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Alteration in haematological and biochemical parameters of *Catla catla* exposed to sub-lethal concentration of cypermethrin

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Abstract A 60-day experiment was carried out to study the effect of sub-lethal concentration of cypermethrin (1/10th of LC_{50}) exposure on haematological and biochemical parameters of the Indian major carp, *Catla catla* fingerlings. Under exposure, the total erythrocyte count, total leucocyte count, haemoglobin content and haematocrit were decreased. All the studied serum parameters viz. total serum protein, albumin, globulin contents and albumin–globulin ratio were significantly decreased in cypermethrin-exposed fishes. A marked increase was recorded in alanine aminotransferase and aspartate aminotransferase activities in liver, whereas lactate dehydrogenase activity of muscle and acetylcholine esterase activity in brain were inhibited in cypermethrin-exposed fish. The membrane transport enzymes (total adenosine triphosphatase, sodium–potassium adenosine triphosphatase and magnesium adenosine triphosphatase) activities were decreased significantly in the gills of *C. catla* exposed to sub-lethal concentration of cypermethrin. The present study indicates that sub-lethal exposure of *C. catla* fingerlings to cypermethrin alters the haematological and biochemical parameters.

Keywords *Catla catla* · Cypermethrin · Haematology · Biochemical parameters

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Introduction

Pesticides have contributed considerably to the human welfare, but their residues often reach ecosystems causing undesirable impact. Hence, pesticides have become an increasingly serious source of chemical pollution to the environment due to their extensive usage in agriculture and public health protection programs. In the late 1970s, synthetic pyrethroid was successfully introduced into the agricultural market as a new generation of insecticides, owing much to their unique ability to kill insects instantly at lower application rates, lower mammalian toxicity and longer stability in outdoor environment.

Cypermethrin, RS-α-cyano-3-phenoxybenzyl (1RS)-cis-, trans-3-(2,2,-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate, is one among the synthetic pyrethroid

insecticides that is extremely effective against a wide range of insect pests in agricultural and sanitary pest control. It is both stomach and contact poison (Jin and Webster 1998), which affect the nervous system of vertebrates and invertebrates by affecting voltage-dependent sodium channels (Vijverberg and van den Bercken 1990) and inhibiting ATPase enzymes (Siegfried 1993).

Cypermethrin is registered for use on a wide array of crops including cotton, cabbage, okra, brinjal, sugarcane, wheat and sunflower in India. Almost 70 % of all sprays used on cotton in Andhra Pradesh in India are pyrethroids, which contain mostly cypermethrin (Jayswal 1989). In aquaculture also, cypermethrin is used against lice infestations in fish (Sommerville 1995; Hart et al. 1997; Braidwood and Hart 1998; Mukherjee et al. 2000). However, the residual cypermethrin, which is released directly into the environment, enters the water body through run-off and affects the aquatic ecosystem. The residual cypermethrin was found to be in the range of 0.001–0.035 μg g^{-1} in soil and 0.022–0.090 ppb in the water of agriculture field in Haryana, India (Kumari et al. 2008).

The toxicity of pyrethroid insecticides to aquatic organisms, esp. fish, has received much attention in recent years. The exposures of cypermethrin alter the biochemical, haematological parameters and enzymes of organ tissue and exert stress on the fish (Das and Mukherjee 2003; Kumar et al. 2007). It also alters the several physiological parameters in *caenorhabditis elegans* (Shashikumar and Rajini 2010). Due to its lipophilic nature, cypermethrin has high rate of gill absorption, thereby rendering fish as most sensitive to this pesticide. Although it has been reported that cypermethrin toxicity is reduced under field conditions in water bodies with abundant particulate material (Hill 1989) and also no lethal effects have been observed after insecticide exposure under field-use conditions (Carriquiriborde et al. 2007), concern exists about the possible sub-lethal effects induced by this pesticide; particularly taking into account that cypermethrin spraying events mainly occur during the breeding season of many fish species and also during stocking of fish seeds into aquaculture system.

