Chapter 5

HISTOPATHOLOGICAL EFFECT OF MONOCROTOPHOS

Contents

5.1 Introduction
5.2 Materials and methods
  5.2.1 Processing of tissues
  5.2.2 Sectioning and staining techniques
5.3 Results
5.4 Discussion
5.5 Summary
5.1 Introduction

The increasing emphasis on the evaluation and monitoring of aquatic ecosystems has highlighted the need to set up appropriate biological indices for these locations. Fish diseases and histopathology, with a wide range of causes, are ever more being used as indicators of environmental stress since they present an explicit biological end-point of past exposure. The aquatic environment is a major sink for potentially hazardous pollutants emitted from agricultural, industrial and domestic sources. Inventory-based chemical monitoring programmes are restricted to identification of a limited range of contaminants and give no information on their biological significance. Recently, greater emphasis has been given to the assessment of the causal relationships between contaminant exposure and observable biological effects in aquatic organisms (De Flora et al., 1991).

In field studies fish can be used as a monitoring tool for the quality of the aquatic environment. Evidently this is done in wild animals, but also the use of semi-field (mesocosm) studies, and more recently, caged sentinels have shown to be successful (Vethaak and Wester, 1996; Harries et al., 1997; Vethaak et al., 2002). The use of fish eco-epidemiology has become predominantly developed in marine and fresh water pollution monitoring programs. Initially, larger animals were screened for visible disease signs/abnormalities on skin and liver in addition to, tissue residue concentrations of toxicants and change in metabolic biomarkers, but as more in depth information was required such as further characterization or screening for early pre-neoplastic changes, histopathology was introduced.

The tool histopathology helps in identifying target organs of toxicity and mechanism of action and this tool was materialised by combining knowledge and experience from fundamental fish biology (anatomy,
physiology, endocrinology), mammalian toxicological pathology and mammalian toxicology. At a quick look this would not seem to be of direct 'ecological relevance'. But other than the specificity of the induced effects histological monitoring has a better sensitivity compared to classical toxicological testing, since effects on the histological level will be visible at lower dosage, compared to toxicological endpoints such as mortality or behavioural changes. Considerable interest has been shown in recent years in histopathological study while conducting sub-lethal tests in fish. Tissue changes in test organisms exposed to a sub-lethal concentration of toxicant are a functional response of organisms which provides information on the nature of the toxicant.

It is generally understood that histopathological biomarkers are important as pointers of the general health of fish and they reflect the effects of exposure to a range of toxicants (Hinton et al., 1992) without any geographic or ecosystem limitations. Acute changes are seen when pollutant levels are amply high, while chronic duration is necessary to determine sub lethal aspects of change (Oost et al., 2003). Many of histological changes persist even after the toxicant exposure has ceased. So that organism's responses to prior toxicity can also be used to determine effects. Responses are relatively easily recognized, provided that proper reference and control data are available.

5.2 Materials and methods

Collection, transportation, acclimation and bioassay of/on *Heteropneustes fossilis* was performed, as described in the chapter I. Sampling was done on 21st day and fish were sacrificed by cervical disjunction. Liver, brain and gill tissues were dissected out and were washed to remove any debris.
5.2.1 Processing of Tissues

The tissue specimens were fixed in 10% neutral buffered formalin for twenty four hours washed thoroughly with water and dehydrated in a graded ethanol series, (20%, 30%, 50%, 70%, 90% and 100%) quickly. Tissues were kept in absolute alcohol for one hour and were cleared using xylene for 3 hours. Infiltrated the tissues in 2-3 changes of molten paraffin of melting point 58-62°C. Tissues were embedded in wax (58-60°C) and made into blocks, which were then labelled and stored in polythene covers.

5.2.2 Sectioning and staining techniques

Thin sections were cut from the block samples manually using a rotary microtome. The thickness of the sections was adjusted to 3-4 μm. Mayer’s egg albumin glycerol (1.1 v/v) was used as an adhesive for fixing paraffin ribbon on to glass slides. Slides were kept overnight. Slides were cleared off paraffin using xylene and sections were hydrated in alcohol series (20%, 30%, 50%, 70%, 90% and 100%) by giving brief dips. Sections were stained using Ehrlich’s haematoxylin and counter stained with 0.5% eosin. Brief dips in 95% and 10% alcohol were given to dehydrate the slides. Finally slides were cleared in xylene. DPX was used as the mounting medium and cover slips were put with out locking any air bubbles. Sections were observed and photographed under light microscope (OLYMPUS BX41TF, Japan).

