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Changes in behavioural and locomotory activities of freshwater fish, *Cirrhinus mrigala* (Hamilton) in response to sublethal exposure of Chlorpyrifos

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Abstract
The aim of the present study was to analyze the impact of sub-lethal toxicity of chlorpyrifos, one of the largest market selling agrochemical on physiological parameters of teleost fish, *Cirrhinus mrigala* addressing the possible causative involvement in behavioural and locomotion responses. Primarily, the acute toxicity (96h LC_{50}) test was carried out and the value calculated by probit analysis was found to be 0.44 mg L^{-1}. Further, one-fifth, one-tenth and one-twentieth of 96 h LC_{50} were selected as sublethal concentrations for sub acute studies. The experiment was carried out for 21 days and the alternative behaviour was recorded in terms of Air Ingulping (AI), Operculum Beat Frequency (OBF), Surfacing Movement (SM), Vertical Hanging (VH) and Tail Beat Frequency (TBF) on duration day 2, 4, 7, 14 and 21. Significant effect of both the concentrations and duration was observed in fishes treated with selected doses of chlorpyrifos. It was found that AI, OBF, SM, VH, TBF was highest on day 7 at 0.08 mg L^{-1} concentration of Chlorpyrifos. The findings revealed that there is a need to control the use of chlorpyrifos because of its toxicity. All the fish avoidance tests proved to be an important predictive and sensitive biomarker in aquatic monitoring and pollution management.

Keywords: Behavioural alterations, Biomarkers, Chlorpyrifos, *Cirrhinus mrigala*, Concentration, Dependent response

INTRODUCTION

Environmental pollutants especially the agrochemicals are posing serious risk to aquatic biota because of their unsystematic use at global scale. Undoubtly, there is an immediate need to monitor the impact of these chemicals especially on the non-targeted species. Behaviour is a sensitive measure of an organism's response to stress including environmental contaminants. It is served as biological indicator which provides a unique perspective linking the physiology and ecology of an organism and its environment. Therefore, behaviour is considered as a comforting tool in ecotoxicology (Drummond *et al*., 1990; Scherrer, 1992; Cohn and MacPhail, 1996; Dube and Hosetti, 2010; Kumar *et al*., 2015). This allows an organism to adjust to any stimuli (external or internal) in order to survive in altering habitat. Contrarily, behaviour is also the outcome of refitting to environmental variables. The use of behaviour responses, such as avoidance behaviour, has been proposed as a quick screening tool for preliminary assessment of toxicity (Slimak, 1997; Loureiro*et al*., 2005; Lukkari*et al*., 2005). These studies are becoming prominent in toxicity assessments in unicellular organisms (Tadehl and Hader, 2001), insects (Martin, 2003), fish (Hansen *et al*., 1999) and even rodents (Dell'Omo*et al*., 1997). Earlier studies conducted had discussed several other behavioural alterations in the fishes as loss of locomotor activity, sometimes rapid and jerky movements of body and fins, increased rate in ventilation, jumping movements towards the surface, sometimes very slow and backward swimming followed by convolutions and depleted rate of feeding in fishes (Rao *et al*., 2004; Rao *et al*., 2005; Cheema *et al.,* 2014).

Chlorpyrifos (O, O-diethyl-O-(3, 5, 6-trichoro-2-pyridyl)-phosphorothiorate) is a chlorinated broad spectrum, largest market selling and multipurpose organophosphate insecticide It is widely used in urban and domestic pest control. Chlorpyrifos is also registered for agricultural uses which include crops, cotton, sugarcane, vegetables, cereals, canola rice, pome fruits, stone fruit, citrus, tropical

fruit and grapes (Berg, 1986; Ali *et al*., 2009). This pesticide needs to be activated into its oxon metabolite by cytochrome P450 in order to become toxic and inhibit AChE activity because this chemical act by interfering with an enzyme known as cholinesterase, which is required for the proper functioning of the nervous system (Ali *et al*., 2009) . Its residues have been detected in human blood, in milk sample from cow, buffalo, dog and ewe (Marrs, 1993; Yasmashita*et al*., 1997) and even in scented roses and their products (Kumar*et al*, 2004).

