7. SUMMARY & CONCLUSION

Present thesis embodies an account of impact of chlorpyrifos toxicity on different concentrations and exposures on *Ctenopharyngodon idellus* (Cuvier & Valenciennes) by assessing behavioural, morphological, histopathological, ultrastructural and biochemical alterations, along with pesticide residue analysis in liver, kidney and gills of the fish.

Chlorpyrifos, a widely used insecticide, is the second largest selling agrochemical in India. Unfortunately, this organophosphate has wide range of hazardous effects on non-target animals, including fish when present in aquatic environments.

*C. idellus* is a fast growing culturable carp, is also used to control aquatic weeds. It is an excellent model for bioassay studies because of its hardy nature and easy maintenance in laboratory.

The fish have been brought from Nanoke Fish Seed Farm, Patiala (Pb), acclimatized in glass aquarium for fifteen days and were subjected to acute and chronic toxicity tests:

A) **Acute toxicity test**

The static toxicity tests were performed for 96 hr to calculate LC$_{50}$ of chlorpyrifos for *C. idellus* using Probit analysis (Finney, 1980) and has been found to be 7.24 µg/l.

B) **Chronic toxicity test**

For chronic toxicity tests, the fish were exposed to 1/3$^{rd}$ (2.41 µg/l) and 1/5$^{th}$ (1.44 µg/l) of LC$_{50}$ of CPF as sub-lethal concentrations for 15, 30 and 60 days. Toxicity test had been aimed to observe various responses in *C. idellus* exposed to CPF:
Summary

a) Morphological studies

The morphological changes in fish exposed to CPF include:

- De-pigmentation and loosening of scales
- Secretion of copious amounts of mucous
- Slight caudal bending and leaning of body towards abdomen
- Blood coagulation in cephalic region in some fishes.

b) Behavioural studies

Behavioural alterations on exposure to CPF include:

- Irregular, erratic and darting movements
- Loss of equilibrium
- Hyper excitability
- Decrease in opercular movements
- Increase in fin movements, air gulping
- Reduced feeding

c) Histopathological studies

Histopathological studies were made on gills, liver and kidney of the exposed fish to characterize the nature of damage caused by chlorpyrifos.

The gills on exposure to CPF for 15 and 30 days at lower concentration showed partially affected secondary lamellae with shortening and curling, increased inter-lamellar space, sloughed off epithelium, hyperplasia and necrosis in pavement cells. At higher concentration, lifting of epithelium form basal membrane, necrosis of epithelial & pillar cells, distortion of secondary lamellae and increased vacuolization have been observed. At some places, primary filament got detached from the base of its attachment to gill arch. On 60 days exposure, more pronounced degenerative changes in the gills, including shortening of secondary lamellae, necrosis, hyperplasia,
hypertrophy of pavement cells, erosion & wasting away of lamellar epithelium, breakage or completely obscured cartilaginous bar and marked up-liftment of epithelial membrane have been noticed.

The histopathological alteration index (HAI) of gills showed necrosis, shortening and curling of secondary gill lamellae as the major lesions. The highest mean assessment value of lesions was of necrosis, followed by deformation of SGL, while the lowest has been observed for aneurism. The plot made on the basis of PCA showed direct relationship between histological alterations observed at different exposures and toxicants concentrations.

Histopathology of liver of CPF exposed fish revealed mild cloudy swelling and necrosis of hepatocytes, sinusoidal dilation, vacuolization and constriction of blood vessel at 15 and 30 days exposure. These alterations progressively intensified at higher concentration and resulted in vacuolar degenerative changes, dilation of sinusoidal spaces and infiltration of RBCs in them, ultimately leading to cell injury. After 60 days exposure, liver of the fish got badly damaged with infiltration of RBCs, hypertrophy, sinusoidal dilation, severe necrosis in the form of karyolysis, pycnosis and karyorrhexis, vacuolar degeneration and intercellular edema.

HAI of liver of the fish revealed main lesions to be necrosis, pycnosis, vacuolization, sinusoidal dilation and nuclear alterations. The highest mean assessment value of lesion has been found for pycnosis, followed by necrosis, while the lowest has been for infiltration of RBC’s. Plot made on the basis of PCA depicted relationship between necrosis and nuclear alteration.