Composition of fixative and stain used for the study

10% Neutral buffered formalin (pH- 7.0) was used as fixative. It is composed of the following ingredients.

Formalin-10ml
Distilled water- 90ml
Sodium dihydrogen orthophosphate (NaH$_2$PO$_4$. H$_2$O) - 400 mg
Disodium hydrogen orthophosphate anhydrous (Na$_2$HPO$_4$) - 650 mg

Ehrlich's Hematoxylin was used for staining. It is composed of following ingredients

- Distilled water- 10 ml
- 100% alcohol- 100 ml
- Glycerin- 100 ml
- Glacial acetic acid- 10 ml
- Hematoxylin- 2 g
- Aluminum ammonium sulphate (or potassium alum) – 20 g

Eosin (0.5%) as counter stain has the following composition

- Eosin- 500 mg
- 70% alcohol- 100 ml

5.3 Results

Liver of the control group of fishes were devoid of any pathological changes and hepatocytes were seen as well arranged structures in histological sections. Liver of the MCP treated animals exhibited various histopathological features such as vacuolated hepatocytes, cell necrosis, pycnotic nuclei, cytoplasmic degeneration and necrosis leading to disintegration of hepatocytes. Small oedema could be found in hepatocytes.

At higher concentration, 6.6 ppm, total loss of architecture of the liver tissue was observed, with more ceroid pigments, more fibrosis and more necrosis. Nuclei were all pycnotic. Other prominent features were swelling of hepatocytes, cytoplasmic degeneration and granulation of cytoplasm. At the medium concentration, 4 ppm, there was an increased occurrence of stromal connective tissue. Vacuolation and some areas of necrosis were also
noted. At the lowest concentration, 2 ppm, observed changes were mainly fibrosis and necrosis.

Brain of the control group of fishes were quite normal without any pathological changes while, brain of the treated animals displayed histopathological features such as galeal cell proliferation, encephalomalacia, total damage to neurons, neuronophagia and loss of neurons. At the highest concentration, 6.6 ppm, galeal cell loss, appearance of cytoplasmic vacuoles, encephalomalacia and neuronophagia were prominent pathological features. At the medium concentration, 4 ppm, encephalomalacia, satellitosis, neuronophagia and disappearance of neurons were observed. At the lowest concentration 2 ppm, focal encephalomalacia and glyosis were the observed features.

Gill of the treated fishes were showing pathological features like hyperplasia, lifting of secondary epithelium, squamous metaplasia, fusion of secondary lamellae, break down of pillar system and hyperaemia of cells. At the highest concentration, 6.6 ppm, areas of hyperplasia, squamous metaplasia, lifting of secondary epithelium and fusion of secondary epithelium was observed as prominent pathological features. At the medium concentration, hyperplasia, fusion of secondary epithelium and break down of pillar system were observed. While for the lowest concentration, 2 ppm, hyperemia, and hyperplasia along with pillar system break down were the main pathological features observed. Slides of histological sections of brain, gill, and liver of *H. fossilis* are given as plates A-Z.

5.4 Discussion

Histopathology is a higher-level response, reflecting prior alteration in physiological and/or biochemical function (Hinton *et al.*, 1992). Studies of responses of stinging catfish gill, brain and liver showed a reduction in cell
membrane integrity, lysosomal function and alterations in the activity of major stress enzymes, at sub lethal levels of monocrotophos exposure, given the liver biochemical activity as reported in previous studies (Rao et al., 2004, 2006) in exposed environments, it was expected that gill and liver histopathology would demonstrate to be sensitive measurement endpoints.

