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RESEARCH ARTICLE

Acute and sublethal effects of organophosphate insecticide chlorpyrifos on freshwater fish *Oreochromis niloticus*

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ABSTRACT

Chlorpyrifos is a widely used organosphosphate insecticide in India. Residue of the insecticide is frequently detected in trace to moderate concentration in food grains and in surface water of different freshwater ecosystems of the country. In this study, 96 h LC_{50} of the technical grade (94% a.i.) and commercial formulation (20% EC) of chlorpyrifos to freshwater fish *Oreochromis niloticus* were determined as 90.0 and 42.0 μg/L based on 2 h actual concentration of chlorpyrifos in water. About 96 h exposure to sublethal concentrations (0, 12.0 and 25.0 μg/L) of the commercial formulation (20% EC) of chlorpyrifos reduced the level of hepatic glycogen, activities of alkaline phosphatase, acetylcholinesterase, and catalase in liver and elevated the level of plasma glucose and activities of hepatic acid phosphatase, aspartate aminotransferase, and alanine aminotransferase in *O. niloticus*. About 28-day exposure to these sub-lethal concentrations caused anemia in fish, while 90 days exposure reduced growth of the fish and carcass concentration of crude protein and crude lipid as compared to control. It was concluded from this study that commercial formulation of chlorpyrifos (20% EC) was highly toxic to *O. niloticus*. Exposure to sub-lethal concentrations of the insecticide could induce oxidative stress and anemia resulting in reduced growth of the fish.

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Chlorpyrifos; LC_{50}; *Oreochromis*; enzymes; plasma; growth; protein

Introduction

Chlorpyrifos is one of the most widely used organophosphate insecticides to control insect pests of vegetables and cereal crops in India. Chlorpyrifos persists in environment for long time owing to its relatively low volatility and degradation under aerobic conditions (NFMS 2008). Sporadically, residue of the insecticide has been reported in trace to moderate quantity in vegetables, crops (Mukherjee 2003, Srivastava *et al.* 2011) and in surface waters of various freshwater ecosystems (Rahmanikhah *et al.* 2011) in India. This has caused a concern for the environment and human health, because the ecotoxicological effects of chlorpyrifos on non-target aquatic organisms and its potential to biomagnify through food chain are yet to be clearly understood.

In recent years, researchers have focused on toxicity studies of chlorpyrifos on aquatic organisms, because of contamination of the aquatic ecosystem by this insecticide and its potential to cause several adverse effects on aquatic community (Xing *et al.* 2010, Bernabó *et al.* 2011, John and Shaike 2015). Laboratory studies indicate that chlorpyrifos is highly toxic to aquatic invertebrates (Narra *et al.* 2012), amphibian tadpoles (Bernabó *et al.* 2011) and fish (Oruç 2010, Xing *et al.* 2012, Paracampo *et al.* 2015). Acute and chronic toxicity of chlorpyrifos varies between species, life stage and sex of aquatic organism. Oruç (2010) reported chlorpyrifos as more toxic to juveniles (96 h LC_{50}: 98.67 μg/L) than adults (96 h LC_{50}: 154.01 μg/L) of *Oreochromis niloticus*. Wast *et al.* (2015) observed sex specific susceptibility of *Poecilia reticulata* to chlorpyrifos in which males exhibited maximum susceptibility, followed by females and mixed population. Acute toxicity of chlorpyrifos to aquatic organisms is also influenced by bioassay systems and exposure period (Tilak *et al.* 2004, Rao 2008, Nwani *et al.* 2013a, Samajdar and Mandal 2015).

But most of these studies are based on active ingredients of chlorpyrifos. Chlorpyrifos is marketed under different commercial formulations. Emulsified concentrate (EC) containing 10–20% active ingredients and 80–90% inert ingredients, is the most common commercial formulation used in agricultural fields. Constituents of the inert ingredients are never divulged by the manufacturer under the veil of trade secrecy. As a result, specific assessments on the toxicity of inert ingredients of the formulated products to non-target organisms are seriously hampered (Cox and Surgan 2006). However, commercial formulations have been found as more toxic to aquatic organisms than their active ingredients (Agostini *et al.* 2010). Demetrio *et al.* (2014) reported that commercial formulation of chlorpyrifos exerted more toxicity on *Daphnia magna* and *Hydra attenuata* than active ingredient (a.i.). Therefore, it is more relevant to determine toxicity of both technical grade containing more than 90% active ingredient and commercial formulation of chlorpyrifos to evaluate impact of inert ingredients, if any, on the toxicity of chlorpyrifos to non-target aquatic organisms (Pereira *et al.* 2009, De Silva *et al.* 2010). Nile tilapia *O. niloticus* has been used as model test organism in the present study due to its worldwide importance in aquaculture as a fish with fast growth,

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hardy nature, capability to tolerate wide variety of environmental conditions (low oxygen, salinity etc.), omnivorous feeding habit, tasty flesh and easy production of fingerlings in captivity. Chlorpyrifos is reported to alter hematological profiles (Nwani *et al.* 2013a, Narra *et al.* 2015), histopathology (Pal *et al.* 2012, Velmurugan *et al.* 2015), hepatic dysfunction (Oruç 2012), growth (Huynh and Nugegoda 2012), neurobehavioural dysfunction (Levin *et al.* 2004, Richendrfer *et al.* 2012), reproductive physiology (De Silva and Samayawardhena 2005), and endocrine dysfunction (Oruç 2010) in fish. It can also impose oxidative (Oruç 2012, Xing *et al.* 2012), genotoxic (Xing *et al.* 2013, 2015, Wang *et al.* 2014), and cytotoxic (Palanikumar *et al.* 2014) stress on fish. Xing *et al.* (2010) reported reduction of acetylcholinesterase and carboxylesterase activity in brain and muscle of the teleost fish *Cyprinus carpio* due to chlorpyrifos exposure. However, effects of chlorpyrifos on nutrient assimilation in fish is not well documented (Huynh and Nugegoda 2012).