The effects of pesticide pollution on non-target organisms (e.g. fish) in the environment can be studied by detecting changes in organisms at the physiological, biochemical or molecular levels, which can provide 'early warning' tools in monitoring environment quality (Crane and Maltby 1991; Miren et al. 2000). These sensitive early warning biomarkers can measure interaction between environmental xenobiotics and biological effects. Inhibition and induction of these biomarkers are a good approach to measure potential impacts of pollutants on environmental organisms (Rendon-von Osten et al. 2005). The analysis of haematological and biochemical parameters in fish can contribute to the assessment of the animal's health and also the habitat conditions (Thrall 2004).

Catla catla contributes a major portion to the freshwater fish production in India. Most of the freshwater fish farms in India are situated in and around the agriculture fields or their source of freshwater is continuously in contact with the agriculture farms in which lot of pesticides, weedicides and insecticides are used. Keeping in view of these aspects, a study was planned to investigate sub-lethal effects of cypermethrin on haematological and biochemical parameters of *C. catla* fingerlings.

Materials and methods

Fish and husbandry

Catla (*C. catla*) fingerlings of 14 ± 1 g were obtained from a fish seed farm at Pen, Raigarh District, Maharashtra, to the aquaculture laboratory complex, Central Institute of Fisheries Education (CIFE), Mumbai, India. Fishes were acclimatized for 2 weeks prior to the experiment. Chlorine-free tap water was used throughout the experiment. The physicochemical characteristics of the test water were as follows: temperature, 27 ± 2.0 °C; pH, 7.4; hardness, 80 mg L^{-1} (as $CaCO_3$); alkalinity, 88 mg L^{-1} (as $CaCO_3$); and dissolved oxygen concentration, 5.2-5.8 mg L^{-1}.

Cypermethrin

The toxicant, Cypermethrin (10 EC) (cyano-(3-phenoxyphenyl)-methyl-(3-C2,2-dichloroethenyl)-2,2-dimethyl cyclopropane carboxylate), a synthetic pyrethroid obtained from Rallis India Pvt. Ltd. was used for determining the median lethal concentration on catla fingerlings at 96 h. The LC_{50} value for cypermethrin was determined in the laboratory starting with range finding test to acute toxicity trials (Reish and Oshida

1987). The 96-h LC_{50} was found to be 4.43 μg L^{-1}. One-tenth concentration of 96 h LC_{50} (0.443 μg L^{-1}) was selected for sub-lethal test trials.

Experimental design

Six rectangular plastic tanks (100 L) were arranged with continuous aeration. Randomly selected ninety catla fingerlings were equally divided into two groups (Group A and B). Both groups were equally divided to form their triplicate. Fishes of Group B were exposed to 1/10th concentration of 96-h LC_{50} for a period of 60 days. Group A fishes were not exposed to pesticide and kept as control. Every day morning, the test solution (water containing 0.443 μg L^{-1} cypermethrin) of Group B was completely renewed with fresh one to maintain the required cypermethrin concentration of 0.443 μg L^{-1}. Similarly, Group A tanks were also completely changed with normal water. The fishes were fed with feed @ 5 % of their body weight twice a day throughout the experimental period.

Haematological studies

Blood was drawn from the caudal peduncle region using sterile 2-mL syringes rinsed first with 2.7 % EDTA solution and was collected in small glass vials coated with 20 μL of 2.7 % EDTA solution. For the estimation of total erythrocyte count and total leucocyte count (TEC, Schaperclaus et al. 1991), 20 μL of blood was mixed with 3,980 μL of red blood cell (RBC) diluting fluid and white blood cell (WBC) diluting fluid (Dacies fluid) in a clean glass vial. The mixture was shaken well to suspend the cells uniformly in the solution. The cells were counted using a hemocytometer (Feinoptik, Blakenburg, Germany) and expressed as:

$$\text{Number of RBC/mm}^3 = N_r \times 10,000$$

$$\text{Number of WBC/mm}^3 = N_w \times 500$$

Where N_r is the total number of red blood cells counted in five squares of the hemocytometer; 10,000 is the factor obtained after taking the initial dilution factor into consideration; N_w denotes the total number of white blood cells counted in 4 squares of the hemocytometer; and 500 is the factor obtained after taking the initial dilution factor into consideration.

