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Full Length Article

Toxicity to Hematology and Morphology of Liver, Brain and Gills during Acute Exposure of Mahseer (*Tor putitora)* to Cypermethrin

Rafiq Ullah[1], Amina Zuberi[1*], Muhammad Naeem[2] and Sana Ullah[1]
[1]*Fisheries and Aquaculture Lab, Department of Animal Sciences, Quaid-i-Azam University, Islamabad, Pakistan*
[2]*Institute of Pure and Applied Biology, Zoology Division, Bahauddin Zakariya University, Multan 60800, Pakistan*
[*]For correspondence: zuberi_amina@yahoo.com

Abstract

The present study was aimed to evaluate the effects of acute dose of Cypermethrin (CYP), an extensively use synthetic pyrethroid, on hematology and morphology of the liver, gills and brain of Mahseer (*Tor putitora*). The effects were assessed on the basis of the previous results of acute toxicity test after exposing fish to acute concentration, 63 µg L^{-1} (LC_{50} of 96 h) of CYP. Light microscopic studies revealed severe histopathological changes in liver, gills and brain tissues. The morphological alterations in liver involved glycogen vacuolation, hemorrhage vacuolation, congestion, fatty infiltration and hepatic necrosis. In gills, it resulted in cellular infiltration, congestion, swollen tip of the gill filament, hetrophilic infiltration and damaged gill while in the brain it caused discoloration, neuronal degeneration, infiltration and severe spongiosis. Blood cell count also showed the toxic effect of CYP, as RBCs count decreased while WBCs count increased with time in the treated group. The results clearly classify CYP as a strong toxic agent for *T. putitora*. © 2015 Friends Science Publishers

Keywords: Cypermethrin; Acute toxicity; *Tor putitora*; Hematology; Histopathology

Introduction

The rapid advancement of industrialization and green revolution has led to a number of environmental problems, aquatic pollution being the most prominent. In Pakistan effluents from industries, wastes from household activities and agricultural runoffs directly discharge into streams, ponds and other aquatic bodies. These pollutants contain infectious pathogens, oil, hydrocarbon, radioactive substances, heavy metals, pesticides, herbicides and different corrosive substances such as acids and bases (Samantha *et al.*, 2005). Yet these sources are used for supplying water to the local masses and culturing of economically important and luscious fish species (Stanitski *et al.*, 2003).

Pesticides are among the major contributor of aquatic pollution. According to Latif *et al.* (2013), there are more than 200 types of organic pesticides, being used in thousands of different products. These pesticides contain a number of heavy metals such as iron, chromium, cadmium, nickel, copper, lead, zinc and manganese etc. These elements ultimately reach the water bodies, and adversely affect the growth, reproduction, physiology and even survival of the non target aquatic organisms including fish (Hayat *et al.*, 2007).

Liver, gills and brain are the most important organs of all vertebrates, as these controls and maintain the most important life activities such as metabolism, detoxification, excretion and respiration etc. The chemical pollutants including heavy metals and pesticides adversely affected the morphology and functions of these organs and disturb the normal physiology of all animals (Atamanalp *et al.*, 2008; Velmurugan *et al.*, 2009a, b; Ali *et al.*, 2014).

In ecotoxicological studies, various biomarkers are used for evaluating stress responses. Recently fish histopathology is gaining importance for rapid assessment of the toxic effects of pollutants in the laboratory. Histopathology can be utilized for the determination of the effect of stressor at biological organization level. According to Latif *et al.* (2013) studying histopathology is an important way to evaluate the effects of pollutants on fish; therefore it is used as a rapid tool for examining the effect of the pollutant in different organs and even tissue of the body.

The hematological parameters also reflect the animal response towards its environment and are useful indicators in assessing the toxic effects of chemicals in aquatic organisms such as fish (Gabriel *et al.*, 2007; Ghaffar *et al.*, 2014). Moreover, surrounding environment where fish performing its activities put forth some impact on their hematological characteristics. Therefore, hematological parameters are also helpful in examining the response of fish against stressors (Gabriel *et al.*, 2007). These parameters have standardized reference values, which can be increased or decreased due to stressor like pesticide and other pollutants and helpful in diagnosing the problem. Fish are directly linked to the aquatic environment, therefore their hematological profile can provide information about their internal body conditions, earlier than any noticeable

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disease indication (Fernandes and Mazon, 2003).

