* exposed to Plant Nutrient

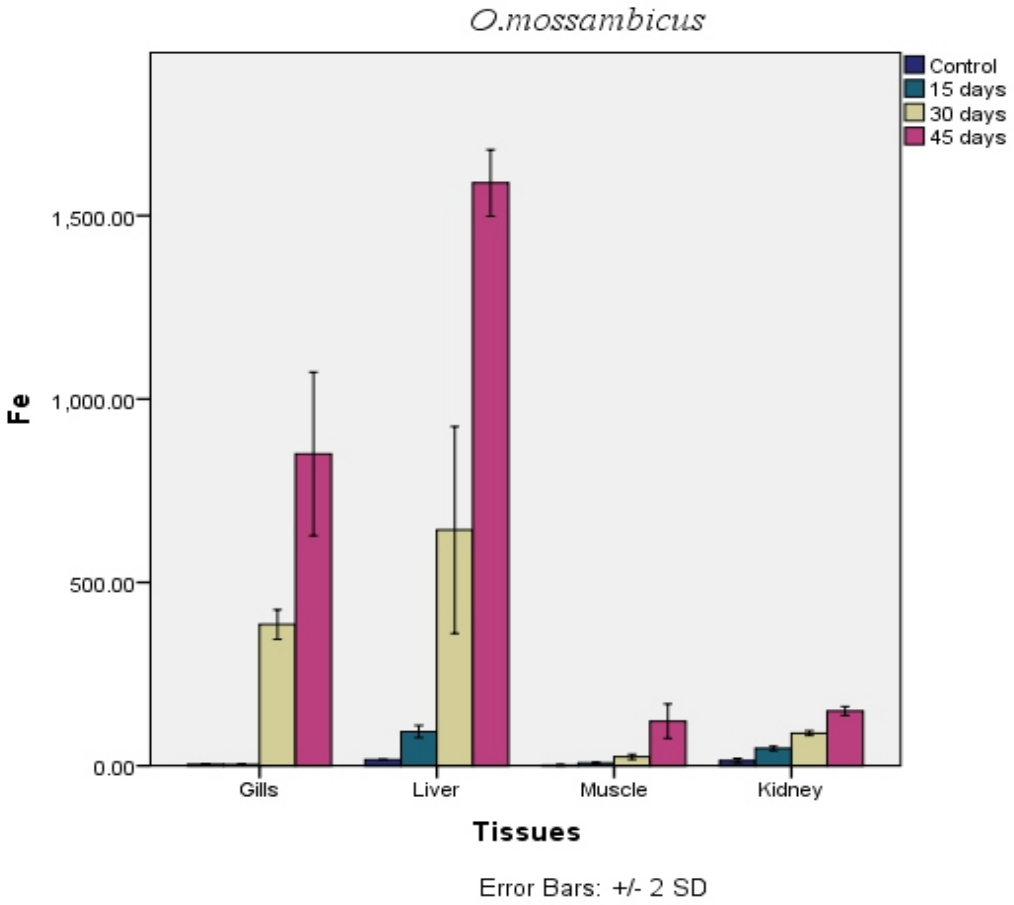


Fig. IV: Average Fe concentration (mg/kg) in the tissues of *L.rohita* exposed to Plant Nutrient

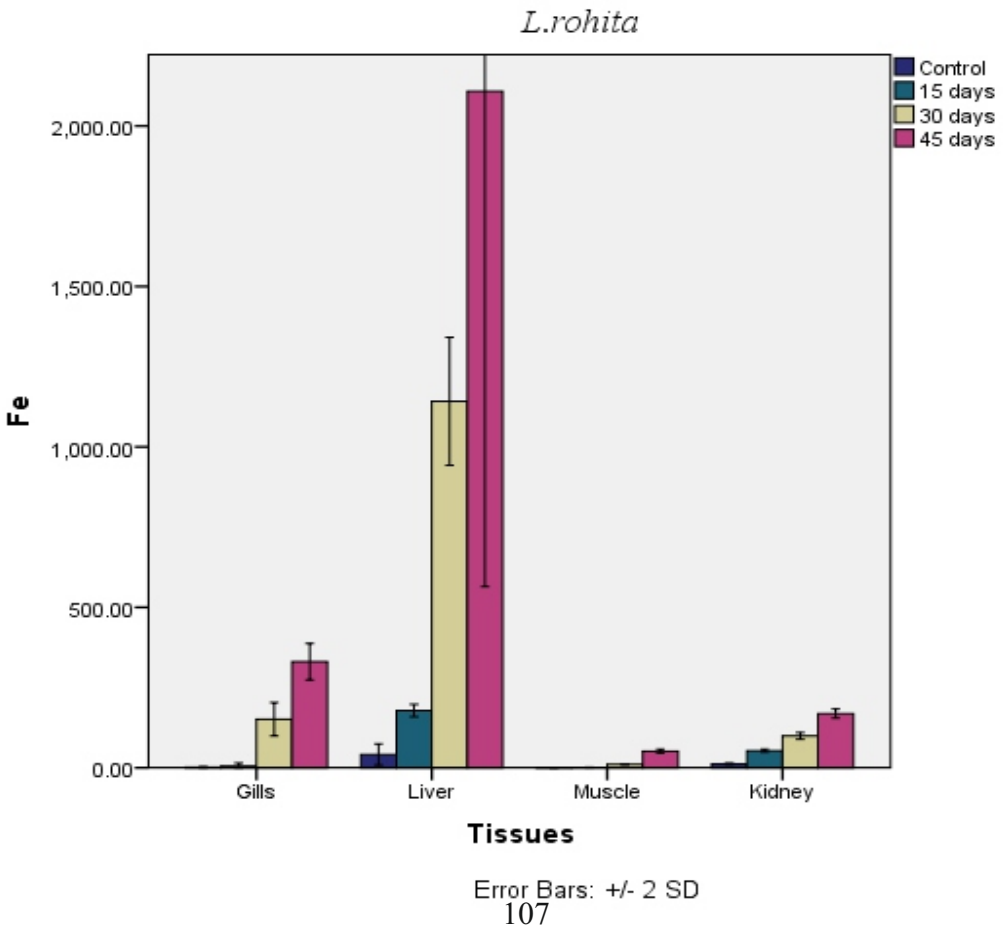


Fig. V: Average Cu concentration (mg/kg) in the tissues of *O.mossambicus* exposed to Plant Nutrient

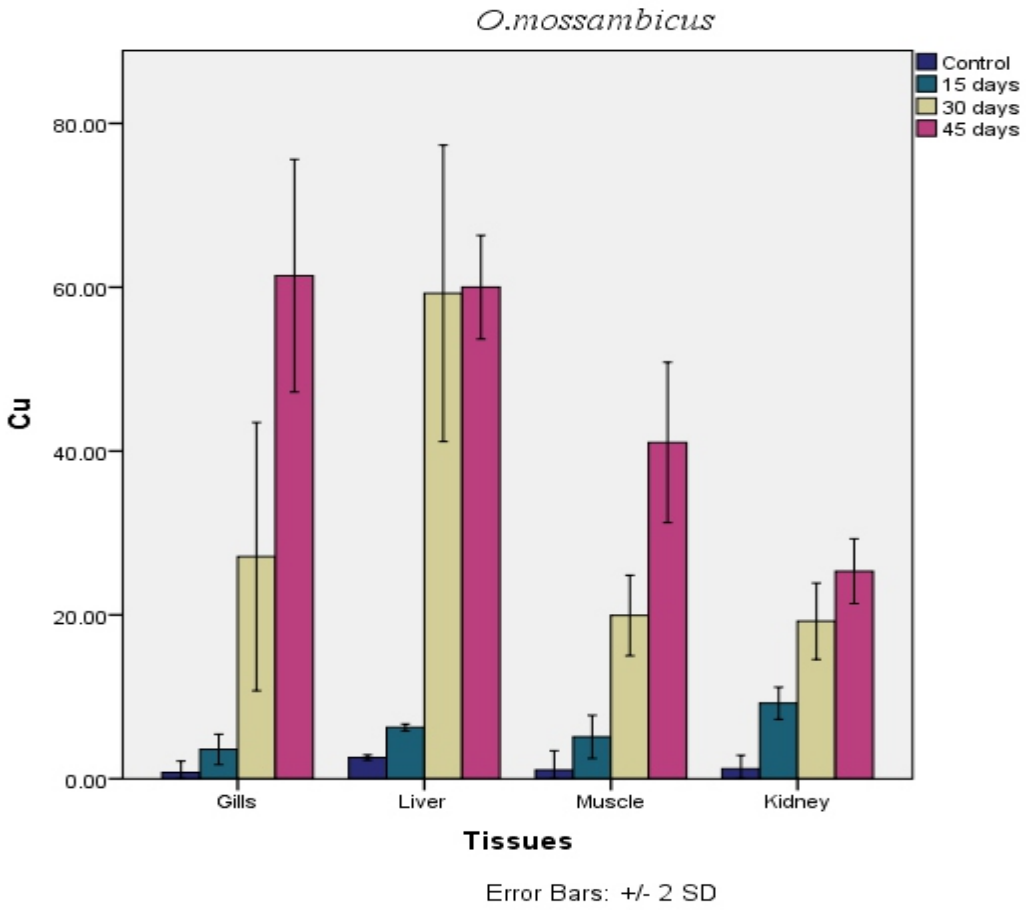


Fig. VI: Average Cu concentration (mg/kg) in the tissues of *L.rohita* exposed to Plant Nutrient

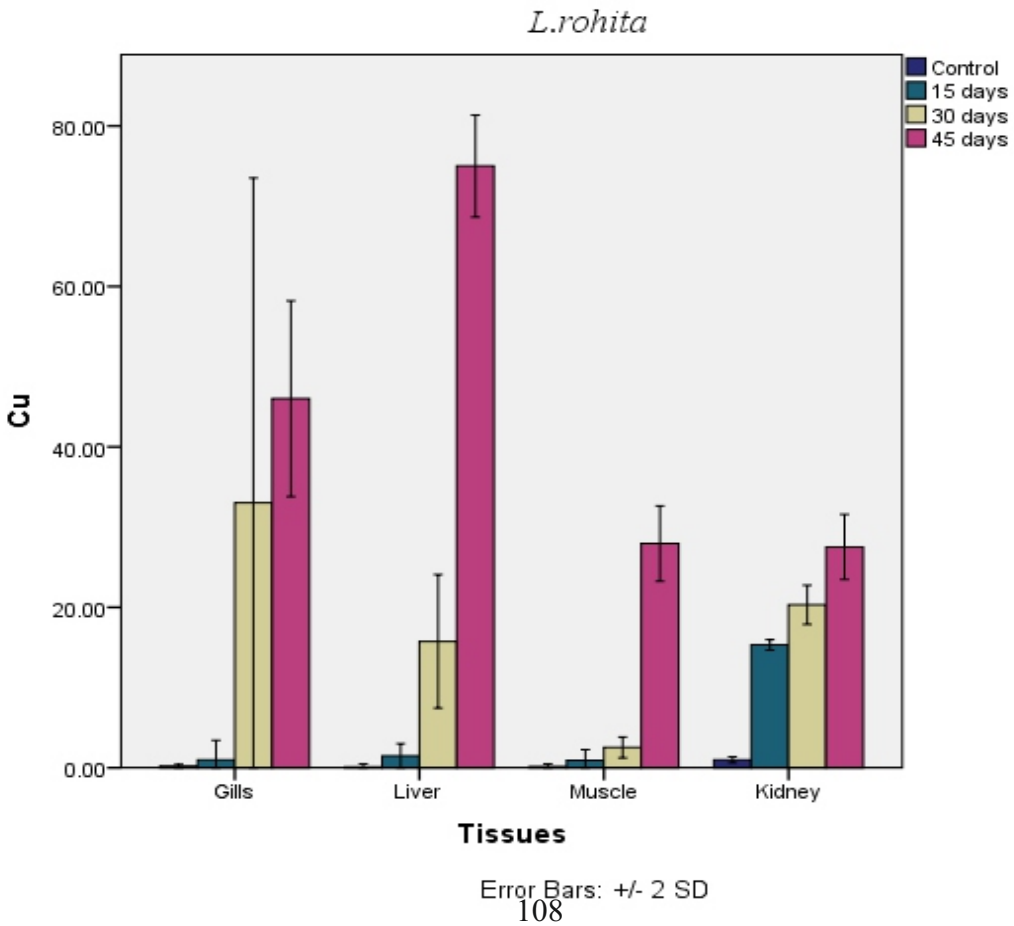


Fig. VII: Average Mn concentration (mg/kg) in the tissues of *O.mossambicus* exposed to Plant Nutrient

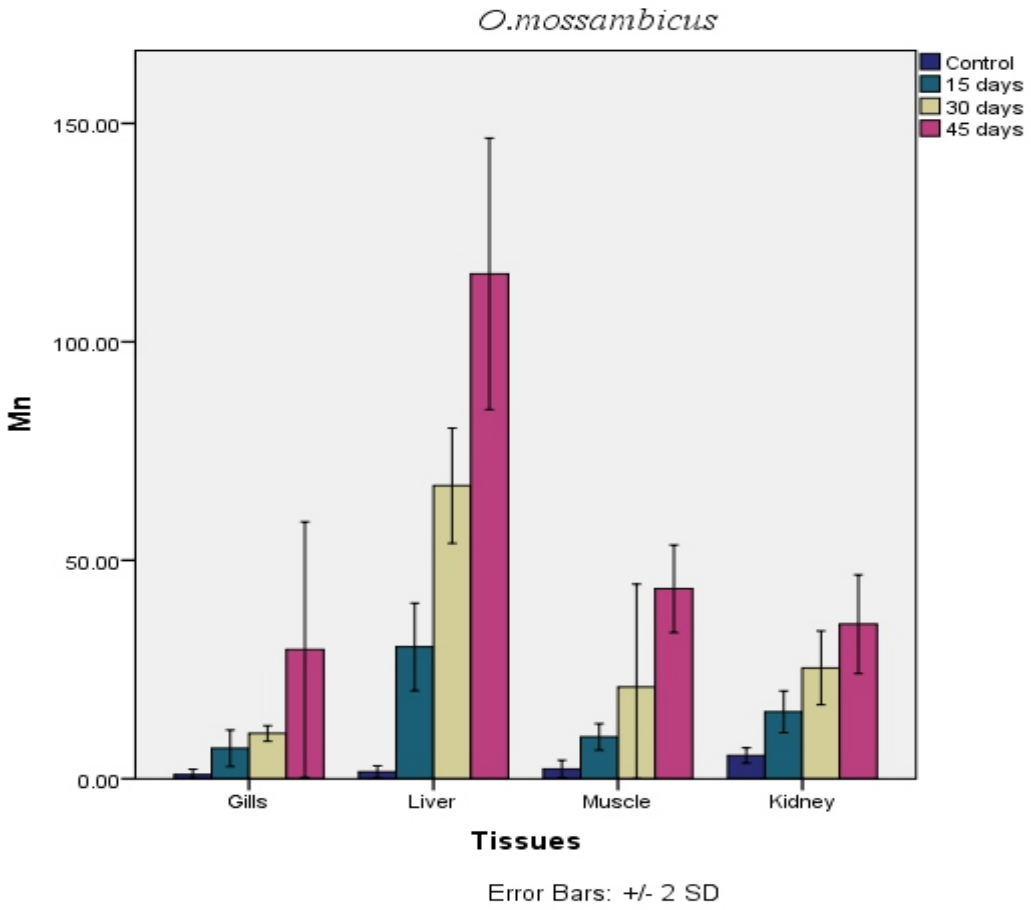


Fig. VIII: Average Mn concentration (mg/kg) in the tissues of *L.rohita* exposed to Plant Nutrient

