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RESEARCH ARTICLE

AGROCHEMICALS INDUCED GENE EXPRESSION ALTERATIONS IN O.MOSSAMBICUS.

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Abstract

The effect of agrochemicals in general and pesticides in particular on non-target organisms has been a source of worldwide attention and concern for decades. Pesticide residues in water are a major concern as they pose a serious threat to biological communities including humans. The present study deals with the sub-lethal effect of four agrochemicals namely insecticide (Imidaclorprid-IMI), Herbicide (Pyraonsulfuron Ethyl-PE), Fungicide (Curzate-CZ) and plant nutrient (Mixture of Trace metal ions-MN) on the alteration in gene expression pattern of various target genes of hypothalamus pituitary gonadal axis (HPG) in *Oreochromis mossambicus*. IMI exposure resulted in an up regulation of the kisspeptin 2 and GnRH-I mRNA level in hypothalamic region, gonadotropin receptors (GtH-Ir and IIr) and estrogen receptor (ERI) in ovary. PE and CZ exposure altered the GtHIIr and Estrogen receptor II (ERII) mRNA levels in brain and ovary. Micronutrient mixture (MN) exposure altered the kiss 2 and ER-I transcript in hypothalamic region of brain and GtH-IIr and ER-I receptor in ovary. The result of the present study implies that agrochemicals has modified HPG axis which ultimately lead to the reduced reproductive fecundity of the fish.

Introduction:-

The endocrine system is comprised of numerous organs throughout the body, which work in tandem with the central nervous system (CNS) to regulate biological processes. Reproduction in fish is controlled by the highly conserved hypothalamus pituitary gonadal (HPG) axis . At its apex are the GnRH neurons in the hypothalamic-preoptic area of the brain that ultimately control the reproduction by integrating information from social and environmental signals with internal information such as nutritional and hormonal state (Maruska and Fernald 2011). The hypothalamic neuron stimulates the anterior and posterior pituitary to regulate the entire system by delivering its hormone to target organ**.** However, due to lack of hypophyseal portal system in teleost, neurohormones show their action by the use of nerve fibers from the preoptic region to the pituitary (Levavi-Sivan B et al., 2010).

The world population is expanding rapidly and is likely to be 8 billion by the year 2025. Limited availability of additional arable land and water resources and the declining trend in crop yields globally will make food security a major challenge in the 21st century (Sadekarpawar and Parikh 2013). 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 (Parikh et al., 2010). Gujarat state, In India is one of the important agro-economic states; hence use of pesticides for the better yield of crops is the routine practice. Due to pesticides toxic properties there is an obvious risk that non target organisms are affected, either at the application site or due to unintentional spreading, at nearby or even distant areas (Akerblom 2004).Chemicals Originating from agricultural activities can enter the nearby aquatic environment through agricultural runoff and disturbs the whole aquatic life through bio-accumulation , one of the non-targeted species which is affected the most are the fishes (Ullah and Zorriehzahra 2014).

Recently, advancement in the field of toxicology is to develop and evaluate new molecular and cellular methods to supplement traditional methods of toxicity testing and risk assessment (Jang et al., 2014).In this context, various studies have been carried out; such as proteomic studies in zebra fish (Biales et al., 2011) exposed to fungicide prochloraz have reported an altered expression of proteins in brain region (Biales et al., 2011); endocrine disrupting activity of endosulphan is been well demonstrated in *Cichlasoma dimerus* on gonadotropin-releasing hormone (GnRH) I, II, and III and β follicle-stimulating hormone (βFSH) activity by Piazza et al., (2011); genetic profiling has also been studied by Weber et al (2013) in zebra fish on exposure of atrazine. Multiple studies have been conducted to assess the effect of pesticides on the HPG axis of fish (Palais et al., 2012; Shenoy 2012; Freeman et al., 2014; Wirbisky et al., 2016). As proposed by Mennigen et al., (2008) the inhibitory effects of agrochemicals on reproduction may be mediated through changes in regulation at the any level of the HPG axis.

Earlier findings have proved the toxicity of IMI (Desai et al., 2014); CZ (Parikh et al., 2015); PE (Upadhyay et al., 2014); and MN (Sadekarpawar et al., 2015 a,b,c), however there is a gap in our understanding of the adverse effect of these chemicals on HPG axis. We hypothesize that, in addition to the toxicity of these agrochemicals, they may have endocrine disrupting property. Hence the objective of the study is to have an insight into the effect of agrochemicals on hypothalamic pituitary gonadal axis (HPG) of O.mossambicus.

Materials and Method:-

Fish:-

Healthy male and female adult O.mossambicus was procured from the pure brooders of length 12±3cm and weight 25±3g. Fishes (5 males and 5 females) were kept in a clean glass aquarium for an acclimation period of 12-15 days in de-chlorinated water 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. They were fed with the commercial available healthy food during the period of study. Animal maintenance and experimental procedures were in accordance with the guideline of A.P.H.A., A.W.W.A. and W.P.C.F. (1998).

Experimental Design:-

After the acclimation period the fishes were divided into 5 groups having 5 females and 5 males (n=10) and 4 replicates were performed for each group. Group I:as control, Group II: Exposure of Insecticide Imidacloprid-IMI (0.074mg/L i.e. $1/10^{th}$ of LC_{50}),Group III Exposure of Herbicide pyrazonsulfuron Ethyl-PE (50 mg/L i.e. $1/10^{th}$ of LC_{50}), Group IV Exposure of fungicide Curzate-CZ (4.9mg/L i.e. $1/10^{th}$ of $LC_{50)}$,Group V Exposure of micronutrient mixture-MN (500mg/L i.e. $1/10^{th}$ of LC_{50}). The exposure was for the period of 14 days and on the 15th day fishes were sacrificed via rapid cervical transection. Tissues from brain (including hypothalamus and pituitary), ovary were sampled and preserved in TRIzol reagent for subsequent RNA isolation.

Total RNA extraction and cDNA synthesis:-

Total RNA was isolated by Trizol method (Invitrogen**)** (Peterson and Freeman 2009) and concentration was measured spectroscopically by Perkin elmer. cDNA was reverse transcribed from 50ng total isolated RNA using Thermo Verso cDNA synthesis kit (AB-1453/B) and PCR for the candidate genes was performed with their specific primers and with standardized condition i.e. denaturation was performed at 95^0C for 1 min, annealing was carried out for different primer according to their respective T_m for 30 sec and extension was carried out at 72^0C for 7 mins with 18srRNA as the reference gene (Table 1).A total of 35 cycles were carried out for all the genes and finally the amplicon obtained were checked on 2% agarose gel and images were taken using ABI gel documentation system. Relative quantification analysis of the PCR products was done using Image J software.

SDS page:-

Hypothalamus was dissected from the brain tissue from each experimental group and was prepared in laemmli SDS (Laemmli 1970) sample buffer, further 10% homogenate was used for total protein estimation assayed using Bradford method (Bradford 1976). Equal amount was loaded on to 15% SDS PAGE gels. These gels were further used for western blot analysis.

Western Blot study:-

30 micrograms of total protein was resolved on 15% SDS-PAGE Tris-glycine gels and transferred to nitrocellulose membranes. Non-specific binding was blocked by incubating the membranes in 5% BSA and 0.1% Tween in Tris-buffered saline (TBS, pH 7.4) for 1 h at room temperature. The blots were subsequently incubated with primary polyclonal antibodies raised in rabbit (kisspeptin 1 and 2, procured as a gift from Prof.Parhar, 1:1000dilution),

overnight at 4°C, with gentle agitation. Blots were washed with TBS containing 0.1% Tween (TBS-T) (4 × 15 min) and then incubated with respective anti rabbit secondary antibodies conjugated with HRP (horse radish peroxidase) for 2 h at room temperature with gentle agitation. After four washes with TBS-T and one wash with TBS; specific bands of immunoreactive proteins were visualized using enhanced chemiluminescence (ECL) reagent (Millipore) in Chemidoc (Alliance Model 4.7).

Estradiol (E_2) Hormonal Assay:-

At the 15^{th} day, blood was collected using caudal peduncle with the help of heparinized syringe. Plasma was separated and circulating steroid levels were measured by Cayman ELISA kit (Cat #582251). Each sample was assayed in triplicates were 100 ul of ELISA buffer was added to all the wells, followed by addition of 50 ul of estradiol standard to each well. Estradiol standard was made using serial dilution from the stock solution (400ng/ml) in 8 tubes.50ul of sample was added to each triplicate trailed by 50ul AChE tracer in each well except for blank and TA (total activity). Finally 50ul of estradiol antiserum was added to each well expect for the well of TA and NSB (Non-Specific binding). The plate was incubated at room temperature on orbital shaker for 1 hr, and was developed using Ellman's reagent. The standard curve and sample concentration was determined using the following formula:

$$\text{logit}(B/B0) = \ln [B/B0/(1 - B/B0)]$$

B/B0 (Sample or Standard Bound/Maximum Bound)
B0- Maximum binding.
B-Sample or Standard Bound.

Statistical Analysis:-

The computed data was analyzed using PRISM 6 Software. One and two way ANOVA followed by DUNNETs multiple comparison were used to the test for significant differences among the individual treatment combinations. Statistical significance was accepted at $*p \leq 0.05$ for all tests.