Among many types of pesticides, pyrethroid accounts 25% sales of pesticides in the world (Zhang *et al*., 2011). They are common in practice due to their low toxicity to avian and mammalian species, but it appears that they are highly toxic to bees, aquatic insects and fish (Aydin *et al.*, 2005). The difference in the rate of metabolic degradation and elimination from the body may be a major factor for the variations in the toxicity of pyrethroid between fish, birds and mammals.

Cypermethrin is an important synthetic pyrethroid that has much higher biological activity and stability than its natural homolog pyrethrum (Khan *et al.*, 2006). It is widely used throughout the world for controlling different types of insect pests of cotton, fruits and vegetables (Khan *et al.*, 2006) copepode parasite infestation (Athanassopoulou *et al.*, 2001), aquatic and terrestrial ectoparasites (Treasurer and Wadsworth, 2004) and for illegal fishing (Khan *et al.*, 2006). It enters the water bodies, mostly through agricultural run offs and affects the non target aquatic organisms such as fish (Werimo *et al.*, 2009; Arjmandi *et al.*, 2010).

Keeping in view the current scenario of polluted aquatic environments, the present study was designed to investigate the adverse effects of cypermethrin on the hematology and histology of liver, brains and gills of *Tor putitora*, economically an important freshwater species of fish commonly used as a stapled food item in Pakistan.

Materials and Methods

Test Animals

About 250 healthy and uniform size seeds of Mahseer (*Tor putitora)*, average body weight 3.23 ± 0.34 g were purchased from Hattian Nursery Unit, Attock Pakistan. These seeds were transported to Fisheries and Aquaculture laboratory, Department of Animal Sciences, Quaid-i-Azam University by closed system live hauling method. The fish were acclimatized for two weeks before the start of the experiment. During acclimatization, water was changed on a daily basis and fish were offered 40% protein diet at 5% body weight.

The water quality parameters like temperature, dissolved oxygen and pH were checked regularly on a daily basis and were ensured to remain in the optimum range. To avoid water foiling dead fish were removed as soon as possible. During acclimatization, ammonia was less than 0.25 ppm while temperature, pH and dissolved oxygen were 22˚C, 8.44, 5.02 mg L^{-1}, respectively.

Preparation of CYP Solution

A stock solution of pesticide was prepared by dissolving 5 mg of technical grade CYP [Cyano (3-phenoxyphenyl) methyl 3-(2, 2-dichloroethenyl)-2, 2-dimethylcyclopropane-carboxylate] in 5 mL of 80% acetone. Then 1 mL solution was taken in 10 mL volumetric flask with the help of micropipette and 80% acetone was added up to mark to make concentration, 1 mg CYP per 10 mL. The flask was shaken well to get homogenous solution. Then further dilutions were made from that stock solution. Based on previous studies on juvenile Mahseer (Atika, 2012) of similar sized, LC_{50} for 96 h, 63 µg L^{-1} was selected for acute study tests.

Experimental Detail and Treatment

The experiment was conducted in semi-static closed system. Healthy and uniform sized fish, regardless of sex, were selected and evenly distributed in six glass aquaria (60 × 30× 30 cm) at a stocking density of 1.5 kg m^{-3}. The experiment was conducted in triplicate. The first three aquaria served as a control group while other three as treated group. All aquaria were fitted with air stones and heaters for constant temperature and dissolved oxygen. After 96 h of acclimatization, fish in the treatment group were exposed to acute concentration, 63 µg L^{-1} (LC_{50} of 96 h) of CYP while the control group received 80% acetone equal to the volume used for exposing CYP in the treatment group. The water of each aquarium changed after every 24 h and concentrations of CYP was restored afresh.

Sample Collection

The experiment was conducted for 4 days. After every 24 h, up to 4 days one fish from every aquarium in the control and treated group was captured with a hand net and sacrificed. Their liver, gills and brain tissues were removed by decapitation and placed in sera (absolute alcohol, formaldehyde and glacial acetic acid in 6:3:1 ratio) for the further histology process.

