Concise Summary

Due to rapid increase in globalization and industrialization the use of agrochemicals for yielding a good variety of crop has raised an alarming situation in normal ecosystem. This use of agrochemicals is due to increase in population which is a worldwide scenario (Parikh *et al.*, 2010). These are used either in the form of insecticide, plant nutrient, fungicide etc. for a better yield of crops, but it affects the non target organism of terrestrial as well as aquatic origin. This is because it easily gets through the agricultural runoff and enters into the nearby water body i.e. lake, pond or river. Agrochemicals are also known to act as EDC in the aquatic ecosystem. Generally, agrochemicals which act as EDCs can work through three mechanisms of action: (i) Agonistic/antagonistic effect ('hormone mimics'), (ii) Disruption of production, transport, metabolism or secretion of natural hormones, and (iii) Disruption of production and/or function of hormone receptors. This classification indicates discrete and separate routes of action of an EDC, depending on the properties of the compound responsible. Studies on estrogenic activities of EDCs have been carried out to monitor the reproductive fecundity for e.g. hermaphroditism and estrogenic responses in rainbow trout; increased blue sac disease and early life stage mortality in Great Lakes salmonid populations alterations in endocrine homeostasis and reproductive fitness such as reduced gonad size, egg size and fecundity and delayed sexual maturity (Hoeger *et al.,* 2005; Zorriehzahra, 2008); depression of plasma sex steroid hormone levels and inhibition of gonadal development (Singh *et al.,* 2010). While many of these responses have been recognized for more than a decade, uncertainty remains with respect to the mechanisms by which these agrochemicals affect reproduction and development. In certain cases, the identity of the responsible chemicals remains unknown; also, when endocrine disruption has been noted, it is not know if this is primarily due to disruption of endocrine homeostasis or if it is the result of other mechanisms of toxicity (Ankley et al., 2009).

The endocrine and reproductive effects of these agrochemicals are believed to be due to their ability to: mimic the effect of endogenous hormones, antagonize the effect of endogenous hormones, disrupt the synthesis and metabolism of endogenous hormones and disrupt the synthesis and metabolism of hormone receptors. They may also bind to these receptors without activating them; this antagonistic accomplishment blocks the receptors and inhibits their action.

Finally, they may also interfere with the synthesis, transport, metabolism and elimination of hormones, thereby decreasing the concentration of natural hormones. The intricate relationship between brain and endocrine action allows adaptability of an organism to the environment while maintaining homeostasis. In vertebrates, the hypothalamus represents a master regulator of homeostasis and is the critical nexus between the nervous and endocrine systems. The hypothalamus mediates responses to homeostatic imbalance mainly through regulation of the pituitary gland, which, in turn, produces hormones that are able to affect systemic change, for example within the gonad. The central role of the hypothalamic–pituitary (HP) axis makes it particularly susceptible and sensitive to perturbation by a variety of environmental contaminants. Chemical disruption of the HP axis will often result in modifications of circulating hormones, leading to an inability to mitigate environmental stress, as well as, direct impacts on reproduction and development, which has been demonstrated to produce population-level impact on fish.

Fishes are an important part of aquatic ecosystem as they interact closely with physical, biological and chemical environment. Fishes provide food source for other animals such as birds, mammals and thus form an integral part of the food web. A lot of research has been carried out to examine the impact of agrochemicals on different aspects such as changes in fish behaviour haematological changes, histopathological alterations, biochemical modifications and enzymes alteration and changes in antioxidant defence system.Previous studies in our lab on different agrochemicals have proven a negative correlation of toxic effect of it to the growth of fish. ***To our knowledge, a comprehensive assessment of gene expression changes in relation to agrochemicals and hypothalamus-hypophyseal impairment in O.mossambicus is lacking. So, the present study is designed to validate the effect of agrochemicals (Imidacloprid-IMI, Curzate-CZ, Pyrazosulfuron ethyl-PE and Micronutrient Mixture-MN) on neuroendocrine regulation in O.mossambicus.***

The hormonal status in the *O.mossambicus* on exposure to agrochemicals was first checked. The fishes were divided into 5 groups: Control as well as CZ, PE, MN and IMI exposed groups. The exposure of the agrochemicals was for 14 days. At the 15^{th} day fishes were sacrificed and blood was collected by the tail ablation method and was stored at -20 °C. The blood was centrifuged and plasma was separated and subjected to analysis of Cortisol, Triidothrionine (T_3), Thyroxine (T_4), Estradiol (E_2) and 11-ketotestosterone using ELISA based kit method. Sublethal exposure of agrochemicals resulted in a decrease in cortisol concentration in response to agrochemical exposure. CZ and IMI resulted into a significant decrease ($p < 0.05$) in comparison to the normal control group while, Mn and PE showed no significant alterations. the observed reductions in the plasma cortisol level is an indication of interrenal exhaustion and an adaptive stress response of the fish by way of maintaining low basal metabolic rate (BMR) under agrochemical toxicity.