Results:-

There was an up regulation of GnRH-I in all the agrochemicals expect in case of herbicide (PE), which showed significant ($*p<0.05$) down-regulation (Fig –1). Kiss2 mRNA expression was found to be was up-regulated maximally (Fig-3) in case of micronutrient mixture (MN) followed by insecticide (IMI) and fungicide (CZ) which was establish to be statistically significant ($*p<0.05$).

On the other hand, the expression pattern of kiss-I revealed down-regulation of its transcript (Fig-2) in all the groups, however the significance ($*p<0.05$) was noted in case of insecticide (IMI), micronutrient mixture (MN) and Herbicide (PE).

GtH-Ir: The expression pattern of receptors were also studied in ovary, among which GtH-Ir was up regulated in all case (Fig-5), but was significantly ($*p<0.05$) elevated in case of IMI and CZ. Analogous results were obtained for GtH-IIr, that showed up regulation among the different class of agrochemicals (Fig-4), but the significant change ($*p<0.05$) was noted under the exposure of MN followed by IMI.

ER-I & ER-II: The expression of estrogen receptor (ER-I & ER-II) was studied in brain and ovary both to witness the fold change in the tissue level gene expression (Fig-6-9).ER-I showed a significant change ($*p<0.05$) of its expression under the exposure of IMI and MN in both tissues. However, there was a down regulation of it when exposed to PE in both the tissues, which was found to be non-significant. However, this pattern of expression was not true for ER-II, which showed significant over expression under the influence of CZ, IMI, PE and CZ, PE in brain and ovary respectively. Other agrochemical exposure in both the tissues showed the alteration, but it was non-influential. Further, western blotting was performed for Kiss 1 and Kiss 2 (Fig- 10, 11, & 15). There was significant down regulation of Kiss 1 expression in all the groups. Correspondingly, Kiss 2 expression was also attributed to be up regulated under the exposure of MN, IMI and CZ ($*p<0.05$).

Accession No.	Gene Name	Sequence	Tm	Species	Amplicon Size (bp)
NM_001113489	Kiss1	FP:CTCAGGGGAACAGACACTCG	59^0C	*Daneo rerio*	400
		RP:GCAAATACCTCAGAGAGGACCA			
NM_001279468.1	Kiss 2	FP:5':GGATCCCAGCCTCTGCTTTT3'	60^0C	*O.niloticus*	214
		RP:5':TCAGGTGGGTACCTCCAGTT3			
AB104861.1	GnRH1	FP:5'CGCCATTTCCTCTCCAGCTTA3'	60.1^0C	*O.niloticus*	180
		RP:5'CGCTACTCCAACAGAGGTCG3'			
AB042422,	GTH Ir	FP:5'ACCTGCTGGAGAGTATCGGT3'	60^0C	*O.mossambicus*	276
		RP:5' AGGCGGTGGAATGGATCTTG3'			
AB042423,	GTH Iir	FP: 5'AAATGCTCCCCAAAGCCAGA3'	60^0C	*O.mossambicus*	214
		RP:5'GCCAGTCTGTGGCTGATTGT3'			
NM_001279770.1	ER-α (Type I)	FP:5'GGAGGTATGCGTAAGGACCG3'	53^0C	*O.niloticus*	85
		RP: 5'GCAGGTCTTTGGCTGGTTTG3'			
NM_001279774.1	ER-β (Type II)	FP:5'CAATGTCATGCATGGGTTGTCT3'	52^0C	*O.niloticus*	198
		RP:5'TCCATGTTGGGGTTGCATCA3'			
AF497908	18srRNA	FP:5′-TATTGTGCCGCTAGAGGTGAA-3′	51^0C	*O.mossambicus*	102
		RP:5′-CCTCCGACTTTCGTTCTTGA-3′			

Table 1: Depicts the forward and reverse primer sequence of candidate genes with its accession number, T_m and amplicon size

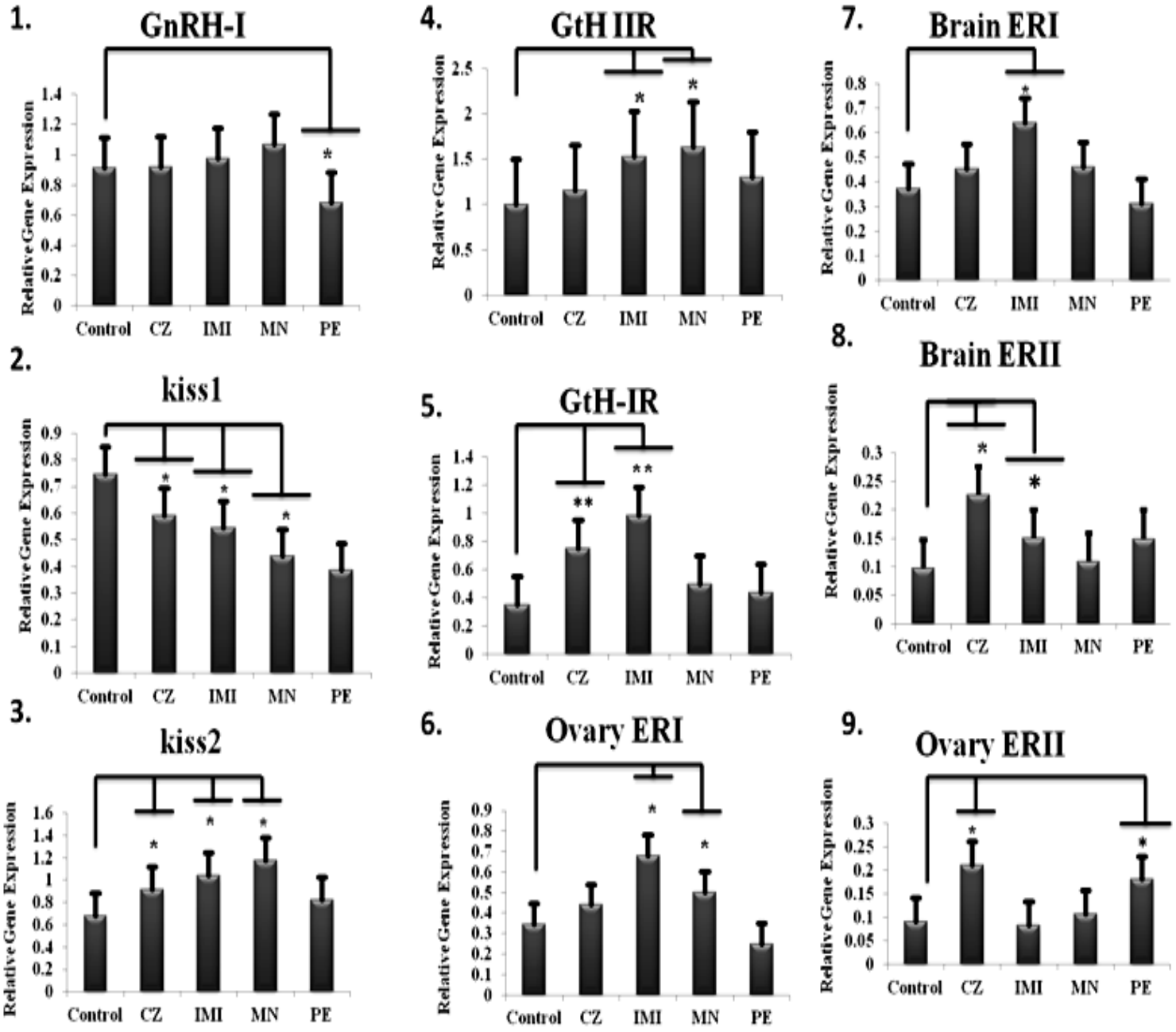

Fig 1-9: Relative gene expression pattern of GnRH-I,Kiss1,Kiss2 in brain hypothalamic region GtH-Ir, GtH-IIr in ovary and ERI , ERII in brain and ovary. (*) denotes level of significance at *p<0.05,p<0.01,***p<0.001.**

