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RESEARCH ARTICLE

Impact of chlorpyrifos on behavior and histopathological indices in different tissues of freshwater fish *Channa punctatus* (Bloch)

Arumugam Stalin[1] · Palani Suganthi[2] · Subramanian Mathivani[3] · Bilal Ahmad Paray[4] · Mohammad K. Al-Sadoon[4] · Varadharajan Gokula[1] · Mohamed Saiyad Musthafa[5]

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Abstract

The present systematic experiment was conducted to estimate the impact of behavioral and pathological indices on freshwater fish *Channa punctatus* exposed to sub-lethal concentration (5 ppm) of an organophosphorus insecticide chlorpyrifos (CPF). Fish were segregated into four experimental groups (G1, control; G2, 10 days; G3, 20 days; and G4, 30 days exposure), each group comprises 15 fish in triplicate. The behavioral and histological changes were assessed in each group. Severe behavioral changes were observed in the 30 days, moderate changes in the 20 days, and mild changes in the 10 days exposure groups respectively when compared with the control group. The pathologic lesions such as inter lamellae space, necrotic lamellae, fused lamellae, and lifting of lamellae epithelium in gills; vacuolation, blood conjunctions, and necrotic hepatocytes in the liver; and lamina propria, fusion of villi, and flattened villi in the intestine were observed. These structural alterations of the gills, liver, and intestine could affect respiration, osmotic and ionic regulation; absorption, storage and secretion; digestion; and absorption of nutrients respectively, which in turn could adversely affect the growth and survival of freshwater fish *Channa punctatus*. This study serves as a biomonitoring tool for the effects of organophosphorus insecticide CPF on the aquatic biota.

Keywords *Channa punctatus* · Gill · Liver · Intestine · Chlorpyrifos · Histology

Introduction

Agriculture is considered as the back bone of India, but the farmers utilize 70% of synthetic chemicals as insecticide and herbicide instead of using biocides during cultivation. Farmers get exposure through inhalation or dermal pore penetration (via Skin lesions or wounds) of these chemicals at the time of spraying. In addition to that, water ecosystem receives synthetic chemical pollutants through agricultural sources via leaching into groundwater table and also adjacent water bodies.

Chlorpyrifos (CPF) [O, O–diethyl–O (3,5,6-trichlor-2-pyridyl) phosphorothioate] is the second largest selling organophosphorus agro chemical in India. According to the US Environmental Protection Agency, approximately 800 registered products on the market contain chlorpyrifos, and they are used for a number of purposes, including pest control for a variety of food crops, turf and ornamental plants, green houses, and sod; indoor pest control; structural pest control; and pet collars (NPIC 2017).

Fish have proved to be of significance as bioindicators of the aquatic environment so-called ecological integrity (Faggio et al. 2014a, b; Gobi et al. 2018; Bartoskova et al. 2013). It can provide quantitative information on the ecological integrity and its health. Therefore, fishes are successful bioindicators. Recently, Marigoudar et al. (2018) and Zahran et al. (2018) reported that the chlorpyrifos treated group exhibits elevated

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✉ Mohamed Saiyad Musthafa
saiyad_musthafa@rediffmail.com

[1] P.G. and Research Department of Zoology, National College (Autonomous), Tiruchirappalli, Tamil Nadu 620001, India

[2] P.G. and Research Department of Zoology, Jamal Mohamed College (Autonomous), Tiruchirappalli, Tamil Nadu 620020, India

[3] Department of Biomedical Science, Bharathidasan University, Tiruchirappalli, Tamil Nadu 620024, India

[4] Department of Zoology, College of Science, King Saud University, Box 2455, Riyadh, PO 11451, Saudi Arabia

[5] P.G and Research Department of Zoology, The New College, Chennai, Tamil Nadu 600 014, India

oxidative stress and distorted activities of superoxide scavenging enzymes in gills and liver tissues of *Oreochromis niloticus*, *Mugil cephalus*, and *Chanos chanos* fingerlings.

Pathological lesions in gills, liver, eye, and brain tissues of various fish species due to exposure of chlorpyrifos were extensively studied by many researchers (Cengiz and Unlu 2006; Ogueji et al. 2013; Marigoudar et al. 2018; Zahran et al. 2018). The chlorpyrifos (CPF) is reported to cause hepatic dysfunction, genotoxicity, and neurobehavioral and neurochemical changes through multiple mechanisms of action (Deb and Das 2013). The prevalence of CPF in the aquatic environment and its potential for adverse effects suggest that it is a good model candidate for studies of organophosphate effects on aquatic animal health (Oruç 2012).