The blood haemoglobin content was analysed following the Cyanmethaemoglobin method using Darbkins Fluid (Qualigens Diagnostics Kit, Mumbai, India). 20 μL of blood was mixed with 5 mL of Darbkin's working solution. The absorbance was measured using a spectrophotometer at wavelength of 540 nm. The final concentration was calculated after comparing with the standard. Haemoglobin content was expressed as gram/dl.

Serum chemistry

Blood was drawn from caudal region of fish without rinsing the syringe with anticoagulants and collected into clean and dry eppendorf tubes. The collected blood was allowed to clot for 45 min in inclined position at room temperature followed by 30-min incubation at 4 °C and then centrifuged at 3 000*g* for 10 min at 4 °C. Serum was collected into sterilized eppendorf tubes and analysed for the different serum parameters in AR. 601, Semi Automatic analyzer (Qualigens Diagnostic kit) using Qualigens kits. The parameters that were analysed by using this instrument were total protein (biuret method using buffered dye reagent and biuret reagent, Qualigens Diagnostic kits) and albumin (bromocresol green binding method, Qualigens Diagnostic kits).

Enzyme assay

At the end of experiment, brain, liver, muscle and gills were collected to carry out enzyme assay. Tissues of the five individuals from each replicate of a group were homogenized in chilled sucrose solution (0.25 M) in Teflon-coated mechanical tissue homogenizer (Remi tissue homogenizer) and centrifuged at 5,000*g* at 4 °C for 10 min. The supernatant was used as an enzyme source for measuring enzymatic activity. All enzyme preparations were carried out on ice. Dilution of the sample was done as and when required.

Lactate dehydrogenase (LDH) (L-Lactate NAD + oxidoreductase; E.C.1.1.1.27) was assayed in 100 mM phosphate buffer (pH 7.5) and 0.1 mM NADH. The reaction was initiated by adding 0.2 M Na-pyruvate and monitored at 340 nm (Wroblewski and Ladue 1995). Alanine aminotransferase (ALT) (L-Alanine-2-oxaloglutarate aminotransferase; E.C.2.6.1.2) was assayed with 200 mM DL-alanine and 2 mM α-ketoglutarate in 40 mM phosphate buffer (pH 7.4) and estimated at 540 nm (Wotton 1964). Aspartate aminotransferase (AST) (Laspartate-2-oxaloglutarate

aminotransferase, E.C.2.6.1.1) was assayed by the same procedure as for ALT except for the substrate, 200 mM DL-aspartic acid instead of DL-alanine (Wotton 1964). Acetylcholine esterase (AChE) (Acetyl hydroxylase, E.C.3.1.1.7) was assayed by using a mixture of 0.07 M phosphate buffer (pH 7.2), 4 mM acetylcholine (pH 4.0) and a substrate buffer mixture (1/10 dilution) (Hestrin 1949; Augustinsson 1957). The mixture was incubated at 37 °C for 30 min. Alkaline hydroxylamine solution was used to terminate the reaction, and HCl (2:1) was added. The colour developed by the addition of 10 % $FeCl_3$ was measured at 540 nm. Total adenosine triphosphatase (ATPase) (Adenosine triphosphate phosphohydrolase, E.C. 3.6.1.3) was assayed in a reaction mixture of 0.1 M Tris–HCl buffer (pH 7.8), 100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$ and 5 mM ATP. The mixture was incubated for 15 min, and then the reaction was terminated by means of 10 % trichloroacetic acid (Post and Sen 1967). Phosphate liberated was estimated at 660 nm (Fiske and Subbarow 1925). A similar reaction mixture was used for the estimation of magnesium adenosine triphosphatase (Mg^{2+}-ATPase) activity (Magnesium Transporting ATPase, E.C. 3.6.3.1) except for reaction termination, 0.1 mL of 1 mM of ouabain was used. Sodium–potassium adenosine triphosphatase (Na^+, K^+-ATPase) activity was estimated by subtracting Mg^{2+}-ATPase activity from total ATPase activity. Total protein contents were analysed from the supernatant of all organ samples (Lowry et al. 1951) for calculating enzyme activities. All the colorimetric assays were carried out using UV–VIS spectrophotometer (E-Merck, Germany).

Statistical analysis

The data obtained from 60-day experiment on sub-lethal toxicity test were statistically analysed by *t* test. A significance level of $P \leq 0.05$ was used.