Fish are able to ingest and retain various xenobiotics dissolved in water via active or passive approaches. Therefore, these can be used to detect and document pollutants released into their environment. *Cirrhinus mrigala* is one of the prime cultured and staple Indian Major Carp generally found in rivers, ponds and reservoirs. Contamination of water bodies at sub lethal levels by chlorpyrifos is common in northern states of India. Hence, the present work focused to analyse the aquatic toxicity of chlorpyrifos with special emphasis on behavioral and locomotion responses using video recording system on the fish, *C. mrigala* exposed to desired doses of commercial grade of this pesticide.

MATERIALS AND METHODS

Toxicant used: The pesticide for the experimental study, technical-grade Chlorpyrifos (50% EC), CAS No. 2921-88-2.1 with the trade name Classic Super manufactured by M/S. Cheminova, India Ltd., 242/P, G.I.D.C Panoli 3941/6, District: Bhaurch, Gujrat was purchased from local market.

Experimental animal: Fingerlings of freshwater fish *C. mrigala* (Hamilton) with an average wet weight 20±0.22g and length 14.3±0.03 cm were procured from the local outlets. The fingerlings were given prophylactic treatment by bathing them twice in 0.05% potassium permanganate ($KMnO_4$) solution for two minutes to avoid any dermal infections. These were then acclimatized for two weeks under laboratory conditions and were provided with diet containing 40% protein at 4% Body Weight (BW) in two installments a day. Dietary ingredients (g Kg^{-1}) were groundnut oil cake: 650 g, rice bran: 42 g, processed soyabean: 276 g, wheat flour: 32 g, and mineral mixture: 10 g. Chlorine free tap water was renewed on alternative day with these physiochemical characteristics (Temp. 25±1.0°C, pH 7 ± 0.33, and DO 6.5 ± 0.33 mg L^{-1}) and lighting schedule of 12 hours of light alternating with 12 hours of darkness (LD12:12) was maintained. The oxygenation was provided with the aerators during acclimatization as well as experimentation period.

Determination of sub lethal concentration: The acute toxicity bioassay to determine the 96h LC_{50} value of chlorpyrifos in fish was conducted in the semistatic system according to the standard methods (APHA, 2012). The stock solution of chlorpyrifos (1.0 ml L^{-1}) was prepared by dissolving 1 ml of it in 1L of chlorine free tap water. A set of ten pre acclimatized fish specimens were randomly exposed to each of the six chlorpyrifos concentration with initial dose starting from 0.0001 mg L^{-1} and the experiment was repeated thrice to obtain the 96h LC_{50} value of the test chemical.

The 96h LC_{50} of chlorpyrifos for the *C. mrigala* was determined as 0.44 mg L^1(with 95% confidence limits), with regression equation y=11.87x-0.5333 and R^2=0.881 following the probit analysis (taking mortality with respect to time and concentration) as described by Finney (1981) (Table 1). Based on the median lethal concentration (96h LC_{50}) value, three sub-lethal concentrations of chlorpyrifos *viz*., sublethal 1 ($1/20^{th}$ of 96h LC_{50}=0.02 mg L^{-1}; Treatment 1) sublethal 2 ($1/10^{th}$ of 96h LC_{50}=0.04 mg L^{-1}; Treatment 2), sublethal 3 ($1/5^{th}$ of 96h LC_{50} =0.08 mg L^{-1}; Treatment 3) were selected for further experimentation. Control (TC) was also maintained throughout the experiment along with the desired doses.

***In vivo* exposure experiment and its procedure:** The experiment for studying the changes in locomotory and behavioural responses was conducted under laboratory conditions (25±1°C) in glass aquarium (35 L capacity). Fish (30 fish in each group with mean wt. 20.1±0.22g) were exposed to $1/20^{th}$ (0.02 mg L^{-1}; T1), $1/10^{th}$ (0.04 mg L^{-1}; T2) and $1/5^{th}$ (0.08 mg L^{-1}; T3) of LC_{50} for 21 days along with the control (TC). All the three treatments and control (TC-T3) were maintained in triplicate in the experimental aquaria and two days starved fishes were released into the aquaria. The desired concentrations (0.02 mg L^{-1}, 0.04 mg L^{-1}, 0.08 mg L^{-1})were maintained with proper aeration using aerators and renewed daily during the entire duration of the experiment and all groups of fishes were provided with the diet (40% protein @4% Body Weight day^{-1}). The altered behavioural and locomotory responses of fishes was monitored at regular interval of day 2, 4, 7, 14 and 21 in the morning at fixed time. Before recording the behaviour, fishes from all the treatments and the control were acclimatized individually for ten minutes in glass aquaria (15×15×15 cm) containing water. The internal three sides of the aquarium and bottom were made opaque by placing white thermocol sheets to avoid the mirror image of the test organism and any visual disturbances. The behaviour of fish was recorded for 5 minutes in a fixed monitoring area with a high resolution camera (SONY, Handycam-HDR-XR550E) mounted 20 cm away from the leftover side of recording aquarium. A minimum of 10 fishes from each test interval were used to evaluate individually for determining their behaviour. All the activities of the test fish were observed carefully and video record-