Recently, the jury at the Superior Court of California in San Francisco ordered the top most agricultural company Monsanto to pay $250 million to Johnson (former school groundskeeper) in punitive damages and about $39 million in compensatory damages. Monsanto's popular weed killer, Roundup, leads to terminal skin cancer, a type of non-Hodgkin's lymphoma [blood cancer that starts in cells called lymphocytes, which are part of the body's immune system] (CNN 2018). In March 2015, the World Health Organization's International Agency for Research on Cancer (IARC) said that the key ingredient in Roundup, glyphosate (organophosphorus agrochemical), is "probably carcinogenic to humans" (IARC, 2017 and 2018).

The study attempts to reconcile apparent trends of deficiency of behavior indices and alterations in the histopathology pointing out that exposure to sub-lethal concentration (5 ppm) of agricultural organophosphorus insecticide CPF is often associated with significant sub-lethal impacts. In this present study, freshwater fish *Channa punctatus* was used as an experimental animal for 30 days under laboratory conditions. The microscopic study of the tissue provided essential knowledge of living cells, as they are the basic building blocks of all living organisms. It examines the structural organization of the cells in different tissues allowing better understanding of morphological aspects and physiological process of different organs.

Materials and methodology

Maintenance of fish

The Riverine fish *C. punctatus* fingerlings were captured from River Cauvery (Tamil Nadu) and kept for acclimatization for a duration of 30 days in a cement pond (0.8 m length, 0.3 m width, and 0.4 m height). The experimental animals were disinfected using potassium permanganate (0.1%) and transferred to a glass aquarium with a 40-L volume of water, and maintained for another 7 days. Commercial pellet feed was given during the entire study period. The aquarium water was changed every day and oxygenated using an air pump. The dead fishes were discarded from the tank immediately. The water in aquarium tank was renewed periodically to maintain water quality (temperature = 28 ± 0.5 °C, pH = 7.5, dissolved oxygen = 6.7 mg/L, salinity = 0.5 ± 0.05 ppt) parameters as per the standard quality recommended by APHA [American Public Health Association] (1998).

Sub-lethal studies (LC50)

Chlorpyrifos (20% EC) from Zeneca Agrochemical Ltd., Chennai, India, was obtained to study the toxic effects in aquatic animals. For sub-acute studies, a few different concentrations of chlorpyrifos such as 1 ppm, 2 ppm, 3 ppm up to 10 ppm were prepared; the concentrations were examined with fishes (15 fish/aquarium). Finally, LC_{50} were identified in 5 ppm concentration.

Experimental design

A total of 60 fishes (15 fishes per aquarium) were separated as four groups.

- Group 1 *C. punctatus*, without any pesticide exposure (control)
- Group 2 *C. punctatus*, on exposure to 5 ppm chlorpyrifos for a period of 10 days
- Group 3 *C. punctatus*, on exposure to 5 ppm chlorpyrifos for a period of 20 days
- Group 4 *C. punctatus*, on exposure to 5 ppm chlorpyrifos for a period of 30 days

The pesticide concentration was controlled by mixing chlorpyrifos in 40 L volume of water and changed everyday without oxygenation.

Behavioral analysis

The control- and pesticide-exposed fishes were observed from a glass aquaria. According to Mishra and Mohanty (2008), the behavioral activities of *Channa punctatus* were observed semiquantatively. The behavioral results were given as no abnormalities (0%), mild abnormalities (< 10%), moderate abnormalities (10 to 50%), and severe abnormalities (> 50%).

Histopathological studies (methodology)

The dissection of gills, liver, and intestine takes place in a sterile condition. Soon after dissection, the organs of *C. punctatus* were fixed in Bouin's fluid and maintained for a period of 48 h. The samples preserved in fixative were dehydrated in a serial manner using alcohol. It was then placed

in xylene for clearing process. At a temperature of 58 °C, the samples were infiltered using paraffin wax (liquid state) and then embedded in paraffin blocks. Using microtome (Weswox MT, Chennai, India), the samples were sliced at a size of 5 μm and stained using hematoxylin and eosin. It was then placed on a slide using DPX (MERK) and examined under a light microscope (Leica DM750, Switzerland).

