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Toxicity of Chlorpyrifos to the Fish *Oreochromis mossambicus*

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Responses to organophosphorous (OP) insecticides by aquatic organisms are broad ranged depending on the compound, exposure time, water quality and species (Eisler 1970; Fisher 1991; Richmonds and Dutta 1992). Now, there is a growing concern worldwide over the indiscriminate use of such chemicals, resulting in environmental pollution and toxicity risk to aquatic organisms (Khan 1996). Currently, about 153 pesticides have been registered and 35 are widely used in the Indian market. Among them, 33 pesticides have been banned based on their persistence and toxicity towards non-targeted organisms, both in India and advanced countries. Hence, production and use of permitted insecticides could increase considerably. Chlorpyrifos, O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl) phosphorothioate insecticide was commercially used for more than a decade particularly to control foliar insects on cotton, paddy fields, pasture and vegetable crops. Since, Chlorpyrifos is an extensively used OP insecticide with an increasing load in the environment, causing adverse effects on non-targeted fish. Chlorpyrifos is the second largest selling OP agro chemical in India (Mathur and Tannan 1999). Hence, a study was undertaken to evaluate the aquatic toxicity of chlorpyrifos with special emphasis on behavioural, morphological, target enzyme interaction and bioaccumulation effects of the toxicant in different parts of the body.

MATERIALS AND METHODS

All the reagents used in the present study were of analytical grade and were used without further purification. The test compound chlorpyrifos, synthesized at Indian Institute of Chemical Technology, was of 99% purity. A complete systematic evaluation of chlorpyrifos was undertaken using *Oreochromis mossambicus (Tilapia)* because of its wide availability and suitability as a model for toxicity testing (Ruparrelia et al. 1986). They were collected from Osmanbagh (Hyderabad) and were brought to the laboratory in large aerated drums. They were acclimatized for 30 days in a cement tank of 8' x 6' x 4' and fed wheat bran daily. Fish weighing 3±1 g were transferred to a 40 L glass aquarium (60 x 30 x 30 cm) for seven days and fed commercial dry feed pellets, for conditioning. The water in the aquarium was renewed daily and was aerated mechanically.

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Determination of 96 hours median lethal concentration (LC_{50}): The acute LC_{50} for chlorpyrifos was determined in the laboratory using the semi-static method (UNEP 1987). The test concentrations 20, 25, 30, 40 and 50 µg L^{-1} were chosen based on initial experiments to determine the lethal concentration (LC_{50}) for 96 hrs. The required concentrations were maintained in 40 L of water by adding the toxicant dissolved in 2 ml of acetone and renewed daily. The aquarium receiving only acetone was the control. Two-day starved fish were released into each aquarium (20 fish) and were exposed to the above concentrations. The mortality record of the fish was maintained (during 96 hr exposure and seven days post-exposure observation) for each concentration. The data consisting of concentration, sample size and percent corrected mortality was used to determine the median lethal concentration (LC_{50}) by using a computer program developed by Reddy et al. (1992a), based on the method of Finney (1953).

In a separate set of experiments, two-day starved fish were divided into five replicates of 50 each and four replicates were exposed to the LC_{50} concentration, along with a control. The fish that survived after 96 hr exposure were transferred into fresh water (day 0) to study the recovery pattern of acetylcholinesterase (AChE) activity, versus the control, for upto 20 days. At 4 d intervals, beginning with day 0, three fish from each control and treatment replicate were decapitated and their brains (including the brain stem but excluding olfactory bulbs and peduncles), and gills were dissected out and homogenised (10% w/v) in 0.1 M phosphate buffer (pH 7.5) using a Potter-Elvehjam homogenizer fitted with a Teflon pestle. The homogenates were centrifuged at 5,000 x g for 10 minutes and the supernatant was further centrifuged at 5,000 x g for 10 minutes. The resultant supernatant was used as the enzyme source for the estimation of AChE activity. All the enzyme preparations were carried out at 4°C. Protein was estimated by the method of Lowry et al. (1951). The AChE activity was estimated in brain and gill tissues by the method described by Ellman et al. (1961).