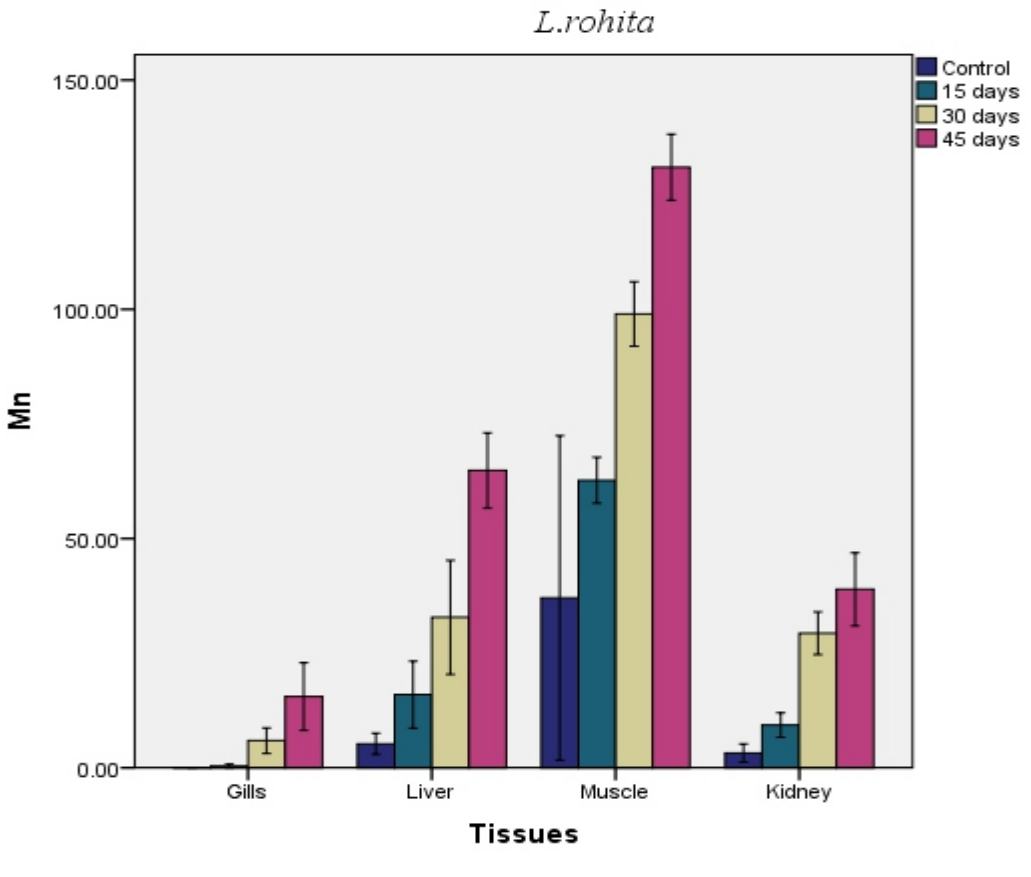


Fig. IX: Bioconcentration factor (BCF) of tissues of *O.mossambicus* exposed to Plant Nutrient

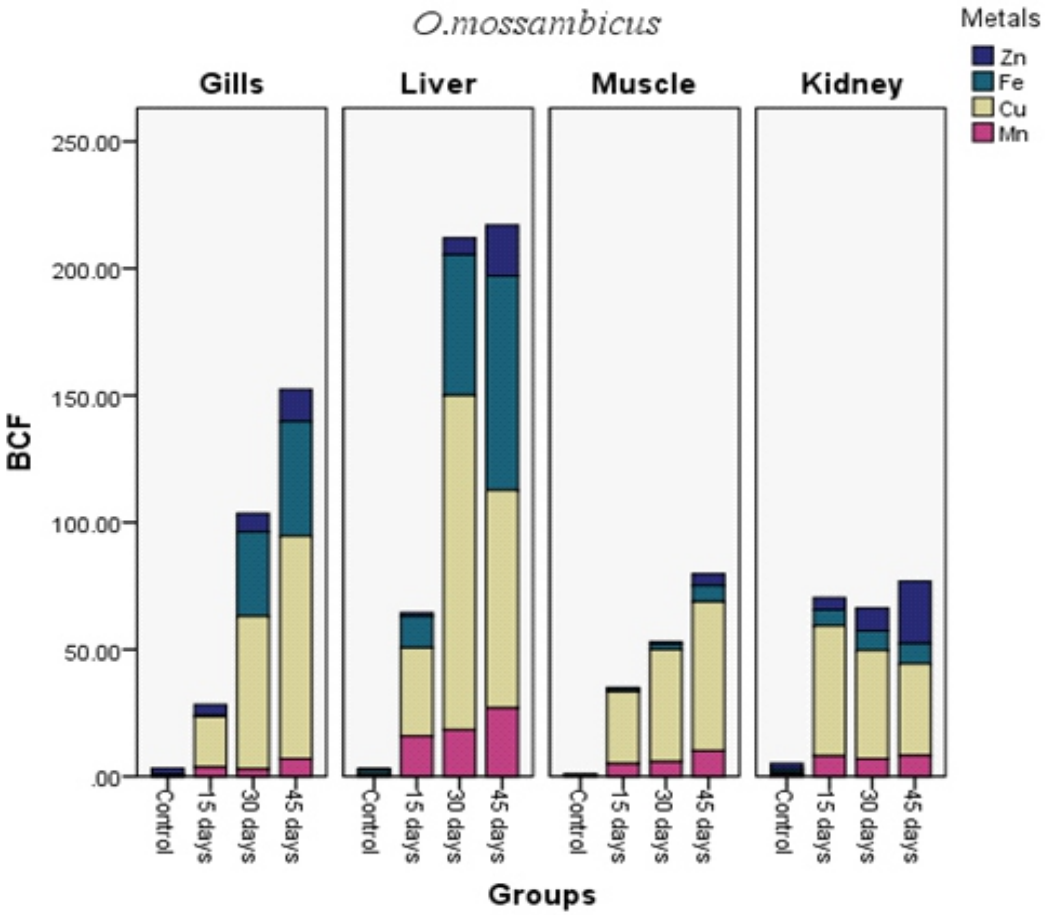
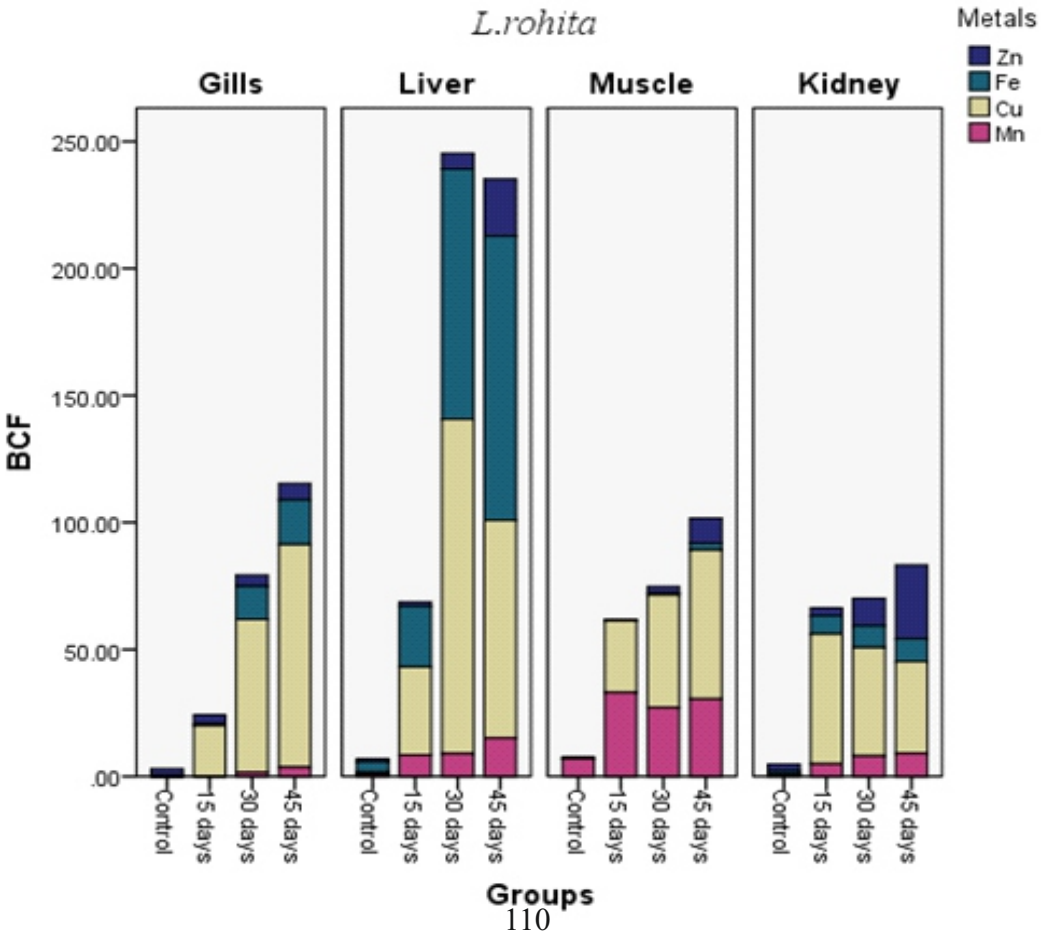


Fig. X: Bioconcentration factor (BCF) of tissues of *L.rohita* exposed to Plant Nutrient



Discussion

The results of the present study strongly revealed that Librel exposure has resulted into time dependent accumulation of metals in teleost. Although both the fishes showed the accumulation of the metals in tissues the most astonishing fact was that no mortality was reported during the experiment. The essential metals, such as iron, zinc, copper and manganese are in higher concentrations, presumably due to their function as co-factors for the activation of a number of enzymes and regulated to maintain a certain homeostatic status in fish.The results of several studies of metal accumulation in fish living in polluted waters showed that considerable amounts of metals may be deposited in fish tissues without causing mortality (Jezierska and Witeska, 2001).In the present study of the two fishes *L.rohita* was found to have more affinity to accumulate metals compared to *O.mossambicus*, which are in agreement with the comparative studies done by Javed (2005 and 2012) who observed significantly higher accumulation of metals in *L.rohita* than *C.mrigala* and *C.catla.* Many studies have focused attention on the dependence between the contents of metals and different species of the fish (Kostecki, 2000; Jezierska and Witeska 2001; Canli and Atli, 2002; Mazon *et al.,* 2002; Salam *et al.,* 2002; Farkas*et al.,* 2003; Luczynska and Tonska, 2006;Ebrahimpour*et al.,* 2010; Promya and Chitmanat, 2011). Furthermore, a variety of species of fish from the same water body are also reported to accumulate different amounts of metals (Usha Rani, 2000; Voigt 2004; Bhattacharya et al., 2006; Senthilkumar and Sajwan, 2007; Sajwan*et al*., 2008; Kumar *et al*., 2011; Mukherjee and Kumar, 2011).Szarek-Gwiazda*et al.,* (2006) have also said that along with the species specificity the trace metal concentration and their binding capacity varywith tissues too. Further, Kumar *et al*., (2011) in their studies have also reported the interspecific metal contamination in the organs of seven fish species along a polymetallic pollution gradient whileRajkowska and Protasowicki (2013) in their studies on distribution of metals in fish tissues have reported that the concentration of trace metals in fish vary significantly among two species pike (*Esoxlucius L.)* and bream (*Abramisbrama L.*).

Metal distribution in various organs is also time-related (Eggleton and Thomas, 2004). In the present study both fishes showed a time dependent significant increase in the metal concentration in all the tissues. The effect of time on metal distribution within the organism is a complex issue due to different affinity of various metals to the tissues of various fish species. Various metals are accumulated in fish body in different amounts. Particularly, accumulation of essential metals

such as iron, zinc, copper, manganese or cobalt is tissue-specific. These differences result from different affinity of metals to fish tissues, different uptake, deposition and excretion rates. The major part of total body loads accumulated at different concentrations of metals in the water and at various exposure times are reported in liver, kidney and gills by Giguere *et al.*,(2004). The results of the present studies are parallel with the earlier reported tissue specific accumulation. Furthermore liver has shown the highest level of accumulation of metals followed by gills while kidney and muscle have shown the least level of accumulation. Liver accumulates high concentrations of metals, irrespective of the uptake route (Tsai *et al.,* 2013). The liver is considered a good monitor of water pollution with metals since their concentrations accumulated in this organ are often proportional to those present in the environment (Dural *et al.,* 2006).Next in order of accumulation were the gills which are in direct contact with water,therefore, the concentration of metals in gills reflects their concentration in water. Moreover, the gill surface is negatively charged which provides a potential site for gill-metal interaction for positively charged metals. Gills are known to contain almost 38-50% of total metal burden (Yousafzai and Shakoori, 2008). Fish can absorb ions through gills, since they have special salt secreting cells, according to Griffitt*et al.,* (2009)gills are involved in the secretion of metals, probably via the secretion of mucus, but when the metal accumulation crosses the excretion threshold limit bioaccumulation exceeds the excretion level. Hence the second highest metal burden observed in the gills of *O.mossambicus* and *L.rohita* is self explanatory mechanism of metal accumulation. Our results are in agreement with earlier reported metal accumulation in tissues of freshwater fish *C.gariepinus* (Kusemiju *et al.,* 2012) *O.niloticus* (Al-Nagaawy, 2008)*inO.mossambicus* (Naigaga, 2002) in *Tor putitora* (Shakoori, and Yousafzai, 2006).

Metal concentrations in the kidneys rise slower than in liver, and usually reach slightly lower values, except for metals such as zinc that show very high affinity to kidneys, therefore the kidneys may be considered a good indicator of pollution too. As reported by Palaniappan and Karthikeyan, (2009) during depuration, kidney metal levels remain high or may even increase for some time, which is related to the role of kidneys as excretory organs.The main function of kidney is washing/filtration of body fluids and to maintain the homeostasis. Javed and Usmani (2013) in their studies on assessment of heavy metals in *Masracembelus armatus* have reported the histopathological alterations in kidney and are of the view that severity of lesions observed results into damage in the uriniferous tubules and hematopoietic tissue which impairs the renal

function and as a consequence heavy metals get accumulated in kidney. In the present study at this point it is difficult to correlate the same as histological alterations are lacking.The present data corroborates with the studies of metal accumulation in the kidney of *O. niloticus* (Abdel-Baki*et al.*, 2011), *Onchorynchus mykiss* of *Carassiusauratus and Cyprinus carpio* respectively (Boeck*et al.*, 2004). Metal uptake and binding has been reported to increase with increase in the metabolic rate (Green &Knutzen, 2003 and Voigt, 2004). Fish liver, kidney and gills are metabolically very active organs to accumulate large quantities of heavy metals. However, fish muscle tended to accumulate less metal (Yilmaz*et al.*, 2007). This result was in agreement with many authors who reported that muscles is not an active organ in accumulation of most heavy metals (Khalil and Faragallah, 2008; Kraiem 2007).Thus, plant nutrient exposure in the present study has probably led to the increase in the metabolic rate particularly for the metabolically important tissues such as liver.Generally, the higher metal concentration in the environment, the more may be taken up and accumulated by fish. In the present study as Librel, the trace element mixture having a commercial formulation of Fe-6.0, Zn-4.0, Mn-0.5, Cu-0.3 (Nutrient % by Wt.Min.) was exposed to fishes. Hence in addition to the background concentration the exposure of plant nutrient adds on to the total concentration leading to overall metal load in the tissue.On the other hand, under conditions of metal contamination, metals tend to deposit in the same organs where they may exert toxic effects. Accumulation of certain metals in fish may be altered in the presence of the others (Ashraj, 2005 and Farkas*et al*., 2003).