Histopathological analysis

According to Mishra and Mohanty (2008), histological variations were observed semiquantatively. The histological results were given as no abnormalities (0%), mild abnormalities (< 10%), moderate abnormalities (10 to 50%), and severe abnormalities (>50%). These results were used in the total study of tissue anomalies. Data are estimated as mean of randomly selected 10 slides from 50 slides (each organ).

Results

The chlorpyrifos (5 ppm) exposed animals were subjected to monitor the behavioral and histological changes at 10, 20, and 30 days.

Behavior

In fact, there is no behavioral abnormalities (0%) seen in the control group. The chlorpyrifos (5 ppm) exposed group shows no (0%) hyperactivity on 10 days, but mild (< 10%) loss of balance, reduction in swimming rate, reduced feed intake, and convulsions were noticed on the 10 days group. Moderate (10 to 50%) loss was noted on day 20 and severe abnormality was noticed on the 30th day of experimental animal (*C. punctatus*) (Table 1).

Table 1 Effect of CPF on the behavior of fish, *Channa punctatus*

Parameter		Exposure to CPF (5 ppm)		
	Control	10th day	20th day	30th day
Hyperactivity	–	–	+	++
Loss of balance	–	+	++	+++
Rate of swimming	–	+	++	++
Rate of food intake	–	+	++	+++
Convulsions	–	+	++	+++

–, None (0%); +, mild (< 10%); ++, moderate (10 to 50%); +++, severe (> 50%)

Histopathological observations

It is essential to interpret that highly CPF-exposed animals show higher histological alterations than lower exposure groups of fish

Gill

In control, gill lamellae were arranged uniformly with inter lamellar space (ILS). The lamellae of each filament were enclosed in epithelial cell (EC) cuticle; endothelial cell (ENC), salt cell (SC), primary gill lamellae (PGL), and secondary gill lamellae (SGL) were observed (Fig. 1 (A1)).

After 10 days of chlorpyrifos (5 ppm) exposure, the gills of fishes showed very common histopathological abnormalities, such as lamellae fusion (LFU), swollen tip of secondary lamellae (STSL), salt cell (SC), and red blood cells (RBC) (Fig. 1 (A2)). Whereas after 20 days, the swollen tips of secondary lamellae (STSL), lifting of lamellae epithelium (LLE), necrotic lamellae (NL), lamellae fusion (LFU), and proliferation of chloride cells (PCC) (Fig. 1 (A3)) were evidenced. After 30 days, the lamellae fusion (LFU), necrotic lamellae (NL), mucoid metaplasia (MM), and clubbed lamella (CL) (Fig. 1 (A4)) histoarchitectural changes were observed.

Liver

In the control group, the histoarchitecture of the liver consists of hepatocytes (HC) containing homogenous cytoplasm with a centrally placed nucleus (N) and vacuolation (V) (Fig. 2 (B1)).

In the CPF (5 ppm) exposed groups, the previously mentioned characteristics were lost. At the 10 days group, histopathological changes including necrotic hepatocytes (NHC) and blood conjunction (BC) were observed (Fig. 2 (B2)). At 20 days, the changes were observed in the histoarchitecture of the liver including necrotic hepatocytes (NHC) and blood veins (BV) (Fig. 2 (B3)). At the end of the treatment on 30 days, histopathological changes were observed such as vacuolation (V) and portal vein (PV) (Fig. 2 (B4)).

Intestine

The control group of *Channa punctatus* shows normal structure of the intestine, which consists of longitudinal muscle layer (LML), lamina propria (LP), serosa (S), submucosa (SM), circular muscle Layer (CML), and columnar epithelium (CE) (Fig. 3 (C1)).

In the CPF (5 ppm) exposed group, the histological changes in the intestine such as hypertrophied epithelial