The AChE experiments were performed in a 96 well-plate consisting 75µl of 0.1 M phosphate buffer pH 7.5, 25µl of DTNB (0.16mM) and 25µl of homogenate (0.5mg) for each well. The reaction was initiated by adding 25µl of the substrate, acetylthiocholine iodide (0.4mM) at 27±1°C, and colour development was recorded continuously for five minutes at 412nm in a spectrophotometer (Molecular Devices, USA; supported by the software, Spectro-max Plus). AChE activity was calculated as µmoles/mg protein/min.

During sub-acute studies a total of sixty fish (20 fish per aquarium) were exposed to 10.4 µg L^{-1} chlorpyrifos for 21 d with test solution being renewed daily. A batch of sixty fish kept in chloripyrifos-free water served as the control. Behavioral and morphological abnormalities and AChE activity in both brain and gill were studied at regular intervals of day -3, -6, -9, -15, and on day-21. A representative sample of gills (pair-III) from either side of the fish were dissected out and placed in 4% glutaraldehyde for 1 hour at 4° C for

SEM studies. After rinsing (2 to 3 times) with phosphate buffer they were placed in a gradient series of alcohol (10-100%) for dehydration. The samples were air-dried, mounted on alumina stubs and coated with gold in an Hitachi HUS-5Gb vacuum evaporator and were observed using an Hitachi S-520 SEM.

The head, viscera and body parts from day 3 to 18 of fish exposed to 10.4 $\mu g\ L^{-1}$ concentrations were frozen in liquid nitrogen for the estimation of chlorpyrifos. The deep frozen tissues were ground and homogenized in petroleum ether and the extract was filtered through anhydrous sodium sulfate. The extracts were passed through an activated Florisil column for clean-up of the sample. The resultant extract was evaporated under reduced pressure and dissolved in 1 ml of acetonitrile for High Performance Liquid Chromotography (HPLC) analysis. Briefly, the HPLC program was operated by using a UV detector with a mobile phase consisting of acetonitrile (65%) and water (35%) in 0.1% acetic acid through a C_{18} (ODS) column with a flow rate of 1.5 ml/min. The obtained peak areas of chlorpyrifos in individual tissue (mg/kg wet tissue) were analyzed with standard peaks.

RESULTS AND DISCUSSION

Acute toxicity of chlorpyrifos on *Oreochromis mossambicus* was carried out by the semi-static method and its LC_{50} value for 96 hrs is presented in Table-1. It is evident from the results that the chlorpyrifos can be rated as highly toxic to fish with an LC_{50} value of 26 $\mu g\ L^{-1}$.

Table 1. Acute toxicity of chlorpyrifos on *Oreochromis mossambicus*

Compound	Regression Equation	Acute Toxicity range 98% confidence limit		Median LC_{50} ($\mu g\ L^{-1}$)
	$Y = (\bar{Y} - b\bar{x}) + bX$	Upper ($\mu g\ L^{-1}$)	Lower ($\mu g\ L^{-1}$)	
Chlorpyrifos	Y=12.62+5.1632x	32.0	19.7	25.97±0.01

Chlorpyrifos exposed fish exhibited abnormal behaviors like erratic swimming and loss of equilibrium. They became lethargic and secreted excess mucous. At the time of death they exhibited transient hyperactivity before collapsing. Similar behavioral changes were reported previously in fish exposed to OP pesticides (Heath 1987).

The AChE activity of brain and gill in control fish were 0.14±0.002 and 0.11±0.003 µmoles acetylthiocholine hydrolised/min /mg protein respectively. However, fish exposed to the LC_{50} (26 $\mu g\ L^{-1}$) for 96 hrs (day-0), indicated 88±4% AChE inhibition in both brain and gill. Fish have ability to overcome the stress of the toxicant during recovery (Figure 1). Based on the regression equation, the time required for 50% recovery of AChE was 8.7 days in brain and 13.8 days in gill and for total recovery 19.4 and 29.2 days were needed

respectively. A similar pattern of AChE inhibition was reported earlier in *Melanotaenia duboulayi* (Kumar and Chapman 1998) and in shrimp *Paratya australiensis* (Abdullah et al. 1995) exposed to chlorpyrifos. It is apparent from the earlier results that the fish can survive even after 90% inhibition of true cholinesterases in brain AChE, when exposed to parathion (Francois and Giler 1990).