Metals in natural waters occur in particulate or soluble form. Soluble species include labile and non-labile fractions. The labile metal compounds are the most dangerous to fish. The order of lability of the metals is Pb> Zn > Co > Ni > Cu >Mn> Fe > Cd (Leermakers *et al.*, 2005). Many data showed that the amounts of metals in the labile fraction, and the share of various metal ions strongly depend on environmental conditions (Witeska and Jezierska, 2003 and Wiechuła *et al*., 2005). In the present study the order of accumulation of metals in water was found to be Fe >Zn>Mn>Cu.The mean concentration of Cu recorded in water in this investigation was below permissible limits, while the mean concentration of Zn, Mn and Fe were above limits (WHO, 2005). This higher concentration could be linked to the presence of synergistic or additive effects other metals

Natural water contains variable amounts of iron despite its universal distribution and abundance. Iron in ground water is normally present in the ferrous or bivalent from (Fe++) or insoluble Iron urban exposure to air. Iron is a trace element required by both plants and animal. It is a vital oxygen transport mechanism in the blood in all vertebrate and some invertebrate animals.The current aquatic life standard is 1.0 mg/l based on toxic effects.Toxicity data for iron are also limited, and effects of dissolved iron on brook trout have been reported to vary widely under different test conditions (Besser*et al.*, 2001).

Metals are non-biodegradable, and once they enter the aquatic environment, bioconcentration may occur in fish tissue by means of metabolic and biosorption processes (Wepener 2004; Yousafzai and Shakoori, 2008; Kaoud and El-Dahshan, 2010). From an environmental point of view, bioconcentration is important because metal ions usually occur in low concentrations in the aquatic environment and subtle physiological effects go unnoticed until gross chronic reactions (e.g. changes in populations' structure, altered reproduction, etc.) become apparent. BCF in the present study has revealed alterations in the tissue specific and metal specific responses. Overall the BCF of liver tissue has resulted into the highest affinity for Cu, Fe and Zn, Whereas BCF of gills showed affinity for Cu and Fe, kidney showed affinity for Fe and Zn and muscle had affinity for Mn. Fish liver as a major detoxifying and storage organ differed from the concentrations detected in the gills, kidney and muscles. Significantly higher levels of all metals in fish liver can be related to the binding of metals to metallothionein that provide detoxification mechanism (Sinaie*et al.*, 2010). Further, Boeck *et al.*, (2004) have reported tissue-specific Cu bioaccumulation patterns and differences in

sensitivity to waterborne Cu in three freshwater fish: rainbow trout (*Oncorhynchu smykiss*), common carp (*Cyprinus carpio*), and gibel carp (*Carassius auratusgibelio*). Copper is an essential element that promotes the activity of certain enzyme systems in the body while iron is a component of haemoglobin which is responsible for the transport of oxygen in the body.

Metal concentrations in water were used to calculate BCFs, which indicated that among examined metals Cu was the most readily absorbed by fish. The presented research has also shown that water was a good source of Fe, which possibly has penetrated into fish organisms mainly through the gills. Manganese was absorbed through muscles of *L. rohita* and through gills of *O.mossambicus*. Depending on the fish species, Cu and Fe showed the highest affinity to: liver, gills, kidney and muscle. Rajkowska and Protasowicki (2013) have observed the highest

BCFs for Fe and Cu fish tissue of two lakes of different trophy in Northwestern Poland. Species difference in heavy metals bioconcentration could be linked to difference in feeding habits and behaviour of the species (Altindag and Yigit, 2005). Although, these two elements may also be toxic to man and animals when ingested in large amount. The variability observed in the fish species is a reflection of different thresholds of metals which are a function of homeostasis. The thresholds of metals in fish can be considered as the concentration level where the metal starts to interfere with the variable physiology of the fish species in such manner that once a particular level of the metal has been sequestered in the body, equilibrium is established between the fish burden and the ambience. The high bioaccumulation factor for Cu and Fe suggests that the concentration of these metal ions serves as a harborage or the fish species have poor mechanisms for digesting and eliminating these heavy metals. The rate of bioaccumulation of heavy metals in organisms depends on the ability of organisms to digest the metals and the concentration of such metals in the river. It also has to do with the concentration of the heavy metal in the surrounding soil as well as the feeding habits of the fish species. Bioaccumulation of naturally occurring substances occurs along a continuum of exposure, and trace amounts of metals, both essential and nonessential, can be found in all biota (Cowgill, 1976; Shearer, 1984). In addition to back-ground accumulation, aquatic biotais also able to regulated internal concentrations of metals through active regulation, storage, or a combination of these two (Rainbow, 1988; Viarengo, 1989; Depledge and Rainbow, 1990). Understanding and predicting bioaccumulation of metals is one of the key requirements in understanding their fate and toxicity in aquatic environments and for environmental protection measures. Bioaccumulation of metals follows a different paradigm relative to neutral organics. For example, metal uptake occurs via specific mechanisms that can often be modified as a result of exposure. Additionaly low-level accumulation at background concentrations is a natural phenomenon, detoxification and elimination of accumulated metals is part of acclimation and toxicity is predominantly associated only with charged cations (Mcgeer*et al.*, 2003).

Simkiss& Taylor (1989) in their studies have proposed various pathways of metal accumulation by aquatic organisms either through lipid permeation, complex permeation, and carrier mediated, through ion channels, ion pumps or endocytosis. The high metal concentration in the tissues and water reported in the present studies thus suggest that possibly few of the mechanism might be working simultaneously, however at this point it is difficult to conclude the exact mechanism for

metal accumulation. Moreover depuration studies were not done and perhaps it will throw more light to validate our data. As there was no mortality reported in the present studies, possibly the fishes are having an inborn mechanism to counteract the potential toxicity of the metals in the tissues, by synthesizing the stress proteins as they are assumed to play a role in the detoxification of heavy metals. Hence, to further take the work a step ahead it was thus thought worthwhile to go for protein profiling.

CHAPTER VI

Proteotoxicity Studies of *Oreochromis mossambicus* And *Labeo rohita* Tissues Induced by Plant Nutrient Librel

INTRODUCTION

For several reasons, fish have attracted considerable interest in studies designed to assess the biological and biochemical responses that organisms have to environmental contaminants (Powers 1989). Fish are particularly useful for assessing water-borne and sediment-deposited toxins, and may provide advanced warning of the environmental contamination potential of new chemicals, or the status of environmental contamination by well-known toxicants. Fish are also particularly good models for studies in which biochemistry and comparative physiology are involved, because they live in diverse habitats and must adapt to environmental parameters and stress, both of which can be easily reproduced under laboratory conditions (Beyer, 1996). The understanding of toxicant uptake, behavior, and responses in fish, therefore, has a high potential for ecological relevance. Because of these aspects, it is important that these organisms be studied in more detail; the development of 'omic techniques in recent years offers the possibility to perform such studies in new and useful ways.

Proteomic analyses provide valuable information, when variations that occur within the proteome of organisms are compared as a consequence of biological perturbations or external stimuli. These stimuli often result in different protein expressions or the redistribution of specific proteins within cells (Martin *et al*. 2001, 2003;Tyers and Mann 2003; Vilhelmsson*et al*. 2004), which can be correlated with environmental contamination, and which may help identify proteins that are altered from pollutant exposure, or may help establish a protein-pollutant toxic mechanism relationship (Lopez-Barea and Gomez-Ariza 2006). An added bonus in these studies is the fact that it is not absolutely necessary to establish the identity of a protein for it to become a successful biomarker of exposure. Indeed, the characteristics of a peptide and the specific conditions under which it occurs are the more pressing concerns (Hogstrand*et al*., 2002). Recent studies have produced protein expression signatures that were characterized in marine

invertebrates, in response to changing salinities and temperatures, and in response to the presence of polychlorinated biphenyls and copper (Shepard *et al*. 2000; Shepard and Bradley 2000; Kimmel and Bradley 2009).

Comparing fish proteomes in different situations is appealing, because changes in proteome expression under complex field situations may disclose which gene products, metabolites, or proteins are most interesting to investigate (Albertson*et al*., 2007). Such comparative proteomic studies are also useful as tools in the investigation of fish development and different ecological situations.The effects of starvation in certain species, such as rainbow trout, have been studied by using comparative protein expression tools (Martin *et al*., 2001). This study was the first to use protein profiling in a nonmodel organism (rainbow trout) to demonstrate, for the first time in teleosts, that proteomics have the potential to assist in studying cellular mechanisms involved in protein degradation. Stentiford*et al*., (2005) reported significant differences in fish caught from polluted and non polluted sites. Their study was an important pilot study conducted in 2005 on flat fish *L. limanda* in which liver lesions and normal tissue from wild populations were investigated using proteomic techniques. This was one of the first studies, in which comparative proteomic expression was utilized as a means to discriminate between tumorous and non tumorous livers in fish. Moreover, the preliminary data from this study was one of the first to suggest that proteomic approaches may be useful in an environmental contamination context for studying fish, and may have potential application to serve as a high-throughput screening approach for disease classification. Further Hogstrand*et al*., (2002) have also used genomics and proteomics for study of the integrated response to zinc exposure in a non-model fish species, the rainbow trout.; Ling *et al*. 2009 have explored the proteomic changes in response to acute cadmium toxicity in gill tissue of *Paralichthysolivaceus*; Karim*et al*., (2011) have looked at the toxins and stress in fish by proteomic analyses and Mezhoud*et al*., (2008) have studied the proteomic and phosphoproteomic analysis of cellular responses in medaka fish (*Oryziaslatipes*) following oral gavage with microcystin-LR.Hither to a few studies are available on the impact of different pollutants upon the elctrophoretic protein pattern in the tissues of fishes. (Muthukumaravel*et al*., 2007; Arockia and Milton, 2007; Yilmaz*et al.,* 2008 and 2011; Sheik *et al.,* 2014).

Proteins are the important biomolecules involved in a widespectrum of cellular functions. They interplay between enzymaticand non-enzymatic proteins to govern the metabolic harmony(Lehninger, 2008). They are also involved in all the majorphysiological events to maintain the homeostasis of the cell.Therefore, the assessment of proteins can be considered as adiagnostic tool to determine the physiological process of cell(Mushigeri, 2003). Proteins play a vital role in the physiology of living organisms. All biological activities are regulated by enzymeand hormones, which are also proteins. If any alteration takesplace in the proteins turn over, it may have an adverse impact ontheir vital and complex groups of biological materials,comprising the nitrogenous constituents of the body and the foodintake; thus they perform different biological functions (Prashanth, 2007).Protein constitutes the building block and the basic molecule for any biochemical reaction. Theyare intimately related with almost all physiological processes, which maintain a simple biochemicalsystem in living condition. The physiological and biochemical alterations observed in an animalunder any physiological stress can be correlated with the structural and functional changes ofcellular proteins. Proteins occupy a unique position in the metabolism of cell because of theproteinaceous nature of all the enzymes which mediate at various metabolic pathways Vidhya and Nair, (2013).Serum or organ proteins of fish are occasionally studied to estimate the toxic potential of many substances including metals. Organisms may respond to chemicals with up or down regulation of serum proteins, which could give a view of defense pattern against them. These changes are of some value in assessing the impact of exposure under natural conditions and may also serve as tools for biological monitoring (Thorpe*et al*., 2007). Agricultural run-off has caused ecotoxicity of the aquatic environment leading to death of targeted and non-targeted organisms (Joseph and Raj, 2011). The genotoxicity of many agrochemicals is still under debate as there are reports which are either positive or negative; plant nutrient is one of them, for which such studies are the rare.

Exposure of Librel has led to hematological, biochemical and has resulted into serious impairment of gonads and liver tissues. Further the metal accumulation is also reported in various tissues of both the teleost fish, leading to alteration in the metabolic activities, and biochemical indices of stress.We preferred to use *O.mossambicus*and*L.rohita* as the test model, mainly because these have been favored by many earlier workers as test models for both cytogenetic and molecular studies; moreover they make an appropriate model as an indicator species in biomonitoring programs (Veeraiah*et al.,* 2013).The development of electrophoretic

techniques makes it possible to detect the protein composition. Several studies have revealed that fish are able to accumulate and retain metals in different fish tissues (Gupta*et al.,* 2005; Batvari*et al.,* 2008; Shukla*et al.,* 2007; Malik *et al.,* 2010;; Kumar *et al.,* 2011; Adhikari*et al.,* 2009; Begum *et al.,* 2009 Veeraiah*et al.,* 2013) and have shown to be time dependent.Hence understanding the protein profile may add more understanding towards the outcome of the previous studies. The potential value of electrophoresis in this study is based on the hypothesis that stress conditions may cause significant qualitative and quantitative changes in the proteins of different tissue exposed to the toxicant. Such changes might reflect an altered antibody synthesis, protein biosynthesis, cellular leakage or perhaps other events resulting directly or indirectly from the stress.However, reports on variations of qualitative tissue proteins are lacking, especially with reference to Librel exposure which is multi micronutrient mixture containing metals (Fe, Cu, Zn, Mn and B). Hence the present study was aimed at adding information on the protein profiling in response to the plant micronutrient using *O. mossambicus* and *L.rohita* as test animal. Hematological, indices, histomorphological, biochemical assayobservations has well established the toxicity of plant nutrient. The present studywas done with the view to look into the protein profiling of the major tissues gills, liver and muscle.

MATERIALS AND METHODS

Freshwater fishes, *O. mossambicus*(12 ± 2 cm, 25 ± 1.9 g) and *L. rohita*(20± 2 cm, 125 ± 5 g) of similar length and weight were brought to the laboratory from a local pond of Vadodaradistrict, stocked in well aerated tanks containing chlorine free water and acclimated for 10 days. They were fed with commercial fish pallets. 30% Water was renewed every alternate day to provide freshwater, rich in oxygen. Ten well-acclimatized fish were transferredfrom the stock to each experimental tank containing 40 L of water exposed to the concentration of 500mg/L in *O.mossambicus* and 600 mg/L in *L.rohita*for the period of 45 days. A control group was also maintained in the same condition for the basic test. After the study period the fishes were sacrificed and tissue was dissected out.

