Bull. Environ. Contam. Toxicol. (2004) 73:933–941

DOI: 10.1007/s00128-004-0516-8

Bulletin of Environmental Contamination and Toxicology

Toxicity and Bioaccumulation of Chlorpyrifos in Indian Carp *Catla catla* (Hamilton), *Labeo rohita* (Hamilton), and *Cirrhinus mrigala* (Hamilton)

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Received: 4 November 2003/Accepted: 30 July 2004

Organophosphates are neutral esters biologically active against insects and hydrolyse in acid or basic medium to give their respective alkyl phosphates and leaving aryl or alkyl groups. Owing to their ester nature, the organophosphates offer fundamental advantages in better control of pests. The physical and chemical properties of a compound determine its behaviour in the environment. Although environmental variables are important in modulating transformation and transport, the basic pattern of persistence and partitioning in the tissues of the organism is fundamentally based on the chemical characters of the compound. The propensity for partitioning between solid and aqueous compartments is a key property on the behaviour of a pesticide in both soil and aquatic environments causing toxic effect and accumulate in the tissues of the fish (Deneer, 1994).

Due to widespread use, these organophosphate pesticides are transported into aquatic environments. Aquatic toxicity has become an integral part of the process of environmental hazard evaluation of toxic chemicals. Generally, the potential impact of pollutants is greater for aquatic organisms (Murty, 1986).

Chlorpyrifos [0,0-diethyl 0-(3,5,6-trichloropyridine-2-yl) phosphorothioate is a member of the organophosphorus (organophosphate) insecticide, displaying its activity to control against a wide range of pests (O'Brien, 1967).

Hence in the present study, an attempt has been made to study toxicity and residue levels in fish tissues viz., brain and liver of *Catla catla, Labeo rohita* and *Cirrhinus mrigala.* Being neurotoxic the residue levels in brain indicate the extent of action of these compounds whereas the liver is the primary site of detoxification which indicates the process of depuration of this compound (O'Brien, 1967). Due to this, the two selected tissues are the important organs as the organophosphate pesticides, are known to inhibit the enzyme acetycholinesterase (O'Brien, 1967; Barron and Woodburn, 1995).

MATERIALS AND METHODS

The Indian major carps *Catla catla, Labeo rohita* and *Cirrhinus mrigala* with a size range of 8-10 cm and 6 to 8 gm in weight irrespective of their sex were obtained from the fish hatcheries. They were acclimatized to the laboratory conditions in well-aerated unchlorinated ground water for two weeks at a room

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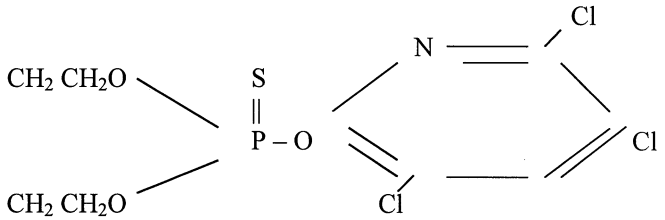

Figure 1. Chlorpyrifos 0,0 diethyl 0(3,5,66 trichloro-2-pyridyl) phosphorothioate CAS 2921-88-2 MW = 350.58

temperature of 28±2°C. During the period of acclimatization the fish were fed daily, with an average of 3% of their body weight. During the period of acclimatization if the number of deaths exceeded 5% in any batch that batch was discarded. Feeding was stopped two days prior to the experimentation.

Toxicity studies were conducted using chlorpyrifos technical grade (100%) formulation supplied by Gayatri Pestichem, Mumbai, India, employing static and continuous flow systems as recommended in the report of the committee on methods for toxicity tests with aquatic organisms (EPA 1975). [Fig.1 Chemical structure of the chlorpyrifos]. Test solutions of desired concentrations were prepared in 95% acetone to yield a concentration of 1 mg/1 ml for technical grade chlorpyrifos and diluted with distilled water to get a working solution. The other precautions such as use of acetone without the toxicant, in control as recommended by EPA (1975) were followed.

For the flow through system, test solutions of desired concentrations were prepared once every 12 hrs in glass reservoirs and let into the test containers through thin-walled polyethylene drip sets. The flow rate was adjusted with regulators such that 4 L of water run through containers in one hour. The conditions of the test medium were: temperature 28±2°C oxygen 8-10 ppm, hardness 80 mg/L, alkalinity – 425 mg/L (as $CaCO_3$) and pH 8.2. All the precautions laid down in the report of committee on toxicity tests were followed (EPA, 1975).