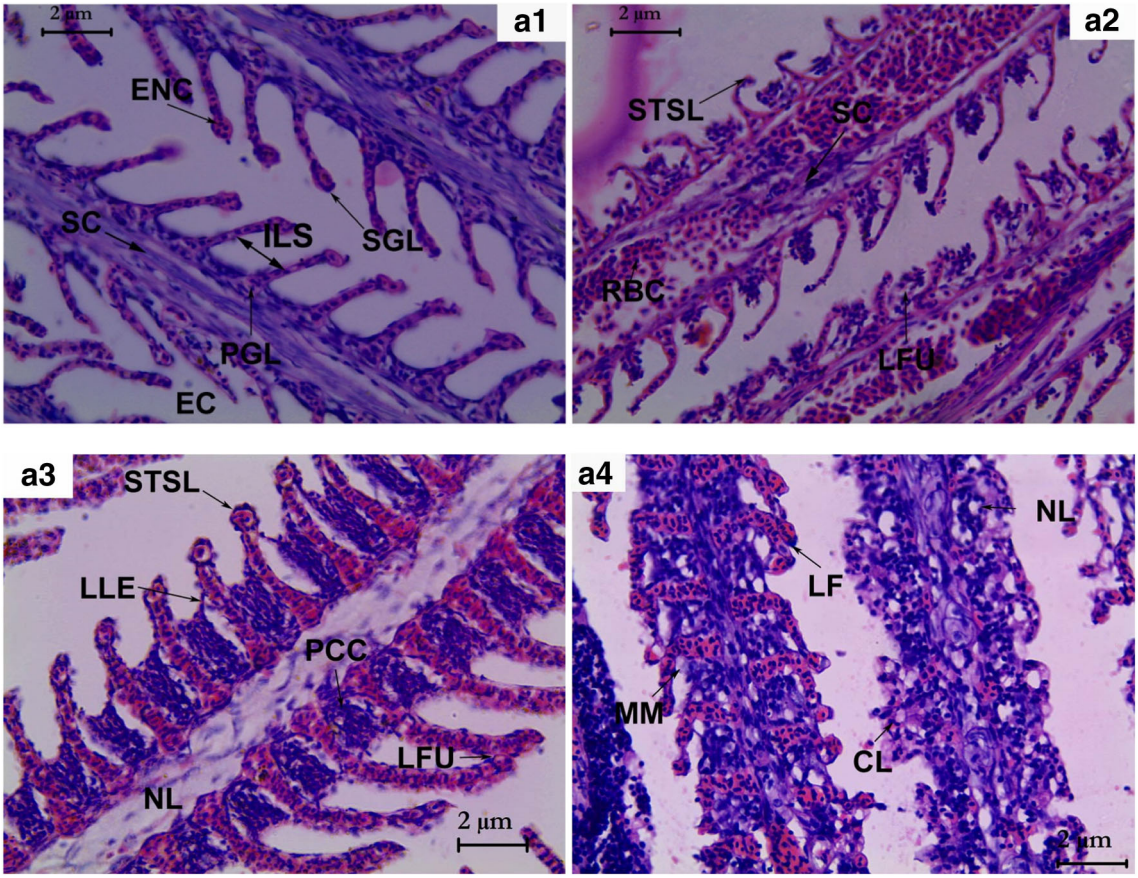

Fig. 1 (A1) Typical organization of the gill of *Channa punctatus* (control). Gill lamellae with uniform inter lamellar space (ILS), epithelial cell (EC), endothelial cell (ENC), primary gill lamellae (PGL), secondary gill lamellae (SGL), salt cell (SC) (× 40 H&E). (A2) Chlorpyrifos induced alterations (at 10 days) in the histoarchitecture of the gills of *Channa punctatus.* Fusion of lamellae (LFU), swollen tip of secondary lamellae (STSL), salt cell (SC), red blood cells (RBC) (× 40 H&E). (A3) Chlorpyrifos induced alterations (at 20 days) in the histoarchitecture of the gills of *Channa punctatus.* Swollen tip of secondary lamellae (STSL), lifting of lamellae epithelium (LLE), necrotic lamellae (NL), fusion of lamellae (LFU), proliferation of chloride cells (PCC) (× 40 H&E). (A4) Chlorpyrifos induced alterations (at 30 days) in the histoarchitecture of the gills of *Channa punctatus.* Lamellae fusion (LF), necrotic lamellae (NL), mucoid metaplasia (MM), clubbed lamella (CL) (× 40 H&E)

cell (HEC), lamina propria (LP), and columnar epithelium (CE) were noticed in 10 days (Fig. 3 (C2)). In 20 days, the experimental group exhibits the changes including cracked clay appearance (CCA), swelling in lamina propria (SLP), and fusion of villi (FV) (Fig. 3 (C3)). In 30 days, the intestine of the exposed fish exhibits cracked clay appearance (CCA), fusion of villi (FV), and flattened villi (FLV) (Fig. 3 (C4)).

The exposed fish groups show severity of lesion when compared with the control fish group and the results were given in Table 2.

Discussion

Behavior

The present study reveals an abnormality in swimming behavior of *C. punctatus* on exposure to 5 ppm CPF for a period of 30 days and severity of changes takes place in hyperactivity, loss of balance, rate of swimming, rate of food intake, and convulsions. The present experiment correlates with the works carried out by different authors.