Histoarchitectural damage in kidney of treated fish illustrated contracted glomerulus, gap between the parietal and visceral epithelium, obstructed lumen of proximal convoluted tubule due to the invasion of nuclei of its ruptured epithelial cells, slight cytoplasmic vacuolization and progressive degeneration of renal tubules on 15 and 30 days exposure. After 60 days exposure, kidney of the fish showed pronounced damage in the form of increased disintegration of glomerulus and its visceral layer, enlargement of bowman’s space, vacuolated proximal tubules with
Summary

pycnotic nuclei, severely necrotic tubular epithelium, dwindling of tubular lumen at some places, ruptured basement membrane of renal tubules and hypertrophy & necrosis of hematopoietic tissue.

From HAI of kidney of *C. idellus*, it could be interpreted that the main lesions include necrosis, vacuolization and contracted glomerulus. The highest mean assessment value of lesion has been observed for necrosis and vacuolization, while the lowest for atrophy. A relationship exists between necrosis, vacuolization and contracted glomerulus as depicted by PCA.

On the basis of total histopathological alteration index of liver, kidney and gills of the fish, the order of histopathological damage has been found to from Liver > Gills > Kidney.

d) SEM studies

In order to ascertain ultrastructural damage caused by the pesticide on liver, kidney and gills of the treated fish, electron microscopic investigations were made.

Studies on gills of the fish exposed to CPF for 15 and 30 days revealed curling and shortening of secondary lamellae, wrinkled epithelial surface, gradual necrosis of lamellar surface, cracks on gill raker and sloughing off epithelium. On 60th day exposure of the fish, direct effect of the toxicant in the form of raised epithelium of primary filament and gill arch has been noticed. These could be due to wrinkled epithelium.

The liver showed necrotic and swollen hepatocytes, loss of fenestration, irregular structure of bile canaliculi and sinusoidal dilation on 15th and 30th day of toxicant exposure. After 60 days exposure period, sinusoidal vacuolization increased with damaged central vein, necrotic and swollen hepatocytes and blebbing on their surface.

Ultrastructural alterations in kidney of the fish exposed include contracted glomerulus, ruptured and necrotic renal tubules on 15th and 30th day exposure. After 60 days exposure, the glomerulus got contracted and degenerated severely. The
vacuolization and necrotic renal tubules increased with increased dosage as well as duration.

SEM studies made on the scale of CPF exposed fish revealed considerably deformed focus and breakage of lepidonts. Some lepidonts have been found to be completely separated or ruptured at 15 and 30 days exposure. Whereas in fish exposed for 60 days, the scale depicted marked cracks and breaks in circuli, damaged radii and tubercles.

The histopathological and ultrastructural alterations in liver, kidney and gills of the fish had a direct correlation with toxicant concentration and exposure time.

The phenotypic alterations observed in erythrocytes in the exposed fish for 15 and 30 days showed the presence of spherocytes, echinocytes, ellipto-echinocytes, rhomboidal shaped cells, fusiform cells and discocytes. There was more clumping, shrinkage and development of lobopodial projections due to protrusion of cytoplasm in the form of protuberances from the surface of cell. On 60th day, more pronounced effects have been observed in the shape of erythrocytes. Fusiform cells, rhomboidal cells, stomatocytes, acanthocytes, discocytes, ovalocytes, and cells with bud formation and folded membrane has also been noticed.

e) TEM studies

On 15 and 30 days of the exposure of the fish to CPF, pavement cells had distorted shape of nucleus and mitochondria and marked contraction of cisternae of GC occurred. In chloride cells, there were abundant mitochondria with distorted shape and size, dilation in between their cristae and tubular network with mild fragmentation. Whereas at higher toxicant concentration, pavement cells showed vacuolization in intercellular spaces, irregular shaped nucleus, formation of vesicles, loss of cisternae and flattened sacs in golgi complex. MRCs had deformed mitochondria in shape and size along with interspersed fragments of tubular network. The severity of damage was more profound at 60 days exposure. The pavement cells were found with completely deformed nucleus, ruptured outer membrane, scattered
cytoplasmic content, flattened cisternae & dilated sac of golgi complex. Chloride cells depicted severely fragmented tubular network, misshaped mitochondria with loss of cristae and fragmented endoplasmic reticulum with scattered cisternae.