Estimation of total protein content

Total protein content was estimated by the modified method of Lowry *et al.*, (1951). In the Lowry method, the Folin–Ciocalteu reactive(FolinandCiocalteu, 1927) (Sigma Co.) was diluted in1volume of distilled water (1:1) and 0.5 mL ofthe diluted reactive was added to 1.0 mL of sample,previously mixed with 5.0 mL of the reactive "C" [50volumes of reactive "A" (2.0% Na_2CO3+0.1 N NaOH)+1 volume of reactive "B" (1/2 volume of 0.5% $CuSO_45H_2O$+1/2 volume of 1.0% $C_4H_4NaO_6$ $4H_2O$)]. Afterthe addition of each reactive, samples were stirred for2 s in a test tube stirrer. Absorbance was measured at660 nm, 30 min after the start of the chemical reactionat room temperaturein a spectrophotometer (ELICO Model SL171) against a blank. The standard graph was plotted by the method of Lowry *et al.*, (1951) with bovine serum albumin supplied by Sigma chemical Company, U.S.A. The values were expressed as mg protein/100 mg wet weight of the tissue.

Protein precipitation by TCA: Before loading on SDS-PAGE, proteins were precipitated by mixing 10% TCA with tissue homogenate prepared in distilled water. Then the samples were kept in ice for 10 min and centrifuged for 10 min at 3000rpm. Again the pallet was mixed with 5% chilled TCA, kept for 10 min and centrifuged at 3000 rpm. The protein pellet was then washed with ethanol/ethyl acetate (1:1) and centrifuged for 10 mins at3000 rpm. The final protein pellets was dissolved in 0.5 N NaOH and used for SDS PAGE.

Preparation of Gel slab:

The glass plate's sandwich was assembled using two clean glass plates and 1mm teflon spacers. The glass plates were sealed with 0.8 % agar solution. Resolving gel solution 12 % (1.5M Tris-

HCl, PH 8.8 -2 ml, 30 % Acrylamide-3.2 ml, 10 % SDS-0.5 ml double distilled water-1.8 ml, TEMED-0.015 ml, Ammonium per sulfate-0.5 ml) was prepared and poured in between the clamped glass plates. To avoid entrapment of any air bubbles, the gel solution was overlaid with distilled water. The plates were left undisturbed for 30 min for polymerization of the gel. Aftergel polymerization, overlaid water was removed and rinsed with stacking gel buffer. Now the 5% stacking gel solution (0.5 M Tris-HCl, pH 6.8-2 ml, 30% Acrylamide-0.8 ml, 10% SDS-0.5 ml,double distilled water -1.2 ml, TEMED -0.015 ml, 1.5% Ammonium per sulfate 0.5 ml) wasprepared and poured over the polymerized resolving gel, comb was inserted carefully. The gel slab was left undisturbed for 15 minutes, after polymerization comb was removed carefully

SDS- PAGE analysis: The precipitated proteins dissolved in 0.5N NaOH was mixed in sample buffer (0.5M Tris-HCl pH-6.8-2 ml, 40% glycerol-1.6ml, 10% SDS-3.2ml, 2-mercaptoethanol-0.8ml, 0.1%(w/v) bromophenol blue-0.4 ml) at the ratio of 3:1 and heated at 60^{o}C for 10 minutes.The SDS-PAGE was performed to analyze protein profile in muscle of control and Librel exposed tissues of different time intervals by using standard method (Laemmeli, 1970). The concentration of acrylamide was 12% and sample extract was loaded in each lane of the gel. The electrophoresis was carried at 100V for 2.5 hrs by watching the movement of the tracking dye and the gel was analyzed with Coomassie blue staining for visualizing protein bands.

Staining Method:

The proteins separated by electrophoresis through SDS-PAGE were fixed by placing the gel in fixation solution (50:10:40 / methanol: acetic acid: H_2O) for 2 hours with gentle shaking. The fixation solution was decanted, and gel was rinsed thrice with double distilled water for 5 seconds. The gel was than stained in the staining solution for 5 minutes with gentle shaking. The developing solution was decanted and the reaction was quenched by washing the in dw for few minutes. Then the gel wasdestained in destaining solution (0.1% CBB R-250/ 40% MeOH/ 10% Glacial Acetic Acid) for 5 minutes and then the gel was kept overnight for the appearance of clear bands. The electrophoretogram gel was preserved in water and then gel documented by Gel Doc (Bio Rad).

Determination of molecular weight of the protein subunits separated on SDS PAGE:

To determine the molecular weight of the individual subunits of the protein, the relativemobility of the individual subunit was calculated by using the following formula.

Relative mobility (Rm) = Distance travelled by individual subunit / Distance travelled by the marker dye

A standard curve is prepared by plotting migration distances ('X'-axis) of known protein standards against their molecular weights ('Y'-axis) on semilog graph paper. From the migration distance of an unknown protein, the molecular weight of the protein was calculated from the standard curve.

Statistical analysis

The values of protein content were statistically calculated using One way ANOVA and post hoc Dunnetts's t test was done to find the significance alterations if any between control and different exposure groups using SPSS software (version 21).

RESULTS

Plant nutrient exposures lead a time dependent significant ($p<0.05$) increase in the protein content in tissues of both the fishes. The calculated values for total proteins along with standard deviation are given in Table IV. The fresh water fishes*O.mossambicus*and*L.rohita*onsubchronicexposure to low sublethal concentration of plant nutrient revealed variation in electrophoretic protein fractions between thecontrol and experimental fishes. As presented in the Fig. I-IIIelectrophoretogramof both species represents anincrease in the intensity of tissue protein subunits compared to control in the both the species at the initial 15th and 30th day of exposure while a decrease at the 45th day. The size of protein was extrapolated by plotting the graph of log(mol.weight) against Rm(Tables I-III).

Polypeptides in muscle ranged from 3.46 to 199.52KDa in bothspecies; in gills 3.46 to 97.4 in O.mossambicus and 3.56 to 43.0 in *L.rohita*; while in liver 3.54 to 43.0 in *O.mossambicus*3.46 to 63.2 in *L.rohita*. In *O.mossambicus*; muscle control showed 5 bands whereas 15th, 30th and 45th day groups showed 5, 6 and 4 bands respectively; gill control showed6 bands whereas 15th, 30th and 45th day groups showed 5 bands each while liver control showed 4whereas 15th, 30th and 45th days groups showed 5, 5 and 4 bands respectively;In *L.rohita,* muscle control showed 6 bands, whereas 9, 9 and 4 bands were seen in 15th, 30th and 45th day groups; gill control showed 5 bands whereas 10, 8 and 8 bands were seen in 15th, 30th and 45th day; while liver control showed 3 bands whereas 4, 6 and 5 bands were seen in 15th, 30th and 45th day. In O.mossambicus; muscle showed depletion of 28.84 and addition of new bands (3.46, 6.6, 14.45 and 29.51); gill showed depletion of (3.54, 40.0, 59.6 and 97.4) and addition of (3.46, 42.3 and 55.3) bands while liver showed while liver showed depletion of (29.1) and addition of (20.1, 22.3, 29.1) bands. In L.rohitamuscle showed depletion of (6.60, 14.45, 20.1 and 24.51) and addition of new bands (3.46, 6.30, 6.45, 13.80, 14.12, 19.95, 28.18); gill showed depletion of (3.54, 14.6, 20.1 and 22.3) and addition of (3.46, 4.2, 6.7, 9.1, 12.2, 14.3, 17.2 and 43.0) bands while liver showed depletion of 29.1 and addition of (3.46, 3.54, 6.5, 21.9, 22.3 and 63.2) bands.

Table I: Time dependent changes in the protein subunits (KDa) of fishmuscle exposed to sublethal concentration of Plant nutrient Librel.

Sr. No.	PM	*Oreochromismossambicus*				*Labeorohita*			
		L I	**L II**	**L III**	**L IV**	**L I**	**L II**	**L III**	**L IV**
1				3.46	3.46				3.46
2	3.5	3.5	3.5						
3						3.54	3.54	3.54	
4							6.30	6.30	
5		6.45	6.45	6.45					6.45
6	6.5								
7					6.6	6.60			
8							13.80	13.80	
9		14.12	14.12						14.12
10	14.3								
11				14.45	14.45	14.45			
12		19.95		19.95			19.95	19.95	
13	20.1					20.1			
14							28.18	28.18	
15		28.84							
16	29								
17				29.51		29.51			
18	43								
19						43.65	43.65	43.65	
20	66								
21	97.4								
22							100	100	
23			199.52	199.52			199.52	199.52	199.52
24	205								

PM-Protein Marker, L I – Control, L II – 15 days, L III-30 days, L IV-45 days

Table II: Time dependent changes in the protein subunits (KDa) of fishgill exposed to sublethal concentration of Plant nutrient Librel.

Sr. No.	PM	*O.mossambicus*				*L.rohita*			
		L I	L II	L III	L IV	L I	L II	L III	L IV
1			3.46	3.46	3.46		3.46	3.46	3.46
2	3.5								
3		3.54				3.54			
4							4.2		4.2
5	6.5								
6							6.7	6.7	6.7
7		8.2	8.2	8.2	8.2	8.2	8.2	8.2	
8							9.1		
9							12.2	12.2	12.2
10	14.3						14.3	14.3	14.3
11						14.6			
12							17.2	17.2	17.2
13	20.1	20.1	20.1	20.1	20.1	20.1			
14						22.3			
15							17.2	17.2	17.2
16	29								
17		40.0							
18			42.3	42.3	42.3				
19	43						43.0	43.0	43.0
20			55.3	55.3	55.3				
21		59.6							
22	66								
23	97.4	97.4							
24	205								

PM-Protein Marker, L I – Control, L II – 15 days, L III-30 days, L IV-45 days

Table III: Time dependent changes in the protein subunits (KDa) of fishliver exposed to sublethal concentration of Plant nutrient Librel.

Sr. No.	PM	*Oreochromismossambicus*				*Labeorohita*			
		L I	L II	L III	L IV	L I	L II	L III	L IV
1								3.46	3.46
2	3.5								
		3.54	3.54		3.54		3.54	3.54	
3	6.5						6.5	6.5	6.5
4		14.2	14.2	14.2	14.2	14.2	14.2	14.2	14.2
5	14.3								
6	20.1			20.1		20.1			20.1
7		21.9					21.9		
8			22.3	22.3				22.3	22.3
9	29								
10			29.1	29.1		29.1			
11	43	43.0			43.0				
12								63.2	
13	66								
14	97.4								
15	205								

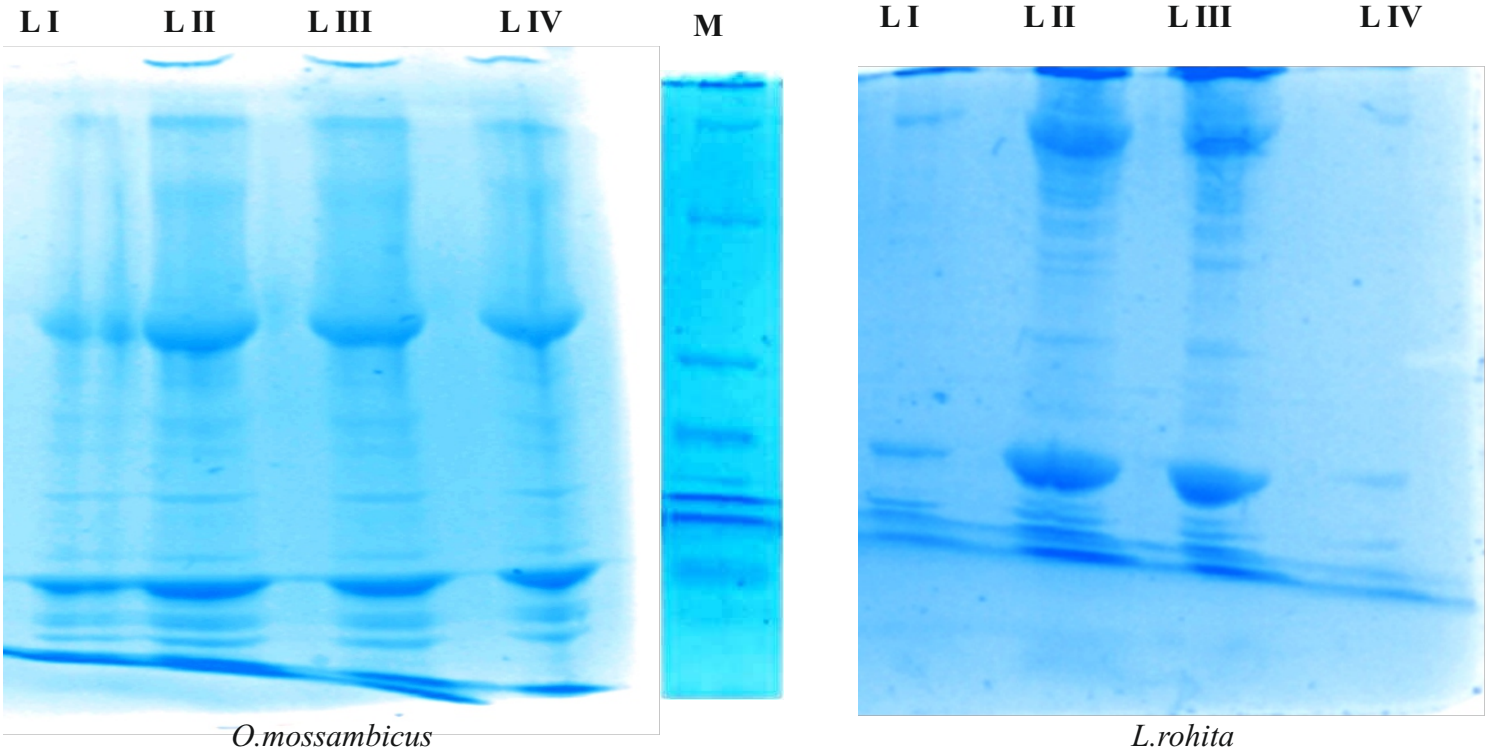
PM-Protein Marker, L I – Control, L II – 15 days, L III-30 days, L IV-45 days

Table IV: Changes in Protein values(mg protein/100 mg wet weight of the tissue) of fish

Sr. No.	*O.mossambicus*				*L.rohita*			
	Control	15 days	30 days	45 days	Control	15 days	30 days	45 days
Muscle	2.33 ± 0.1	5.8* ± 0.3	15.9 ± 0.5*	17.3± 0.23*	3.4± 0.78	7.3± 0.84*	20.4± 0.25*	22.2± 0.2**
Gill	5.25± 0.2	8.55± 0.08	9.11± 0.08*	16.23± 0.08	8.82 ± 0.8*	12.3± 0.25	19.22± 0.2*	32.86± 0.12
Liver	6.22± 0.7	15.2± 0.3*	19.22± 0.5	25.66± 0.08	12.1 ± 0.9*	15.2± 0.08	20.3 ± 0.2*	37.1 ± 0.3*