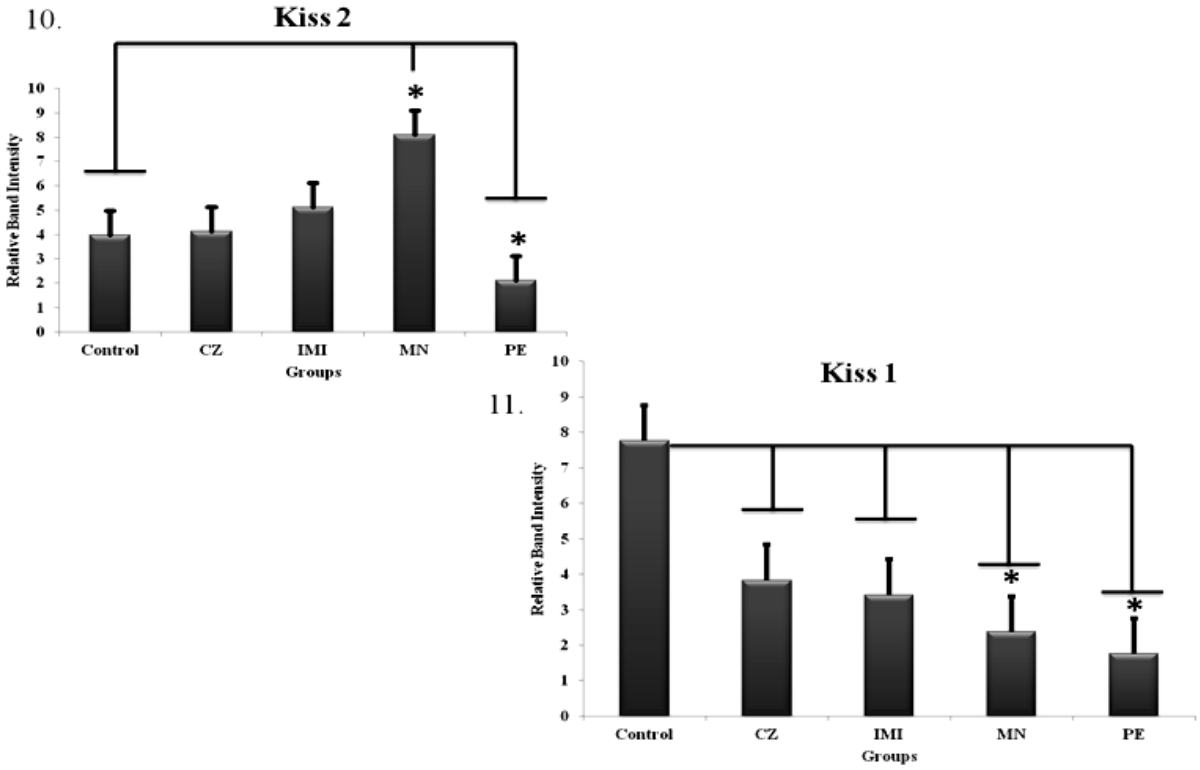

Fig 10 & 11: Relative band density of Kiss 1 and Kiss 2 of 4 groups compared to control. (*) denotes level of significance at *p<0.05, **P<0.01, ***P<0.001

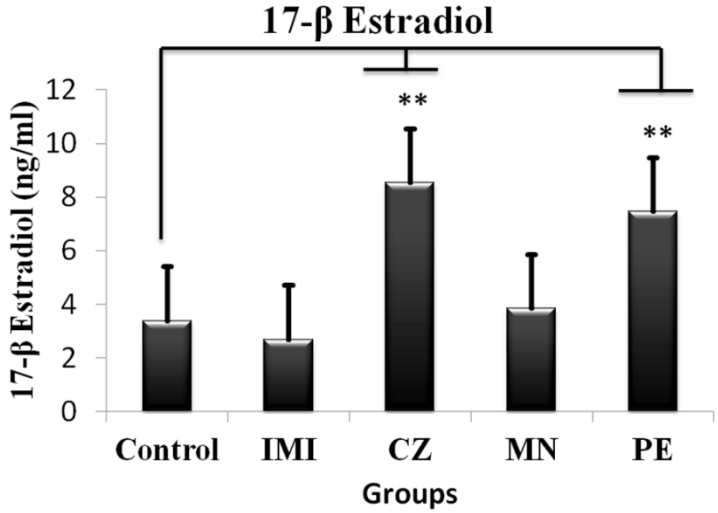

Fig 12: Graph showing the Plasma estradiol level (ng/ml) of *O.mossambicus* . (*) denotes significance at *p<0.05, **p<0.01, ***p<0.001

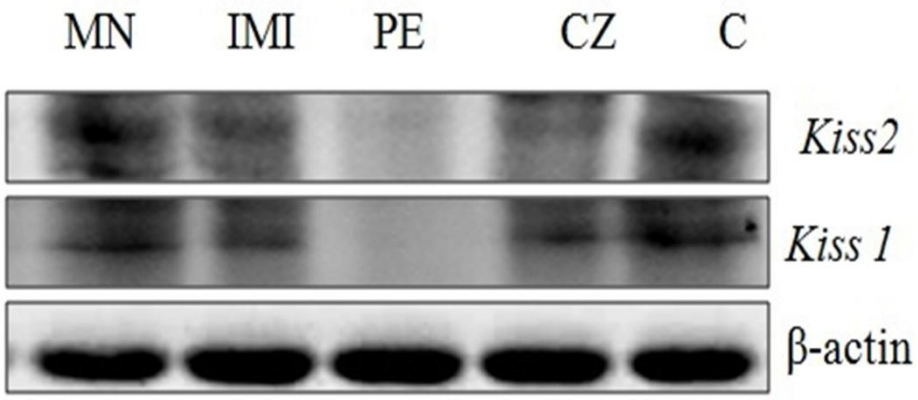

Fig15: Western blot analysis of kisspeptin 1 and kisspeptin 2. B-actin was taken as internal control for densitometric analysis

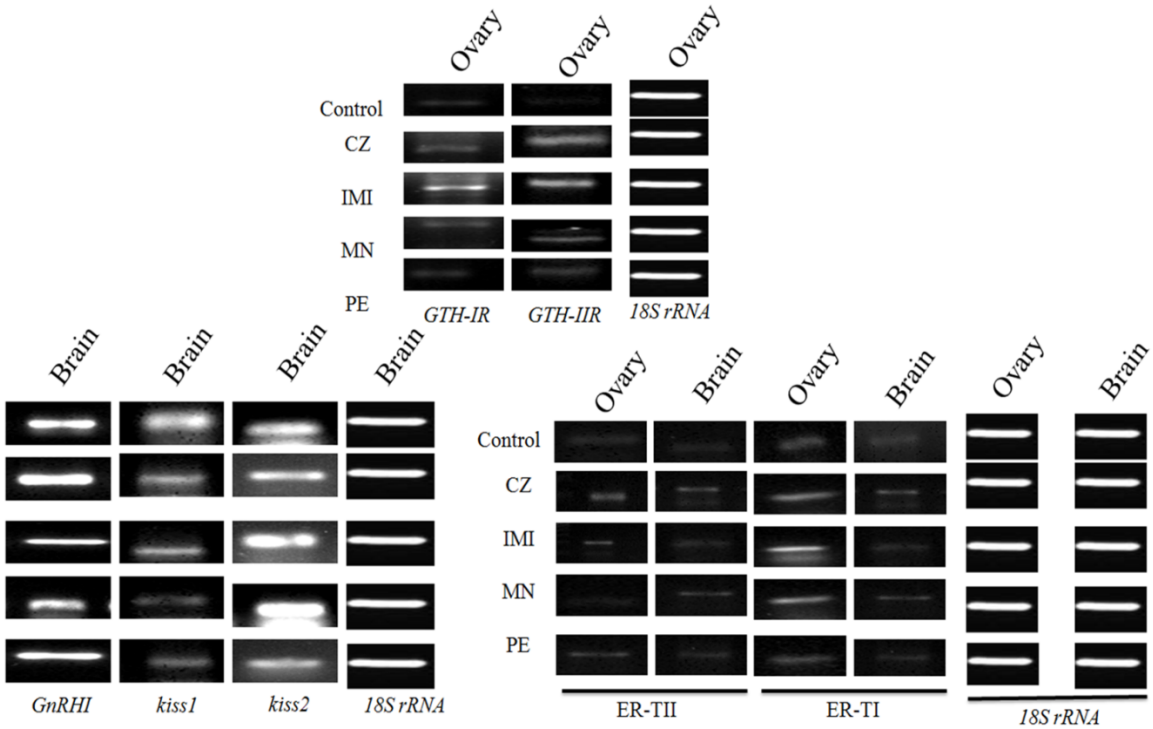

Fig 14:Gene expression profile on agarose (2%) of Kisspeptin 1 & 2, GnRH I-Gonadotropin Releasing Hormone, GtHIr & GtHIIr-Gonadotropins Hormone Receptor, ERI& ERII Estrogen receptor.

Discussion:-

The increase in the spray of agrochemicals has resulted in elevation of toxicity in the environment that has end result in bioaccumulation from one trophic level to the other, ultimately affecting the humans (Ribeiroa et al., 2005; Guoa et al., 2008; Singh and Singh 2008). Cichlids are one of the remarkable models of study, ranging from developmental to toxicological studies (Fujimura and Okada 2007). In the present study, *O.mossambicus* was taken as the model organism with the aim to find the endocrine disrupting properties of the agrochemicals by studying the key genes responsible for the altered physiology on exposure of the widely used agrochemicals in the state of Gujarat.

In the present study, of all the agrochemicals exposed, the expression of GnRH-I was found to be up regulated except in case of PE, where significant down regulation was found. Similarly, Kiss2 mRNA expression was also found to be significantly up regulated under the exposure of MN, IMI and CZ, implying that it is exerting its effect by up-regulating GnRH-I and Kiss2 neurons. Teleost particularly *O.mossambicus* are known to have three isoforms of GnRH (GnRH-I ,GnRH-II ,GnRH-III) that are distributed in various tissues, till now GnRH-I is known to regulate HPG **(**Sempere et al., 2012**)**. As reported by Oakley et al ., (2009) GnRH I neurons are known to be regulated by kiss 2 neurons of discrete nuclei of hypothalamus in some teleost, thus intimately regulating each other (Parhar et al., 2004; Clarkson et al .,2010). In the present study MN exhibited the maximum alteration in the Kiss2 gene expression pattern , this may be because it is an amalgamation of trace metal ions (Zn^{2+},Fe^{2+},Cu^{2+},B^{+},Mn^{+}) suggesting the synergistic or individual action of metal ions (Mebane et al., 2012; Sadekarpawar et al., 2015). . IMI belongs to neonicotinoid group, the increase in GnRH-I and Kiss2 on IMI exposure proves an additional role of IMI apart from its usual mode of action (Gibbons et al., 2015; Crosbya et al., 2015; Desai and Parikh 2013).