Marked hepatic ultrastructural alterations in CPF treated C. idellus at 15 and 30 days exposure include deformed nuclei surrounded by a cluster of clumped mitochondria, cytoplasm characterized by swollen misshaped mitochondria with vacuolization and distorted cristae and dilated & fragmented RER, appearance of small glycogen granules, large number of lipid droplets, along with few lysosomal elements in hepatocytes. At higher concentration of CPF, the alterations in the cellular components were relatively more profound with microvilli in the perisinusoidal area, perisinusoidal stellate cell, numerous dilated RER in the cytoplasm of the hepatocytes forming coiled circular structures and granular ribosomes scattered in the cytoplasm. On 60 days exposure, alterations such as formation of crescent shaped nucleus along with condensed chromatin, severe fragmentation of ER, vacuolization, scattered glycogen granules, severe mitochondrial malformation (clumped mitochondria with loss of cristae, vacuolization of outer membrane of mitochondria, condensation of its matrix), and disorganization of RER and loss of ER in some cells were observed.

Studies on kidney of CPF treated fish on 15th day exposure depicted major alterations in tubular epithelial cells such as clumped and swollen mitochondria with loss of cristae & defragmented basilar invaginations around it, dilated cisternae of ER and golgi complex. At 30 day exposure, shrinked nucleus with the condensation of chromatin, clumped and swollen mitochondria with loss of cristae at some places, frequent appearance of deformed mitochondria and some of them became elongated, and loss of cristae have been noticed. Alterations in kidney on 60th day at higher concentration of CPF were severe damage of epithelial cells characterized by degeneration of nucleus, vacuolization in the cytoplasmic matrix and intercellular spaces, autophagic vacuoles, severely misshaped mitochondria with loss of cristae, dilated and highly fragmented ER and dilated sacs & cisternae of golgi complex.
f) **Biochemical studies**

In all the organs, significant decrease in protein and glucose content has been seen throughout the experiment with increase in exposure period and concentration of the pesticide. Depletion in protein content indicated the requirement of large amounts of protein under a toxic stress to compensate the energy demand and the metabolic requirements.

AChE activity got inhibited with increase in the exposure period and chlorpyrifos concentration in liver, kidney and gills of the fish. Maximum inhibition by 15.05 folds has been noticed in liver on 60th day exposure at higher concentration of the toxicant. AChE Inhibition in all the tissues especially in liver and gills revealed the potency of the chlorpyrifos to impair the synaptic transmission mechanism.

The remarkable changes in activity of LDH suggested the impaired oxidation of carbohydrates through TCA cycle. Variations in ALT, AST, ACP and ALP enzyme activities indicated the role of the tissue in detoxification processes under chlorpyrifos toxicity and tissue injury. The toxicity resulted in impaired metabolism leading to disturbed homeostasis.

Fluctuating levels of antioxidants (LPO and GSH) and activity of antioxidant enzymes (CAT, SOD, GST) on exposure for 60 days showed the efficiency of antioxidant system to defend the insecticide-induced stress and the inability of the organism to overcome the toxic effect of CPF for long exposure.

The results suggest that the exposure of CPF enhanced ROS production in liver, kidney and gills of *C. idellus*, and antioxidant defense system was incapable of scavenging the generated ROS. The fluctuation in CAT and SOD activities in liver, kidney and gills of the fish indicates an elevated antioxidant status attempting to neutralize the adverse effect of ROS.

Considerable decline in tissue GSH level during exposure to CPF might be due to an increased utilization of GSH, which get converted to oxidized glutathione. Depleted GSH level indicated enhanced risk of oxidative stress due to reduced cell
protection ability. Toxicant exposed fish tissues try to remove the ROS by direct conjugation with GSH or by means of GST. The observed declined GSH activity also indicates the exhaustion of phase II biotransformation, confirmed by the fluctuating GST activity in liver, kidney and gills of the fish.