Objectives of the present study were to determine acute toxicity of technical grade (94% a.i.) and commercial formulation (20% EC) of chlorpyrifos to Nile tilapia *O. niloticus*, and to evaluate stress produced by short term and long term exposure to sub-lethal concentrations of the commercial formulation from biochemical, hematological and growth parameters of the fish. Acute toxicity tests were carried out on both technical grade and commercial formulation of chlorpyrifos and intensity of toxicity between the two forms of chlorpyrifos was compared. However, experiments on biochemical, hematological and growth parameters of the fish *O. niloticus* were carried out using only sub-lethal concentrations of commercial formulation (20% EC) of chlorpyrifos, because this form is used in agricultural fields.

Materials and methods

Test specimen and chemicals

Specimens of *O. niloticus* (mean length 4.61 ± 0.24 cm and mean weight 2.64 ± 0.16 g) were procured from Kulia fish farm, Kalyani, West Bengal, and were allowed to acclimatize to the test conditions for 96 h before use. The fish were fed a balanced diet containing 30% crude protein during acclimatization. Technical grade chlorpyrifos (94% active ingredient of O,O-diethyl O-3,5,6-trichloro-2-pyridinyl- phosphorothioate) was obtained from Krishi Rasayan Group of Companies, Kolkata (India) and the emulsified concentrate (20% EC) of chlorpyrifos was procured under the brand name Dursban® from the Dow Agro Sciences India Pvt. Ltd.

Experimental design

Altogether four experiments were carried out in this study using Nile tilapia *O. niloticus* as test organism. The first experiment was 96 h acute toxicity bioassays to determine lethal concentrations of the technical grade (94% a.i.) and commercial formulation (20% EC) of chlorpyrifos. Based on 96 h LC_{50} value of chlorpyrifos (20% EC) three sublethal concentrations of chlorpyrifos were determined. Three separate experiments were conducted with these sublethal concentrations of chlorpyrifos. These experiments included 96 h bioassays on biochemical parameters, 28-day bioassays on hematological parameters, and 90-day bioassays on growth of *O. niloticus*. Deep tube-well water stored in an overhead tank (Temperature 32° ± 3 °C, pH 7.2 ± 0.1; free CO_2 2.31 ± 0.31 mg/L; dissolved oxygen 7.03 ± 0.2 mg/L; total alkalinity 128.18 ± 3.37 mg/L as $CaCO_3$; total hardness 139.23 ± 11.01 mg/L as $CaCO_3$) was used as the test medium in all experiments.

Table 1. Range of concentrations of chlorpyrifos (μg/L) used.

Active ingredient (94%)											
Nominal	0	30	50	70	90	110	130	150	170	190	210
Actual after 2 h	0	25	42	58	75	91	108	125	141	158	174
% Recovery	–					83 ± 0.2					
Emulsified concentrate (20% EC)											
Nominal	0	5	10	20	40	60	80	100	120	130	140
Actual after 2h	0	4	9	18	35	53	71	88	106	115	124
% recovery	–					88 ± 2					

Acute toxicity bioassays

Acute toxicity bioassays were conducted, following the static bioassay procedures of APHA (1995), in 15 L glass aquaria each holding 10 L of water and five acclimatized fish. Separate bioassays were carried out with technical grade (94% a.i.) and formulation (20% EC) of chlorpyrifos. The range of concentrations of chlorpyrifos used for these bioassays have been presented in Table 1. Three replicates were set for each concentration including a water control and a solvent control containing 0.1 ml/L acetone, because technical grade chlorpyrifos was dissolved in acetone and the maximum amount of acetone present in the highest concentration of technical grade chlorpyrifos tested was less than 0.1 ml/L. The experiment was conducted for 96 h. Mortality of the fish was noted every 24 h and the dead fish were removed. No food was supplied during the tenure of bioassay to avoid interference of excretory products of the fish with the test chemical. Lethal concentrations of chlorpyrifos at which 50% mortality of the test fish occurred (LC_{50}) and its 95% confidence limits were estimated for 96 h from the mortality data using EPA-Probit analysis version 1.5 statistical software based on probit analysis method of Finney (1971). Criteria of Mayer and Ellersieck (1986), Schmuck *et al.* (1994), and Demetrio *et al.* (2014) were applied to compare LC_{50} values between the active ingredient and formulation.