Further 3 fish from each aquarium were captured and anesthetized with MS222 (70 mg L^{-1}) and bled by caudal vein puncture and blood was collected for RBCs and WBCs counting.

Histopathology Study

Rosety *et al.* (2005) method was adopted for the preservation of tissues and preparation of slides for histopathological assessment. After staining with hematoxylin and eosin, the slides were mounted with Canada balsam. Cover slips were placed on these and were kept in an incubator overnight. The extra Canada balsam was removed with xylene. The prepared slides of liver, gills and brain tissues from both control and treated groups were studied under OPTIKA B-350 Microscope. Photography was done by using AIPTEK digital camera.

Hematological Study

Blood cell counting: Blood was diluted by using commercially available diluting solutions (dilution for RBC, 1:200; and for WBC, 1:20) and blood cells were counted with the help of Neubauer hemocytometer.

RBCs and WBCs counting: Under light microscope counting chamber was adjusted and observed the smallest squares in the large center square where red cells lies. The chamber was examined at 10 X magnifications for the evenness of the red blood cell distribution. The 40X objective was turned in place; focused and the cells were counted in the designated squares.

For WBCs counting 400 µL of WBCs solution was taken in tube and a drop of blood was added to the WBCs solution. WBCs were counted with the help of hemocytometer. The cell counting was done under light microscope at 100 X magnification and only those cells touching the upper and left-hand boundary lines of the main squares were counted. For WBCs count, cells in each square chambers were counted and then average per chamber was calculated. By using this average number, WBCs per cubic mm was calculated.

Results

Histological Changes in Body Parts

Hematological and Histopathological changes were observed in the gill, liver, and brain of juvenile *T. putitora* when exposed to an acute concentration of CYP for 96 h. The liver of fish in the control group revealed normal appearance having hepatocyte of polygonal shape, central nuclei and granulated cytoplasm. Exposure of CYP for 96 h caused glycogen vacuolation, congestion, hemorrhage vacuolation, fatty infiltration and hepatic necrosis (Fig. 1). No change was observed in the fish gills from the control group, while the CYP exposure severaly damaged the gills by causing cellular infiltration, congestion, swollen tip of the gill filament, hererophilic infiltration (Fig. 2). The histopathological examination of the fish brain from control group showed normal morphological structure while in CYP treated group discoloration, neuronal degeneration, mononuclear infiltration and severe spongiosis of the brain were obvious (Fig. 3).

Hematological Changes

In a control group of fish, no significant changed in the number of RBCs during 24, 48, 72 and 96 h were observed. The RBCs value showed decreasing trend with time in CYP exposed group of fish (Table 1), therefore significantly ($P < 0.001$) higher value was observed at 24 h and lower value was observed at 96 h. The WBCs count of juvenile fish significantly increased after exposure to an acute concentration of CYP (Table 1). The values in the control group of fish at 24, 48, 72 and 96 h were fluctuating between 10.54 and 11.86 × 10^3 m^{-3}. After exposure to CYP, the WBCs count was significantly increased and shown an increasing trend with time and maximum value (36.11 ± 1.78 × 10^3 m^{-3}) was observed at 96 h.

Table 1: Variations in RBCs and WBCs count of juvenile *Tor putitora* at different time period after exposure to an acute concentration of CYP

Time (h)	RBCs (10^6 m^{-3})		WBCs (10^3 m^{-3})	
	Control	Treated	Control	Treated
24	1.71±0.07^{a}	1.26±0.10^{bc}	11.29 ±4.8^{d}	20.73 ±1.77^{c}
48	1.65±0.16^{a}	1.21±0.12^{cd}	11.86 ± 0.47^{d}	27.33 ±1.84^{bc}
72	1.76±0.16^{a}	0.90±0.06^{cd}	10.55 ±1.12^{d}	33.89±3.27^{ab}
96	1.59±0.18^{ab}	0.83±0.12^{d}	10.63 ±0.76^{d}	36.11±1.78^{a}

Data are represented as Mean ± SE. (n=15). Means followed by the different letter within the column are significantly different (P<0.05)