Results

The TEC, TLC, haemoglobin content and haematocrit levels were significantly reduced ($P \leq 0.05$) in cypermethrin-exposed group of fishes when compared to control group after 60 days (Table 1). All the serum parameters viz. total protein, albumin, globulin contents and albumin–globulin ratio were significantly decreased ($P \leq 0.05$) in Group B fishes compared to Group A fishes (Table 2). The LDH activity in muscle of Group B fishes was significantly reduced ($P \leq 0.05$) by 36.84 % in compare to Group A fishes (Table 3). AChE activity in brain was inhibited in cypermethrin-exposed fish (0.39 ± 0.02) in comparison with control fish (0.59 ± 0.03) (Table 3). The activities of ALT and AST enzymes in liver of fish exposed to cypermethrin were significantly enhanced ($P \leq 0.05$) by 30.16 % and 32.47 %, respectively, in comparison with control group of fishes (Table 3). The activities of membrane transport enzymes in gill tissues of catla are given in Table 4. A significant reduction ($P \leq 0.05$) in the level of adenosine triphosphatase, ATPase, Na^+, K^+-adenosine triphosphatase and Mg^{2+}-adenosine triphosphatase activities in gill (42.56, 41.58 and 44.78 %, respectively) of cypermethrin-exposed fish was observed compare to the control.

Discussion

Haematological and biochemical profiles of blood can provide important information about the internal environment of the organism (Li et al. 2010). In the present study, exposure of fishes to sub-lethal concentration of cypermethrin showed remarkable alterations in haematological and physiological stress markers. The significant decrease in total erythrocyte counts, total leucocyte count, haemoglobin concentration and haematocrit value was observed in fishes exposed to a sub-lethal concentration of cypermethrin for 60 days. Decrease in haemoglobin concentration, TEC and haematocrit may be the indicator of anaemia. The similar finding was reported in *Cyprinus carpio* (Dorucu and Girgin 2001) and *Labeo rohita* (Das and Mukherjee 2003) exposed to cypermethrin. Jee et al. (2005) observed reduction in erythrocytes count, haemoglobin concentration and haematocrit value in Korean rockfish (*Sebastes schlegeli*) exposed to cypermethrin and they opined that it was because of the destructive action of cypermethrin on cell membrane. They also reported that decline in RBC count, haemoglobin concentration and haematocrit value presumably reflected erythrocyte haemolysis and/or irreparable damage of gill morphology and function. The decrease in haemoglobin concentration may be because of either an increase in the rate at which

Table 1 Alteration in haematological parameters of *C. catla* exposed to sub-lethal concentration of cypermethrin for 60 days

Treatment	TEC ($\times 10^6$ cells/mm^3)	TLC (10^4 cells/mm^3)	Haemoglobin (g %)	Haematocrit (%)
Group A	$2.68^a \pm 0.05$	$4.18^a \pm 0.10$	$10.57^a \pm 0.35$	$33.17^a \pm 1.16$
Group B	$2.11^b \pm 0.05$	$3.36^b \pm 0.13$	$6.60^b \pm 0.21$	$17.17^b \pm 0.22$

Values are expressed as mean ± SE. Mean value ($n = 3$) in column bearing different superscripts varies significantly ($P \leq 0.05$)

Table 2 Change in serum biochemical parameters of *C. catla* exposed to sub-lethal cypermethrin concentration for 60 days

Treatment	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	Albumin–globulin ratio
Group A	$4.48^a \pm 0.28$	$2.09^a \pm 0.08$	$2.40^a \pm 0.19$	$0.88^a \pm 0.02$
Group B	$2.77^b \pm 0.20$	$1.03^b \pm 0.02$	$1.73^b \pm 0.18$	$0.61^b \pm 0.06$

Values are expressed as mean ± SE. Mean value ($n = 3$) in column bearing different superscripts varies significantly ($P \leq 0.05$)

Table 3 Alterations in enzyme activity of anaerobic pathways (lactate dehydrogenase in muscle), neurotransmission (acetyl choline esterase in brain) and protein metabolism (alanine aminotransferase and aspartate aminotransferase in liver) of *C. catla* exposed to sub-lethal concentration of cypermethrin for 60 days