Residue analysis of chlorpyrifos

Residue analyzes of chlorpyrifos were made in samples of water collected from the aquaria used for acute toxicity bioassay. Concentrations of chlorpyrifos in water after 2 h of exposure were measured by chromatographic methods. About 250 ml of sample water was collected from each aquarium in a 500 ml conical flask. 25 g sodium chloride (NaCl) was added to it and then partitioned with 50 ml mixture of Hexane and Dichloromethane (80:20) three times and organic phase was collected over anhydrous sodium sulfate in a conical flask and evaporated in a rotary evaporator. The volume was made up to 10 ml with ethyl acetate. The extract

obtained was filtered with the help of syringe filter using 25 mm, 0.22 μ nylon filter paper, and transferred into the vials for estimation of chlorpyrifos in a Gas Chromatograph equipped with ECD detector (Agilent 6890 N) equipped with wide bore HP column (HP-5, 30 m, 0.32 mm ID, and 0.25 μm film thickness), Electron Capture Detector (ECD) and a 7683 B Series auto injector. N_2 gas was used as a carrier. Concentration of chlorpyrifos was quantified from the calibration curve prepared from standard chlorpyrifos concentrations, using Chemistation software. Limit of detection (LOD) and limit of quantification (LOQ) of the instrument was respectively 0.005 and 0.15 ppm. Nominal concentrations (N) used in the experiments and their respective actual concentrations (A) determined by above method after 2 h of exposure have been given in Table 1.

Experiments on biochemical parameters

Experiments on biochemical parameters were also carried out in 15 L glass aquaria, each containing 10 L of water and five fish. Three sub-lethal concentrations of the chlorpyrifos (20% EC) used for these experiments were control (0.0 μg/L), a low dose (12.0 μg/L), and a high dose (25.0 μg/L) of chlorpyrifos (20% EC). The low and high dose of chlorpyrifos were approximately 25 and 50% of the 96 h LC_{50} value of the nominal concentration of chlorpyrifos (20% EC) to *O. niloticus*, respectively. There were three replicates for each concentration. Fish specimens were sampled after 96 h of exposure, rinsed in deionised water and dried in blotting paper. Blood was collected directly from the heart of the sampled fish with micro syringe and was used for glucose estimation. Simultaneously, liver was dissected out from the sampled fish, weighed, homogenized and the homogenate was used for analysis of glycogen content, alkaline and acid phosphatase, aspartate and alanine aminotransferase, acetylcholinesterase, and catalase activities of the liver. Plasma glucose was determined by the method of Hyvarinen and Nikkila (1962) and liver glycogen was determined by the method of Carroll *et al.* (1956). Protein in liver tissue was determined by the method of Lowry *et al.* (1951), while activities of the enzymes acid and alkaline phosphatase, aspartate and alanine aminotransferase, acetylcholinesterase and catalase were determined respectively by the method of Walter and Schutt (1974), Reitman and Frankel (1957), Ellman *et al.* (1961), and Luck (1974).

Experiments on hematological parameters

These experiments were also conducted in 15 L glass aquaria without any alterations in treatments and experimental conditions as described above in experiment for biochemical parameters. However, the experiment was carried out for 28 days. Half of the fish were sampled from each aquarium at the end of 14 days and rest half was sampled at the end (28 days). Blood was drawn from the sampled fish by the technique described above and was used to determine different hematological parameters. Hemoglobin (Hb%) was determined by cyanomethemoglobin method following Dacie and Lewis (1968), packed cell volume (PCV) or Haematocrit (Hct) was determined by Wintrobes method or Macromethod (Dacie and Lewis 1968) and total erythrocyte count (TEC) and total leucocyte count (TLC) was done by Neubauer's improved double hemocytometer using RBC and WBC diluting fluid, respectively.

Experiments on growth

Bioassays were conducted in 400 L outdoor cement vat each with a 3-cm thick layer of uncontaminated soil mixed with cow dung at the bottom. The vats were then filled with water and kept undisturbed for one month, which ensured growth of planktons as the natural food for the test fish. Thirty numbers of fingerlings of *O. niloticus*, irrespective of sex, were stocked in each vat and were acclimatized for one week before the start of the experiments. Altogether nine vats were arranged according to randomized block design so that fingerlings could be reared in three replicates for each of the three test concentrations (0, 12.0, and 25.0 μg/L) of chlorpyrifos (20% EC). Treatments of the chlorpyrifos were made on day 1 of the experiment (initial treatment) and 20% of the test medium was renewed at 10 days interval. The experiment was continued for 90 days. During the experiments the fish were hand fed, daily at 8.0 A.M. and 4.0 P.M., a formulated diet containing 30% crude protein up to apparent satiation of the fish. Observations were made daily on the behavior and mortality of the experimental fish. Water samples were collected from each vat every 15 days and dissolved oxygen, free carbon dioxide, temperature, total hardness and total alkalinity of the sampled water were determined by standard method (APHA 1995). All fish sampled at the end of 90 days and length (cm) and weight (g) of the sampled fish were recorded. Three sampled fish from each vat were subjected to biochemical analyzes to determine crude protein, crude lipid and ash content of the fish following the AOAC method (Helrich 1990). Growth was determined from percent increase in weight, specific growth rate (SGR), feed conversion ratio (FCR) and apparent net protein utilization (ANPU) using standard formulae (Bagenal 1978, Castell and Tiews 1980, Adams and Mclean 1985, Steffens 1989).