Kisspeptins are a group of peptides that stimulate GnRH release and are required for puberty and maintenance of normal reproductive function. Studies in teleosts have revealed the presence of multiple kisspeptin forms (Kiss1, Kiss2) in the brain. It has been suggested that there is a double site of Kisspeptin action in the brain, either in the hypothalamic-hypophyseal region or in the median eminence, an area located outside the blood brain barrier (Nocillado et al., 2008). The important role of Kiss 1 has also proved to regulate gonadotropin secretion, proving the pivotal role in regulation of reproduction (Akazome et al., 2008) .In the present study, mRNA expression and western blot analysis has confirmed the expression of kiss 1 in *O.mossambicus*, and mRNA expression of Kiss 1 was found to be significantly downregulated under PE exposure, suggesting its pleotropic role (unpublished data) in other than regulating the HPG axis. However, the exact mechanism by which it happens is still illusive. Immune-histochemical studies can shed more light on the same.

GnRH-I upon activation acts on anterior cells of pituitary, thus initiates the release of GtH-I (LH like) and GtH-II (FSH like) peptides. This in turn binds to its receptor (GtH-Ir, GtH-IIr) present either on testes or ovary (Yaron et al., 2003; Chen and Fernald 2008). It has been demonstrated that sex reversal is influenced in a large number of teleost species exposed to agrochemicals that have estrogenic effect (Cheshenko et al., 2008; Scholz and Kluver 2009;Hachfi et al., 2012;Frye et al., 2012). IMI and MN exposure resulted in impairment of gonadal activity and the outcome was that on 15^{th} day the dissected fish showed only the presence of ovary, suggesting the possibility of sex reversal phenomena which was corroborated by earlier reported reduction in Gonadosomatic index (GSI) (Sadepawar and Parikh 2013; Desai 2013). Receptor profile of GtH-Ir was found to be up regulated under all agrochemicals exposed, but the significance was reported only in case of IMI and CZ, while the GtH IIr expression was significantly higher in IMI, MN exposed groups. Our results are in accordance with earlier reported sex reversal in various teleost species (Jobling et al., 2002; Orlando et al., 2004; Kortenkamp 2007; Brown et al., 2016).

ER-I and ER-IIr which are analogous to mammalian estrogen receptors α and β (Nelson and Habibi 2013; Nagler et al., 2007) were also studied in ovary and brain as they illustrate negative feedback directly or indirectly (Guiguen et al., 2010;). Among all the agrochemicals exposed, there was significant up regulation of ER-I in IMI and MN in ovary and brain, possibly governing the action by some downstream signaling mechanism (Selin et al., 2009), confirming the endocrine disrupting action of these chemicals. ER-II did not show same pattern of regulation as CZ and PE exposure resulted in a significant up regulation of ER-II mRNA in both brain and ovary, while IMI accredited the higher expression of ER-II only in brain tissue. CZ being the mixture of cymoxanil and mancozeb, has resulted into constitutive receptor activation leading to its up regulation (Coumailleau et al., 2015), which may be due to its mimicking action as that of estrogen. Apart from the conventional studies done on various groups of herbicide (Parikh et al., 2014), very few studies are accounted for the negative effects of PE on any organism. PE

which belongs to the group of sulfonylurea too expressed the parallel effect as that of CZ, suggesting its mimicking role to that of estrogen which was well supported by an increase in plasma level of 17- β estradiol. Our result are parallel to the result of Kim et al.,2014; Servili et al., 2011.

Conclusion:-

Among all the agrochemicals examined, in brain IMI, MN exerts its effect by up-regulating Kiss-2 and GnRH-I transcripts, altering the HPG axis. While PE acts via acting on Kiss-1 neurons in hypothalamic region of brain. Ovary, receptor profile was well being altered by IMI, CZ and MN, which illustrated higher GtHIr and GtH-IIr mRNA levels respectively. Increased expression of ER-I by IMI ,MN and that of ER-II by PE, CZ suggests its mimicking role of estradiol (E_2). Hence, from the results of the current investigation, it can be concluded that all agrochemicals could be potential endocrine disrupting chemicals. The exact mode of action needs to be delineated by further investigations. The results obtained can be considered as a preliminary hint about these chemicals on HPG axis and encourages in-depth analysis of the mechanisms involved therein.

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EFFECTS OF AGRO-CHEMICALS ON ANTIOXIDANT ENZYMES AND LIPID PEROXIDATION IN *OREOCHROMIS MOSSAMBICUS* AND *LABEO ROHITA*

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ABSTRACT

In the present work, an attempt has been made to investigate the mechanisms of agrochemicals (Imidacloprid-IMI and Curzate-CZ) induced oxidative stress (OS) in key organs of agrochemicals exposed two teleost fishes. Major complications arise when the stressor is very effective and the body starts expressing mechanisms of stress response. They include lipid peroxidation (LPO) and expression of various antioxidant machineries like Glutathione S transferase (GST), Catalase (CAT), Superoxide dismutase (SOD) and Glutathione Peroxidase (GPx) as well as scavengers such as reduced Glutathione (GSH) and ascorbic acid (AA). Agrochemical stress significantly increased AA and GSH content in liver, kidney and gills in a dose dependent manner while, GPx level was found to be increased in gills and liver only. Organ-based GST assay reflected a dose dependent significant increase in liver, kidney and gills. Thus, from the present study it can be concluded that the response of antioxidant enzymes (SOD, CAT, GPx, and GST) and non-enzymatic antioxidant/scavengers (AA and GSH) showed that the both the teleost are under severe OS and that the agro-chemicals are acting as potent free radicals generators. LPO level proves that extensive lipid peroxidation has occurred on exposure of the agro-chemicals. And that both the antioxidants interact in a concerted manner to eliminate ROS and prevent damage to cellular components. This suggests that IMI and CZ at levels below median lethal concentration are capable of causing oxidative damage in *Oreochromis mossambicus* and *Labeo rohita*.

Keywords: *IMI, CZ, Oxidative stress, Reactive Oxygen species, Agrochemicals.*

INTRODUCTION

Agrochemicals in the form of insecticides, herbicides and fungicides are used extensively throughout the world. They are playing a pivotal role in meeting the food, cotton fibre and tobacco demand of escalating population and control of vector-borne diseases. Although they furnish some benefits for crop, they entail a number of risks and problems. Pesticide misuse in various sectors of the agriculture often has been associated with health problems and environmental contamination worldwide (Remor *et al.*, 2009). Misuse of highly toxic pesticides, coupled with a weak or a totally absent legislative framework in the use of pesticides, is one of the major reasons for the high incidence of pesticide

Developing countries use only 20% of the world's agrochemicals, yet they suffer 99% of deaths from pesticide poisoning (Atreya, 2008). The unregulated release of agricultural chemicals especially pesticides into water bodies have caused environmental problems to all classes of organisms in the aquatic habitat. The aquatic ecosystem is under threat of biodiversity loss due to indiscriminate use of pesticides. The application of environmental toxicology studies on non mammalian vertebrates is rapidly expanding, and for aquatic system, fish have become an indication for the evaluation of the effects of noxious compounds (Mensah *et al.*, 2014).Pesticides at high concentrations are known to reduce the survival, growth, and reproduction of fish and produce many visible effects on fish (Joseph and Raj, 2010).

Pesticide 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

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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. Major complications arise when the stressor is very effective and the body starts expressing stress response, that include lipid peroxidation (LPO) and expression of various antioxidant mechanisms like GST, CAT, SOD and GPx and scavengers such as GSH and AA.

Several studies demonstrated that changes in the levels of antioxidant enzyme activities can be used as possible biomarkers in different aquatic organisms (Slaninova *et al*., 2014; Sepperumal and Saminathan, 2014). These enzymes are biomarkers of tissue damage, thus their bioassay can serve as a diagnostic tool for assessing the functions of vital organs. Hence, in the present work, an endeavor has been made to explore the pesticides induced OS, in various key organs of pesticide exposed fishes on lipid peroxidation as well as their antioxidant defense mechanisms as this aspect of the toxicity data for this new group of insecticides for aquatic invertebrate are far from enough.

MATERIALS AND METHODS

Experimental designs

Freshwater teleosts, *O. mossambicus* and *L. rohita* of similar size in length and weight (12 ± 2 cm; 25 ± 1.9 g) and (25 ± 3 cm; 110 ± 5 g) respectively were brought from a local pond of Baroda district. Animals were transported to laboratory in large aerated plastic container and were acclimatized in glass aquaria containing 50 liter of well aerated dechlorinated tap water (with physic-chemical characteristics: pH 6.5- 7.5, temperature 25±3ºC and dissolved oxygen content of 7-8 ppm) for ten days. During an acclimatization period of 10 days, the fish were kept under natural photoperiod (10:00 and 16:00h) and fed two times a day with commercial pellet diet. The acclimatized healthy fishes of both sexes were selected randomly for the studies Based on the result of the 48 h LC_{50}, 30 tilapia fish were divided in 3 groups, 10 fish for each group:

• Group 1 served as control without any treatment of Agro-chemicals.