Gills are generally considered good indicators of water quality, being used as models for studies of environment impact, such as of xenobiotics (Rankin et al., 1982; Fanta et al., 2003) being models for environmental impact assessment (Mallat, 1985; Evans, 1987; McKim and Erickson, 1991; Laurent and Perry, 1991; Bonga and Lock, 1992). For fish, gills are crucial organs for their respiratory, osmoregulatory and excretory functions. Respiratory distress is one of the early signs of pesticide poisoning (McDonald, 1983). A high rate of absorption of pesticide through gills also makes fish a vulnerable non-target organism of its toxicity (Srivastav et al., 1997).

Tissue damages brought about by waterborne pollutants can be easily observed because the fish gills come into immediate contact with the environment. The gill surface is more than half of the entire body surface area. In fish the internal environment is separated from the external environment by only a few microns of delicate gill epithelium and thus the branchial function is very sensitive to environmental contamination. Hence, fish serve as excellent bioassay animals for toxicological impact studies and have been widely used for this purpose. Water pollution induces pathological changes in fish. As an indicator of exposure to contaminants, histology represents a useful tool to assess the degree of pollution.

The teleost fish gill is covered by a complex epithelium whose function is regulated by perfusion through an intricate vascular system. In
addition to being the site of gas exchange for these aquatic animals, the gill epithelium possesses transport steps which mediate active and passive movements of ions, counteracting dissipative movements down electrochemical gradients between the fish’s blood and water. These same transport steps play major roles in acid-base regulation and excretion of unwanted nitrogen in the form of ammonia. A variety of aquatic pollutants produce gross histopathological changes of the gill epithelium, which are often associated with osmoregulatory, acid-base, or hemodynamic malfunction. Since similar pathways and receptors are common to a variety of human tissues, which are affected by environmental pollutants (e.g., kidney, intestine, liver, blood vessel etc.), the fish gill presents an apt model which may be used to examine general epithelial pathologies induced by toxic substances.

In this study, desquamation, necrosis, hyperplasia of epithelial cells, squamous metaplasia and fusion of the secondary lamellae were observed in the gills after exposure to monocrotophos. Karan et al., (1998) observed lesions such as epithelial hyperplasia and lifting of secondary lamellae on the gills, swelling at the tips of several secondary lamellae, and club-shaped secondary lamellae as responses to pesticides. Erkmen et al., (2000) reported the lifting of epithelial layer from gill lamellae, necrosis and degeneration of secondary lamellae, shortening of secondary lamellae, and club shaped lamellae in the gills of *Lepistes reticulatus* exposed to pesticide.

Epithelial necrosis, secondary lamellae showing fusion and lifting of epithelium have also been showed in other species (Cengiz and Unlu, 2002, 2003). Ortiz et al., (2003), reported fusion of the secondary lamellae, increased rising of the branchial epithelium and intraepithelial oedema in gills of fish after an accidental discharge of lindane. The observed epithelial
Histopathological effect of monocrotophos

Chapter 5

Histopathological effect of monocrotophos

Histopathological effect of monocrotophos

necrosis and desquamation of the gill epithelium are direct responses to the action of pesticide. The defense responses observed are lifting up of the epithelium and hyperplasia. The lifting up of the epithelium increases the distance through which the toxicant has to travel to reach the blood stream (Cengiz, 2006). Collapse of the pillar cell system was observed in this study which lead to the breakdown of vascular integrity with a release of large quantities of blood that push the lamellar epithelium outward (Alazemi et al., 1996). Such effects on respiration (Schwarzbaum, 1988) and consequent lower levels of oxygen in the tissues impair the health of fish.

Gill hyperplasia might serve as a defensive mechanism leading to a decrease in the respiratory surface and an increase in the toxicant-blood diffusion distance. But the defense responses will take place at the expense of respiratory competence of the gills and finally, the respiratory impairment must outweigh any protective effect against pollution uptake (Cengiz, 2006). Degenerative changes, such as gill epithelial cell necrosis, observed in this study and lesions of this sort are believed to mirror the direct deleterious effects of irritants rather than a compensatory response to pollutants (Mallatt, 1985).