Impairment of thyroid function was demonstrated by the significant decrease ($p < 0.01$) in plasma T_3, T_4 and TSH levels on CZ and IMI exposure. Thyroid titer in PE exposed group resulted into a significant increase ($p < 0.05$) in T_3, T_4 and TSH level. An insignificant decrease in T_3, T_4 and TSH level in MN exposed group compared to the normal control group. Reduced thyroid titers on exposure of CZ and IMI is mediated through direct or indirect effects via specific nuclear receptors and increase in T_3, T_4 and TSH levels in the plasma of *O. mossambicus* following PE exposure probably suggests the onset of an increased metabolic activity triggered by the pesticide exposure.

As far as sex steroids are concerned, agrochemicals exposure resulted into a significant increase ($p < 0.05$) of plasma estrogen and testosterone levels in CZ exposed group of fish. PE exposure resulted into significant increase (119%) in estrogen with a decrease testosterone in comparison to the normal control group. On exposure of IMI and MN the estrogen titer was found to decrease whereas, there was an increase in the titer of Testosterone in comparison to the normal control group. Overall, the set of responses suggests that the PE is androgen antagonists and estradiol agonists, whereas, IMI and MN are androgen agonists and estradiol antagonists and the mode of action of CZ suggest that in agonists to estradiol as well as testosterone (**Chapter I**).

After the hormonal assay alterations, next aim was to check the candidate gene expression pattern of HPG, HPI and HPT axis. On 15^{th} day, the fishes were sacrificed and tissue specific gene expression pattern was checked. In brain, the marker genes, such as GnRH-I, kiss 1 and kiss 2 was analyzed. Among all the agrochemicals exposed, a significant reduction in GnRH-I and Kiss 2 mRNA expression was obtained exposed to PE, While IMI, MN and CZ resulted in an increase in expression of GnRH-I and kiss 2. In fish GnRH-I is known to be regulated by negative feedback of circulating hormonal levels through its receptor located in hypothalamus and pituitary. PE being a herbicide belonging to the group of Sulphonyl urea has elevated the levels of hormones (Estradiol and Testosterone Chapter I) which directly confirms the mechanism, suggesting that the GnRH-I fibers present in the pituitary, probably through its primary hypophysiotropic role has a strong correlation between GnRH-I expression in brain and gonadal activity.

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. MN exhibited the maximum alteration in the Kiss2 gene expression pattern, possibly due to the nature of MN, which is an amalgamation of trace metal ions (Zn^{2+},Fe^{2+},Cu^{2+},B^{+},Mn^{+}) proposing the synergistic or individual action of metal ions. IMI belongs to neonicotinoid group, is known to exert its effect by blocking the actylcholinesterase activity in brain. In the present study, the increase in GnRH-I and Kiss2 proves the genotoxic potential of IMI in altering the activity of gonads and thereby on reproduction apart from its usual mode of action.

A significant down-regulation of kiss 1 under PE exposure, suggests its non-essential role for reproduction. However, the exact mechanism by which it happens is still illusive. Immune-histochemical and cloning studies will be able to shed more light on the same. These results thereby suggest that of all the agrochemicals; PE, IMI and MN are capable of interfering with kiss 2 and GnRH system thereby altering the HPG axis. Western blot analysis was done to confirm the expression of kiss 1 in *O.mossambicus*. The study has confirmed the expression of the kisspeptin for the first time. However its role in HPG axis still needs to explored.

*T*he expression pattern of *GtH-Ir* was studied in ovary and testis, under all the exposure the ovary and testis exhibited an up regulation in *GtH-Ir* in IMI and CZ group. *GtH-IIr*, up regulation was observed in ovary and testis for all the class of agrochemicals, but the significant change (*$p<0.05$) was noted under the exposure of MN and IMI. An up regulation in GtHs suggest either an operation of kiss 2 or PKC mediated pathway leading to an increase in GtHrs culminating into either vitellogenesis/spermatogenesis in the gonads.