A decline ($P < 0.05$) in rate of swimming after 4 days was studied in *Cyprinus carpio*, when exposed to chlorpyrifos, as the concentration increases from 0.1 to 2.2 mg/L (Xing et al. 2015). On exposure to chlorpyrifos, the Japanese medaka shows abnormality in the swimming behavior (Khalil et al. 2013). The Indian Major Carp (*C. catla*) shows balance loss, frequently surfacing, abnormal swimming, and common jerking when exposed to methyl parathion at a dose range of 1 to 10 ppm (Selvi and Ilavazhahan 2012). The feeding behavior and floating of fish (*Cyprinus carpio*) were observed to be normal, when exposed to terbutryn for a period of 90 days at different concentrations (0.02, 0.2, and 2.0 µg/L) (Velisek et al. 2011).

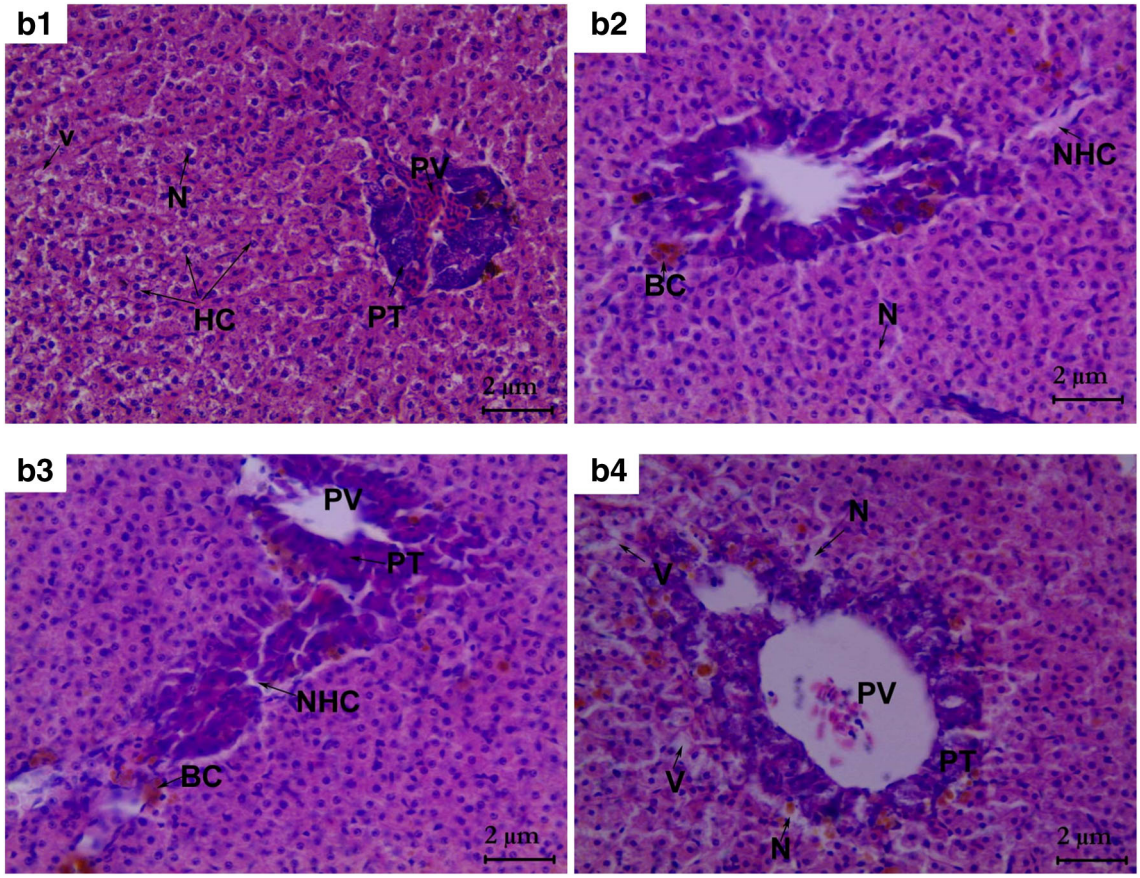

Fig. 2 (B1) Typical organization of the liver of *Channa punctatus* (control). Vacuolation (V), nucleus (N), hepatocytes (HC) (× 40 H&E). (B2) Chlorpyrifos induced alterations (at 10 days) in the histoarchitecture of the liver of *Channa punctatus.* Necrotic hepatocytes (NHC), blood conjunction (BC), nucleus (N) (× 40 H&E). (B3) Chlorpyrifos induced alterations (at 20 days) in the histoarchitecture of the liver of *Channa punctatus.* Necrotic hepatocytes (NHC), blood veins (BV) (× 40 H&E). (B4) Chlorpyrifos induced alterations (at 30 days) in the histoarchitecture of the liver of *Channa punctatus.* Vacuolation (V), portal vein (PV) (× 40 H&E)