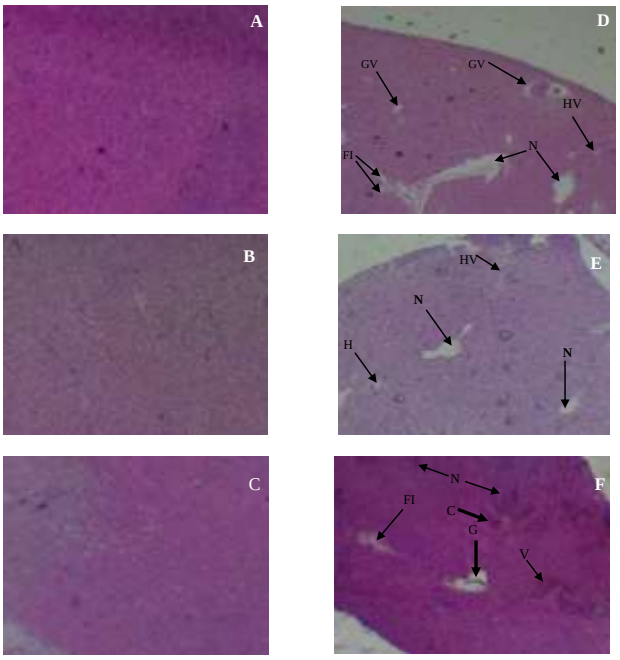

Fig. 1: Microphotograph of liver tissues, A, B and C show normal structure in all control groups while D, E and F shows glycogen vacuolation (GV), hemorrhage vacuolation (HV), congestion (C), fatty infiltration (FI) and hepatic necrosis (N) in all treated groups

Discussion

In ecotoxicological studies, histopathology is gaining importance for rapid evaluation of the toxic effect of pollutant and considered as an important tool for examining the effect in different organs and even tissue of the body (Latif *et al.*, 2013). Many investigators reported lesions in different organs of fish in response to various chemical contaminants like heavy metals and pesticide (Omitoyin *et al*., 2006; Ayoola and Ajani, 2008; Velmurugan *et al.*, 2009a, b). In the present study, the histopathological examination of the brain, gill, and liver tissues of juvenile mahseer (*Tor putitora)* in response to CYP revealed that the liver and gills were the organs most affected compared to other organs.

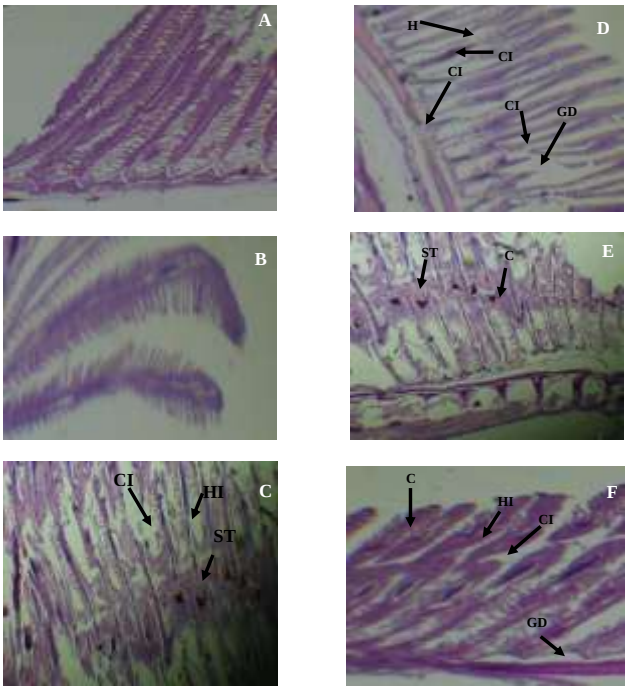

Fig. 2: Microphotograph of gills, A and B showing normal structure in all control groups while C, D, E and F showing cellular infiltration (CI), congestion (C), swollen tip of the gill filament (ST), hererophilic infiltration (HI) and gill damage (GD) in treated groups