Other predominant pathological responses of H. fossilis gills involved basal epithelial cell proliferation and extensive proliferation of mucous cells following exposure to monocrotophos. This is consistent with observations of proliferation of gill mucous cells and basal epithelial cells following exposure to organic contaminants (Spies et al., 1996; Teh et al., 1997). A hyper secretion of mucus is considered a defense response to contaminant exposure rather than a direct effect of toxicants (Mallatt, 1985). Mucous cells contain mucins, polyanions composed of glycoproteins that can be efficient in trapping toxicants and aid in the prevention of toxicant entry.
into the gill epithelium (Perry and Laurent, 1993). Although mucous cell proliferation may be helpful in reducing toxicant entry, the end result is an increase in the distance for gas exchange along the secondary lamellae, potentially reducing the efficiency of gas exchange and causing hypoxic conditions (Ultsch and Gros, 1979).

In general, the circulatory system transports required nutrients to different tissues of an animal's body. As the control of rate of blood flow and its allocation to different tissues in a fish is based principally on homeostasis of respiratory system, the cardiovascular responses could be induced as a consequence of the damaged respiratory system, which in turn would badly affect the health of fish and put their life at risk. The morphological findings in this study indicate a string of symptoms in fish gill, which confirms severe damage of fish gills and severely influence normal physiological activities.

The liver holds responsibility for vital functions of basic metabolism and it is also the key organ of accumulation, biotransformation and excretion of contaminants in fish, together with degradation and bioactivation of pesticides (Triebeskorn et al., 1994, 1997). The assessment of biochemical and histological changes in fish liver has become an important tool for monitoring environmental exposure of fish to contaminants in experimental studies. As mentioned earlier, majority of the insecticides are biotransformed to metabolites by the liver, through enzymes from the soluble fractions of mitochondria and microsomes, and in some cases the metabolites are more toxic than the original product. Thus, liver is the organ that contains the major concentration of organophosphorus residues (Bender, 1969; Ansari and Kumar, 1987a) and that undergoes different levels of damage as a consequence of this process.
Liver of the monocrotophos exposed group of fishes displayed an array of pathological traits such as vacuolation of hepatocytes, pycnotic nuclei, cytoplasmic degeneration and necrosis leading to disintegration of hepatocytes. There were many regions in the liver where cells were highly vacuolated, leading to a foamy aspect. Shrunk and pycnotic nuclei indicate that the cells became hypo functional, and at the end, necrosis was extensive. Ram and Singh (1988) also showed similar changes in *Channa punctatus* exposed to pesticide carbofuran for six months. The widespread vacuolation might be likely due to accumulation of glycogen in hepatocytes (Wester and Canton., 1986).

At the highest concentration, 6.6 ppm, liver showed total loss of architecture with symptoms including disarray, connective tissue damage, granulation and vacuolation of the cytoplasm, and hypertrophy of the nucleus, necrosis, pycnosis, fatty infiltration, and glycogen depletion. The chain of pathological symptoms portrayed above certainly indicates that the liver is the chief detoxification organ in the organisms. So, the widespread vacuolation of the liver might be a common response in fish hepatocyte to stressor (Chun-Yang Liao, 2006). Leaner and Mason (2004) experimented on toxicant induced stress responses in Sheephead minnow (*Cyprinodon variegates*) and reported that exchange between the blood and the internal organs was relatively slow, with maximum uptake in the liver and gill occurring next to dietary exposure, which demonstrated that fish's liver and gill were common target of aquatic pollutant's action.

Alterations associated with the brain to subtle exposure of monocrotophos are not reported in the accessible literature. The present experiment revealed that monocrotophos can be neurotoxic, as proved by the histopathological changes observed. Brain of the treated fish exhibited
glyosis or glial scarring which is a reactive cellular process that occurs after injury to the central nervous system. As with scarring in other organs and tissues, the glial scar is the body’s mechanism to protect and begin the healing process in the nervous system. Although the glial scar does a good job at controlling and suppressing further physical damage, many neuro-developmental inhibitor molecules are secreted by the cells within the scar that prevent complete physical and functional recovery of the central nervous system.

Brain also showed condition of neuronophagia, or phagocytosis of neurons, in which a dying neuron is surrounded by glial cells. Vacuolation may have been due to glycolysis leading to microsomal and mitochondrial dysfunctions. Another pathological feature exhibited by brain was focal encephalomalacia, which in fact is the softening of the brain associated with inadequate cerebral blood flow. Satellitosis, a condition marked by an accumulation of neuroglia cells around the neurons of the central nervous system; often as a prelude to neuronophagia, was also observed in the brain of monocrotophos treated H. fossilis.