Histopathology

Gill

In aquatic organisms, gills are the organs, which serve as an utmost surface area in contact with the external ecosystem and receive dissolved toxicants from the water. Gills play a pivotal role in respiration, osmoregulation, acid balance, and excretion of nitrogenous waste products. The constant intake of toxicants through water which disturbs the chloride cells of gills in the aquatic animals and also leads to discomfort of respiratory pigment to transport dissolved oxygen in the water and prolonged exposure leads to anorexia condition.

The several histological alterations in the gills were noticed in the present study such as fusion of lamellae (LFU), swollen tip of secondary lamellae (STSL), salt cell (SC), red blood cells (RBC), necrotic lamellae (NL), lifting of lamellae epithelium (LLE), proliferation of chloride cells (PCC), mucoid metaplasia (MM), and clubbed lamella (CL) in *C. punctatus* exposed to 5 ppm CPF for about 30 days.

Katuli et al. (2014) and Rosety-Rodrıǵ uez et al. (2002) reported that the impact of diazinon and sodium dodecyl sulfate leads to severe necrotic lamellae in gill tissues of *Rutilus rutilus* and *Scophthalmus maximus*. Similarly, diazinon exposure in gills of *Scatophagus argus* exhibited edema, epithelial lifting, curling of secondary lamellae, shortening of secondary lamellae, and lamellar fusion (Ghasemzadeh et al. 2015).

Our findings are consistent with the histopathological work carried out by Marigoudar et al. (2018), on Mullet fish fingerlings. The authors reported that the CPF highest dose of 0.80 µg/L elicited changes in gill architecture of fish which included the following: hyperplasia, lifting and fusion of epithelium in the secondary lamellae, degeneration cells in primary lamellae, and necrosis of secondary lamellae. Similarly, in the fingerlings of *C. chanos*, degeneration of cells and necrosis in primary lamellae and lifting and fusion of epithelium in secondary lamellae were observed at higher concentrations of CPF (1.92 µg/L).

Zahran et al. (2018), noticed histopathological changes in the gills of Nile tilapia (*Oreochromis niloticus*) on exposure to