Pilot experiments were conducted to determine the concentrations causing 10 to 90% mortality for 24, 48, 72 and 96 hrs of the test fish. For each concentration 10 fish were tested and the experiment was repeated thrice. Probit analysis (Finney, 1971) as recommended by Roberts and Boyce (1972) was followed to calculate the LC_{50} values.

In the present investigation, three kinds of chromatographic methods were employed, Thin layer Chromatography (TLC), Column Chromatography (CC) and Gas liquid Chromatography (GLC). TLC is for the confirmation of pesticide residues, CC is for clean-up whereby the residue is made free from co-extractives

by differential adsorption running through a column packed with absorbants and the GLC for estimation of residues of the pesticide from tissues of fish.

After exposure to sublethal concentration of chlorpyrifos technical grade ($1/5^{th}$ of 96 hrs LC_{50}) for 8 days, the fish were sacrificed for the analysis of residues. The fish tissues of brain and liver were extracted by the modified method of Mills and Olney (1977) incorporated in the pesticide analytical manual (PAM). 1 g of brain and liver tissues of the three fish was separately blended with 4 g of anhydrous sodium sulphate [pre-extracted in a soxhlet column with analytical grade hexane] and were homogenised in a tissue homogeniser with minimal quantity glass triple distilled water. Later, it was extracted in 2:1 hexane : acetone taken as 2 ml for each gram of tissue carried out for an hour in horizontal shaker, at gentle speed and any spillage was avoided.

The hexane, acetone extract was washed with double distilled water and dehydrated using analytical grade sodium sulphate and stored in stoppered glass vials in the refrigerator for further processing for analysis. The remaining hexane extract was concentrated to about 1 ml transferred directly to a florisil column (obtained from Sigma Chemical Company, PR grade 60-80 mesh) prepared according to Mills and Olney (1977).

The method of Murty et al. (1980) was followed for the analysis. Silica gel (obtained from Merck Company) was washed with copious amount of glass triple distilled water in a Buchner funnel to remove inorganic phosphate. The TLC plates were prepared by spreading the above washed silica gel to give 250 μ thick on plates of size 5 x 20 cm. They were dried for one hour and activated at 180°C for 15 min before use.

The concentrated extract of 1 ml as above and technical pesticide for reference as standard were spotted using micropipettes (5-20 μl) on thin layer chromatographic plates prepared as above (Murty et al. 1980). The spotted plates were developed in a TLC chamber until the solvent front reached 10 cm height from the starting point. Then the above plates were removed from the chamber, air dried, kept in a hot air oven for 30 minutes. Later they were taken, cooled and were sprayed with a mixture of solution containing molybdate antimony tartrate and 2% Ascorbic acid (Murty et al. 1980). Appearance of blue spots on the TLC plates and also a standard reference Rf value was taken as confirmation of the residue of the pesticide.

For residue analysis the precautions laid down by Thompson (1974) were followed. Gas chromatography was carried out by Hewlett Packard Model 5840 with FID (Flame Ionisation detector) and glass coiled column (6 μ x ¼" as internal diameter) packed with 10% SE 30 as 80-100 mesh chromosorb WAW. The column temperature was 280°C, injector and detector temperatures were 300°C and 300°C respectively. Nitrogen was used as the carrier gas at 50 ml/min and an online integrater provided the retention time and area of each peak. 5 μ of

each sample was analysed thrice with and without adding known amounts (100 mg/g of sample) of chlorpyrifos as an internal standard. The analytical conditions were attenuation, slope sensitivity 0.5 area of rejection in millions and carrier gas Nitrogen at 2 kilo pascals per cm^2. Peak retention times and areas were calculated using a microprocessor connected to the GLC instrument.

Table 1. LC_{50} values µg/L of technical grade (TG) of chlorpyrifos to *Catla catla, Labeo rohita* and *Cirrhinus mrigala* using static and continuous flow (CF) systems

S.No.	Name of the Fish	Toxicity Method	Hours of Exposure			
			24 h	48 h	72 h	96 h
1	*Catla catla*	SM	510	460	420	350
		CF	460	380	350	300
2	*Labeo rohita*	SM	740	660	560	470
		CF	580	480	400	300
3	*Cirrihinus mrigala*	SM	940	840	760	650
		CF	850	750	660	550

SM = static method. CF = Continuous flow through method. Each value is mean of 5 individual observations; method tested for chi-square and are significant at $p < 0.05$.

RESULTS AND DISCUSSION

The 96 hr LC_{50} values for static and continuous flow (CF) systems for the fish *Catla catla, Labeo rohita* and *Cirrhinus mrigala* are given in Table 1.