Statistical methods

The data of biochemical, hematological and growth parameters were subjected to single factor ANOVA followed by least significant difference (LSD) test to verify significance of difference between treatments at 5% level of probability (Gomez and Gomez 1984).

Results

Acute toxicity

96 h LC_{50} value of technical grade (94% a.i.) and formulation (20% EC) of chlorpyrifos to *O. niloticus* has been presented in Table 2. The formulation was found more toxic to *O. niloticus* than the technical grade chlorpyrifos. Fish exposed to chlorpyrifos exhibited symptoms of loss of equilibrium. They

Table 2. 96 h LC $_{50}$ values (μg/L) of chlorpyrifos and 95% confidence limit (CL) to freshwater teleost *Oreochromis niloticus*.

	Nominal		Actual (2h)	
Chlorpyrifos	LC_{50}	95% CL	LC_{50}	95% CL
Technical (94% a.i.)	109	90–129	90	75–107
Formulation (20% EC)	47	33–65	42	30–57

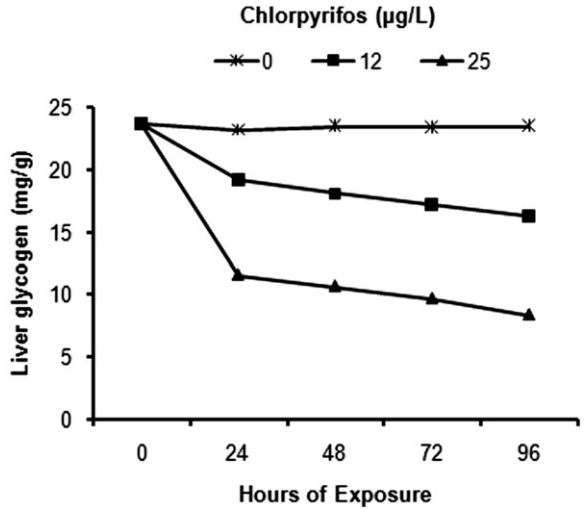

Figure 1. Effects of chlorpyrifos (20% EC) on liver glycogen of *O. niloticus*.

frequently came to the surface of water and gulped air. In exposures to high concentrations, the fish exhibited spiral swimming. After an initial increase, the opercular movements of the exposed fish steadily decreased with the increase in exposure period of the insecticide. Ultimately, fish sunk to the bottom and died with mouth open wide. Copious secretion of mucus was found on the surface of gill of the dead fish. Symptoms of heavy internal hemorrhage were also observed around pharynx of the dead fish.

Effects of sub-lethal concentrations of chlorpyrifos

Liver glycogen and plasma glucose

Changes in liver glycogen and plasma glucose levels of control and chlorpyrifos (20% EC) treated *O. niloticus* have been presented in Figures 1 and 2. The results demonstrate a significant decrease ($p < 0.05$) in hepatic glycogen level (Figure 1) and increase in plasma glucose level (Figure 2) in fish exposed to chlorpyrifos as compared to control. The effects were dose dependent. The higher the concentration of chlorpyrifos, lower the level of hepatic glycogen and the higher the plasma glucose level.

Hepatic enzymes activities

Effects of chlorpyrifos on hepatic enzyme activities of *O. niloticus* have been presented in Table 3. Results indicated that chlorpyrifos exposure significantly elevated ($p < 0.05$) hepatic acid phosphatase, aspartate aminotransferase, and alanine aminotransferase level of *O. niloticus*. In contrast, the activities of hepatic alkaline phosphatases, acetylcholinesterase, and catalase were reduced in chlorpyrifos treated groups in comparison to control. Significant differences in activities of

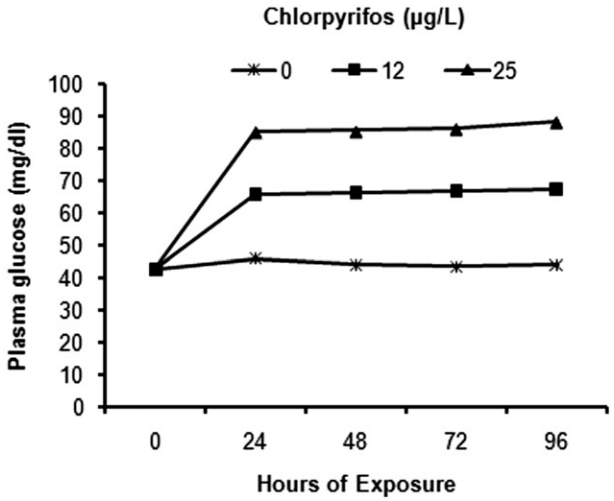

Figure 2. Effects of chlorpyrifos (20% EC) on plasma glucose of *O. niloticus*.

Table 3. Changes in hepatic enzymes of *O. niloticus* after 96 h exposure to chlorpyrifos (20% EC).