ings were used for analyzing the changes in the behaviour and locomotion pattern of the fish. The behavioural alterations of the fishes in control as well as chlorpyrifos treated fishes *i.e*T1-T3 and TC were recorded and evaluated in terms of Air Ingulping or mouth opening (AI), Operculum Beat Frequency (OBF), Vertical Hanging (VH), Surfacing Movement (SM), Tail Beat Frequency (TBF). All the frequencies of the changes were recorded per minute(min^{-1}).

RESULTS

General behaviour

Control fish (Treatment TC): Fingerlings maintained as control showed a fairly compact locomotor behaviour covering one third of the tank, these were active for feeding and alert to slightest of the disturbance with their well synchronized movements. Fingerlings were observed to scrap the bottom surface. When started, they instantly formed a tight school that was maintained briefly. They were sensitive to light and moved to bottom and corner of the tank when light was passed into the tank. Except a less response to form a dense school towards the end of the study, no other extraordinary behaviour was observed. The behaviour did not significantly vary between the control group, therefore, these results were taken as standards for the entire experiment.

Exposed fingerlings (T1-T3): Exposed fingerlings exhibited disrupted school behaviour, migration/localization to the bottom of the test chamber (aquaria) and independency in swimming. The locomotion behaviour was observed to be disturbed on the first day itself and the fish occupied twice the area than that of the control group. They were spread out and appeared to be swimming independent of one another. Irregular, erratic and darting movements followed this with imbalanced swimming activity and loss of equilibrium followed by hanging vertically in water. The fishes also exhibited peculiar behaviour of trying to leap out from the pesticide medium, which can be viewed as an escaping phenomenon. Maximum surfacing phenomenon was observed on the day 2 and 4 wherein the fish frequently come to the water surface. Respiratory disruption (cough, yawn) was observed with a rapid and repeating opening of mouth and opercular coverings. Partially extended fins and singlewide opening of the mouth and opercula coverings of mouth and opercula coverings accompanied with a state of excitement was mostly observed on day 7. The swimming behaviour also showed disturbed response, they seemed to lose their equilibrium following jerky, sudden, rapid, non directed spurt of forward movement. The fish progressively became lethargic as it showed signs of tiredness, weakness, apathy and secreted excess mucus all over the body. Fish showed no response to external stimuli as light and touch followed by drowning to the

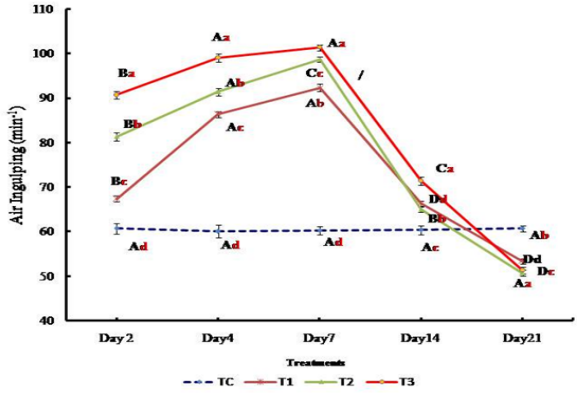

Fig. 1. *Frequency of Air Ingulping (min^{-1}) (Mean± S.E of mean) of Cirrhinus mrigala fingerlings on various time duration exposed to selected doses of test chemical chlorpyrifos. (TC=no pesticide, T1= 0.02mg L^{-1}, T2=0.04mg L^{-1}, T3=0.08mg L^{-1})(Mean with the capital letter on the same line are not significantly different. Values with the same letter in red color at the same point are not significantly different).*