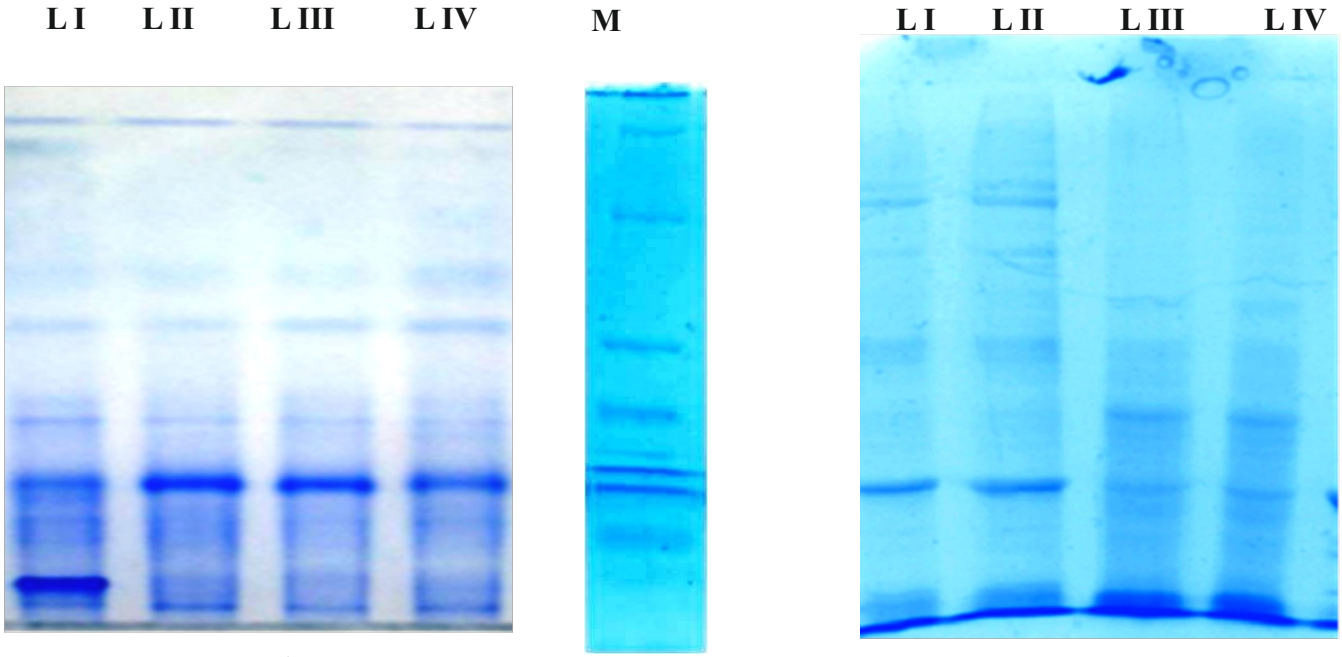
***** *The mean difference is significant at the 0.05 level;* ****** *The mean difference is significant at the 0.01 lev*

Fig. I: Electrophoretogram showing in protein subunits of Fish muscle exposed to sublethal concentration of Plant Nutrient Librel.



O.mossambicus *L.rohita*

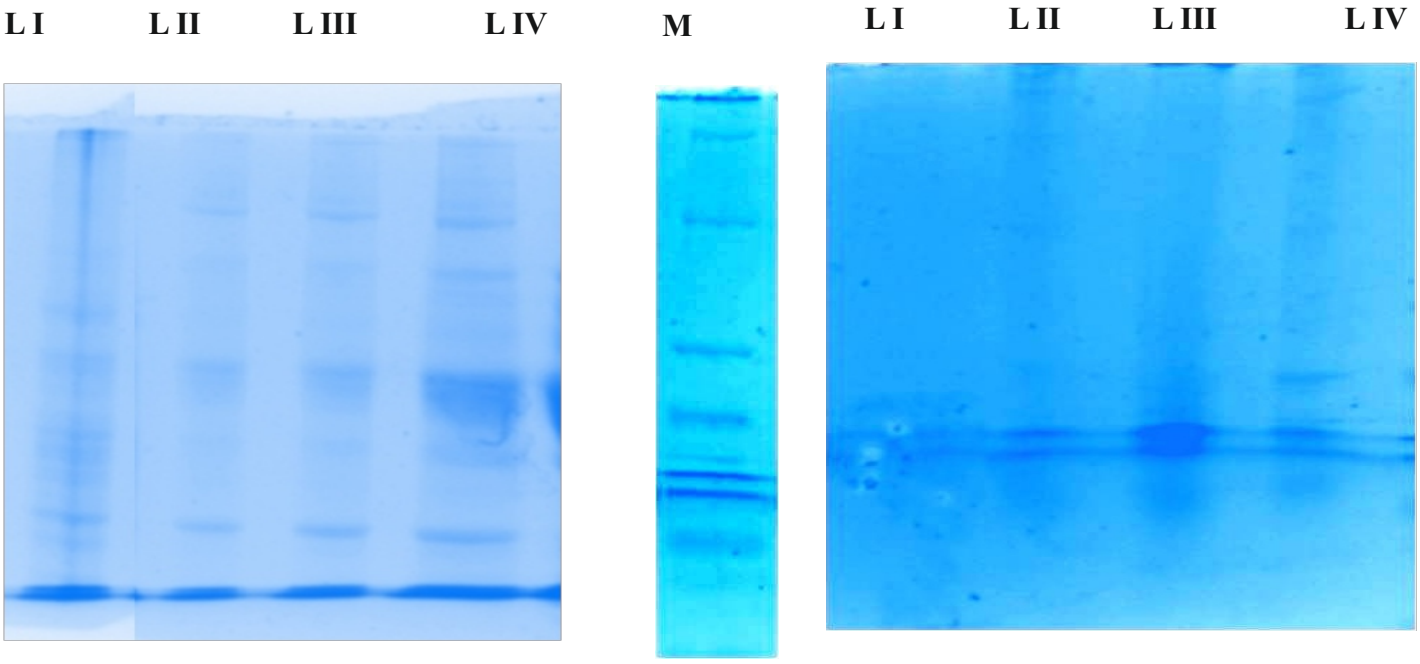
Fig. I: Electrophoretogram showing in protein subunits of Fish Gill exposed to sublethal concentration of Plant Nutrient Librel.



O.mossambicus *L.rohita*

L I - Control; L II - 15 days group; L III - 30 days group L IV - 45 days group

Fig. I: Electrophoretogram showing in protein subunits of Fish muscle exposed to sublethal concentration of Plant Nutrient Librel.



O.mossambicus *L.rohita*

L I - Control; L II - 15 days group; L III - 30 days group L IV - 45 days group

DISCUSSION

Fish are one of the major sources of protein for human beings and the nutritional value of fish depends on their biochemical composition like protein, amino acids, vitamins, mineral contents, etc (Deka*et al.,* 2012). The present study was done with the view to study protein composition by means of electrophoretic patterns of proteins fractions in tissuesof *O.mossambicus* and *L.rohita*. The clinical value of the protein analysis by electrophoresis depends upon whether a given change represents an adaptation to stress conditions or a failure in the supportive physiological and biochemical mechanisms of the animals (Muthukumaravel, 2007). In the present study there was a time dependent significant increase in the total protein content of both the fishes. This appraisal of the quantitative estimation of proteincontent in tissues ofLibrel exposed fishes indicates that apparently both species induced time dependent mild to drastic alterations as compared with the controlsto face the stress cause by the exposure. The present trend is justifiablein the wake of mechanical tissues (muscle and gills) intended for mobility while the liver being highly metabolic organ. Thaker and Mohammed, (2008) found similar results in the gills of clam *Pseudontopsiseuphraticus* exposed to 0.4 mgHg/l for 21 days; Thaker and Farhan (2009) found the same in soft tissue due to zinc exposure.

Further there was a species specific difference in the protein fractions when SDS-PAGE was performed. The electrophoretic profileof both the species revealed an increase in the intensity of bands upto 30 days while a decrease at 45th day. This initial increase in the intensity and then a decrease could be attributed to the activation of certain proteins and then a sudden deactivation. Moreover according to Thaker and Farhan (2009) the increase in the band intensity suggests that such responses possibly represent a common reaction to heavy metals.This increase in new bands of protein was gradual and synchronous with exposure. In *O.mossambicus*, muscle of control group showed 5 bands whereas 15th, 30th and 45th day groups showed 5, 6 and 4 bands respectively. At 15th day 14.3, 19.95 and 28.84 KDa proteins were depleted whereas a new polypeptides 20.41 and 199.52 were found. On the other hand at 30th and 45th day new polypeptides 3.46, 14.45, 29.51, 199.52and 3.46, 6.6 and 14.45 were observed, whereas there was a depletion of 3.5, 14.12, 19.95 and 28.84 protein subunits. In *L.rohita*, muscle*s* of control group 6 bands were, whereas 9, 9 and 4 bands were seen in 15th, 30th and 45th day groups. All the bands that were observed except subunit 3.54 and43.65 were new in the experimental groups.

Whereas the new bands 6.30, 13.80, 19.95, 28.18, 43.65, 64.56, 100 and 199.52 were observed at the 15th and 30th day exposure while 3.46, 6.45, 14.12 and 199.52 at 45th day. Likewise there were overall increase in the number of bands in the gills, however, the intensity of bands was more in muscles compared to gills. It is suggested that the occurrence of new protein in the muscle has been transported through blood stream or they could have been synthesized in the muscle itself. It is inferred that these protein fractions could be stress proteins to overcome the toxic effect of heavy metal (Salahudeen*et al.,* 2014). Such an increase in the protein bands in muscle and gill tissues of *O. mossambicus* exposed to 10 % sub lethal concentration of cadmium for a period of 10 dayshas been reported by Muthukumaravel (2007).Hence in the present study the appearance of new protein fractions in muscle could be stress proteins to overcome the toxic effect of Librel.

Along with the addition of new band there was a depletion of the protein fractions (3.5, 14.12, 19.95, 20.41 and 28.84kDA) and (6.60, 14.45, 20.1 and 29.51) in *O.mossambicus* and *L.rohita*that were found in the control group was observed. This reduction of proteins could be due to the toxic impact on the protein synthetic pathway (Venkataramana*et al.,* 2006) or due to the depletionof reserve proteins to overcome to stress by the plant nutrient, or due to the consumption of energy through erratic movement caused due to toxicant stress (Veeraiah, 2013) since muscles are the vulnerable organ to environmental stress. Further this may led to a rise in free amino acid levels due to the breakdown ofprotein for energy and impaired incorporation of amino acids inprotein synthesis. Thus, energy generated in this way may be possibly utilized for the metabolic purposes. Same observations were made by Salahudeen and coworkers (2014) in *O.mossambicus* in their studies of chronic urea toxicity. These changes in the protein banding pattern in response to exposure to pesticides may be attributed to the changes in the turnover (synthesis/degradation of various proteins).

The expression of low molecular proteins in the experimental groups was a curious observation in all the tissues of both the species which was highest in liver. The agrochemicals may inhibit the expression of some genes (or) activate the others to produce specific mRNAs which may subsequently be translated into specific proteinscalled stress induced proteins (Yamashita *et al.,* 2010; Singh, 2010).Among them are members with low molecular weight, such as metallotionein and ubiquitin, as well as ones with masses of 27, 32, 60, 70, 90, and 110 kDa(Del

Razo*et al.,* 2001). In this particular study the low molecular weight proteins that were activated could be due to the stress and according to Bouskill (2006) metals induces the expression of low molecular weight cysteine-rich, metallothioneins. Moreover, *in vitro* or *in vivo* in a variety of model systems has been shown to cause the induction of a number of the major stress protein families such as heat shock proteins (Hsp). Moreover the toxicants may affect the hormonal balancewhich could directly or indirectly affect the tissue protein levels (Prasad *et al.,* 2002; Ogueji and Auta 2007; Ahmad *et al.,* 2012; Vidhyaand Nair, 2013). Thus, protein depletion in tissues constitutes aphysiological mechanism with an important role in providingenergy to cope with the stress situation.

Proteins play a vital role in the physiology ofliving organisms. All biological activities are regulated by enzymeand hormones, which are also proteins. If any alteration takesplace in the proteins turn over, it may have an adverse impact ontheir vital and complex groups of biological materials, comprising the nitrogenous constituents of the body and the foodintake; thus they perform different biological functions (Prashanth, 2007).The primary structure of a protein molecule with its amino acid sequence, is genetically determined and it is very likely that the specific folding and cross-linking of polypeptide chain results largely, if not entirely, from the primary structure (Kluger and Alagic, 2004). The reasoning is that 'the primary structure dictates the secondary, tertiary and quaternary structures (conformation) in any given environment. The existence of multiple forms of proteins has interested many biochemists and biologists (Powell *et al.,* 2000; Hahn, 2002; Hahn *et al.,* 2006). In addition to the multiple forms resulting from the differences in the primary structure of the fundamental protein unit, there are also multiple forms arising due to other reasons. For example, one type of multiple molecular form results from the molecules of proteins having the same primary structure which exists in several physico-chemical forms when the structure gets influenced by the environment. These are termed as 'conformational forms` (Taipel and Koshland, 1971; Maiorov and Abagyan, 1997). The development of electrophoretic techniques makes it possible to detect the protein composition. The potential value of electrophoresis in this study is based on the hypothesis that stress conditions may cause significant qualitative and quantitative changes in the proteins of different tissue exposed to the toxicant.

Further significant alteration in the respiratory stress enzymes observed (Chapter IV) authenticates that the change in protein bands is suggestive of the probable genotoxic potential of the plant nutrient. Most of the toxic chemicals that produce genotoxic effects have been known to form reactive oxygen species as well as electrophilic free-radical metabolites that interact with DNA to cause disruptive changes (Chandra and Khuda-Bukhsh, 2004).The present observations on protein represents an initial step in the process of determining the expression of the DNA, in the form of protein products, affected by the exposure of Librel, because expression of certain classes of proteins (e.g., metallothionein, stress proteins) are known to be affected in very specific ways upon exposure that produce genotoxic stress in an organism (Chou *et al.*, 2001).

The present study may provide an insight in rate of turnover of various proteins alterations at cellular and subcellular levels and changes in the biological properties of fish in reference to plant nutrient. Our study has lend the findings of induction of species specific new protein bands upon exposure of trace element mixture, Hence one possible mechanism is that, when fish is trying to adjust in *invitro* environment small fluctuations of protein band is seen, while on the hand it is also trying to combat the stress which is being produced/given. So, these metal ions which are resent in high amount is accumulated in respective tissuewhich lead to activation/expression of certain stress genes which are either not produced or are at low level in normal condition. So in reference to this study, we hypothesized the expression of metellothionine(ranges from 4-14kDa) and stress activated serum factor (SASF ranges from 50-100kDa) as proposed by Jaso- firedmann*et al.*,(2000) which may have been induced by the organism's physiology to get rid of stress. Although, further insights into the identification of bands are needed, which can be confirmed by Western blotting that involves higher specificity of antigen-antibody reaction. It can thus be concluded that electrophoretic analysis provides a very useful method for certain aspects of biology that it can be used as an additional tool to evaluate environmental stress on animals with success. Although the appearance and disappearance of protein bands may or may not be directly related to cytogenetic changes that occur after exposure to metals (Chandra and Khuda-Bukhsh, 2004) particularly in view of the current studies in proteomics is indicating that protein banding patterns represent very complex biochemical interactions at both the molecular and cellular levels—the data on change in protein features noted during our study have been included for their possible future use and academic interest in the field of toxicology.