• Group 2 were treated with low dose of IMI and CZ (LC_{50} / 10).

• Group 3 were treated with high dose of IMI and CZ (LC_{50} / 20) for a period of 21 days.

Each concentration was replicated two times. Constant amount of the test chemical and test media were changed every 24 hours to maintain the toxicant strength and the level of dissolved oxygen as well as to minimize the level of ammonia during experiment. The fishes were fed once in a day throughout the duration of the sub-lethal toxicity tests.

Preparation of the tissue samples for the study

At the end of the experiment (21 days) the fish were carefully netted to minimize stress, and weighed. Tissues such as liver, kidney, gills and muscle were carefully removed, wiped thoroughly, washed in chilled PBS After noting the total weight of the tissues, the desired amount of the tissues were weighed and used for quantitative analysis of the enzymes.

Estimation of AA was done following the method of Roe and Oesterling (1944); GSH was estimated by the method of Ellman and Fiches (1958); SOD was determined using the method of Kakkar *et al*., (1984); CAT level was determined using the method of Maehly and Chance (1955); GPx was estimated by the method of Rotruck *et al*. (1973); GST was determined using the method of Beutler *et al*. (1986) and LPO was estimated by the method of Niehaus and Samuelson (1958).

Statistical Analysis

The statistical analysis was carried out using the software Graph pad prism 5 packages. For determining the significant difference between different treatments in biochemical parameters, Two-way ANOVA followed by Tukey's test for multiple comparisons between different concentration of IMI and CZ was done. Significance level (P value) was set at 0.05 in all tests.

RESULT

The effects of IMI and CZ exposure on various parameters of OS at high and low dose in *O. mossambicus* and *L. rohita* are shown in Figure 1 to 4. All the tissues, gills, liver, kidney and muscle showed significantly ($P<0.05$) elevated AA activity compared to control (Figure 1 A-D). There was significant $P<0.05$) variation in GSH content between treated groups and within tissues on exposure of IMI and CZ (Figure 1 E-H). Among the tissues, gills, liver and muscle illustrated significant ($P<0.05$) elevated activity compared to control but the kidney in both the treated groups showed significant ($P<0.05$) reduced activity compared to control. A dose dependent increase in the activity of SOD and CAT was found to in gills, liver and kidney of *O. mossambicus* and *L. rohita* on exposure of CZ and IMI (Figure 2 A-H) An increased GST activity ($P<0.05$) was seen in liver, but kidney and muscle expressed a significant ($P<0.05$) decrease in the GST (Figure 3 A-D). GPx activity too revealed an overall significant change ($P<0.05$), there was an increased activity in liver, kidney and muscles of the treated groups compared to control. Whereas gills, on exposure to IMI and CZ showed a decreased GPx activity compared to control (Figure 3 E-H). A significant elevation in LPO was noted in *L. rohita* and *O. mossambicus* on IMI exposure, however a non-significant increase was observed in *O. mossambicus* on exposure of CZ (Figure 4 A-D).

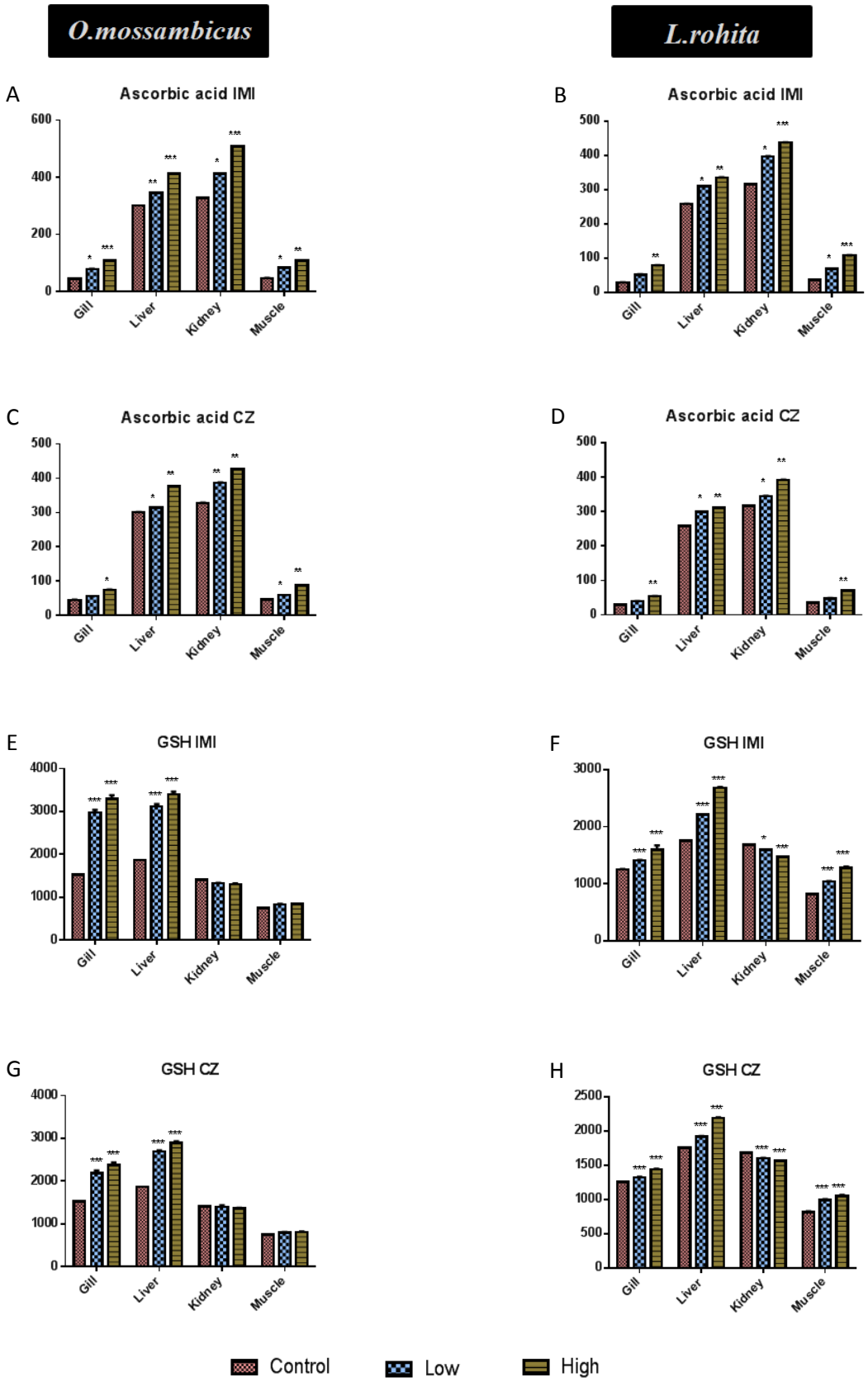

Figure 1. Effect of IMI and CZ on AA and GSH activity (mean ± SEM) in *O.mossambicus* (A, C, E, G) and *L. rohita* (B, D, F, H).