Parameter	0.0 μg/L	12.0 μg/L	25.0 μg/L
Alkaline phosphatase[a]	11.02 ± 0.04^a	9.05 ± 0.08^b	6.66 ± 0.09^c
Acid phosphatase[a]	6.73 ± 0.06^a	7.91 ± 0.07^b	9.06 ± 0.11^c
Aspartate aminotransferase[b]	2.39 ± 0.02^a	3.07 ± 0.06^b	3.77 ± 0.04^c
Alanine aminotransferase[b]	5.77 ± 0.04^a	6.98 ± 0.03^b	9.90 ± 0.08^c
Acetylcholinesterase[c]	0.74 ± 0.01^a	0.49 ± 0.01^b	0.28 ± 0.02^c
Catalase[d]	13.52 ± 0.16^a	10.90 ± 0.22^b	8.34 ± 0.08^c

[a]μg PNP/mg tissue protein/30 min.
[b]μM pyruvate/mg tissue protein/h.
[c]μM acetylthiocholine iodide/mg tissue protein/min.
[d]μM H_2O_2 decomposed/mg tissue protein/min.
Data are mean ± SD ($n = 3$); means with dissimilar superscripts in the same row indicates least significant difference between the means at $p < 0.05$.

hepatic enzymes were noted between the two sublethal concentrations (12.0 and 25.0 μg/L) of chlorpyrifos.

Hematological parameters

The changes in hematological parameters of *O. niloticus* exposed to control and different doses of chlorpyrifos have been summarized in Table 4. The results showed a significant ($p < 0.05$) decrease in the values of total erythrocyte count, hemoglobin concentration, packed cell volume, mean cell hemoglobin with the increase in concentration and duration of chlorpyrifos exposure. Besides, a significant decrease ($p < 0.05$) in mean cell volume was also observed in chlorpyrifos exposures in comparison to control; but mean cell volume (MCV) value was comparable between 12.0 and 25.0 μg/L till 14 days ($p > 0.05$), while MCV value significantly decreased in 25.0 μg/L as compared to 12.0 μg/L after 28 days exposure. Total leucocyte count increased in exposed fish in comparison to control. In addition, mean corpuscular hemoglobin concentration declined gradually in chlorpyrifos treated groups though the difference between 0.0 and 12.0 μg/L was insignificant ($p > 0.05$) after 28 days of exposure.

Effects on growth

Long term exposure to different sublethal concentrations of chlorpyrifos (0.0, 12.0, and 25.0 μg/L) on mortality, feed

Table 4. Changes in hematological parameters of *O. niloticus* exposed to cypermethrin.

Parameters	Days of exposure	0.0 μg/L	12.0 μg/L	25.0 μg/L
Total erythrocyte count (10^6/mm^3)	14	2.88 ± 0.08^a	2.74 ± 0.05^b	2.44 ± 0.06^c
	28	2.88 ± 0.03^a	2.55 ± 0.08^b	2.42 ± 0.04^c
Hemoglobin concentration (g/dl)	14	8.71 ± 0.06^a	7.72 ± 0.06^b	6.42 ± 0.09^c
	28	8.82 ± 0.07^a	7.25 ± 0.06^b	5.78 ± 0.11^c
Packed cell volume (%)	14	26.34 ± 0.07^a	23.96 ± 0.13^b	20.53 ± 0.17^c
	28	26.64 ± 0.14^a	22.27 ± 0.19^b	20.17 ± 0.07^c
Mean cell volume (fl/cell)	14	91.38 ± 2.13^a	87.47 ± 1.56^b	84.28 ± 1.52^b
	28	92.49 ± 0.88^a	87.27 ± 1.86^b	83.49 ± 1.09^c
Mean cell hemoglobin (pg/cell)	14	30.24 ± 0.92^a	28.18 ± 0.58^b	26.37 ± 0.54^c
	28	30.63 ± 0.29^a	28.39 ± 0.96^b	23.94 ± 0.70^c
Mean corpuscular hemoglobin concentration (g/dl)	14	33.09 ± 0.31^a	32.22 ± 0.10^b	31.29 ± 0.21^c
	28	33.11 ± 0.39^a	32.54 ± 0.41^a	28.67 ± 0.59^b
Total leucocyte count (10^3/mm^3)	14	23.54 ± 0.13^a	24.89 ± 0.07^b	26.08 ± 0.13^c
	28	23.98 ± 0.16^a	26.35 ± 0.08^b	27.03 ± 0.08^c

Data are mean ± SD ($n = 3$); means with dissimilar superscripts in the same row indicates least significant difference between the means at $p < 0.05$.

Table 5. Changes in growth parameters and mortality of *O. niloticus* exposed to chlorpyrifos (20% EC) for 90 days.

Parameters	0.0 μg/L	12.0 μg/L	25.0 μg/L
Initial weight (g)	2.64 ± 0.16	2.64 ± 0.16	2.64 ± 0.16
Weight gain[a] (%)	129.75 ± 4.94^a	64.17 ± 3.69^b	42.55 ± 3.97^c
FCR[b]	3.04 ± 0.10^a	4.01 ± 0.24^b	5.27 ± 0.48^c
SGR[c] (%/d)	0.92 ± 0.02^a	0.55 ± 0.03^b	0.39 ± 0.03^c
PER[d]	1.10 ± 0.04^a	0.83 ± 0.05^b	0.64 ± 0.06^c
HSI[e]	1.02 ± 0.03^a	1.67 ± 0.04^b	1.88 ± 0.06^c
ANPU[f] (%)	21.29 ± 0.56^a	13.23 ± 0.67^b	9.16 ± 0.68^c
Mortality (%)	3.02 ± 0.53^a	18.41 ± 2.18^b	23.26 ± 1.22^c