5 µl of standard chlorpyrifos solution was injected into the instrument and also the sample under test. Then using the graph, the areas of chloropyrifos were measured as internal standard peaks in each case of analysis that was made and compared to the pesticide content as residue with standard as follows:

$$\text{Chlorpyrifos content per cent by mass} = \frac{m_1 \times A_1 \times A_3 \times P}{m_2 \times A_2 \times A_4}$$

The estimated chlorpyrifos was expressed as µg/g wet weight of the tissue that were tested.

The results of the 100 x Rf values of chlorpyrifos technical grade in four different solvent systems are given in Table 2A & 2B.

The results of the gas liquid chromatographic analysis in the tissues of the brain and liver of the fish *Catla catla, Labeo rohita* and *Cirrhinus mrigala* are given in Table 3.

The reported LC_{50} values for different fishes exposure to chlorpyrifos were given in Table 4.

Table 2A. Pesticide standard confirmation and the tissue chromatogram.

Absorbant	:	Silicagel
Solvent	:	Hexane + Acetone (90 + 10 v/v)
Front	:	10 cm
Spray reagents	:	Molybdate antimony tartrate; 2% Ascorbic acid
Time	:	30 minutes
Period of exposure	:	8 days
Colour of the spot	:	Blue

Table 2B. Rf values of standard in different solvent systems.

Solvent system	Ratio v/v	100 x Rf
Hexane + Acetone	9 : 1	45
Hexane + Benzene	1 : 1	85
Hexane + Acetone	1 : 1	87
Hexane + Benzene	4 : 6	48

Tissue	100x Rf (Hexane : Acetone 1:1)
Brain	85
Liver	82

Table 3. Residue levels of organophosphate pesticide chlorpyrifos in Brain and Liver tissues of *Catla catla, Labeo rohita* and *Cirrhinus mrigala* exposed to sublethal concentration of chlorpyrifos technical grade for 8 days.

S.No.	Fish	Organ	Amount of residue mg/g
1	*Catla catla*	Brain	0.28
		Liver	0.09
2	*Labeo rohita*	Brain	0.38
		Liver	0.09
3	*Cirrhinus mrigala*	Brain	0.11
		Liver	0.08

* The results are average of three observations.

Gas chromatography – "Hewlett Packard model 5840, with FID and glass coiled column 6 μ x ¼" as internal diameter.

Column is packed with 10% SE as 80-100 mesh chromosorb WAW (Temp 280^0C, injedtor and detector temperatures were 300^0C respectively).

Table 4. Reported LC_{50} values of chlorpyrifos technical grade.

Name of the Fish	LC_{50} value	hrs of exposure	Reference
Bluegill (*Leponis microchirus*)	1.7 – 4.2 µg/L	96 hr	Mayer & Ellersieck, 1986
Bluegill (*Leponis microchirus*)	10 µg/L	24 hr	Mayer & Ellevsieck, 1986
	2.4 µg/L	96 hr	Johnson and Finley, 1980
Carp (*Cyprinus carpio*)	10 µg/L	96 hr	Phipps & Holcombe, 1985
	1.3 µg/L	72 hr	Dutt & Guha, 1988
Channet cat fish (*Ictalums punctatus*)	280 µg/L	96 hr	Johnson & Finley, 1980
Cut throat trout (*Onchorhincus clarki*)	18 µg/L	96 hr	Johnson & Finley, 1980
Europian cel (*Anguilla anguilla*)	540 µg/L	96 hr	Ferrando et al. 1991
Fathead Minnow (*Pimephales promelas*)	203 µg/L	96 hr	Holcombe et al. 1982
Rainbow trout (*Onchorhynchus mykiss*)	24 µg/L	96 hr	Mayer and Ellersieck, 1986
Lake trout (*Salvelinus namaycush*)	419 µg/L	96 hr	Mayer and Ellersieck, 1986
Fathead minnow (*Pimephales promelas*)	806 µg/L	96 hr	Holcombe, 1985
Gold fish (*Carrassius auratus*)	542 µg/L	96 hr	Holcombe, 1985
Roach (*Rutilus rutilus*)	250 µg/L	96 hr	Douglas and Bell, 1990

In the present study, the toxicity is in the order of *Catla catla* > *Labeo rohita* > *Cirrhinus mrigala.* The toxicity varies from species to species (Table 4). The toxicity of chlorpyrifos results from initial metabolic activation to form chlorpyrifos oxon, with subsequent inactivation of acetylcholinesterase (ACh.E) at neural junctions, the inactivation of ACh.E often occurs by oxon phosphorylation of the enzyme active site. In some fish species, ACh.E can become irreversibly inhibited through dealkylation of the phosphorylated ACh.E, a process that renders, it resistant to hydrolysis (Chambers and Chambers, 1989). The species related differences in the sensitivity of brain were noticed by Yamin et al. (1994); Siddiqui et al. (1988 & 1989); Nemesok (1987); Jarvinen et al. 1983 and Olson, 1980.