The effect of chloripyrifos at 10.4 μg L^{-1} on percent inhibition of brain and gill AChE activity was estimated at regular intervals (3 days) for upto 21 d are presented in Figure 2. Initially, brain AChE activity was inhibited comparatively less than gill on day-3, but subsequently brain exhibited more inhibition and reached maximum on day-15. It is evident from the earlier reports that the inhibition of AChE activity in brain (73.6%) was greater than in gill (41.3%) when *Cyprinos carpio* were exposed to fenvalerate (Reddy et al. 1992b). There was no significant difference in brain and gill AChE activity of control fishes during the same intervals.

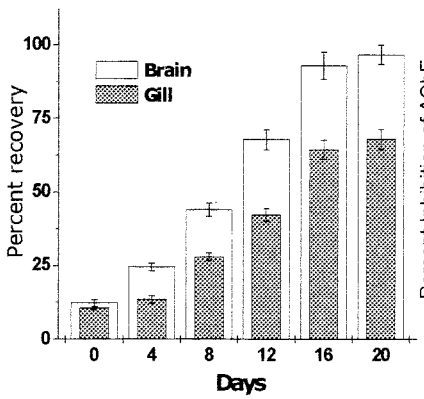

Figure 1. Recovery patterns of brain and gill AChE activity after exposure to 26 μg L^{-1} chlorpyrifos.

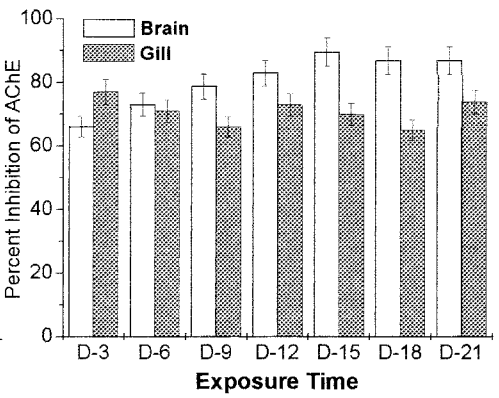

Figure 2. Inhibitory patterns of brain and gill AChE activity during exposure to 10.4 μg L^{-1} chlorpyrifos. (D= Days)

Chlorpyrifos is known to bio-accumulate in the tissues of test organisms and estimates of tissue concentrations may be more valuable for the assessment of situations in the natural environment (Kumar and Chapman 2001). Hence, the accumulation of toxicant in different parts of the body was estimated using HPLC and presented in Figure 3. It is evident from the results that accumulation (126 to 154 mg/kg) in viscera was highest throughout exposure. The rate of accumulation in head and body regions ranged from 12.9 to 39.0 mg/kg and 13.3 to 182.3 mg/kg respectively. The order of accumulation in tissues was viscera> body>head.

Respiratory distress is one of the early symptoms of pesticide poisoning (Murthy 1986) and gills take part in metabolism and elimination of xenobiotics (Dutta et al. 1996). Hence, the sub-acute effect of chlorpyrifos on

gill morphology of fish was studied. Direct deleterious effects of the toxicant in the form of necrosis and abnormalities to gill lamellae were evident. A thick coat of mucus covered the entire gill filaments and lamellae were observed during the exposure period.

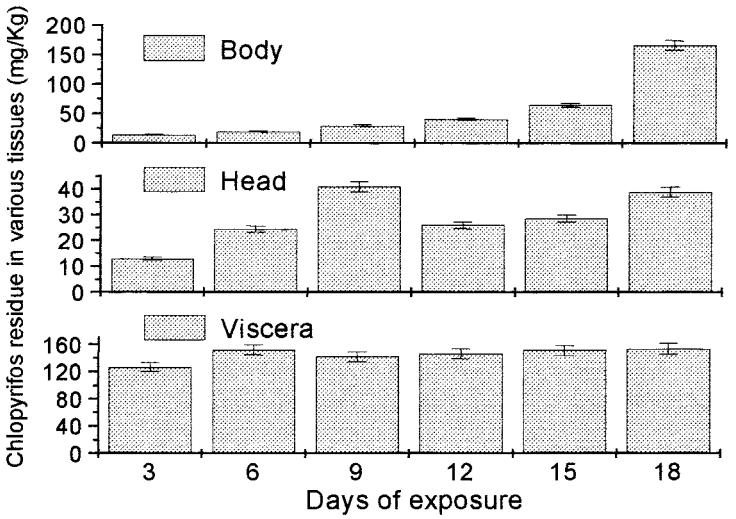

Figure 3. Bioaccumulation of chlorpyrifos in body, head and viscera of *Oreochromis mossambicus*, during exposure to 10.4 μg L^{-1} for a period of 18 days.