[a]Weight gain (%) = [{Final wt (g)–Initial wt (g)}/Initial wt (g)] × 100.
[b]Feed conversion ratio = Food given/Weight gain.
[c]Specific growth rate (%/d) = {(log $_e$ W_2 − log$_e$ W_1)/T} × 100 where, W_1 = initial body weight (g) and W_2 = final body weight (g); T = days of exposure.
[d]Protein efficiency ratio = Increase in weight of fish (wet weight)/Weight of protein in feed (dry weight).
[e]Hepatosomatic index = [{Wet weight of liver(g) without gall bladder}/Wet body weight] × 100.
[f]Apparent net protein utilization (%) = (Net increase in carcass protein/Amount of protein consumed) × 100.
Values are mean of three replicates ± SD; means with dissimilar superscripts in the same row indicates least significant difference between the means at $p < 0.05$.

Table 6. Proximate composition (% wet weight basis) of carcass of *O. niloticus* exposed to chlorpyrifos (20% EC) for 90 days.

		Final		
	Initial	0.0 μg/L	12.0 μg/L	25.0 μg/L
Crude protein	11.18 ± 0.31	15.51 ± 0.15^a	12.97 ± 0.20^b	12.06 ± 0.17^c
Crude lipid	2.76 ± 0.13	4.81 ± 0.12^a	3.58 ± 0.11^b	3.16 ± 0.09^c
Ash	2.10 ± 0.06	4.07 ± 0.09^a	2.41 ± 0.07^b	2.25 ± 0.05^c

Values are mean of three replicates ± SD, means with dissimilar superscripts in the same row indicates least significant difference between the means at $p < 0.05$.

utilization and growth performance of *O. niloticus* have been presented in Table 5 There was significant variation in mortality between the concentrations. As compared to control, mortality of the fish significantly increased at 12.0 and 25.0 μg/L of chlorpyrifos exposure. The results clearly indicate that pulse treatment of chlorpyrifos for ninety days significantly reduced growth rate of the exposed fish in comparison to control. The results showed significant decrease ($p < 0.05$) in weight gain percentage (WG%), specific growth rate (SGR), protein efficiency ratio (PER), and apparent net protein utilization (ANPU) with the increasing concentrations of chlorpyrifos. Feed conversion ratio (FCR) and hepatosomatic index (HSI) of the chlorpyrifos exposed fish was significantly higher than that of control ($p < 0.05$). Growth reduced with the increase in concentration of chlorpyrifos. Proximate compositions of carcass of the sampled fish have been presented in Table 6. The proximate composition analysis of carcass of *O. niloticus* at the end of 90 days in outdoor trial indicate increase of all parameters (crude protein, crude lipid and ash content) as compared to initial values. The rate of increase was significantly lower in chlorpyrifos exposed fish than the control ($p < 0.05$).

Table 7. 96 h LC_{50} values of chlorpyrifos to different species of fish.

Test species of fish	LC_{50} value (μg/L)	Reference
Oreochromis mossambicus	82	Kunjamma *et al.* (2008)
Gambusia yucatana	85	Rendón-von Osten *et al.* (2005)
Oreochromis niloticus	90	This paper
Cnesterodon decemmaculatus	105	Paracampo *et al.* (2015)
Cyprinus carpio	149	Li *et al.* (2013)
Oreochromis niloticus	154	Oruç (2010)
Cyprinus carpio	160	Halappa and David (2009)
Poecilia reticulata	176	Sharbidre *et al.* (2011)
Pimephales promelas	200	Belden and Lydy (2006)

Discussion

Acute toxicity

Results of the present study indicate that 90 μg/L (2 h actual concentration in water) is the 96 h LC_{50} value of chlorpyrifos (94% a.i.) for the freshwater fish *O. niloticus*. This value is similar to values reported by many other workers (Table 7). On the other hand, 96 h LC_{50} value of the commercial chlorpyrifos (20% EC) for *O. niloticus* is only 42 μg/L (2 h actual concentration in water). It clearly indicates that the commercial formulation of chlorpyrifos (20% EC) is more toxic than the technical grade (94% a.i.) chlorpyrifos. Similar differences in toxicities between commercial formulation and technical grade chlorpyrifos were reported by Tilak *et al.* (2001), De Silva *et al.* (2010), and Demetrio *et al.* (2014) for *Labeo rohita*, *Perionyx excavatus* and *Daphnia magna* respectively. The high toxicity of the formulated products might be due to added inert ingredients (Cox and Surgan 2006). Mayer and Ellersieck (1986) assumed formulation as more toxic when quotient (ratio of LC_{50} between technical and formulation) was more than 1, while Schmuck *et al.* (1994) considered formulation as more toxic when quotient was more than 2. In this study, quotient was found as 2.32. However, Demetrio *et al.* (2014) proposed to