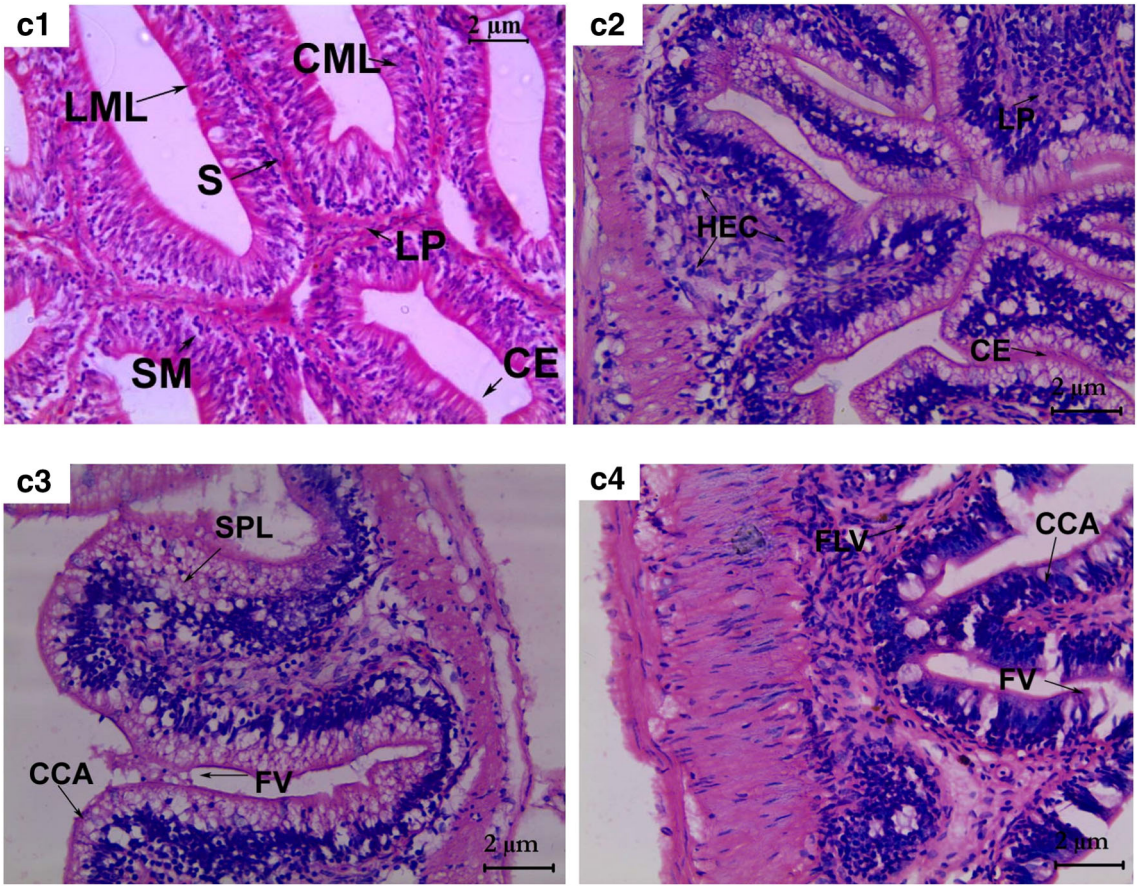

Fig. 3 (C1) Typical organization of the intestine of *Channa punctatus* (control). Longitudinal muscle layer (LML), lamina propria (LP), serosa (S), submucosa (SM), circular muscle layer (CML), columnar epithelium (CE) (× 40 H&E). (C2) Chlorpyrifos induced alterations (at 10 days) in the histoarchitecture of the intestine of *Channa punctatus.* Lamina propria (LP), columnar epithelium (CE), hypertrophied epithelial cel (HEC) (× 40 H&E). (C3) Chlorpyrifos induced alterations (at 20 days) in the histoarchitecture of the intestine of *Channa punctatus.* Cracked clay appearance (CCA), swelling in lamina propria (SLP), fusion of villi (FV) (× 40 H&E). (C4) Chlorpyrifos induced alterations (at 30 days) in the histoarchitecture of the intestine of *Channa punctatus.* Cracked clay appearance (CCA), fusion of villi (FV), flattened villi (FLV) (× 40 H&E)

two different dose of CPF. Fifteen grams per liter of CPF caused damage in the histoarchitecture of secondary lamellae such as curved and fused tips of secondary lamellae, decreased interlamellar space, mucous accumulation inside secondary lamellae, epithelial hyperplasia at the base of secondary lamellae, and necrosis severity at the tip of primary lamellae. Whereas, fivefold higher dose, i.e., 75 µg/L of CPF exposure, exhibited the necrosis of epithelium, deformation of secondary lamellae, epithelial hyperplasia at the base of secondary lamellae and at the tip of primary gill lamellae, and clubbed tips of primary lamellae.

The damages reported by Devi and Mishra (2013), in the gills of *Channa punctatus*, included the hypertrophy and proliferation in the erythrocytes of cartilaginous core (HPC), complete destruction of the secondary lamellae, and lifting of epithelial cells (LE) at a dose rate of 0.538 µL/L CPF for a period of 7 days exposure.

Karmakar et al. (2015) observed fusion of primary and secondary lamellae, epithelial hyperplasia, curling of secondary lamellae, and decrease of inter lamellar cell mass (ILCM) with increasing malathion concentration (10, 50, and 100 µg/L) in gills of fish (*Labeo rohita*).

Selvi and Ilavazhahan (2012) studied the histological sections of gills in the fingerlings of *C. catla* treated with methyl parathion (1 to 10 ppm), and reported numerous changes such as fusion of secondary lamellae as a result of hyperplasia, elongated secondary lamellae with club-like structures, edema at the secondary lamellae and swelling of the epithelia cells, and observance of mucoid metaplasia distinctly.

Liver

In this experiment, the liver showed the most prevalent histological characteristics being necrotic hepatocytes (NHC), blood conjunction (BC), vacuolation (V), and portal vein (PV) on exposure to 5 ppm of CPF for about 30 days.