Under sublethal exposure to chlorpyrifos for a period of 8 days, it was observed that *Labeo rohita* tissues accumulated more amount of residue when compared to other two fish, *Catla catla* and *Cirrhinus mrigala* (Table 3). The brain tissue

accumulated more residue than the liver due to the detoxification mechanism forming the metabolites in the three fish. TLC confirmation of the residues of chlorpyrifos appeared as blue spots, prominently in brain and less prominently in liver in the present study. Comparatively *Cirrhinus mrigala* accumulated lesser amount of residues which is due to the habitat effect, being bottom dweller, its exposure as well as uptake both are reduced. Barron and Woodburn (1995) stated that fish and other aquatic organisms rapidly absorb chlorpyrifos from water, with reported uptake rates ranging from 2-60 $mL/g^{-1}/h^{-1}$ and elimination half lives of 0.5 – 4 days. Deneer (1993 & 1994) suggested that elevated bioconcentration in guppies and stickle backs greater than sublethal concentrations caused by inhibition of chlorpyrifos bio-transformation, which in turn lead to slow elimination. USEPA (1992) reported the average concentrations of chlorpyrifos in fish at industrial and background monitoring sites in the United States were 0.004 and 0.0004 mg/kg respectively. Chlorpyrifos uptake, clearance and elimination half life values in rainbow trout (*Oncorhynchus mykiss*) were 14.4 mL g^{-1} h^{-1} and 66 hr respectively (Murphy and Lutenske, 1986) and in eels (*Anguilla anguilla*) were 2 mL g^{-1} h^{-1} and 81.5 hr (Douglas and Bell 1990). Pharmacokinetic studies in fish have shown that chlorpyrifos is rapidly absorbed from exposure to water and metabolised extensively (Giesy et al. 1999).

Liver is the main detoxifying organ containing relatively high levels of enzyme for the process and also the first organ to face the effect of pesticides being carried through the portal circulation resulting in the greater accumulation of chlorpyrifos (Byford et al. 1986). Being Neurotoxic (O'Brien, 1967) and detoxification, the present work confers the accumulation as residue which leads to the process of magnification in the food chain. Thus, it was natural that concomitantly with the discovery of chlorpyrifos, research was initiated that dealt with potential toxicological and environmental issues (Giesy et al. 1999). Hence the danger of carry over from aquatic system to terrestrial system when such fish are consumed.

In disease management of aqua farming, the chemical treatment is contemplated. The use of organophosphates like chlorpyrifos results to reach a level of concentration being toxic or sometimes may be sublethal. In such a situation due to concentration of below lethal, the accumulation can be possible and fish are subjected to more stress, avoid feeding which is detrimental for growth as well as possibility of magnification in the food chain.

REFERENCES

Barron MG, Woodburn KB (1995) Ecotoxicology of chloropyrifos. Rev Environ Contam Toxicol 144:1-93

Barron MG, Plakas SM, Wilga PC (1991) Chlorpyrifos pharmacokinetics and metabolism following intravascular and dietary administration in channel catfish. Toxicol Appl Pharmacol 108:474-482

Byford RL, Lockwood JA, Smith SM, Harmon CW, Johnson CC, Luther DG, Morris HF, Jr., Penny AJ (1986) Insecticide residues in cattle treated with cypermethrin, chlorpyrifos, piperonyl butoxide. Bull Environ Contam Toxicol 37:692-697

Chambers JE, Chambers HW (1989) An investigation of acetyl-cholinesterase inhibition and aging and choline acetyl transferase activity following a high level acute exposure to paraoxon. Pestic Biochem Physiol 33:125-131

Deneer JW (1993) Uptake and elimination of chlorpyrifos in the guppy at sublethal and lethal aqueous concentrtions. Chemosphere 26:1607-1616

Deneer JW (1994) Bioconcentration of chlorpyrifos by the three-spined stickleback under laboratory and field conditions. Chemosphere 29:1561-1575

Douglas MT, Bell IB (1990) The bioaccumulation and depuration of chlorpyrifos by the eel (*Anguilla anguilla*). Tech. Rep. GHE-T-28. DowElanco, Indianapolis, IN

Dutt N, Guha RS (1988) Toxicity of few organophosphorus insecticides to fingerlings of bound water fishes, *Cyprinus carpio* (Linn.) and *Tilapia mossambica* (Peters). Indian J Entomol 50: 403-421

EPA (1975) Committee on methods for toxicity tests with aquatic organisms: Methods for acute toxicity tests with fish, macro invertebrates and amphibians US EPA 660/3-75-009-61pp.