The excessive mucus secretion and lifting up of the epithelium and clavate lamella was noted as a defense response toward the toxicant (Mallatt 1985). Formation of large sub-epithelial spaces within the secondary lamellae was observed on day-3 (Fig.-4B) in comparison to the control (Fig.-4A). The bulging of lamellae at the terminal ends; lesions and erosions at the base of lamellae were observed on day-12 (Fig. 4C). Extensive fusions of secondary lamellae with a thick coat of mucus on the gill filaments were prominent on day-18 (Fig.-4D).

The shape of the gill rakers was severely affected appearing to be thinner and elongated on day-6 (Fig.-4E). Further destruction was observed in subsequent exposure tenure (day-12, -15 and -18) with deep lesions and erosions, thereby destroying the basic structure of gill rakers and arches (Fig.-4F, G and H). Related descriptions were noticed in other species of fish *Herotilapia multispinosa* when exposed to fenthion. Concentration of 1.1 and 7.2 mg/L resulted in gill lesions, including hyperplasia and desquamation of the epithelium and thrombosis in the secondary gill lamellae, upon 96 hr exposure (Jauch 1979). Similar observations like necrosis and exudation were noted in the gills of *Lepomis macrochirous* exposed to 0.05mg L^{-1} malathion (Richmonds and Dutta 1989) and to different concentrations of diazinon (Dutta et al. 1997).

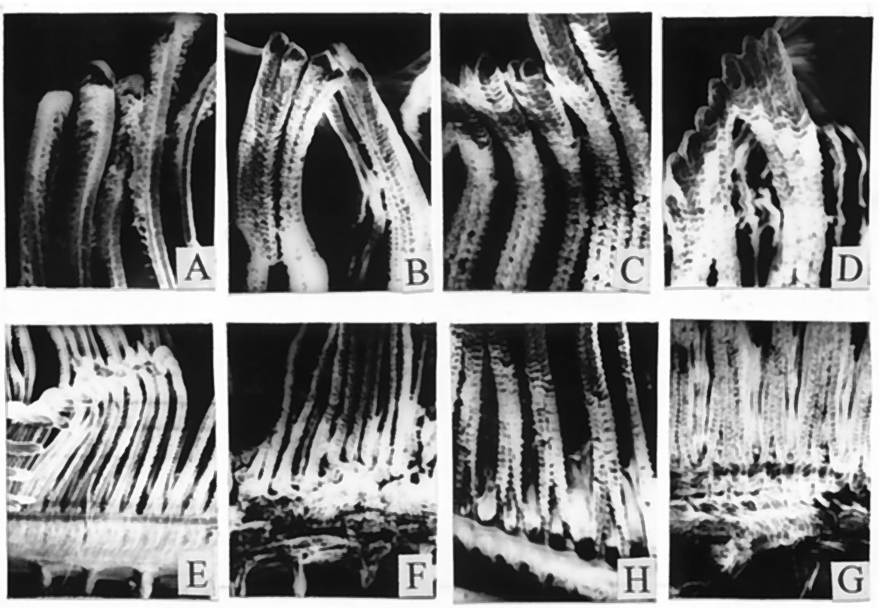

Figure 4. Scanning Electron Microscope photographs of the gills exposed to 10.4 μg L^{-1} of chlorpyrifos at different exposure intervals (Scale: 1cm represents 75μ in A, C; 110μ in B, G, H; 60μ in D; 220μ in E and 185μ in F).

The study has indicated that affects of chlorpyrifos in the fish *Oreochromis mossambicus* reflected not only direct toxicity but also indirect effects by its accumulation in various tissues. The prime focus in this study was the accumulation of chlorpyrifos in viscera, body and head. However, the individual secondary metabolites were not considered. Further experiments are necessary for identifying the metabolites and their quantities. The effects of chlorpyrifos on other aquatic species must be determined before extensive usage of this insecticide is permitted in India.