All the histopathological changes indicate that the exposure to sublethal concentrations of monocrotophos caused destructive effect in the gill, liver and brain tissues of H. fossilis. Tissue alterations, such as those observed in this study may result in severe functional problems, eventually leading to the death of fish. The findings of the present histological investigation reveal a direct correlation between pesticide exposure and histopathological disorders observed in tissues. Generally, gill and liver pathological data suggest that degenerative changes were the most prevalent and sensitive changes observed following exposure of H. fossilis to monocrotophos. All effects that were observed in the gills, brain and liver
induce behavioural changes as a consequence of the decrease in the general state of health of *H. fossilis*. This will, together with the inhibition of plasma cholinesterase as observed in the present study, negatively influence the prospects of survival of these fish in the natural environment. They will face difficulties in detecting, identifying, and responding in proper way to chemical stimulation in nature.

5.5 Summary

In analogy with mammalian toxicology where it is firmly established, fish histopathology ought to have a place in the toxicology toolkit, in particular when the endangered aquatic environment is an issue. Various modern techniques in pathology can equally be applied to fish and these will contribute to its analytical power. As a conclusion, the findings of the present histological investigations demonstrate a direct correlation between monocrotophos exposure and histopathological disorders observed in tissues. Monocrotophos, an extensively used agrochemical in India has caused histopathological changes in *Heteropneustes fossilis* (Bloch) at sub lethal levels of exposure. When these pathological endpoints are measured in combination with other parameters like enzyme responses, haematological parameters, membrane studies and bioconcentration studies, a clearer picture of the complex interactions between anthropogenic and natural environmental modifiers will emerge.
Chapter 5

Histopathological effect of monocrotophos

Brain exposed to 6.6 ppm MCP showing neuronophagia (40x)

Brain exposed to 2 ppm MCP showing encephalomalacia (40x)

Brain exposed to 6.6 ppm MCP - neuronophagia and disappearance of neurons (40x)

Brain exposed to 6.6 ppm MCP showing total loss of neurons (40x)

Brain of *H. fossilis* - control (40x)
Chapter 5

Histopathological effect of monocrotophos

Brain exposed to 6.6 ppm MCP showing vacuolation (40x)

Brain exposed to 4 ppm MCP showing satellitosis (40x)

Brain exposed to 4 ppm MCP showing neuronophagia (40x)

Brain exposed to 4 ppm MCP showing disappearance of neurons (40x)

Gill of *H. fossilis* - control (20x)

Gill exposed to 6.6 ppm of MCP showing hyperplasia (40x)

Stress responses of stinging carfish *Heteropneustes fossilis* (Bloch) to organophosphorus insecticide Monocrotophos
Chapter 5

Histopathological effect of monocrotophos

Gill exposed to 6.6 ppm of MCP showing hyperplasia (40x)

Gill exposed to 2 ppm of MCP showing hyperaemia (20x)

Gill exposed to 6.6 ppm of MCP showing lifting of secondary epithelium (40x)

Gill exposed to 6.6 ppm of MCP showing fusion of epithelium (40x)

Gill exposed to 4 ppm of MCP showing fusion of epithelium (40x)

Stress responses of stinging catfish Heteropneustes fossilis (Bloch) to organophosphorus insecticide Monocrotophos
Histopathological effect of monocrotophos

Chapter 5

Gill exposed to 2 ppm of MCP showing hyperplasia (40x)

Liver of *H. fossilis* - control (40x)

Liver exposed to 6.6 ppm of MCP showing high necrosis, total loss of architecture and increased ceroid pigments. (40x)

Liver exposed to 6.6 ppm of MCP showing total loss of architecture. (40x)

Liver exposed to 4 ppm of MCP showing necrosis (40x)

Liver exposed to 4 ppm of MCP showing necrosis (40x)
Liver exposed to 2 ppm of MCP showing mild necrosis (40x)

Liver exposed to 4 ppm of MCP showing vacuolation (40x)
Stress responses of stinging catfish *Heteropneustes fossilis* (Bloch) to organophosphorus insecticide Monocrotophos