GENERAL CONSIDERATION

The world population is expanding rapidly and will likely be 8 billion by the year 2025. Limited availability of additional arable land and water resources and the declining trend in crop yields globally makes food security a major challenge in the 21st century (Hinrichsen, 1998). To achieve the required huge increases in food production, greater emphasis in application of fertilizers and improvements of soil fertility are indispensable. Today more than 100 elements are known to man, out of whom less than 20 elements are essential for vigorous and healthy growth of plants. (White and Brown, 2010).

The unscrupulous use of agrochemicals, which broadly include; herbicide, insecticide, and fungicide are, currently approved for release by the U.S. Environmental Protection Agency (EPA), with the advent of "Green Revolution" (Boon and Bridge 2003). The environmental impact of pesticide use has been discussed much due to its widespread use in parallel with the modernization of agricultural operations and indiscriminate permeation of the ecosystem with these pesticides. It is apparent that human chemical additions have introduced or increased environmental stress for aquatic organisms and fishes, in particular. Pesticides are toxicants capable of affecting all taxonomic groups of biota, including non-target organisms, to varying degrees depending on physiological and ecological factors. Many pesticides are resistant to environmental degradation so that they persist in treated areas and thus their effectiveness is enhanced.

Dispersal of pollutants in the atmosphere results in treatment of natural terrestrial areas while water run-off transfers pesticide quantities to fresh water areas, and ultimately the oceans and thus their effect comes in aquatic organisms. In India, the use of pesticides in agriculture has significantly increased during the past 3 decades. The agriculture run-off in the aquatic environment leads to massive killing of fish and hence warrants close attention. There is vast amount of scientific information available on different pesticides' toxicity on different fishes in India (Khare et al., 2000; Tilak et al., 2005; Atamanalp et al., 2002 and 2003; Murugan, 2006; Kunjamma, 2008; Desai et al., 2010 and 2011, Parikh et al., 2009 and 2012).

There are 7 essential plant nutrient elements defined as micronutrients [boron (B), zinc (Zn), manganese (Mn), iron (Fe), copper (Cu), molybdenum (Mo), chlorine (Cl)] which are found in plant in relatively small amounts (Hochmuth *et al.,* 2010). A deficiency of any one of these obstructs its normal yields resulting in complete crop failure. Soils deficient in their ability to

supply micronutrients to crops are alarmingly widespread across the globe (White and Zasoski, 1999; Kabata-Pendias, 2011). Fertilizers containing trace elements (such as boron, copper, manganese, zinc, and cobalt) — in small quantities are called as micronutrient fertilizers. It is called *micronutrients* as they are needed only in minuscule amounts, these substances are the "magic wands" that enable the plants to produce enzymes, hormones and other substances essential for proper growth and development (Yoshida, 2008). They are available in two forms, chelated and nonchelated (Modaihsh, 1997). Most of the micronutrients are in chelated form, as they are absorbed quickly and easily by the crop thus providing effective organic nitrogen to overcome stress conditions and boosting up energy metabolism in the plant.

According to the projections, food production on presently used land must be doubled in the next two decades to meet the food demand of the growing world population. To achieve the required huge increase in food production, greater emphasis in application of fertilizers and improvements of soil fertility are indispensable. Presently, in many developing countries, poor soil fertility, low levels of available mineral nutrients in soil, improper nutrient management, along with a lack of concern for plant genotypes having high tolerance of nutrient deficiencies or toxicities are considered to be the major constraints contributing to food insecurity, malnutrition and ecosystem degradation (Malakouti and Balali, 2004).

India was dependent on external food supplies in the early 1960s. Micronutrient Fertility Mapping for Indian Soils have been well explored by Singh (2008) and have reported that the concentration of different micronutrients, variation in the fertility status of soils in different states of India varies from moderately low to very low. To meet the growing demand for food, fiber and fuel, high yielding cultivars were introduced. These high yielding crop cultivars were highly responsive to fertilizers. Thus, slowly the soils were exhausted of their nutrients. Application of major nutrients (nitrogen, phosphorus and potassium) became common, therefore the crops started responding to micronutrient fertilizers. Concerted efforts have been made through the All India Coordinated Research Project on Micronutrients to delineate the soils of India regarding the deficiency of micronutrients. Based on the extent of deficiency, cultivated area, and crop removal, the micronutrient fertilizer demand in 2025 is projected using sufficiency and maintenance approaches (Gupta, 2005).

Trace elements which are present at low concentrations (mg kg-1 or less) in agroecosystems, include copper (Cu), zinc (Zn), manganese (Mn), iron (Fe), molybdenum (Mo), and boron

(B) are essential to plant growth. Except boron, these elements are also heavy metals, and are toxic to plants at high concentrations.

Indian agriculture is now in an era of multiple plant nutrient deficiencies. To meet this deficiency application of trace elements in the form of fertilizers or micronutrients have been used rampantly whereas remediation of soils contaminated with metals is not addressed (Zhenli *et al.,* 2005). Soil microorganisms are the first living organisms subjected to the impacts of metal contamination. Furthermore repeated use of such metal-enriched chemicals, fertilizers, and organic moieties cause contamination of aquatic ecosystem by surface runoff leading toxic effect to non target organisms especially freshwater fishes. A frequently overlooked agrochemical is the plant nutrients added for biofortification of the soil. These nutrient supplementations, though enhancing food production, can have disastrous effects on the aquatic ecosystem as they readily leach out in the surface run off. A classical example of this condition is the death of alligators in Lake Griffin, Florida in 2000. After this incident it was realized that not only pesticides, but plant nutrients can have detrimental effects on the environment. Unlike pesticides, which directly kill the organism/s, these plant nutrients may boost the growth of one organism and cause imbalance in the ecosystem leading to extinction of one or more species.

The resultant widespread and indiscriminate use of plant nutrients may have far reaching effects on organisms in the higher trophic levels of the food chain. Many studies suggest that the trace elements either alone or in various combinations do show the toxicity. Plant nutrients are known to be available in various trade names. One of them is the Librel which because of its stability, solubility and its compatibility with wide range of herbicides, fungicides, insecticides and other crop care products has been used extensively. **Librel™,** rapidly soluble chelated micronutrient mixture, manufactured by DuPont is used in most of the agricultural, horticultural fruit crops to correct nutrient deficiency. It's stability and solubility in water leads to rapid crop absorption and optimum biological performance. Though its solubility is useful to the agricultural crops, it is easily carried to the natural water bodies with agricultural run offs ultimately impairing the non-targeted aquatic biota, including fishes. There are no toxicity reports available in fish or other aquatic organisms for Librel, a plant nutrient.

Moreover, the studies conducted till date have been focused on the metal toxicity, but their role in minute quantities through the fertilizers by way of plant nutrients are not well

documented. As fish production had always been associated with agriculture, it is mandatory to explore and understand the effects of plant nutrient supplementation on fish health so that one can take remedial actions to improve fish health, both in terms of ecology as well as economics. **Hence, the aim of the present work is to look into the toxicity of the plant nutrient Librel ™ on edible fishes as they are major part of the human diet and it is therefore not surprising that numerous studies have been carried out on metal pollution in different species of edible fish.**

The toxicity study is essential to find out toxicants limit and safe concentration, so that there will be minimum harm to aquatic fauna in the near future. Among the several aspects of toxicity studies, the bioassay constitutes one of the most commonly used methods in aquatic environmental studies with suitable organisms. The necessity of determining the toxicity of substances to commercially aquatic forms at the lower level of the food chain has been useful and accepted for water quality management. Fish is usually affected by toxicants in aquatic environment. The moment effects on the exposed fish is well pronounced, abnormal behavior such as incessant gasping for air, backward swimming and secretion of mucus on the skin of fish would set in (Omitoyin *et al*., 2006). Furthermore, fish appear to possess the same biochemical pathways to deal with the toxic effects of endogenous and exogenous agents as do mammalian species (Lackner, 1998). It is important to examine the toxic potential of pesticides on fish since they constitute an important link in food chain and their contamination by pesticides imbalances the aquatic system.

The acute toxicity of agrochemicals on fish has involved the determination of the LC_{50}, which are the concentrations that kill 50% of group fish under specified conditions. Acute toxicity tests are short-term tests designed to measure the effects of toxic agents on aquatic species during a short period of their life span (Ebramhimpour *et al.,* 2010). The observed LC values and 95% confidence limits in static tests are shown in the 48 hours median lethal concentration (LC_{50} values) reported were 5083.77 and 5997.82 $mg.l^{-1}$ with the line of best fit 26.9x – 99.7 and 42.63x – 1.61E2 respectively for *O.mossambicus* and *L.rohita*. The results indicated that *O.mossambicus* was more sensitive to plant nutrient compared to *L.rohita.*

Mortality is obviously not the only end point to consider and there is growing interest in the development of behavioural markers to assess the lethal effects of toxicants. Behaviour is usually a very complicated phenomenon through which the animal capable of adjusting it

various functions to a constant or changing environment. In aquatic toxicology however, the nexus of behavioral sciences with the study of toxicants has only become prominent within the last 5 decades (Kane *et al.*, 2005). Compared to control reduced activity was exhibited during early hours of exposure at all the concentrations in the both the species. Most of the fish which died during the experiment exhibited symptoms of poisoning such as change in colour as well as behaviour. Initially decreased swimming activity, restlessness, jerky movements, hyper secretion of mucus, opening of mouth for gasping, losing scales, decreased abnormal hyperkinetic activity, loss of equilibrium by swimming sideways, finally fishes collapsed and died at higher concentrations. This behavioural abnormality was more prominent in *O.mossambiccus* compared to *L.rohita.*

The purpose of the acute toxicity test with the fish species under laboratory conditions was to help in the assessment of possible risks to similar species in natural environments. It is also aids the determination of possible water quality criteria for regulatory purposes and for use in correlation with acute testing of other species for comparative purposes. From the acute study investigation we conclude that plant nutrient Librel was toxic to both the fishes and based on the LC50 values *O.mossambicus* was more sensitive compared to *L.rohita.* It was established through this study that short-term exposure to plant nutrient resulted in negative alterations in behavior and mortality.

The impact of the toxicant can be well understood by analyzing either blood or serum of the fish, because blood is a pathophysiological reflector of whole body (Sharma and Singh, 2004; 2006). Haematological system is the most important vital system which reflects the total health status in any organism as it provides movement for useful and unuseful components. It is the only transport medium for gases during respiration along with the transport of nutritive materials and other important biomolecules to various parts of body. Any alteration in animal's body like in liver, kidney, brain, digestive system or any infection is reflected in the haematological system.

In the present study, reduction in hemoglobin was accompanied by lowest PCV value in *O.mossambicus*. Moreover an increase in WBC count; reflect the occurrence of leucocytosis in the treated fish samples. This was perhaps, a typical defensive response of the fish against a toxic invasion or probability may be of leukemia (Sudha, 2012). This decrease in the erythrocyte count or in the percent of PCV indicates the worsening of an organism state and developing anemia as they are positively correlated. The anemia could be due to the

destruction of RBC triggered by the influx of micronutrient into the erythrocytes and may also be of hemolytic type of RBC. Similar observations were also reported by Tilak *et al.,* (2007), Saravanan *et al.,* (2010) and Saeed *et al.,* (2012). In contrast *L.rohita* has shown increased levels of RBCs, Hb and PCV values which could be due to an increased loss of scales and haemorrhage. Moreover here the duration of exposure also plays an important role. With the increase in the exposure period *L.rohita* showed, erratic swimming, air gulping, loss of reflex, loss of scale, haemorrhage and molting. They finally settled at the bottom motionless with slow opercular movement. Such results were also reported by Ayotunde and his coworkers (2004) in *O.niloticus* when exposed to drumstick. MCV, MCH and MCHC increased considerably with time compared to control. However the increase in these indices can be attributed to direct or feedback responses of structural damage to RBC membranes resulting in hemolysis and impairment in hemoglobin synthesis, stress related release of RBCs from hemopoietic organ and hypoxia, induced by micronutrient exposure.

Glucose is one of the most important sources of energy for the animals and has been studied as an indicator of stress caused by physical factors in particular pollutants (Manush *et al.,* 2005). An increase in the levels of glucose in fishes in response to micronutrient mixture exposure indicates quantum of stress imposed on fish during subchronic toxicity and its physiological attempts to overcome it. Glucose is synthesized from hepatic tissue proteins and amino acids due to the intensive glycogenolysis induced due to stress (Almeida *et al*., 2001). The stressors first activate the chromaffin cell present in the wall of the cardinal veins and in some cases the heart and the kidneys of the teleosts (Mazeaund and Mazeaund, 1981), which in turn releases the adrenalin and small amount of nor-adrenalin that stimulates the conversion of liver glycogen into blood glucose and the utilization of the glucose by muscle. Umingel, (1977) reported that blood sugar has a direct co-relation to metabolism. On the other hand the increase in blood sugar noticed could also be attributed to the differences in the respiration and the activity (Ghosh, 1987). The progressive accumulation of the blood glucose reported in this investigation revealed that both the fishes become hyperglyceamic. According to Coles (1980) increased blood glucose concentration results from an imbalance between the hepatic output of glucose and the peripheral uptake of sugar. Though there are no reports on diabetes in fishes, however stress imposed upon the fish during the toxicity trial might be the possible reason for hyperglycemia. In the present study, exposure of librel at different time period caused an increase in the blood glucose level leading to lethargy.

The proteins are most diverse bio-molecules which are of prime importance in biochemical reactions and cellular structures. Serum proteins have immunological properties in fishes and other animals as well as in human (Kumar and Dahiya, 2013). Serum proteins were found to decrease due to Librel exposure in the present study. This could be attributed to renal excretion or impaired protein synthesis or due to liver disorder (Kori-Siakpere, 1995; 2008). Proteins are mainly involved in the architecture of the cell. During chronic period of stress they are also a source of energy. During stress conditions fish need more energy to detoxify the toxicant and to overcome stress. The decrease in serum protein level in fish exposed for longer duration in the present study may be due to the low assimilation of food (Sharma and Singh, 2009). Albumin, globulin and A/G ratio have found to be decrease with the increase duration of micronutrient exposure. Measurement of albumin, globulin, and total protein in serum or plasma is of considerable diagnostic value in fish, as it relates to general nutritional status (Schaperclaus *et al.,* 1992). Serum protein, albumin, and globulin were significantly lower in Tilapia and Rohu. These results may be due to the disturbances in the liver protein metabolism due to micronutrient toxicity, as was found to be the case with other contaminants (Dange and Masurekar 1984; Abdel-Tawwab *et al.,* 2007a; b).