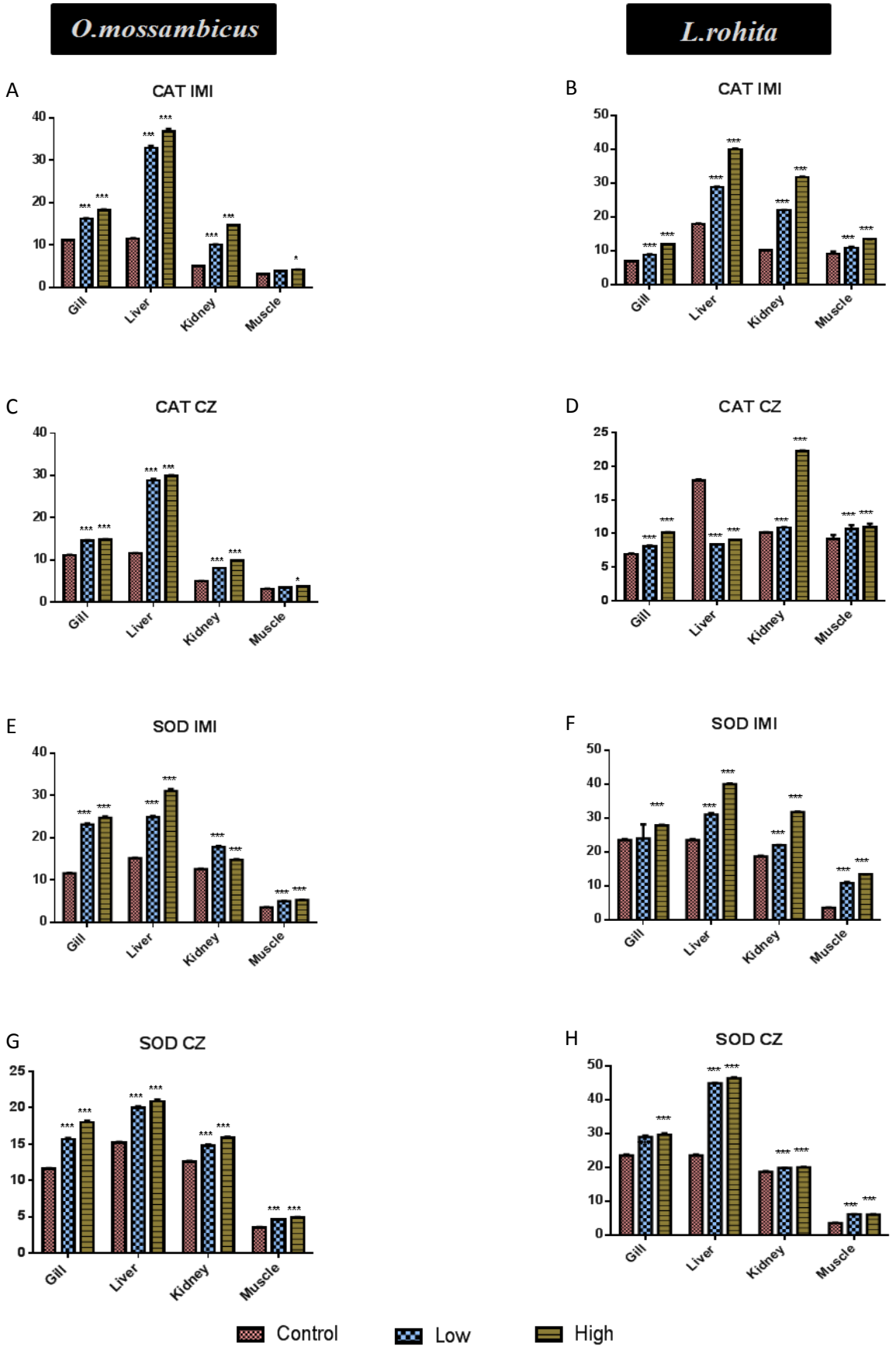

Figure: 2. Effect of IMI and CZ on CAT and SOD activity (mean ± SEM) in *O.mossambicus* (A,C,E,G) *and L.rohita* (B, D, F, H).

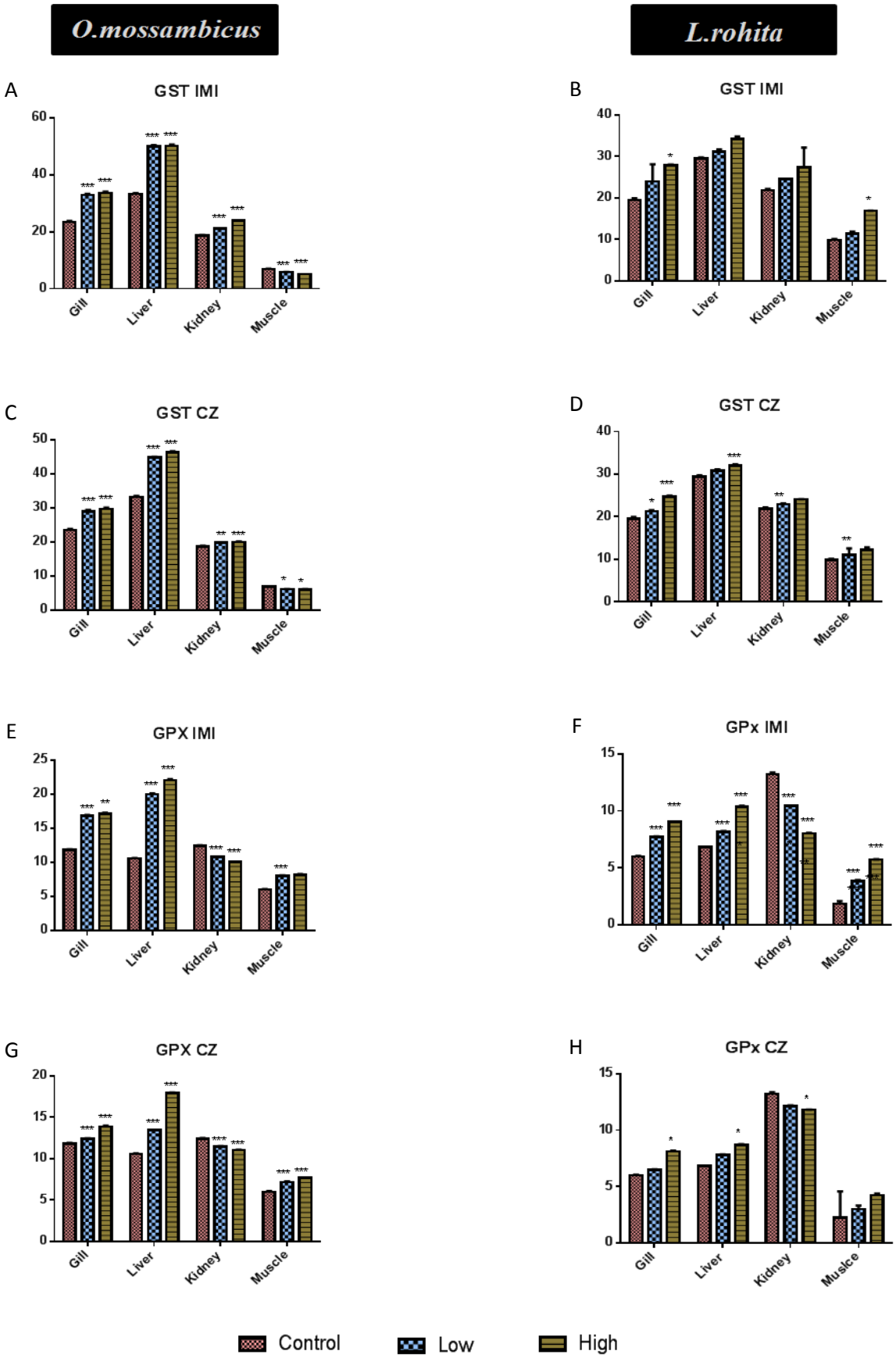

Figure 3. Effect of IMI and CZ on GST and GPx activity (mean ± SEM) *in O.mossambicus* (A, C, E, G) *and L.rohita* (B, D, F, H).

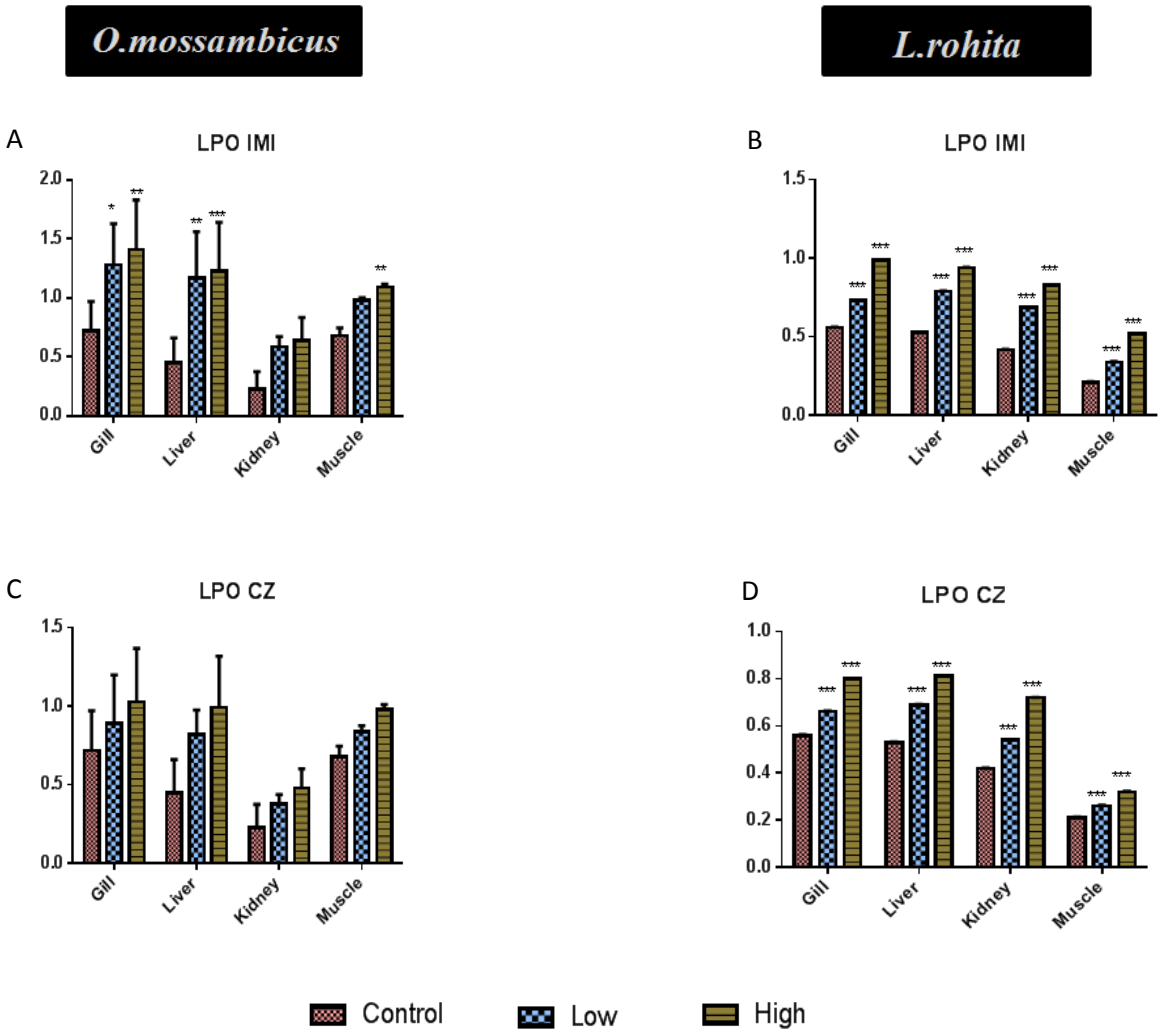

Figure: 4. Effect of IMI and CZ on LPO activity (mean ± SEM) of *O. mossambicus* (AS, C) and *L. rohita* (B, D).