Similar findings were reported in previous studies such as treatment of Nile tilapia (*Oreochromis niloticus*) with 15 µg/L chlorpyrifos. The liver of *C. punctatus* exposed to CPF doses of 0.538 µg/L and 1.46 µg/L for a duration of 7 days showed

Table 2 Histopathologic observations of teleost fish, *Channa punctatus*, exposed to CPF

Tissue histopathology	Control	Exposure to CPF (5 ppm)		
		10th day	20th day	30th day
Gill				
Fusion of lamellae (LFU)	–	+	++	+++
Swollen tip of secondary lamellae (STSL)	–	–	+	+
Lifting of lamellae epithelium (LLE)	–	+	++	+++
Necrotic lamellae (NL)	–	+	++	+++
Proliferation of chloride cells (PCC)	–	–	+	++
Mucoid metaplasia (MM)	–	–	–	+
Clubbed lamellae (CL)	–	–	–	+
Liver				
Necrotic hepatocytes (NHC)	–	+	++	+++
Blood conjunction (BC)	–	+	++	++
Vacuolation (V)	–	+	++	+++
Intestine				
Hypertrophied epithelial cell (HEC)	–	+	+	++
Cracked clay appearance (CCA)	–	+	+	++
Swelling in lamina propria (SLP)	–	+	++	+++
Fusion of villi (FV)	–	–	+	++
Flattened villi (FLV)	–	+	++	+++

–, None (0%); +, mild (< 10%); ++, moderate (10 to 50%); +++, severe (> 50%)

hypertrophy of hepatocytes, nuclear hypertrophy (NH), hepatocellular vacuolation, necrosis, degeneration of hepatocytes (DH), cytoplasmic vacuolation, and fibrosis (Devi and Mishra 2013). Karmakar et al. (2015) also reported increasing number of pyknotic nuclei in the exposed liver of *Labeo rohita* as the concentration of malathion increases from 10 to 100 μg/L in 10 days of experimental period. The damages reported by Begum and Mithra (2015) in the liver of *Heteropneustes fossilis* included hemorrhage, formation of vacuoles, and degeneration of hepatocytes in the liver exposed with 1.07 ppm malathion for a period of 21 days. Maksymiv et al. (2015) reported that the various impacts of sencor (herbicide) lead to hypertrophy, degeneration, and dystrophic lesions in hepatocytes of gold fish (*Carrasius auratus*) due to a 4-day exposure at 7.14 mg/L concentration. In common carp, 40-day exposure of CPF (1.16 to 116 μg/L) and atrazine (4.28 to 428 μg/L) exhibited pathological lesions in the liver such as hydropic degeneration, vacuolization, pyknotic nuclei, and fatty infiltration. Furthermore, gills reflected varied degrees of epithelial hypertrophy, telangiectasis, edema with epithelial separation from basement membranes, general necrosis, and epithelial desquamation (Xing et al. 2012).

Intestine

The changes seen in the experimental animal *C. punctatus*, on exposure of 5 ppm of chlorpyrifos at regular intervals of 10th, 20th, and 30th day, explain the continuous damage of intestine which in turn cause severe abnormalities in digestion process and it leads to lethal condition with increasing exposure period.

Senapati et al. (2013) noticed that the histopathological alterations in the intestine of *Anabas testudineus* after herbicide (Almix 20WP) toxicosis included degeneration of columnar epithelial cells, degeneration of lamina propria, and prominent luminal mucus secretion. Sharma et al. (2001) reported similar histological alteration in the intestine of *C. mrigala* due to toxicological effects of different pesticides. The intestinal damages reported by Ghanbahadur and Ghanbahadur (2012) such as the destruction of mucosa and particularly the columnar epithelial cells in the intestine of *Rasbora daniconius* are due to endosulfan toxicity (Ghanbahadur and Ghanbahadur 2012). The aforementioned reports are consistent with our present findings. The degeneration of mucosal epithelium, damages of lamina propria, and submucosa of the intestine may affect the secretion of digestive enzymes into the lumen of the alimentary canal.

Conclusion

The accumulation of CPF in organs examined in the present study was gill > liver > intestine. These structural alterations of the gill, liver, and intestine of the freshwater fish

C. punctatus could affect the vital physiological functions such as osmotic and ionic regulation in gills, absorption, storage, secretion, and respiration which in turn could adversely affect the growth and survival of freshwater fish C*hanna punctatus*. This study proved that even low concentration (5 ppm) of CPF cause pathology to the fish and it serves as a biomonitoring tool for the effects of organophosphorus insecticide CPF on the aquatic animals.

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