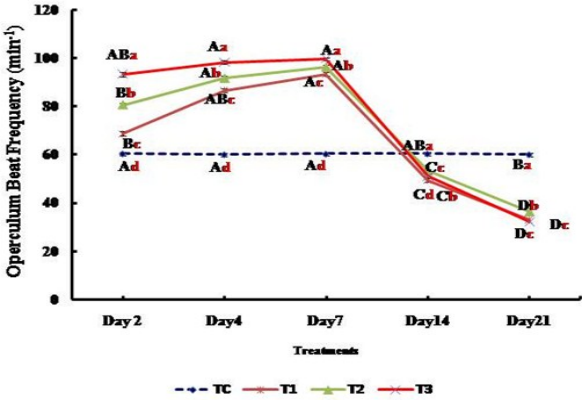

Fig. 2. *Operculum Beat Frequency (min^{-1}) (Mean± S.E of mean) of Cirrhinus mrigala fingerlings on various time duration exposed to selected doses of test chemical chlorpyrifos (TC=no pesticide, T1= 0.02mg L^{-1}, T2=0.04mg L^{-1}, T3=0.08mg L^{-1}) (Mean with the capital letter on the same line are not significantly different. Values with the same letter in red color at the same point are not significantly different).*

Table 1. Median lethal concentration (LC_{50}) of Chlorpyrifos on *C. mrigala.*

Compound	Regression equation	95% Confidence limit for doses		Median Lethal Concentration(LC_{50}) (mg L^{-1})
		Lower limit	Upper limit	
Chlorpyrifos	y=11.87x-0.533 R^2=0.881	0.369	0.519	0.44

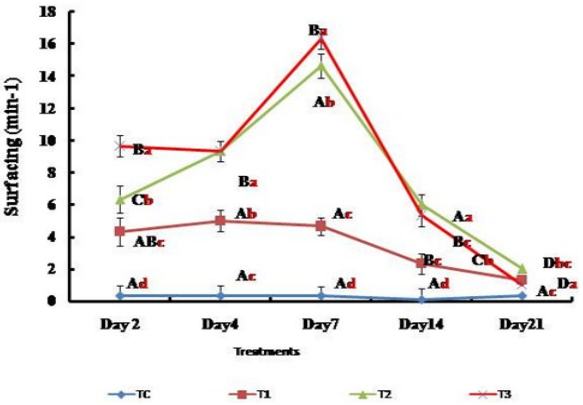

Fig. 3. *Surfacing activity (min^{-1})(Mean± S.E of mean) of Cirrhinus mrigala fingerlings on various time duration exposed to selected doses of test chemical chlorpyrifos (TC=no pesticide, T1= 0.02mg L^{-1}, T2=0.04mg L^{-1}, T3=0.08mg L^{-1}) Mean with the capital letter on the same line are not significantly different. Values with the same letter in red color at the same point are not significantly different).*

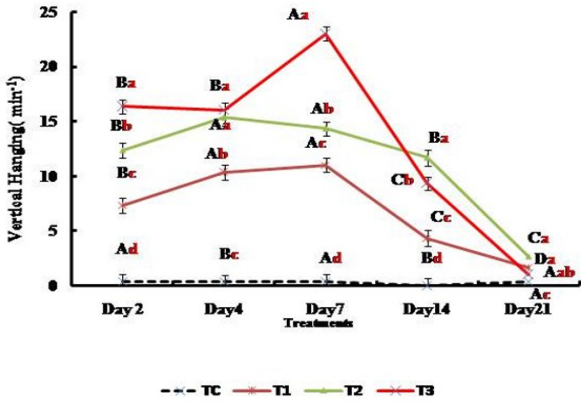

Fig. 4. *Frequency of Vertical Hanging activity (min^{-1}) (Mean± S.E of mean) of C. mrigala fingerlings on various time duration exposed to selected doses of test chemical chlorpyrifos (TC=no pesticide, T1= 0.02mg L^{-1}, T2=0.04mg L^{-1}, T3=0.08mg L^{-1} (Mean with the capital letter on the same line are not significantly different. Values with the same letter in red color at the same point are not significantly different.)*

bottom after 7th day. The mobility of the fingerlings in higher concentration of pesticide (T3) was observed to decrease more by the action of toxicant from day 8 to 21 in comparison to the control. After the day 8, the fingerlings in Treatment T2 and T3 showed redness in the skull region visible due to induction of transparency in skin hence clearly depicting blood coagulation in cephalic region, caudal bending, seizures (muscular contraction and tremor), totally disturbed buoyancy and secretion of profuse amount of mucous all over the body.