Treatment	LDH activity	AChE activity	ALT activity	AST activity
Group A	$0.38^a \pm 0.01$	$0.59^a \pm 0.03$	$2.42^b \pm 0.10$	$8.13^b \pm 0.37$
Group B	$0.24^b \pm 0.02$	$0.39^b \pm 0.02$	$3.15^a \pm 0.16$	$10.77^a \pm 0.54$

Values are expressed as mean ± SE. Mean value ($n = 3$) in column bearing different superscripts varies significantly ($P \leq 0.05$)

Enzyme activities are expressed as follows: LDH as µmol of pyruvate utilized/mg protein/min, AchE as µmol of acetyl choline hydrolyzed/mg protein/min at 37 °C, ALT as nmol of sodium pyruvate released/mg protein/min at 37 °C, AST as nmol of oxaloacetate released/mg protein/min at 37 °C

Table 4 Alterations in the activity of membrane transport enzyme (adenosine triphosphatase, ATPase, Na^+, K^+-adenosine triphosphatase and Mg^{2+}-adenosine triphosphatase activity) as µg of phosphorus/mg protein/min at 37 °C in gill of *C. catla* exposed to sub-lethal concentration of cypermethrin for 60 days

Treatment	Total ATPase activity	Mg^{2+} ATPase activity	Na^+-K^+ ATPase activity
Group A	$68.04^a \pm 4.04$	$46.25^a \pm 2.04$	$21.79^a \pm 2.02$
Group B	$39.08^b \pm 2.93$	$27.02^b \pm 1.30$	$12.05^b \pm 1.68$

Values are expressed as mean ± SE. Mean value ($n = 3$) in column bearing different superscripts varies significantly ($P \leq 0.05$)

haemoglobin is destroyed or a decrease in the rate of its synthesis.

The change in differential leucocyte counts is recognised as sensitive indicator of environmental stress (Cole et al. 2001). In the present study, TLC was significantly reduced in fishes exposed to sub-lethal dose of cypermethrin. Similar findings were reported by Dorucu and Girgin (2001) in *Cyprinus carpio* and Nath and Banerjee (1996) in *Heteropneustes fossilis* following poisoning with cypermethrin. Li et al. (2011) also reported decrease in TLC in rainbow trout exposed to propiconazole. This might be due to the destruction of haematopoeitic tissue or reduced synthesis of leucocytes by chronic exposure to sub-lethal concentration of cypermethrin for long time.

A significantly low ($P \leq 0.05$) serum protein, albumin and globulin levels in Group B fishes exposed to cypermethrin may be attributed to stress-mediated mobilization of these compounds to fulfil an increased demand for energy by the fish to cope up with the detrimental conditions imposed by the toxicant. Similar finding was reported in rohu (Das and Mukherjee 2003) and Korean rockfish (Jee et al. 2005) exposed to cypermethrin. Vani et al. (2011) also reported significant decrease in serum protein, albumin and globulin in catla exposed to sub-lethal concentration of deltamethrin.

The albumin has been reported to be an osmoregulator of blood volume, an easily available protein reserve and a protein transporter (Anderson et al. 1979). Dutta (1996) described that hyperactivity caused by deltamethrin (a synthetic pyrethroid) may lead to the utilization of this easily accessible protein reserve-fraction containing albumin, resulting in a decreased quantity. Another possible reason for the lowered amount of albumin may be a decreased albumin synthesis in the hepatocytes (Dutta et al. 1993). In the present study, similar findings have been noticed in fishes exposed to sub-lethal concentration of cypermethrin. The minimum albumin–globulin ratio in Group B fishes may be mainly due to heavy breakdown of albumin under cypermethrin stress. Nayak et al. (2004) reported a similar result upon permethrin (a pyrethroid insecticide) exposure to *Labeo rohita*. According to their opinion, the reduction in total serum protein, albumin, globulin and albumin–globulin ratio was strong indicators of an immunosuppressive effect of the pesticide.