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REFERENCES

Abdullah AR, Kumar A, Chapman J (1995) Inhibition of acetylcholinesterase in the Australia freshwater shrimp, (*Paratya australiensis*) by profenofos. Environ Toxicol Chem 13:1861-1866

Dutta HM, Munshi JSD, Roy PK, Singh NK, Adhikari S, Killius JS (1996) Ultrastructural changes in the respiratory lamellae of the catfish, *Heteropneustes fossilis*, after sub-lethal exposure to malathion. Environ Pollut 9:329–341

Dutta HM, Munshi JSD, Roy PK, Singh NK, Motz L, Adhikari S (1997) Effects of diazinon on bluegill sunfish, *Lepomis macrochirus*, gills: scanning electron microscope observations. Exp Biol Online 2:17

Eisler R (1970) Acute toxicities of organochlorine and organophosphorous insecticides to estuarine fishes. US Bur Sport Fish Wild Tech Pap 46:12

Ellman GL, Courtney KD, Andres Jr. VV, Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:88-95

Finney DJ (1953) Probit Analysis 2nd Edition. Cambridge University Press, Cambridge, England

Fisher SW (1991) Changes in the toxicity of the three pesticides as a function of environmental pH and temperature. Bull Environ Contam Toxicol 46:197-202

Francois G, Giler B (1990) *In vitro* inhibition of acetylcholinesterase from four marine species by organophosphorous pesticides and carbamates. Bull Environ Contam Toxicol 45:243-249

Heath AG (1987) Behavior and nervous system function in water pollution and fish physiology. CRC Press, Inc Boca Raton, Florida, p 181-196

Jauch D (1979) Gill lesions in cichlid fishes after intoxication with insecticide fenthion. Experientia 35:371-372

Khan K (1996) Integrated pest management and sustainable agriculture. Farmer and Parliament 30:15-17

Kumar A, Chapman JC (1998) Profenofos toxicity to the eastern rainbow fish (*Melanotaenia duboulayai*). Environ Toxicol Chem 17:1799-1806

Kumar A, Chapman JC (2001) Profenofos residues in wild fish from cotton-growing areas of New South Wales, Australia. J Environ Qual 30:740-750

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with folin phenol reagent. J Biol Chem 193:265-275

Mallatt J (1985) Fish gill structural changes induced by toxicants and other irritants: a statistical review. Can J Fish Aquat Sci 42:630–648

Mathur SC, Tannan SK (1999) Future of Indian pesticides industry in the next millennium. Pesticide Information 24:9-11

Murthy AS (1986) Toxicity of Pesticides to Fish, Vol. 2 CRC Boca Raton, Florida

Reddy PJ, Krishna D, Suryanarayana Murty U, Jamil K (1992a) A microcomputer FORTRAN programme for rapid determination of lethal concentration in mosquito control. *Computer Applications in Biosciences* 8: 209-213

Reddy PM, Philip GH, Bashamohideen M (1992b) Regulation of AChE system of fresh fish, *Cyprinus carpio*, under fenvalerate toxicity. Bull Environ Contam Toxicol 48:18-22

Richmonds C, Dutta HM (1989) Histopathological changes induced by malathion in the gills of bluegill, *Lepomis macrochirus*. Bull Environ Contam Toxicol 43:123-130

Richmonds CR, Dutta HM (1992) Effect of malathion on the brain acetylcholinesterase activity of bluegill sunfish *Lepomis macrochirus*. Bull Environ Contam Toxicol 49:431-435

Ruparreila SG, Verma Y, Kasyap SK, Chatterjee BB (1986) A new approach for the use of standard fish toxicological study. In: Dalela RC, Madhysta MN, Joseph MM (Eds.) Environmental Biology, Coastal Ecosystem. The Academy of Environmental Biology, Muzzafarnagar, India, p 89-92

UNEP (1987) Test of the acute lethal toxicity of pollutants to marine fish and invertebrates. Reference methods for Marine Pollution Studies No.43 (draft). UNEP (United Nations Environment Programme) Regional Seas Report, October 1987. FAO, IAEA