Specific behaviour: The variations in the frequency of specific behavioural responses in the *C. mrigala* exposed to varying doses of chlorpyrifos for varying period of time and in control are presented in Fig. 1, 2, 3, 4 and 5. Evidently, all the treated groups of specimens had higher frequency of alteration in all behavioural and locomotory parameters as AI, OMF, SM, VH, TBF as compared to the control. The frequency of all such behavioural patterns increased progressively with increase in period of exposure and concentration of chlorpyrifos. Throughout the experiment period the variation of behavioural and locomotory responses was found significantly higher in T3 in comparison to T2, T1 and TC. Values were compared between durations within concentration as well as concentration within duration. It was also observed that the treatment TC showed no significant variation within different periods whereas treatments T1, T2 and T3 showed significant differences within the same row. When all the treatments were compared between concentration within the duration, they showed a significant (P<0.05) difference. The highest AI frequency of 101.33±0.66 was observed on day 7 in sublethal treatment T3 and 91.33±0.06 in T2 and lowest frequency (59.66±0.03) was observed in TC throughout the study period. Similarly, the frequency of other behavioural responses as OBF, SM, VH and TBF showed maximum values of 99.96±0.33, 16.33±0.38, 23.00±0.57; respectively on 7th day again in treatment T3 and lowest values (58.99±0.66, 0.33±0.00, 0.00±0.00, 20.00±0.57) in control (TC) in which no chlorpyrifos was added. Subsequently, a general trend of increase in all behavioural and locomotion responses were noted from Day 2 to day 7 and a decreasing trend thereafter.

DISCUSSION

The assessment of toxicity of Chlorpyrifos with reference to aquatic biota, especially fish is utmost important as this is being in use for more than a decade on different agricultural crops and subterranean termites (Rao *et al*., 2004). Acute toxicity (96 h LC_{50}) of chlorpyrifos for the freshwater fish, *C. mrigala* was found to be 0.44 mg L^{-1}. This data clearly indicates that chlorpyrifos can be rated as highly toxic to fish. In earlier studies, 96 h LC_{50} of chlorpyrifos in mosquito fish, *Oreochromis mossambicus* (Tilapia) and *Gambusia affinis*were calculated as 0.0259 mg L^{-1} and 0.297 mg L^{-1}, respectively (Rao *et al.*2003, 2005). It is inferred from the present study that chlorpyrifos is highly toxic to freshwater fish, *C. mrigala.* Exposure of sublethal doses of chlorpyrifos on fingerlings of *C. mrigala* showed remarkable alterations in locomotion and behavioural parameters.

The fingerlings maintained in normal freshwater (treatment TC) behaved in usual manner *i.e.,* they were very active with their well co-ordinated movements. They were alert to the slightest disturbance but the exposed fingerlings showed loss

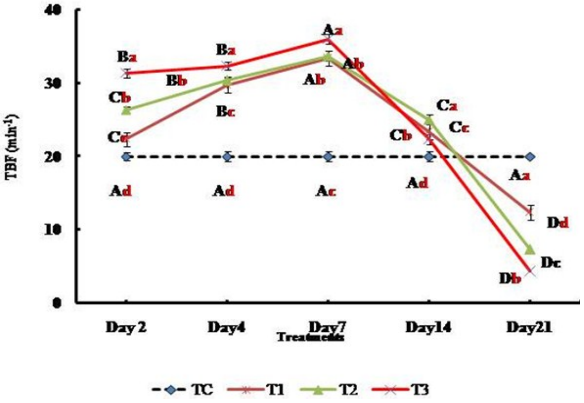

Fig. 5. *Tail Beat Frequency (min^{-1})(Mean± S.E of mean) of C.mrigala fingerlings on various time duration exposed to selected doses of test chemical chlorpyrifos (TC=no pesticide, T1= 0.02mg L^{-1}, T2=0.04mg L^{-1}, T3=0.08mg L^{-1})(Mean with the capital letter on the same line are not significantly different. Values with the same letter in red colour at the same point are not significantly different).*

of equilibrium, loss of pigmentation, blood coagulation in cephalic region, dullness of body colour, caudal bending (scoliosis), profuse mucus secretion, abnormal operculum movement, and difficulty in breathing with abnormal swimming pattern causing stress condition followed by sluggishness.This type of stress condition was also reported to cause a change in the haematology and biochemical parameters of fish surviving in pesticide polluted water which is remarkable tool to access the physiological and pathological stress in fish too (Bhatnagar *et al*., 2017). Gills have the ability to metabolize and eliminate xenobiotics compounds and mucous cells play role in excretion to a considerable extent (Dutta, 1995). Toxicants that enter through permeable gill epithelium might as well be excreted through mucous secretion. Therefore, it can be inferred that the accumulation and increased secretion of mucus in the fish exposed to toxicant may be adaptive responses against corrosive nature of the pesticide. Caudal bending was noticed in all the toxicant concentrations with time, which greatly retarded the normal swimming pattern. Caudal bending might be a type of paralysis, which was due to the inhibition of muscular AChE which further led to the blockage of neural transmission.