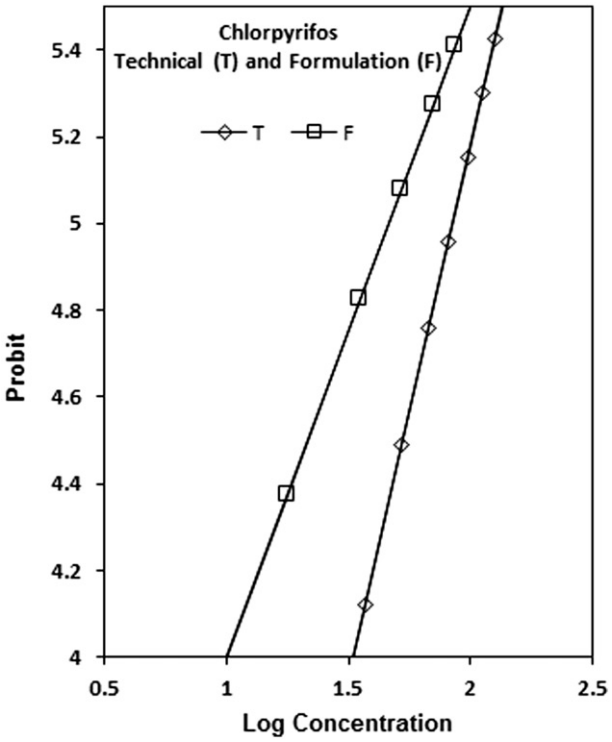

Figure 3. Regression lines for log concentration versus probit mortality of *O. niloticus* following 96 h exposures to technical (T) and formulation (F) of chlorpyrifos.

accept the criteria of quotient as valid only when the concentration effect lines were parallel. In the present study the slopes of different species were not perfectly parallel between technical grade and formulation (Figure 3).

Biochemical parameters

The decreased hepatic glycogen content and increased plasma glucose level in chlorpyrifos-treated *O. niloticus* observed in present study are clear indications of increased glycogenolysis and inhibition on glycolytic pathway as probable adaptive strategies against pesticidal stress. Similar results were reported in *Clarias batrachus* (Narra *et al.* 2015) and *Clarias gariepinus* (Nwani *et al.* 2013b). The elevated plasma glucose levels could meet the demand of excess glucose to satisfy the metabolic needs raised due to chlorpyrifos stress (Nwani *et al.* 2013b). Stress induces conversion of glucose into pyruvate in the glycolytic pathway, then pyruvate is metabolically converted to acetyl-CoA in aerobic tissues, which could be utilized as a precursor for synthesizing the cholesterol and fatty acids in the Kreb's cycle (Nelson and Cox 2002, Tiwari *et al.* 2012).

The present study recorded the decrease in hepatic alkaline phosphatase (ALP) and increase in acid phosphatase (ACP) activity in *O. niloticus* due to exposure of sublethal concentrations of chlorpyrifos. Similar effects were reported in *Clarias batrachus* (Narra *et al.* 2011) for ALP and in *Gambusia affinis* for ACP (Khan and Sharma 2012) exposed to chlorpyrifos. Alkaline phosphatase is crucial in carbohydrate metabolism and transport of phosphorylated intermediates across the cell membranes (Vijayavel and Balasubramanian 2006). Acid phosphatase is a lysosomal enzyme catalyzing the hydrolysis of phosphate esters in acidic medium. Altered levels of acid and alkaline phosphatases activity within the tissue indicate a shifting of energy breakdown pathway from normal ATPase system to phosphatase system with increased phosphorylase activity. Activated phosphatase then induce release of inorganic phosphates from phosphate esters (Khan and Sharma 2012). Liver alkaline phosphatase inactivate phosphorylase enzymes and induces glycogen synthesis. Depletion of alkaline phosphatase in liver thus may lead to breakdown of glycogen. Shaikila *et al.* (1993) explained that acidosis might cause inhibition of alkaline phosphatase activities in liver of *Sarotherodon mossambicus* as an adaptive measure to get the energy via anaerobic breakdown of glycogen. Several studies reported increased activities of alkaline phosphatase in serum of fish due to the leakage of these enzymes from liver into blood stream as a result of damage of hepatocytes and consequent hepatotoxicity of cypermethrin (El-Sayed and Saad 2008, Firat *et al.* 2011, Meenambal *et al.* 2012). On the other hand, increase in acid phosphatase activity of chlorpyrifos exposed fish may be due to hepatocellular damages (Khan and Sharma 2012).

The activities of both aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were found to be increased, as compared to control, on all exposure periods irrespective of chlorpyrifos concentrations tested. Similar increase in AST and ALT were reported in liver of *Clarias batrachus* (Narra *et al.* 2011) and plasma of *Cyprinus carpio* (Banaee *et al.* 2013) due to chlorpyrifos treatment. AST and ALT are crucial to mobilize L-amino acids for gluconeogenesis and work as link between carbohydrate and protein metabolism in fish under stress. Increased activities of hepatic AST and ALT of chlorpyrifos exposed *O. niloticus* indicate increase in protein catabolism and hepatocellular damages.