Urea is synthesized from NH_4^+ and HCO_3 in the liver via the ornithine-urea cycle (OUC). Urea may also formed by the degration of uric acid or arginine. Elasmobranchs utilize the ornithine-urea cycle whereas teleost synthesize urea by uricolysis or arginolysis. Few teleostean species synthesize significant amounts of urea in response to environmental conditions that limit ammonia excretion (*O.a. grahami,* Randall *et al.,* 1989*; Opasanus beta,* Walch *et al.,* 1990; *Heteropneustes fossilis,* Saha and Ratha, 1989). Teleost fishes are primarily aminotelic but their blood contains significant amount of urea and indeed in some teleost it may account for 20% or more of total nitrogen excreted (Joshi, 2002). Creatinine is another nitrogenous waste product that is eliminated by kidneys when excretion is suppressed in renal insufficiency. The high levels of blood urea and creatinine result either from increase breakdown of tissue or dietary or impaired excretion or increased synthesis or decreased urinary clearance by the kidney or decrease degradation of these compounds (Adham *et al.,* 2002). The present studies suggest that micronutrient exposed fish adapt glomerular dysfunction rather than tubular insufficiency as blood levels of urea and creatinine depends largely on glomerular function. In consistent with this explanation of decreased total protein level with micronutrient exposure urea is the end product of protein catabolism in mammals but in fish ammonia is the end product of protein, so the marked increase in blood urea

nitrogen could be attributed to impaired excretion of urea through kidney which is supported by increase in blood creatinine level, a more sensitive and specific indicator of impaired kidney function (Amin and Hashem, 2012). Haematological studies revealed that under Libral exposure blood parameters showed alterations and that both the fishes are sensitive to the micronutrient. Hence, the presence of micronutrient in waterways surrounding the agriculture fields could have adverse impact on the survival of the fish.

Measurements of condition factor, which relates weight and length, and organo-somatic indices, which indicate the proportional sizes of target organs, are standard procedures in fish physiology studies and are used as indicators of the well-being of individual organisms (Di Giullo *et al.,* 2008). Gonadosomatic Index (GSI) is the percentage ratio of the gonad weight and body weight used to determine fecundity among fish (Janz et al, 1997). It is generally used as an indicator of fish sexual condition. In the present studies Plant nutrient exposure resulted into a time-dependent decrease in the GSI, suggesting that the effects were expressed at long time exposure. Furthermore, in both the species it was females which showed significant changes compared to males, implying that the females are more sensitive. To authenticate the GSI results the histological observations were also performed. Fish exposed to the plant nutrient showed progressive thinning and degeneration of ovarian wall which was apparent at the 30th and 45th day. Oocytic stages were not intact. Degeneration of germinal epithelial cells of oocytes caused vacuolation. At the 30^{th} and 45^{th} day more vacuolated follicular epithelium and degenerative cytoplasm was observed. The cytoplasm showed vacuolization at the periphery of oocyte which gradually extended towards the centre. Moreover the study has shown insignificant changes in the testicular GSI of fishes. Though the changes were insignificant but the histological makeup of testes was time dependent. Progressively there was an increase in the vacuolization, disorganization and distortion of seminiferous tubules. At the 45th day of exposure condensation of spermatocytes besides inflammation and inter-tubular vacuolation was very much prominent. As reported by some investigators (Sokal *et al*. 1985, Ruby *et al*.1986, 1987, Gaber *et al*. 2013) testicular inflammation has been documented as one of the common responses on the aquatic animals exposed to environmental toxicants. Testis in fish represents the most dynamic organ having a high cell turnover during the reproductive period which makes it vulnerable to a wide variety of chemical toxicants. These changes in the histological make clearly states that reduced GSI in the present study may be due to lowered gonadal activity under plant nutrient stress and impairment of the production of steroid hormones which might have arrested the formation of germ cells and cause degeneration or necrosis (Sharma et al. 2012).

Liver is the metabolic organ. It is a target for the metabolism in the fish body, the liver index (HSI) is a useful biomarker to detect the hazardous effects of the environmental stressors (Pait, and Nelson, 2003). In the present study a significant increase in the HSI was documented on exposure of the plant nutrient. Pesticides are metabolized in the liver through cytochrome P450 system through hepatotoxic intermediates (Das and Gupta, 2013). The teleost liver is one of the most sensitive organs with regard to showing alterations in histoarchitecture, biochemistry, and physiology following exposure to various types of environmental pollutants (Roy and Bhattacharya, 2006). Brusle and Anadon (1996) stated that, fish liver histology could serve as a model for studying the interactions between environmental factors and hepatic structures and functions. The present study documents pathologic changes in fish treated with Plant nutrient for three different time intervals. The degree of pathology gradually increased during the entire days of experiment which exhibited time-dependent changes. The effect on the cell outline resulted in the mild vacuolation in the cytoplasm which were much more prominent at the 15th day. Hemorrhage of blood vessels in hepatic sinusoids was seen which eventually resulted to its blockage and affecting the metabolic activity. Thus our results are in agreement with the results of those mentioned by Liao et al. (2006) when exposed medaka (Oryzias latipes) to sublethal exposure of methylmercury chloride, Roy and Bhattacharya (2006) when exposed *Channa punctatus* to arsenic, and van Dyk et al. (2007) when exposed *Oreochromis mossambicus* to cadmium and zinc.

Condition factor is one of most important parameters, which throws light on the physiological state of fish in relation to indication of the onset of sexual maturity (Salam and Davies, 1994). It has been used as an indicator of health in fishing biology studies since the beginning of the 20 century, such as growth and feeding intensity (Honeyfield *et al.*2008). Condition factor decrease with increase in length (Badawy,1998, Barakat, 2004). The condition factor provides information on the variation of fish physiological status and may be used for comparing populations living in certain feeding, climate and other conditions (El-Kabbany *et al*.2000, Gupta *et al.*2002, El Nameki, and Badawy, 2005). Therefore, condition factor can be used to determine the feeding activity of a species to determine whether it is making good use of its feeding source (Gupta *et al.*2002, Radwan, and Atalla, 2005, El Nameki, and Badawy, 2005).

Condition factors, when low or having declined, may be interpreted as a depletion of energy reserves, such as stored liver glycogen and body fat. Putting down all the changes together

obtained in the HSI, GSI as well as K it can be concluded that the fishes are more susceptible to the plant nutrient which has resulted into the metabolic and reproductive damage/fecundity which is apparent in sequential changes observed in histomorphological structures of liver as well gonads of the test organism.

Agro-chemicals exposure can lead to oxidative stress (OS) through unregulated generation of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, hydroxyl radical, peroxyl radicals and singlet oxygen. ROS are produced during normal process in the cell. Under normal conditions antioxidant systems of the cell minimize damage caused by ROS. When ROS generation increases to an extent that it overcomes the cellular antioxidant systems, the result is oxidative stress. "Oxidative stress" is a situation when steady-state ROS concentration is transiently or chronically enhanced, disturbing cellular metabolism and its regulation and damaging cellular constituents (Lushchak, 2011b; Nishida, 2011). On the other hand "Reductive stress" may be defined in with the only difference that steady-state ROS concentration is decreased (Lushchak, 2011). The damage caused by the ROS can be measured through cellular markers which includes various adaptive cellular responses such as increased concentrations of non-enzymatic and enzymatic antioxidants as well as by the measurements of cellular damage, such as perturbed redox balances, lipid peroxidation and DNA oxidation (Di Giulio, 1991). The antioxidant defense system includes enzymes such as superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) (Droge, 2002) and the cellular damage caused by malondialdehyde (MDA) content, which is known as an end-product of lipid peroxidation.

The source of these parameters are the indicators responding to the environmental effects and can also serve as markers for toxicant exposure in fish. The plasma transaminase ALT, AST, as well as acid and alkaline phosphatases can be used to establish the tissue damage of the liver and kidney (Nemcsok *et al.*, 1981; Nemcsok and Boross, 1982). Cellular damage releases the Alanine transaminase (ALT) and Aspartate transaminase (AST) into blood stream and the levels of these enzymes have the potential to indicate hepato-toxicity and histopathological changes (Kumari *et al.*, 2011). In the present study the alterations in the ROS parameters, LPO, transaminases and phosphatases were evident. An increase in the ROS such as superoxide anion radical (O^{2-}), hydroxyl radical (OH^{-}), and hydrogen peroxide ($H_2O_2^{-}$) are known to be removed by the enzymes SOD, GSH and CAT respectively (Cao, *et al.*, 2010). The activities of the enzymes usually increases as an adaptive response to free radical overload during moderate oxidative stress by means of an increased synthesis. However, a severe oxidative stress suppresses glutathione levels

due to the mutilation of adaptive mechanism (Sevcikova *et al.,* 2011; Zhang *et al.,* 2004). GSH depletion may reduce the cellular ability to scavenge free radicals which affects the general oxidative potential of the tissue. The increase in GSH and SOD in the study suggests their role in combating the buildup in the ROS content. At the same time the decrease in the CAT activity is probably due to the cell damage and its inability to counter effect the agrochemical toxicity (Sivaperumal and Sankar, 2008). Charge of AA is well established as a scavenger of free radical and is regarded as a resourceful antioxidant (Stegeman, 1992). AA content in the present study was found to be increased significantly ($p<0.001$) compared to control in liver and kidney. As AA has a central position in curing the impaired condition occurred during the agrochemical exposure, the increased AA content thereby is a self explanatory mechanism adopted by the teleost fish.

Lipid peroxides (LP) are products of oxidatively damaged lipids resulting from lipid peroxidation reactions induced by ROS whose quantification by thiobarbituric acid represents the extent of oxidative damage (Meister and Anderson, 1983). Increase on LPO is always known to be parallel with SOD increase, which is again due to an enhanced production of superoxide anion radical. These alterations in the antioxidant enzymes and scavengers clearly depicts that oxidative stress was obvious in the exposed fishes which in turn damaged lipids which is proved by the alterations in the LPO levels.

Transaminases play an important role at the junction between the carbohydrate and protein metabolism by interconverting the strategic compounds viz; ketoglutarate, pyruvate and oxaloacetate on one hand and alanine, aspartate and glutamate on the other hand. Alterations in transaminases has been reported exhibiting an important role in carbohydrate and amino acid metabolism in various tissues of fish (Lushchak, *et al.,* 2001; Jomova and Marian, 2011; Khalaf, *et al.,* 1985; Dhanapakiam *et al.,* 2006). The increased transaminase activity in the gills, muscles and kidney might be due to increase in transamination reaction i.e. transferring of NH_2 group from amino acid to a ketoacid. Documented evidences showed that transamination and transdeamination reactions are prominent under stress condition (Rajender *et al.,* 1986; Dhanapakiam *et al.,* 2006).

Alkaline phosphatase is an ubiquitous transport enzyme present in almost all tissue of an organism especially in cell membrane. Firat *et al.,* (2011) have reported that ALP may increase due to the cellular damage in the liver and that high levels of these enzymes usually in an indicative of necrosis in the liver of animals. Time dependent increase in the ALP activity is in agreement with the earlier reported work of Das and Mukherjee 2003 in *Labeo rohita*, Jee *et al.,*

(2005) in Korean rockfish (*Sebastes schlegeli*), Borges *et al.*, (2007) in fish Bagre (*Rhamdia quelen*) and El-Sayed and Saad, (2008) in Nile tilapia (*Oreochromis niloticus*). The researchers concluded that necrosis of liver and subsequent leakage of this enzyme into blood stream might be responsible for increase of this enzyme in blood. The results of the present studies indicate that the activities of certain biomarkers in *O.mossambicus* are more sensitive to the plant nutrient than those in *L. rohita* suggesting the differences in the defense capacity of the teleost fish and that the uptake and elimination pathways differ substantially among tissues.

Metal concentration in aquatic ecosystems are usually monitored by measuring their concentrations in water, sediments and biota (Ergul et al., 2008) which generally exist in trace levels in water and attain considerable concentration in sediments and biota (Unlu et al., 2008). Trace metals including both essential and non essential elements have a particular significance in ecotoxicology, since they are highly persistent and all have the potential to be toxic to living organisms (Storelli*et al*., 2005). These pollutants when compared with other types of aquatic pollution are less visible but its effects on the ecosystem and humans are intensive and very extensive due to their toxicity and their ability to accumulate in the aquatic organisms (Edem*et al*., 2008). Some metals are known to be toxic even at low concentrations, including chromium, lead, cadmium, arsine and mercury (Nguyen et al 2005). While others, such as copper, iron, zinc, manganese and cobalt, are known to be essential elements and play important roles in biological metabolism at very low concentrations but at the same time if excess or deficit can disturb biochemical functions in both humans and animals (Yilden, 2003). As metals, unlike organic pollutants, are nonbiodegradable their content has steadily increased in water and subsequently accumulated in sediments, plants, fishes, and even in humans (Che et. al, 2006).Studies on trace metals in rivers, lakes, fish and sediments (Tüzen, 2003; Canli and Atli, 2003; Karadede et al., 2004; Ozmen et al., 2004; Begum et al., 2005; Ansari et al., 2005; Tuzen and Soylak, 2007; Fernandes et al., 2008; Ozturk et al., 2008; Pote et al., 2008; Praveena et al., 2008 and Turkmen et al., 2009) have been a major environmental focus especially during the last decade. Sediments have been reported to form the major repository of metals in aquatic system while both allochthonous and autochthonous influences could make a concentration of trace metals in the water high enough to be of ecological significance (Oyewo and Don-Pedro, 2003).

Many studies have been carried out to determine the level of metals in the fish since it is considered as one of the richest sources of protein and unsaturated omega-3 fatty acid for human (Rani , 2000; Chandrasekar et al., 2003; Karadede et al.,2004; Amaraneni 2006;

Waqar, 2006; Agarwal et al., 2007; Mendil and Uluozlu 2007; Ploetz et al., 2007; Yang et al., 2007 and Yilmaz et al., 2007; Bhuvaneshwari et al., 2012).In the previous chapters the toxicity of Librel has been proved by detecting the alterations in the heamatological parameters as well as the resultant stress response in various tissues of the fish. The study was further geared towards determining the accumulation of the trace metals in the fish tissues as well as in the water with the view to establish the comprehensive evaluation of metals in various tissues i.e. liver, kidney, gills and muscles. And to move a step ahead determination of the BCF for the target tissues was also carried out.

Time dependent increase in the metal content of the tissues as well as water was observed. Amongst two fish species, *L.rohita* exhibited significantly higher ability to amass metals than *O.mossambicus*. The order of pattern of accumulation of metals in the tissues was liver>gills>kidney>muscle. Individual metal concentration assessment exhibited higher values of Fe in *L.rohita* than *O.mossambicus* . Organ wise concentration that tracked the order for Fe was: L>G>K>M. The second highest trace metal in order was Zn in both the fishes, where *L.rohita* revealed higher concentration compared to *O.mossambicus*. Organ wise accumulation that followed the order for Mn concentration was K>G>M>L. Next in the order was Cu, and it was *L.rohita* which exhibited higher concentration compared to *O.mossambicus*. Organ wise accumulation that followed the order for Cu accumulation was: L>G>M>K. Mn exhibited the least concentration, where *L. rohita* accumulated higher Mn compared to *O.mossambicus*. Organ wise accumulation that followed the order for Mn accumulation was: M>L>K>G. To have an insight for the interspecific differences Mann-whitney test was performed in the tissues of both the teleost fish over the period of time where all trace elements showed altered affinities in the studied fish tissues and did not differ significantly between the tissues except kidney. The overall relationship among the various elements was calculated by Pearson correlation co-efficient. In water, all metals showed positive correlation with the other metals. While the inter tissue correlation expressed differential correlations for *O.mossambicus* and *L.rohita*. overall the positive correlation was expressed for Fe, Zn and Cu whereas, Mn had overall negative correlation with the other three metals. The order of BCF for trace metals was Cu>Fe>Mn>Zn for both the fishes. However, when tissue BCF was compared the order was for *O.mossambicus* and *L.rohita*

- Cu: liver>gills>muscle>kidney
- Fe: liver>gills>kidney>muscle

- Zn: liver>kidney>gills>muscle

except for Mn which differed in both the fishes. For *O.mossambicus* it was liver>muscle>kidney>gills and *L.rohita* muscle>liver>kidney>gills. The overall results from the metal accumulalation studies have proved that the metal accumulation was time dependent, species specific and organ specific. Moreover, of the two species it was observerd that *O.mossambicus* was able to withstand the metal load more compared to *L.rohita*. The high metal concentration in the tissues and water reported in the present studies thus suggest that possibly few of the mechanism might be working simultaneously, however at this point it is difficult to conclude the exact mechanism for metal accumulation. Moreover depuration studies will throw more light to validate our data. As there was no mortality reported in the present studies, possibly the fishes are having an inborn mechanism to counteract the potential toxicity of the metals in the tissues. It has been assumed that stress proteins play a role in the detoxification of heavy metals. It was thus thought worthwhile to go for protein profiling.