DISCUSSION

Detoxification path at tissue level can be detected by biochemical markers of OS (Van der Oost *et al.*, 2003). The first line of defence to oxidative stress is the use of antioxidant scavengers, such as AA (vitamin C), vitamin E, uric acid, carotenoid and GSH. In the present study agrochemical exposure has significantly increased AA content in liver, kidney and gills. AA protects host cells against harmful oxidants released into the extracellular medium. It can act as a hydrogen carrier as it has an essential role in the metabolism of protein; fats and carbohydrates. Due to its anti-oxidant role and as a part of redox buffer system increased AA is probably inhibiting the oxidative metabolism and preventing the production of electrophilic metabolites and is able to scavenge harmful free radical metabolites/ ROS. Hence, the high level of AA observed in the present study by agro-chemical induced stress condition is reasonable. The indication to detoxifying enzymes is reported to be accompanied by increase in AA content of liver, kidney and gills, which stimulate detoxification of toxicant, suggestive of liver, kidney and gills to be the sites of detoxification. Our results are in agreement with earlier reported elevated AA content in *O. mossambicus* (Guha and Khuda-Bukhsh, 2002); in *Clarias batrachus* (Kaviraj and Gupta, 2014) and *Puntius ticto* (Ganeshwade, 2011). Glutathione is a tri peptide that is mainly present in cells in its reduced form (GSH), which basically acts as an intracellular reductant and neucleophile (Vardharajan, 2010). It functions in the synthesis of proteins and DNA, free radical scavenging, as an essential cofactor of several enzymes, and as a defence against oxidizing molecules and potentially harmful pesticides (Dorval and Hontela, 2003). In the present study there was a significant increase in the GSH activity in liver and kidney on exposure of agrochemicals in a dose dependent manner. Among the tissues GSH level was found to be highest in the liver compared to other tissues which may be due to an adaptive mechanism to oxidative stress in its synthesis which can be provided for the increased GSH activity. However, a depletion of GSH was observed in kidney and muscles at low dose of IMI and CZ which illustrates that severe oxidative stress may have suppressed GSH levels due to loss of adaptive mechanisms leading to the oxidation of GSH to GSSG.

The second cellular mechanism to remove excess ROS and avoid oxidative damage is maintained through enzymatic defence strategy. These enzymatic defences include glutathione peroxidase (GPx), glutathione-s-transferase (GST), catalase (CAT) and superoxide dismutase (SOD). GPx is an enzyme with peroxidase activity and broad substrate spectrum (Lushchak, 2012). This enzyme is known to protect the fish from the damage caused by H_2O_2 and reduces it to lipid hydroperoxides (Mieiro *et al.*, 2011 Banaee, 2013). An increase in GPx activity in liver, kidney and gills is probably eliminating the access of H_2O_2 and lipid hydrogen peroxide produced in the fish exposed to agro-chemicals. Similar results have been observed in the liver of *Cyprinus Carpio* (Vinodhini and Narayana, 2009) and in the kidney of *S. senegalensis* (Velma and Tchounwou, 2011; Oliva *et al.*, 2012), in *C. batracus* (Bhattacharya and Bhattacharya, 2007)

As proposed by Tsangaris *et al.* (2007), GPx is not only an important component of antioxidant defence system but its response is known to be accompanied by the action of other anti oxidants (GST) and Scavenger (GSH) molecules. GST is one of the major phase II, GSH-dependent ROS- electrophilic xenobiotic detoxifying enzyme (Comakli *et al.*, 2011) by making the xenobiotic chemicals more hydrophilic for transportation or excretion. When severe oxidative damage prevents the primary antioxidants from functioning GST can still remove the harmful substance, allowing the cell to regain homeostasis (Perl-Treves and Perl, 2002). In animals the toxicant conjugate is marked for excretion, GST is therefore considered a 'detoxification enzyme' rather than a traditional antioxidant (Adeyemi, 2014; Mani *et al.*, 2014)**.** The elevated levels of GST in the present studies indicate the shift towards a detoxification mechanism under agro-chemical exposure. There is more GST activity in hepatic tissue compared to kidney and gills, which is due to effective role of liver in agrochemical detoxification (Wassmur *et al.*, 2010; Haluzová *et al.*, 2011). The increase in the activity of GST reported in the present study indicates the biotransformation pathway used a protective response in fish towards exposure to an oxidative stress induced by agro-chemicals (Wengu *et al.*, 2009; Dabas *et al.*, 2012 and Anushia *et al.*, 2012).

Among the enzymes that compromise the defence system against toxicity also includes superoxide dismutase (SOD) and catalase (CAT) (Otitoju, 2005). Superoxide dismutase, the first enzyme in the line of antioxidant defense, responsible for catalyzing the conversion of the superoxide ions into water and molecular oxygen via catalase. Antioxidant enzymes are used by the organisms as natural endogeneous protection against the generation of ROS (Metwalli and El-Megd, 2002). SOD catalyses the destruction (dismutation) of superoxide free radicals produced during oxidation of pesticide (Otitoju and Onwurah, 2007). The action of SOD therefore results in the protection of the biological integrity of cells and tissues against the harmful effects of superoxide free radicals (Van der Oost *et al*., 2003). To ameliorate the damage caused by the hydroxyl radicals formed from superoxide radical and hydrogen peroxide, organisms have evolved mechanisms to regulate the concentrations of the two reactants. The present study revealed that SOD and CAT activities in the liver, kidney and gills of *O.mossambicus* and *L.rohita* exposed to agrochemicals increased significantly. Thus, the induction of SOD and CAT may be a physiological adaptation for the elimination of ROS generation (Gad, 2011). As reported by (Sadekarpawar *et al.*, 2014), an increased in SOD is followed by a parallel increase in CAT, since both enzymes are linked functionally and occur in tandem. Similar results have been observed in the *Sparus aurata*, *Oreochromis mossambicus*, *Labeo rohita* and *Carassus auratus* (Gull *et al.*, 2004; Zhang *et al.*, 2004; Sun *et al.*, 2006; Correia et al., 2007; Sivaperumal, 2008; Zaidi and Soltani, 2010). Considering the results for each tissue, it was found that the liver showed the highest SOD and CAT antioxidant activity compared to kidney and gills. Both enzymes appeared to have an important role in combating the generation of superoxide radical ($O2^-$) and hydrogen peroxide (H_2O_2) from the intense metabolic activity characteristic of liver.

All the major biomolecules like lipids, proteins, and nucleic acids may be attacked by free radicals, but lipids are probably the most susceptible to peroxidative damage (LPO) (Ray and Akhtar, 2002). LPO has been identified as one of the basic deteriorative reactions in cellular mechanisms of the agro-chemical induced OS in fresh water fishes (Vardharajan, 2010). A dose dependent increase in the level of LPO was observed in liver, kidney and gills of the fish exposed to IMI and CZ. The elevated levels of LPO in the gills and livers of in response to 21 days of exposure agrochemicals that were observed during the present study suggest that production of ROS is increased, which could be associated with the metabolism of the agrochemicals leading to the peroxidation of membrane lipids in the tissues. Researchers have reported previously LPO induction by pesticides such as alachlor (Peebua *et a.l*, 2007), malathion (Chandra 2008), and butachlor (Farombi *et al.* 2008) in fishes. Our results are also corroborated with previous studies reported by other investigators (Oruc, 2010; Kavitha and Rao, 2008; Sharbidre *et al.*, 2011; Lopez-Lopez *et al.*, 2011). The impairment of enzymatic antioxidant system can facilitate the accumulation of free radicals that might be responsible for increased lipid peroxidation with agrochemical exposure.

CONCLUSION

Thus, from the present study it can be concluded that the response of antioxidant enzymes (SOD, CAT, GPx, and GST) and non-enzymatic antioxidant/scavengers (AA and GSH) confirmed that the teleosts are under severe oxidative stress. The enzymatic and the non-enzymatic antioxidant machinery are interacting in a concerted manner to eliminate ROS and prevent damage to cellular components. This suggests that IMI and CZ at levels below median lethal concentration are capable of causing oxidative damage in *O. mossambicus* and *L. rohita*.Nevertheless, concerted efforts in reducing the use of agrochemicals and

implementing natural remedies for pest encroachment through organic farming can help in resolving the problems of agrochemical pollution.