ER-I and ER- II expression resulted into a significant up regulation (*$p<0.05$) on exposure of IMI, MN and CZ in brain, ovary and testis. While, PE exposure resulted in an in significant down regulation of ER I expression in brain, ovary and testis. Nonetheless, this pattern of expression was not true for ER-II, which resulted in a significant (*$p<0.05$) up regulation under the influence of PE in ovary, with an in significant up regulation in testis and brain. CZ being the mixture of cymoxanil and mancozeb, has resulted into constitutive receptor activation leading to its up regulation, which may be due to its mimicking action as that of estrogen. Apart from the conventional studies done on various groups of herbicide, 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.

In teleost estrogen signaling is mediated through three ER subtypes and each subtypes is likely to show differential responses (cAMP, MAPK, directly activation of transcription factors) to ligands which ultimately results in a deleterious effect on those pathways to affect the physiological functions. In the present study, there was a down regulation of MAPK suggestive of activation of either of the two canonical pathway. However, full elucidation of mechanistic molecular pathways by which agrochemicals are modulating the estrogen signaling requires a better understanding of distinct roles of each ER subtypes.ARI and ARII was found to be significantly (*$p<0.05$) increase on exposure of CZ in brain, ovary and testis with a parallel increase on exposure of IMI only in ovary, probably impairing the gonadal function which has lead to 17 B estradiol (E2)/ 11-keto testosterone (11kt) imbalance

Furthermore regression analysis was done to check the interrelationship of kiss2 gene with candidate HPG axis genes. IMI exposure resulted in significant association of AR-II with kiss-2 gene expression ($R^2=0.93$,*$p<0.05$), similarly PE exposed groups also showed significant association of GnRH-I, GtH-Ir, ER-I, AR-I and AR-II with kiss 2 mRNA expression ($R^2>0.9$,*$p<0.05$). Fishes exposed to CZ showed significant dependency of GnRH-I, GtH-IIr and AR-II on Kiss-II with a $R^2>0.93$,*$p<0.05$, while MN groups also showed dependency of GnRH-I, GtH-Ir, AR-II on kiss 2 genes ($R^2>0.92$,*$p<0.05$).

The candidate gene studied for HPI axis was Glucocorticoid receptor (GR) in vital tissues (liver, brain, kidney, gills, thyroid, ovary and testis). Of all the agrochemicals, only IMI exposure resulted in a significant (*$p<0.05$) down regulation of GR in all the tissues along with a significant (*$p<0.05$) down regulation in testis and ovaries of fish exposed to CZ. The exposure of IMI and CZ resulted in sensitization of cortisol receptor and may be its peptide (cortisol), substantiating the receptor down regulation probably due to its self regulation.

In case of HPT axis, TSH-βr was studied in thyroid tissue where a significant (*$p<0.05$) up regulation was noted under the exposure of PE, while IMI showed up regulation of receptor expression but was non-significant. In contrast, exposure of CZ and MN resulted in a in significant down regulation of TSH-βr. Hence from the present study one can conclude that the agrochemicals have resulted into an overall alteration into HPT axis thereby affecting the overall synthesis or release of the thyroid hormones.

So from the present work it can be concluded, agrochemicals exposure invoked alterations in the expression of genes associated with HPG, HPI and HPT axis. A strong link is thus determined between the measured up regulation/down regulation of specific genes and indicates the potential of using gene expression in toxicological studies as markers. Furthermore, the result of this study illustrates the potential risk of agrochemicals to non-target organisms in aquatic environment and indirectly to human health. However, immune-histochemistry, micro array and cloning of the downstream genes will provide insights into better understanding of the mechanisms governing the effect of agrochemicals on the canonical pathways of the three axis (**Chapter II**).

The information gathered from the present investigation is thus valuable and has probed the extent to which the agrochemicals have an impact at genetic level. These data can be especially important in aquatic systems for which the fate and effect of many chemicals are largely unknown.

The gene expression studies have postulated the possible targets of agrochemicals; however with the help of advance technologies the exact target based mechanism can be validated:

- Like microarrays can be done because they gauge changes in the expression of a large suite of genes, allowing simultaneous screening of multiple biological processes. Thus, gene microarrays will help to detect biological responses.

- Tissue localization of candidate markers can be done with the help of Immuno-histochemistry which will throw more light on the detection of biomarker in *O.mossambicus* on exposure to the agrochemicals.

- Sequencing analysis can be done to screen out the possible point mutations occurring in the promoter region of the genes. Further, *in-situ* hybridization can be employed for specific localization of DNA/RNA sequences to screen out possible mutations.