Toxicants have been shown to cause alterations in the activities of many enzymes concerning to cellular energy metabolism (Sebert et al. 1993; Almeida-val et al. 1995). In the present investigation, alanine and aspartate amino transaminase activities were significantly ($P \leq 0.05$) enhanced in liver tissue of fishes, which were exposed to sub-lethal concentration of cypermethrin. Similar increase in AST and ALT activities in hepatic and gill tissues of *Clarias batrachus* on the exposure to cypermethrin was reported by Begum (2005). Sharma (1999) opined that the rise in the activities of transaminases suggests enhanced protein catabolism and hepatocellular damage in the organism. Similarly, the increase in transaminase activity in catla exposed to sub-lethal concentration of cypermethrin might be due to the enhanced protein catabolism and hepatocellular damage.

In the present study, inhibition in the muscle LDH activity was observed when fishes were exposed to sub-lethal concentration of cypermethrin. The decrease in LDH activity suggests a reduction in the conversion of lactate to pyruvate, thereby leading to the accumulation of lactic acid. The decrease in LDH activity with a consequent increase in the levels of lactic acid suggests the predominance of anaerobic segment, glycolysis. Sivakumari et al. (1997), while working on cypermethrin toxicity, have explained that changes in dehydrogenase activity in pesticide-treated fishes may be due to severe cellular damage leading to the release of these enzymes and impaired carbohydrate and protein metabolism.

Acetylcholine esterase (AChE) is one of the most widely used enzymes as a biomarker for environmental pollution. Acetylcholine is synthesized in nervous tissue by enzyme choline esterase. Acetylcholine is rapidly destroyed by choline esterase, a group of related enzymes that are hydrolytic in action (Stowe 1969). The highly decreased brain AChE activity shown by Group B fishes might be due to the inhibitory effect of cypermethrin. Our results are in agreement with those obtained in *Labeo rohita* (Marigoudar et al. 2009; Das and Mukherjee 2003) and *Channa punctatus* (Kumar et al. 2009), where exposure to cypermethrin showed reduced brain AChE activity.

ATPases, a membrane bound enzyme group, are responsible for the movements of different ions across the cell membrane. In fish, various toxicants enter through gill surface by diffusion. An interaction with the membranes may disrupt the osmotic and ionic regulation of the cell membrane permeability, mainly due to the inactivation of the ATPases in the brancheal epithelial cells (Chhaya et al. 1997). The gill ATPase activity had been used as a sensitive biomarker for the assessment of pollutant-induced damage to the osmoregulatory and acid–base regulatory systems in the gills (Stagg et al. 1992).

The activities of total ATPase, sodium–potassium ATPase and magnesium ATPase were significantly decreased in gill tissue of fishes exposed to sub-lethal concentration of cypermethrin (Group B). Similar to this study, inhibition of the gill ATPase activity upon exposure to cypermethrin is well documented in *Cirrhinus mrigala* (Prashanth and David 2010) and *Labeo rohita* (Das and Mukherjee 2003). The decrease in their activities indicates the demolition of cellular ionic regulations in the organ of fish as reported earlier (Renfro et al. 1974; Schmidt Nielson 1975). This disruption may be due to toxic effect of cypermethrin on passive movement of ions (Chandravathy and Reddy 1995). Further, greater imbalance caused to the gill structures may also be probable reason for changes observed in ATPases activities. At cellular level, pesticide interacts with the ATPases and the interaction mainly depends on the cell surface area (David 1995). The inhibition of Na^{+}–K^{+} ATPase is probably due to the disturbance in Na^{+}–K^{+} pump, resulting in an uncontrollable entry of Na^{+} ions into the cell along

the concentration gradient, with water molecule following along the osmotic gradient. This process may cause swelling of the cell and finally membrane ruptures (Chandravathy and Reddy 1995). The inhibition of Mg^{2+} ATPase, an ion-specific ATPase, could be attributed to the loss of sodium and potassium ions due to cellular leakage into the body fluids. Non-availability of substrates like ATP molecules may also result in the inhibition of these ion-specific ATPases (Chandravathy and Reddy 1995).

Finally, from the present investigation, it can be concluded that the exposure of *C. catla* fingerlings to the sub-lethal concentration of cypermethrin caused significant alterations in its haematological and biochemical parameters. This kind of stressor for a longer period might substantially reduce the growth and immunity of exposed fishes and ultimately it may affect the aquaculture production.

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