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RESEARCH ARTICLE

A COMPARATIVE ASSESSMENT OF TRACE METAL ACCUMULATION IN *OREOCHROMIS MOSSAMBICUS* AND *LABEO ROHITA* EXPOSED TO PLANT NUTRIENT LIBRELTM

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ABSTRACT

Metal pollution from multifarious sources like effluents from industries, agricultural runoff and untreated sewage system has adverse effects on aquatic ecosystem. Metallo-pesticides, including insecticides, fungicides, and herbicides are also known to contain various metals that can increase metal accumulation. 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. The study correlated the level of metal ions (Fe, Cu, Zn, Mn) in tissues and in water in a time dependent as well as dose dependent manner in both the species of teleost (O.mossambicus and L.rohita). Further Bioconcentration factor added metal based affinity towards tissue. Hence, the study gears up and proves that among both the fishes tested on exposure to micronutrient mixture, O.mossambicus was found to be more sturdy i.e it was able to with stand more metal load compared to L.rohita in a time dependent manner.

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 bio accumulate in aquatic ecosystems (Censi *et. 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). Metal pollution from multifarious sources like effluents from industries, agricultural runoff and untreated sewage system has adverse effects on aquatic ecosystem. Intensification of agriculture practices to meet the demand has resulted in increased release of a wide range of agrochemical compounds to the environment (Desai and Parikh, 2013). 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 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). 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). 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).

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, and 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 commercial food during acclimation and experimental period. Acclimatized fishes were exposed to water containing the test chemical at the concentration of 300 mg/L (1/20th of LC50 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 every day 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:

Bio-concentration factor (BCF) =Concentration of M in dry fish tissue (mg/kg)

Concentration of M in water (mg/L)

MPI = (CF1x CF2 x----- CFn)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.

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

The result of metals determined in the water at different exposure periods are presented in Table I-VII 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 (I-IV). 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. Fe exhibited highest concentration in liver of both the fishes. Individual metal concentration assessment exhibited higher values of Fe in *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±2.33mg/Kg). Organ wise accumulation that followed the order for Zn concentration was 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. Mn exhibited the least concentration, where *L. rohita* (131±3.60 mg/Kg) accumulated higher Mn compared to *O. mossambicus* 3.50±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 Table I-IV 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 coefficient and data is presented in the form of a matrix (Table VI&VII).

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 showed negative correlation with each other. In the Liver of 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 a negative correlation with each other.

Table I Concentration of Zn in water and selected tissues of fish exposed to sub lethal 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 II Concentration of Fe in water and selected tissues of fish exposed to sub lethal 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 III Concentration of Cu in water and selected tissues of fish exposed to sub lethal 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 IV Concentration of Mn in water and selected tissues of fish exposed to sub lethal 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.

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 kidney Zn, Fe and Mn showed positive correlation with each other 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). 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 V 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

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).

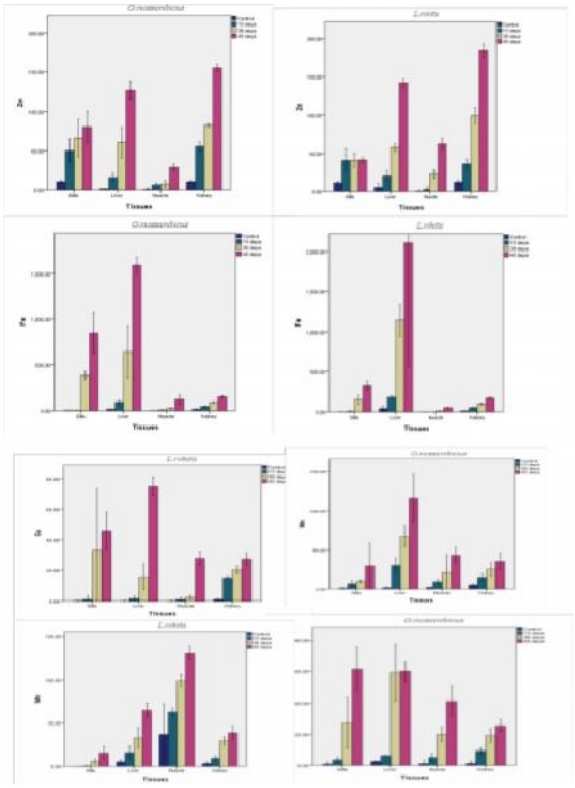

Figure 1 Average concentration of various trace metals in the tissues of *Orechromis mossambicus* and *Labio rohita* exposed to plant nutrient Liberal. (a) zn concentration (mg/kg) in the tissues of *Orechromis mossambicus* (b) zn concentration (mg/kg) in the tissues of *Labio rohita* (c) Fe concentration (mg/kg) in the tissues of *Orechromis mossambicus* (d) Fe concentration (mg/kg) in the tissues of *Labio rohita* (e))Cu concentration (mg/kg) in the tissues of *Orechromis mossambicus* (f) Cu concentration (mg/kg) in the tissues of *Labio rohita* (g) Mn concentration (mg/kg) in the tissues of *Orechromis mossambicus* (h) Mn concentration (mg/kg) in the tissues of *Labio rohita.*

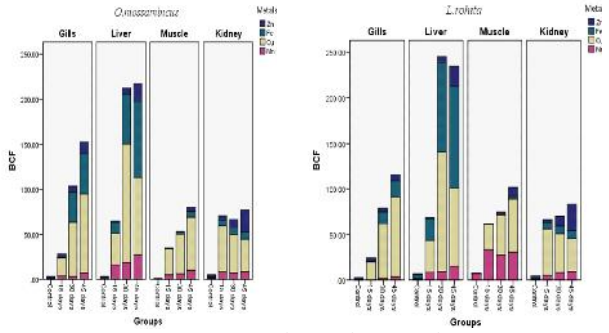

Figure 2 Bio concentration factor of tissues of *Orechromis mossambicus* and *Labio rohita* exposed to plant nutrient Liberal. (i)BCF of tissues of *Orechromis mossambicus* (j) BCF of the tissues of *Labio rohita*

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, 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, Jezierska and Witeska 2006, Nimick et al., 2007, Akan et al., 2012, Naz and javed, 2013). 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 Voigt, 2004 Javed 2005) who observed significantly higher accumulation of metals in *L. rohita* than *C. mrigala* and *C. catla.* Moreover, a variety of species of fish from the same water body are also reported to accumulate different amounts of metals (Senthilkumar and Sajwan, 2007; Sajwanm *et al*., 2008). Metal distribution in various organs is time-associated (Eggleton and Thomas, 2004).

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. In the current study both fishes showed a time dependent significant increase in the metal concentration in all the tissues. These differences result from different affinity of metals to fish tissues, different uptake, deposition and excretion rates (Giguere *et al.,* 2004). Of all the tissues, liver has shown the highest level of accumulation of metals followed by gills while kidney and muscle have shown the least level of accumulation. Significantly higher levels of all metals in fish liver can be related to the binding of metals to metallothionein that provide detoxification mechanism (sensi *et al.,* 2010). As reported by Szarek-Gwiazda *et al.,* (2006) that along with the species specificity the trace metal concentration and their binding capacity vary with tissues too. 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 and as the gill surface is negatively charged it provides a potential site for gill-metal interaction for positively charged metals. As reported earlier fish can absorb ions through gills, since they have special salt secreting cells and 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) *in O.mossambicus* (Naigaga, 2002) in *Tor putitora* (Shakoori, and Yousafzai, 2006). Metal concentrations in the kidneys rise slower than in liver, hence the low concentration of ions in the kidney is the present work is well justified. Kidney is also one of the active metabolically important organs next to liver. Metal uptake and binding has been reported to increase with increase in the metabolic rate (Green & Knutzen, 2003 and Voigt, 2004). The present data corroborates with the studies of metal accumulation in the kidney of *O. niloticus* (Abdel- Baki *et al.,* 2011), *Onchorynchus mykiss* of *Carassius auratus and Cyprinus carpio* respectively (Boeck *et al.,* 2004).

Muscle tissue exhibited the least accumulation of all the tissues, This result are in agreement with many authors who reported that muscles is not an active organ in accumulation of most heavy metals (Yilmaz *et al.*, 2007 and Kraiem 2007, Khalil and Faragallah, 2008;). 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. 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. Metals are non-biodegradable, and once they enter the aquatic environment, bioconcentration may occur in fish tissue by means of metabolic and bio sorption processes (Yousafzai and Shakoori, 2008; Kaoud and El-Dahshan, 2010). From an environmental point of view, bio concentration 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 (sensi *et al.*, 2010). Species difference in heavy metals bio concentration is linked to difference in feeding habits and behaviour of the species (Altindag and Yigit, 2005). The variability observed in the fish species is a reflection o

f 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. 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.

CONCLUSION

The overall results from the metal accumulation studies have proved that the metal accumulation was time dependent, species specific and organ specific. Moreover, of the two species it was observed that *O.mossambicus* was able to withstand the metal load more compared to *L.rohita*.

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