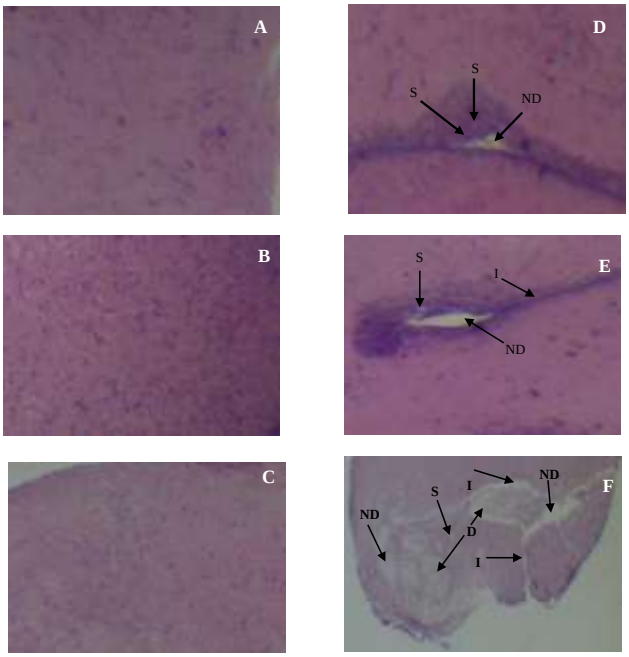

Fig. 3: Microphotography of brain tissues, A, B and C show normal structure in all control groups while D, E and F shows discoloration (D), neuronal degeneration (ND), infiltration (I) and severe spongiosis (S) in treated groups

In vertebrate including fish, liver is the main organ that play important role in detoxification of pesticides. During metabolism, liver has the ability to break down these harmful substances, but beyond a certain limit these toxic compounds disturb the regulating mechanism of the liver and cause morphological alteration (Brusle *et al*., 1996). The histopathological changes observed in the present study were glycogen vacuolation, hemorrhage, fatty infiltration, hepatic necrosis and congestion (Fig. 1). The glycogen vacuolization and fatty infiltration in the liver indicate the accumulation of fat and imbalance between rate of synthesis and release of substance in hepatocytes (Gingerich,1982), whereas necrosis in some part of liver may appeared due to extra work load on hepatocyte during detoxification of CYP (Patel and Bahadur, 2011). Many investigators reported the similar or different level of morphological changes in the liver in response to CYP in various fish species (*Clarias gariepinus*, Velmurugan *et al*., 2009a; *Labeo rohita*, Sarkar *et al*., 2005; *Heteropneustes fossilis*, Joshi *et al*., 2007). The inconsistency in results may be due the fact that degree of lesion depends upon on the concentration and duration of exposure of pesticide (De Oliveira *et al*., 2002). Nevertheless, many insecticides cause specific or non-specific histopathological damage. For example, histopathological lesions in the liver tissue of freshwater fish (*Cirrhinus mrigala*) (Velmurugan *et al*., 2009b) and common carp (*Cyprinus carpio*) (Banaee *et al*., 2011) were observed after 10 and 30 days exposure to sublethal concentrations of dichlorvos and diazinon insecticides, respectively while other researchers reported the same histopathological alterations in different tissues of fish treated with diazinon (Banaee *et al*., 2011), deltamethrin (Cengiz, 2006), fenitrothion (Benli and Ozkul, 2010).

It is well established that in fish, gills are the main organs through, which water enter into the body along with pesticides. According to Rankin *et al*. (1982) gills are a good indicator for studying the water quality and used as a model for the evaluation of environmental impact. Pesticides first affect the gills and once inside the body, may damage various other organs of fish. When the gills are damaged, the hypoxic condition occurs due to alteration in gas exchange mechanisms (Das and Mukherjee, 2003). In the present study no significant changes were observed in the gill tissues of the control group of fish, while fish exposed to CYP for 96 h showed cellular infiltration, congestion, swollen tip of the gill filament, hererophilic infiltration and gill damaged. Cypermethrin cause necrosis, hyperplasia of primary epithelial cells, oedema, epithelial hypertrophy, epithelial lifting, fusion of secondary lamellae and desquamation in the gills of African catfish *Clarias gariepinus* (Velmurugan *et al*., 2009a), while Zeta CYP caused morphological changes like hyperplasia, lifting of the epithelial layer from gill lamellae, shortening of secondary lamellae, exudation and necrosis in the gills of

Lebistes reticulates (Caliskan *et al*., 2003). Moreover, in grass carp *Ctenopharyngodon idella* fenvalerate caused bulging of primary gill lamellae tips, atrophy and complete fusion of secondary gill lamella, club shaped secondary gill lamellae and severe necrotic changes in the epithelial cells (Tilak *et al*., 2001).