The movement of fish towards the bottom of the tank after addition of chlorpyrifos clearly indicates the avoidance behaviour of the fish. The increase in opercular movement and corresponding increase in frequency of surfacing of fish clearly indicates that fish quickly shifts towards aerial respiration (by obtaining atmospheric oxygen surfacing) because fish tried to avoid contact with the toxicant through gill chamber (Santhakumar and Balaji, 2000). Increased operculum movement probably helps in reducing absorption pesticide through gills (Venkata and Nagaraju, 2014). The increased ventilation rate by rapid, repeated opening and closing of mouth and opercula coverings accompanied by partially extended fins (coughing) was observed in the present study. This could be due to clearance of the deposited mucus debris in the gill region for proper breathing (Prashanth *et al*., 2005).Behavioural changes like irregular and erractic swimming, jerky movements indicated the loss of equilibrium. This might be due to the damage of the region in the brain associated with the maintenance of equilibrium (Rao and Rao, 1987). Surfacing phenomenon shown by the fish might be to gulp maximum possible air to ease the tension (Kumar, 2010). Similar type of surfacing phenomenon of the fish was also observed under cyanide exposure due to hypoxic condition of the fish reported earlier by Radhaiah and Rao (1988).These kinds of termers were also observed in fish, *Sarotherodan mossambicus* exposed to dimethorate (Kalavathy *et al*., 2001).

The hyperexcitability of the fish invariably in the sub-lethal exposure to free pesticides may probably be due to the hindrance in the functioning of the enzyme AChE in relation to nervous system (Prashanth, 2003). It leads to accumulation of acetylcholine, which is responsible for prolonged excitatory post synaptic potential. This may first lead to stimulation and later cause a block in the cholinergic system. The results of our investigations were in agreement with the observations of Rao *et al.* (2005). They reported mosquito fish, *Gambusia affinis* exposed to the higher concentrations of monocrotophos exhibited abnormal behaviours such as erratic swimming with jerky movements, loss of equilibrium, and secretion of a plenteous amount of mucus from the whole body. Inhibition of brain AChE activity was an early process of sublethal exposure to chlorpyrifos in hybrid catfish (Chawanrat*et al.,* 2007). Its acute as well as chronic effects have also been found to disturb the metabolism, physiological as well as genetic parameters in the fresh water fishes *C. mrigala* and *Channa punctatus* (Bhatnagar *et al*., 2016; Nwani*et al*., 2010).Overall impairments in fish behavioural responses and morphological deformities under the study periods may be due to inhibition of brain and musculature AChE activity by chlorpyrifos-oxon via biotransformation of sequestered chlorpyrifos in the storage organs. Chlorpyrifos (CPF) inhibits AChE due to the effects of their active oxygen analog chlorpyrifos-oxon (CPF-oxon) (Timchalk *et al.,* 2002). Sequestered chlorpyrifos might have been biotransformed to their active oxygen analog chlorpyrifos-oxon via a desulfuration reaction initiated by cytochrome P450 (Amitai *et al.,* 1998; Poet *et al.,* 2003; Ali*et al*., 2009) dearylation reaction utilizing the same enzymes and A-esterase (Poet *et al.,* 2003). Fur-

thermore, physiological reactions, such as activation of biotransformation enzyme systems in the presence of xenobiotic substances enable the organisms to survive in sub-acute exposures.

Conclusion

The technical-grade Chlorpyrifos was found to be toxic to fishes even at sublethal concentration (*i.e.* 1/20th of LC_{50} = 0.02 mg L^{-1}), which indicates apprehension about the potential hazards of Chlorpyrifos to aquatic organisms. The present investigations indicated that behavioural alterations are sensitive tools for demonstration of toxic effects of this chemical in body of fish. Further, the alterations in locomotion activity showed the suppressed condition of fish in toxic environment. All these alterations in physiological parameters in the present study may provide early warning signals for the determination of toxic levels of pesticide used in the fields and its deterioration into the nearby ponds.

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