**g) Pesticide residue analysis**

The residue level of CPF in liver, kidney, gills and muscles of *C. idellus* for different exposures periods was evaluated.

The residue level of the CPF in liver, gills, kidney and muscles of *C. idellus* on 60th day at higher concentration has been found to be 679.3 µg/l, 370.9 µg/l, 255.6 µg/l and 184.3 µg/l respectively with increase in the following order: Gills > Liver > Kidney > Muscles.

**Conclusion**

Based on 96 hr LC50, it has been observed that *C. idellus* is more sensitive to chlorpyrifos. This value is of great practical utility for regulating the discharge of CPF from any of the source into fresh water bodies, for protecting the fish life at early stages, so that the fish production may be maintained and increased.

Histopathological alterations observed in liver, kidney and gills of the fish support biochemical changes and gas chromatographic findings of increased levels of chlorpyrifos residue in the organs.

Ultrastructural changes observed in liver, kidney and gills of the fish could be correlated to corresponding alterations noticed in the histopathology and activities of marker enzymes and antioxidants. At both toxicant concentrations, activities of dehydrogenase and alanine aminotransferase as markers of cytosolic glycolysis and protein metabolism got altered, indicating disturbances to the cellular metabolism. Thus, the observed cytopathological lesions of tissues can be used as sensitive biomarkers for chlorpyrifos contamination. Particularly, correlation exists between aminotransferases activity and mitochondrial integrity in the tissues, as any
modification in mitochondrial structure is bound to alter associated enzyme system, further confirmed by TEM.

Decreased phosphatase (ACP & ALP) activity in liver, and elevated activity in kidney and gills could be attributed to altered structure and integrity of cell organelles as evidenced by TEM studies; and as a consequence of oxidative stress.

The increase in LPO level could be the result of impairment in antioxidant enzymes due to enhanced ROS formation, resulted in cell membrane damage and cellular dysfunction. This is further evidenced by ultrastructural alterations like loss of functional unit of mitochondria, peroxisomes and endoplasmic reticulum in liver, kidney and gills of the fish.

The findings revealed different tissue responses in liver, kidney and gills of the *C. idellus* exposed to chlorpyrifos. The initial increase in SOD activity might be due to increased generation and overproduction of reactive oxygen species, while decreased SOD activity was due to direct damage of its protein structure by pesticides and enhanced amount of hydrogen peroxide.

On the basis of fluctuation observed in antioxidants in different tissues of the fish exposed to pesticide, it could be concluded that GSH depletion seems to enhance the risk of oxidative stress due to reduction in cell protection ability as increased peroxidative overload could be the result of high SOD activity, thus helping to restore susceptibility and to adapt to oxidative stress. Present study revealed the tissue specific adaptive response to protect cell against the oxidative stress.

Gills have been found to be the most sensitive organ to oxidative stress in comparison to liver and kidney. These differences could be because of different rates of free radicals generation & different antioxidant potential in the tissues. The antioxidant system of the gills is not as robust as that of liver and kidney, which increases its vulnerability towards ROS.

In the present findings, the pesticide residue levels found in the organs studied can be correlated with increased levels of activities of alanine aminotransaminase and
Summary

alkaline phosphatase, especially in liver where the desulfuration of chlorpyrifos to chlorpyrifos-oxon takes place. Total histopathological alterations index also supports the gas chromatographic findings of increased level of chlorpyrifos residues in the organs. CPF residue has been found maximum in liver followed by gills and kidney, parallel to this, liver has been found to show maximum histopathological damage followed by gills and kidney.

Various alterations in liver, kidney and gills of the fish have been found to be directly correlated with toxicant concentration and exposure period.

It could be concluded that chlorpyrifos is a potent metabolic obstructer, AChE inhibitor, oxidative stress inducer to *C. idellus* even at sublethal concentrations and found to be highly toxic even at low concentration. Further, the findings reinforce the importance of histopathological analysis, ultrastructural and biochemical alterations, altogether serving as tool for a more precise evaluation of toxicological effect of chlorpyrifos.