In the present study, the light microscopy of the brain of control and pesticide exposed group of fish showed that beside liver and gills, CYP also affected the brain structure and caused discoloration, neuronal degeneration, mononuclear infiltration and severe spongiosis. All these symptoms are the clear indication of brain damage. This agreed with the observations of other scientist who reported generalized spongiosis and severe congestion in brain of African catfish *Clarias gariepinus* in response to an acute concentration of CYP and Gramoxone (Omitoyin *et al*., 2006; Ayoola and Ajani, 2008). All the histopathological observations in the liver, gill and brain tissues of *T. putitora* after acute exposure of CYP indicated the destructive effect of this pesticide. These histopathological modifications could result in severe physiological problems, those eventually end to the death of fish.

Fish as an aquatic vertebrate, is in direct contact with the aquatic environment that could put forth some impact on the hematological characteristics (Gabriel *et al*., 2007). Therefore, the hematological profile of blood can provide the information about the body internal condition of an animal earlier than any noticeable indication of disease. Many investigators studied the effects of toxicants on the hematology of different fish species and reported various degrees of hematological changes and suggested that reduction in hemoglobin, RBC and PCV are related to oxygen carrying capacity of the blood (Adhikari *et al*., 2004; Gabriel *et al*., 2007).

In this study the RBCs count of *T. putitora* showed significant decreased after exposure to acute concentration of CYP, while no significant difference at different periods was observed in control group of fish (Table 1). The RBCs count in the control group of fish at different time period ranged 1.59- 1.76 x 10^6 m^{-3}. The values lies within the range observed in various other species (Maheswaran *et al.*, 2008; Vasantharaja *et al*., 2012) but somewhat lower than 2.94±0.4 x 10^6 m^{-3} observed in *Labeo rohita* (Adhikari *et al.*, 2004). After exposure to CYP, RBCs count showed decreasing trend and lowest value (0.83 × 10^6 m^{-3}) was observed at 96 h. It was suggested that the reduction in the RBCs counts during treatment, may be due to the development of hypoxia that lead to either increase in destruction of RBS or decrease in the genesis of RBCs due to non-availability of Hb content in cellular medium (Akinrotimi *et al.*, 2012; Vasantharaja *et al*., 2012).

In vertebrates including fish WBCs are related to the defense mechanism and consist of lymphocytes, thrombocytes, monocytes and granulocytes. Monocytes and granulocytes play function in the removal of injured cell debris, while lymphocytes related to the production of antibodies (Wedemeyer and Mcleay, 1981). It was observed that the counted value of WBCs of juvenile mahseer significantly increased after exposure to acute concentration of CYP (Table 1). In treated group, WBCs count showed positive relation with time and reached to maximum (36.11×10^3 m^{-3}) level at 96 h. Adhikari *et al.* (2004) also observed a significant increased in number of WBCs in *L. rohita* due to CYP and carbofuran treatment. Like our results, other scientists also observed increased in WBCs counts in response to similar pesticide (*C. mrigala*, Vasantharaja *et al*., 2012) and other pollutants like methyl mercury (*Hoplias malabaricus*, Ribeiro *et al*., 2006). The increase in number of WBCs in the present study or others reports in response to different type of toxicants may be related to stimulation of immune system due to tissue damage or may be related to compensatory response of lymphoid tissues to circulating lymphocytes (Shah and Altindag, 2004).

The results of this study clearly indicated that CYP at acute concentration is toxic to juvenile mahseer *Tor putitora*. Hence, restrictions on the indiscriminate use of pesticide can play a role in decreasing the wild population of fish in natural water bodies.

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