Reduction in acetylcholinesterase activity in hepatic tissues of *O. niloticus* due to chlorpyrifos exposure observed in the present study bear similarity with the findings of previous workers studying the same in different fish tissues *viz.* brain of *Gambusia affinis* (Rao *et al.* 2005), brain, gill (Rao *et al.* 2003), and gonad (Oruç 2010) of *O. niloticus*, brain of *Tandanus tandanus* (Huynh and Nugegoda 2012), liver of *Chanos chanos* (Palanikumar *et al.* 2014), brain of *Anabas testudineus* (Tam *et al.* 2015), and brain of Rainbow trout (Topal *et al.* 2016) exposed to chlorpyrifos. Chlorpyrifos like other organophosphate insecticides can disrupt the activities of acetylcholinesterase by irreversible binding to its serine hydroxyl group and thus inactivate the enzyme (Oruç 2010). Acetylcholinesterase inhibition in neuromuscular junctions and cholinergic synapses induces accumulation of acetylcholine, which finally leads to disturbances related to nerve impulse propagation and interferes energy metabolism in the nervous system (Thompson and Richardson 2004, Da Cuna *et al.* 2011).

Present results indicate that chlorpyrifos exposure reduces catalase activity in liver of *O. niloticus*. This finding is supported by reports on effects of chlorpyrifos on liver of *Chanos chanos* (Palanikumar *et al.* 2014) and *O. niloticus* (Oruç 2012); liver and gills of *Cyprinus carpio* (Xing *et al.* 2012) and brain, liver, gills of *Poecilia reticulata* (Sharbidre *et al.* 2011).

Catalases are available within peroxisomes of most cells and known as oxidative biomarker enzymes to be involved in detoxification of hydrogen peroxide (H_2O_2) into molecular oxygen (O_2) and water (Oruç 2012, Palanikumar *et al.* 2014, Bhavan *et al.* 2015). Reduction of catalase activity in the present study indicates chlorpyrifos induced oxidative damage to cells.

Hematological parameters

Changes in hematological parameters are considered as valuable indices of the physiological status of an organism in response to toxicant (Nwani *et al.* 2013b). Reduction of total erythrocyte count, hemoglobin content (Hb), packed cell volume of *O. niloticus* exposed to chlorpyrifos, observed in the present study, suggested a probable outcome of erythrocyte hemolysis (Samajdar and Mandal 2015) and resembled similar effects in *Cyprinus carpio* (Ramesh and Saravanan 2008), *Channa punctatus* (Ali and Kumar 2012), *Labeo bata* (Samajdar and Mandal 2015), and *Clarias batrachus* (Narra *et al.* 2015). Reduction of hemoglobin (Hb) content might be due to rapid oxidation of hemoglobin (Hb) to methemoglobin or liberation of O_2 radically induced by the toxic stress of chlorpyrifos (Ramesh and Saravanan 2008). Besides, significantly lower values of mean cell volume (MCV), mean cell hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) in chlorpyrifos exposed *O. niloticus* indicate anemic state (Samajdar and Mandal 2015). In this study, elevation of total leucocyte count due to chlorpyrifos exposure corroborated the findings of Ramesh and Saravanan (2008) in *Cyprinus carpio* and Samajdar and Mandal (2015) in *Labeo bata*. Ramesh and Saravanan (2008) suggested a chlorpyrifos induced leucocyte hypersensitivity, which resulted in increased total leucocyte count and consequent immunological reactions to liberate antibodies to combat against toxic stress of chlorpyrifos.

Effects on growth and biochemical composition

Ninety days exposure to chlorpyrifos reduced growth of *O. niloticus* in the form of decrease in weight gain percentage (WG%), specific growth rate (SGR), protein efficiency ratio (PER), apparent net protein utilization (ANPU) and increase in FCR as compared to control. Huynh and Nugegoda (2012) also observed negative effects on growth and feed utilization of chlorpyrifos exposed *Tandanus tandanus*. Increase in hepatosomatic index of chlorpyrifos exposed fish in the present study, might be due to increased hepatic function during stress as also evident from the enzymatic activities discussed above. Decreased body weight (reduction in weight gain) may also have relationship with decrease in food intake probably to minimize metabolic energy need for digestion during stress (Al-Rudainy 2015). However, reduction in crude protein level appeared as the main effects on reduction in all growth parameters of *O. niloticus*, because synthesis of protein is required for elaboration of tissue, which in turn affects FCR, PER, and ANPU. Reduction in the level of crude protein in the body of fish is a common effect of organophosphate insecticides (Yaji and Auta 2007). It is assumed that protein and lipid are catabolized to generate more energy to combat against stress.

Conclusions

Results of the present study clearly reveal that the commercial formulation of chlorpyrifos (20% EC) is highly toxic to freshwater fish *O. niloticus* as compared to technical grade chlorpyrifos (94% a.i.). It is established from this study that chlorpyrifos is an acetylcholinesterase inhibitor. Mortality of *O. niloticus* under acute exposure of chlorpyrifos is, therefore, probably mediated through impaired nerve impulses. Even short term exposure (96 h) to sub-lethal concentrations (12.0 and 25.0 µg/L) of chlorpyrifos resulted in increased glycogenolysis, protein catabolism and oxidative damages to *O. niloticus*. The present study further reveals that if the exposure to such sub-lethal concentrations is continued it can cause anemia, reduce growth and deposit less amount of protein and lipid in the body of fish.

Disclosure statement

No potential conflict of interest was reported by the authors.

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