Proteomic analyses provide valuable information, when variations that occur within the proteome of organisms are compared as a consequence of biological perturbations or external stimuli. These stimuli often result in different protein expressions or the redistribution of specific proteins within cells (Martin *et al*. 2001, 2003; Vilhelmsson *et al*. 2004; Tyers and Mann 2003), which can be correlated with environmental contamination, and which may help identify proteins that are altered from pollutant exposure, or may help establish a protein-pollutant toxic mechanism relationship (López-Barea and Gómez-Ariza 2006) . An added bonus in these studies is the fact that it is not absolutely necessary to establish the identity of a protein for it to become a successful biomarker of exposure. Indeed, the characteristics of a peptide and the specific conditions under which it occurs are the more pressing concerns (Hogstrand*et al*. 2002). Recent studies have produced protein expression signatures that were characterized in marine invertebrates, in response to changing salinities and temperatures, and in response to the presence of polychlorinated biphenyls and copper (Bradley *et al*., 1985; Shepard *et al*. 2000; Shepard and Bradley 2000; Kimmel and Bradley 2001).

Protein constitutes the building block and the basic molecule for any biochemical reaction. Theyare intimately related with almost physiological processes, which maintain a simple biochemicalsystem in living condition. The physiological and biochemical alterations observed in an animalunder any physiological stress can be correlated with the structural and functional changes ofcellular proteins. Proteins occupy a unique position in the metabolism

of cell because of the proteinaceous nature of all the enzymes which mediate at various metabolic pathways Vidhya and Nair, (2013).Serum or organ proteins of fish are occasionally studied to estimate the toxic potential of many substances including metals. Organisms may respond to chemicals with up or down regulation of serum proteins, which could give a view of defense pattern against them. These changes are of some value in assessing the impact of exposure under natural conditions and may also serve as tools for biological monitoring (Thomas *et aI*., 2007). Agricultural run-off has caused ecotoxicity of the aquatic environment and that leads to death of targeted and non-targeted organisms (Joseph and Raj, 2011). The genotoxicity of many agrochemicals is still under debate as there are reports which are either positive or negative; plant nutrient is one of them, for which such studies are the rare.

Exposure of Librel led to hematological, biochemical and has resulted into serious impairment of gonads and liver tissues. Further the metal accumulation is also reported in various tissues of both the teleost fish, leading to alteration in the metabolic activities, and biochemical indices of stress. We preferred to use *O.mossambicus* and *L.rohita* as the test model, mainly because these have been favored by many earlier workers as test models for both cytogenetic and molecular studies; moreover they make an appropriate model as an indicator species in biomonitoring programs (Manna, 1984; Martins *et al*., 2000 and 2002; Veeraiah et al., 2013). The development of electrophoretic techniques makes it possible to detect the protein composition. Several studies have revealed that fish are able to accumulate and retain metals in different fish tissues (Verma et al., 2005; Samanta et al.,2005; Sharma and Agarwal, 2005Hayat et al., 2007; Karthikeyan et al., 2007; Veeraiah et al., 2013) and have shown to be time dependent. Hence understanding the protein profile may add more understanding towards the outcome of the previous studies. The potential value of electrophoresis in this study is based on the hypothesis that stress conditions may cause significant qualitative and quantitative changes in the proteins of different tissue exposed to the toxicant. Such changes might reflect an altered antibody synthesis, protein biosynthesis, cellular leakage or perhaps other events resulting directly or indirectly from the stress. However, reports on variations of qualitative tissue proteins are lacking, especially with reference to Librel exposure which is multi micronutrient mixture containing metals (Fe, Cu, Zn, Mn and B). Hence the present study is aimed at adding information on the protein profiling in response to the plant micronutrient using *O. mossambicus* and *L.rohita* as test animal. Our earlier chapters have included all the major tissues as they were with the view to look into the overall physiology of the fish and to establish the toxicity of the plant nutrient in

fish. Now, as its toxicity has been established, keeping into mind fish as a major protein diet and muscle being edible part which is consumed, the study of its composition was imperative and its biological monitoring should be done to ensure continuous safety of the fresh water food. Hence in the present studies only muscle tissues were taken into consideration.

Plant nutrient exposures lead a time dependent significant ($p<0.05$) increase in the protein content in muscle of both the fishes. The fresh water fishes *O.mossambicus* and *L.rohita* on subchronic exposure to low sublethal concentration of plant nutrient revealed variation in electrophoretic protein fractions between the control and experimental fishes. As presented in the electrophoretograms of both species represents an increase in the intensity of muscle protein subunits compared to control in the both the species at the initial 15th and 30th day of exposure while a decrease at the 45th day. The size of protein was extrapolated by plotting the graph of log (mol.weight) against R_f. Polypeptides in muscle ranged from 3.46 to 199.52KDa in both *O.mossambicus* and *L.rohita*.

In *O.mossambicus*, muscle of control group showed 5 bands whereas 15th, 30th and 45th day groups showed 5, 6 and 4 bands respectively. At 15th day 14.3, 19.95 and 28.84 KDa proteins were depleted whereas a new polypeptides 20.41 and 199.52 were found. On the other hand at 30th and 45th day new polypeptides 3.46, 14.45, 29.51, 199.52and 3.46, 6.6 and 14.45 were observed, whereas there was a depletion of 3.5, 14.12, 19.95 and 28.84 protein subunits. In *L.rohita,* muscle*s of control group 6 bands were*, whereas 9, 9 and 4 bands were seen in 15th, 30th and 45th day groups. All the bands that were observed except subunit 3.54 and43.65 were new in the experimental groups. Whereas the new bands 6.30, 13.80, 19.95, 28.18, 43.65, 64.56, 100 and 199.52 were observed at the 15th and 30th day exposure while 3.46, 6.45, 14.12 and 199.52 at 45th day. Likewise there were overall increase in the number of bands in the gills, however, the intensity of bands was more in muscles compared to gills. Liver protein bands expressed the least in protein bands. Overall, the increase in new bands of protein was gradual and synchronous with exposure. It is suggested that the occurrence of new protein in the muscle has been transported through blood stream or they could have been synthesized in the muscle itself. It is inferred that these protein fractions could be stress proteins to overcome the toxic effect of heavy metal (Salahudeen et al., 2014).Furthermore, few protein fractions which were absent in the tissues of might have detracted the proteins. The reduction of proteins could be due to the impact on the protein synthetic pathway or due to the depletion of reserve proteins to overcome to stress caused by heavy metal stress.

.

Our study has lend the findings of induction of species specific new protein bands upon exposure of trace element mixture, Hence one possible mechanism is that, when fish is trying to adjust in *invitro* environment small fluctuations of protein band is seen, while on the other hand it is also trying to combat the stress which is being produced. So, these metal ions which are present in high amount is accumulated in respective tissue which lead to activation/expression of certain stress genes which are either not produced or are at low level in normal condition. So in reference to this study, we hypothesized the expression of metellothionine (ranges from 4-14kDa) and stress ac which may have been induced by the organism's physiology to get rid of stress. Although, further insights into the identification of bands are needed, which can be confirmed by Western blotting that involves higher specificity of antigen-antibody reaction. It can thus be concluded that electrophoretic analysis provides a very useful method for certain aspects of biology that it can be used as an additional tool to evaluate environmental stress on animals with success.

The important observations can be summarized:

- Comparative study on acute toxicity and behavioural responses of plant nutrient concluded that plant nutrient Librel was toxic to both the fishes and based on the LC50 values *O.mossambicus* was found to be more sensitive compared to *L.rohita*. It was established through this study that short-term exposure to plant nutrient resulted in negative alterations in behavior and mortality. Further the toxic effect in the fishes observed was the cumulative manifestation of the changes in the physicochemical factors and plant nutrient exposure. Hence, precautions must be taken when it is used in fish inhabiting areas since the excess application can affect the life of organisms living near the farming area and cause the high mortality among them.

- Hematological and biochemical alterations in *oreochromis mossambicus* and *labeo rohita* exposed to plant nutrient provided valuable information in the assessment of fish health and in monitoring stress responses. The exposure of *O.mossambicus* and *L.rohita* to sublethal concentrations of Librel caused marked variations in the blood indices of both the fishes an suggesting the toxic nature of the micronutrient mixture. Furthermore, the results also unveil that under experimental condition, blood parameters of *O.mossambicus* and *L.rohita* were sensitive to Librel . It also points to the fact that hematological and biochemical studies are vital for assess the overall health of fish subjected to plant nutrient exposure. It also suggest that waterways

surrounding the agricultural fields should be monitored which can help in protecting the non target organism from the toxic effects of the agrochemicals.

- Gonadosomatic and Hepatosomatic Indices of Freshwater Fish *Oreochromis mossambicus* in Response to a Plant Nutrient obtained indicated adverse effects on the gonads as well as on liver weight. From the present work one can conclude that the plant nutrients are capable of causing the metabolic and reproductive damage in fish. This was apparent in sequential changes that were observed in histomorphological structures of liver as well gonads suggesting a reduction in the reproductive fecundity of the test organism. However the effects whether reversible or irreversible can be deduced only after the conduction of the recovery studies. GSI and HSI alterations were accompanied by histopathological changes in liver, ovary as well as in testis. This study is the first histological evidence of reproduction disturbance related to a micronutrient Librel^{TM} and the high levels of gonad and liver histopathology recorded raise concerns about the long-term health of fish populations.
- To have an understanding of oxidative stress response of trace element mixture at subchronic level. Species and tissue-specific (liver, kidney, muscle and gill) defences were studied. Alterations in the antioxidants, lipid peroxidation, transaminases and phosphatases have suggested that the fish was in oxidative stress as a result of exposure of the micronutrient mixture. Thus from the present studies it can be concluded that the plant nutrient exposure has led to the alterations in the antioxidants, lipid peroxidation, transaminases and phosphatase, and that the alterations in the parameters in the target tissue (i.e., liver, gill, and kidney and muscles) is due to the damage leading to dysfunction. The results of the present studies also indicate that the activities of certain biomarkers in *O.mossambicus* are more sensitive to the plant nutrient than those in *L. rohita* suggesting the differences in the defense capacity of the teleost fish and that the uptake and elimination pathways differ substantially among tissues. Such a study is thus important as aquatic systems are the ultimate fate of the metals and the fishes are the ultimate target which would be exposed to the metals in the combined form.
- In an attempt to have an insight into the tissue specific and species specific concentration of metals into the fish after establishing its acute and subchronic toxicity as the study was geared towards determining the accumulation of the trace metals in the fish tissues as well as in the water with the view to establish the comprehensive evaluation of metals in various tissues i.e. liver, kidney, gills and

muscles. The high metal concentration in the tissues and water reported in the present studies suggest that there are possibly of multiple mechanisms working simultaneously, however at this point it is difficult to conclude the exact mechanism for metal accumulation. Moreover depuration studies were not done and perhaps it will throw more light to validate our data. As there was no mortality reported in the present studies, possibly the fishes are having an inborn mechanism to counteract the potential toxicity of the metals in the tissues, by synthesizing the stress proteins as they are assumed to play a role in the detoxification of heavy metals.

- Hence, to further take the work a step ahead it was thus thought worthwhile to go for protein profiling. The present study may provide an insight in rate of turnover of various proteins alterations at cellular and subcellular levels and changes in the biological properties of fish in reference to plant nutrient. Our study has lend the findings of induction of species specific as well as tissue specific new protein bands upon exposure of trace element mixture, Hence one possible mechanism is that, when fish is trying to adjust in i*nvitro* environment fluctuations of protein band probably leads to activation/expression of certain stress genes which are either not produced or are at low level in normal condition, which may have been induced by the organism's physiology to get rid of stress. Thus the present study obviously indicates that the presence of trace amount of the plant nutrient in the water is toxic to fishes and it has caused significant alterations in the electrophoretic protein pattern in the different tissues. Therefore the information obtained may be noteworthy for management and monitoring of agricultural contamination in aquatic environment. Although, further insights into the identification of bands are needed, which can be confirmed by Western blotting that involves higher specificity of antigen-antibody reaction.

In a nutshell the present study has established the hematological, biochemical and respiratory stress response along with the reproductive and proteomic studies the toxic potential of the plant nutrient. However, it has now opened avenues for exploration of various signatures of different mechanistic pathways which are operative under stress. Hence, genotoxicity can be done to guarantee the overall effect, consequently for that the expression of metallothioneins can be checked by doing polymerase chain reaction (PCR) and extending it by performing western blotting .This will provide three evidences :

1. That there is an induction of a gene which is upregulated upon metal stress condition.

2. And the same mRNA is translated to form the mature protein. This is because in a stress physiology it is not necessary that if a gene is expressed its protein counterpart will be necessarily be formed.
3. The specificity will lend us to know which among the group of metallothioneins are activated upon exposure to Librel and will also tell us if there is a species specific difference in its expression or not.

   At tissue level, immuno-histochemistry can be added upon to check the cytoskeletal machinary, which will notify us that how far damage is caused by this type of chemicals. This study will give us a clear cut ratio of cellular change to that of genetic imbalance. Finally, continuous monitoring of effluents of agricultural runoff should be done because the use of micronutrient cannot be stopped as it is necessary for the growth of plants. But the problem arises at two level firstly the farmers extensively use this for better yield of crops and secondly when it gets off to the nearby water body and is acquainted with non target organisms it starts to get accumulated. For example the primary target of this type of chemicals is the edible fishes, this in directly affects human beings as they eat, so in particular we can say there is a sum total of materials as well as energy that are transfer from one tropic level to the other (fish to Humans) i.e with energy, the extra unwanted material is also transferred which is nothing but the metals that are accumulated in fish. This is due to the fact that the accumulation rate is directly proportional to the duration of its exposure and is inversely proportional to its excretion rate. Metal accumulation in humans eventually leads to development of physiological disorders which are deleterious to human health. So one need to have a regular check on the use of this type of chemicals as well as on the nearby aquatic ecosystems. The information obtained may be noteworthy for management and monitoring of agricultural contamination in aquatic environment.

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