Ferrando MD, Sancho E, Andreu-Moliner, E. (1991) Comparative acute toxicities of selected pesticides to *Anguilla anguilla*. J Environ Sci Health B26:491-498.

Finney DJ (1971) Probit analysis 3rd Ed., Cambridge Univ. Press, London/ New York.

Giesy JP, Solmon KR, Coats JR, Dixon KR, Giddings JM and Kenega EE (1999) Chlorpyrifos: Ecological Risk assessment in north America Aquatic Environment. Rev Environ Contam Toxicol 160:1-129.

Holcombe GW, Phipps GL, Tanner DK (1982) The acute toxicity of Kelthane, dursban, disulfoton, pydrin and permethrin to fathead minnows. Pimephales promelas and rainbow trout *Salmo gairdneri*, Environ Pollut 29: 167-178.

Johnson WW, Finley MT (1980) Handbooks of acute toxicity of chemicals to fish and aquatic invertebrates. U.S. Fish Wildl Serv Resour Pub 137. 98 pp.

Mayer FL(Jr.), Ellersieck MR (1986) Manual of acute toxicity. Interpretation and database for 410 chemicals 66 species of fresh water animals. Resource publication 160 U.S. Fish and Wild Life Science, Washington DC.

Mills, Olney (1977) Pesticidal Analytical Manual, Food and Drug Administration, Washington D.C., 1-5.

Murphy PG, Lutenske NE (1986) Bioconcentration of chlorpyrifos in rainbow trout (*Salmo gairdneri* Richardson). DowElanco, Indianapolis, IN.

Murty AS, Rajabhushanam BR, Christopher K and Ramani AV (1980) Improved Ammonium Molybdate method for thin layer chromatographic detection of organophosphate residues. J Assoc Off Anal Chem 63:756-757.

Murty AS (1986) Toxicity of Pesticides to fish Vol. I and Vol. II, C.R.C. Press Inc. Bocaraton. 483 pp and 355 pp.

O'Brien RD (1967) Insecticides action and metabolism. Academic Press, London, New York 332pp.

Phipps GL, Holcombe GW (1985) A method for aquatic multiple species toxicant testing: acute toxicity of 10 chemicals to 5 vertebrates and 2 invertebrates. Environ Pollut Ser A Ecol Biol 38:141-157.

Roberts M, Boyce CBC (1972) Methods in microbiology (7-A ed). Norris, J.R. and Ribbows, 479 pp. D.W. Academic Press, New York

Thomson 1974 Analysis of pesticide residues in Human and environment samples supplied by FAO, Washington DC, USA.

Tilak KS, Janardhana Rao NHK, Jhansi Lakshmi S (1991) Toxicity of technical, commercial formulations of four different ratios of Endosulfan, Malathion and carbofuran to the fresh water fish *Labeo rohita* (Hamilton). J Ecotoxicol Environ Monit 1:19-22.

Tilak KS, Marina Samuel G (2001) Impact of Chloropyrifos, an organophosphate compound on fresh water edible fish *Catla catla,* Asian Jr of Microbiol Biotech Environ Sci 3(4):327-329

USEPA: United States Environmental Protection Agency (1992) Worker protection standards, Washington, DC, p.36

Van Wijngaarden R, Leeuwaugh P, Lucassen WGH, Romijin, K, Randay R, Van der Velde R, Welligenburg W (1993). Acute toxicity of chlorpyrifos to fish a new and aquatic invertebrates (1993). Bull Environ Contam Toxicol 51:716-723

Van Wijngaarden R, Leeuwaugh P (1993) Relationship between toxicity in laboratory and parol: an ecotoxicological study with chlorpyriphos. Med Fac Landbouww Rijksuniv Gent 54:1061-1069.

Welling W, de Vries JW (1992). Bioconcentration kinetics of the organophosphorus insecticide chlorpyrifos in guppies (*Poecilia reticulata*). Ecotoxicol Environ Saf 23:64-75.