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SHORT PAPER

Behavioural Responses of the Freshwater Fish, *Cyprinus carpio* (Linnaeus) Following Sublethal Exposure to Chlorpyrifos

Ramesh Halappa[1], Muniswamy David[1,*]

[1] Karnatak University's Karnatak Science College, Environmental and Molecular Toxicology Division, Department of Zoology, Dharwad 580 001, Karnataka, India.

* Corresponding Author: Tel.: +91.9845709815; Fax: +91.8362744334;
E-mail: davidkcd@rediffmail.com

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Abstract

Common carp fingerlings were exposed to different concentrations (0.120 to 0.200 mg/L) of an organophosphate pesticide, chlorpyrifos (20% EC) for 96 h. The acute toxicity (LC_{50}) of chlorpyrifos by static renewal (semi-static) bioassay test was found to be 0.160 mg/L. One-seventh (0.0224 mg/L) and one-fourteenth (0.0112 mg/L) of the 96 h LC_{50} were selected as sublethal concentrations for subacute studies. The fish were exposed to both the sublethal concentrations for 1, 7 and 14 days and were allowed to recover in toxicant free medium for seven days only after 14th day of exposure. Behavioural responses and morphological deformities were studied in the experimental periods. Fish in toxic media exhibited irregular, erratic and darting swimming movements, hyper excitability, loss of equilibrium and sinking to the bottom. The carp were found under stress, but mortality was insignificant in both the sublethal concentrations. Caudal bending was the main morphological alteration during the exposure periods. The behavioural and morphological changes may be due to the inhibition of acetylcholinesterase (AChE) activity. Inactivation of AChE activity results in excess accumulation of acetylcholine (ACh) in cholinergic synapses leading to hyperstimulation and cessation of neuronal transmission (paralysis). Impaired behavioural responses and morphological deformities were observed even under recovery periods. This may be a consequence due to the inhibition of brain and muscular AChE activity by chlorpyrifos-oxon via biotransformation of bioaccumulated chlorpyrifos in the tissues.

Keywords: Chlorpyrifos-ethyl, common carp, acute toxicity (96 h LC_{50}), behavioural anomalies, caudal bending, recovery.

Introduction

Recent evidence indicates that fish, an extremely valuable resource, are quickly becoming scarce. One consequence of this scarcity is the increasing concern for fish survival and a growing interest in identifying the levels of various chemical pollutants, which are safe for fish and other aquatic life. Pesticides are among the most hazardous chemicals to men and ambient. Insecticides are extensively used to protect agricultural crops against the damages caused by pests. However, these chemicals may reach other ecological compartments as lakes and rivers through rains and wind, affecting many other organisms away from the primary target. Only 0.1% reaches the specific target (Rand and Petrocelli, 1984; Aguiar, 2002). The injuries of insecticides to aquatic environments are incontestable. The significant increase of chemical emissions in the water resources has lead to deleterious effects for aquatic organisms (Livingstone, 2001; Matsumoto *et al*., 2006).

Organophosphates (OPs) have become the most widely used class of insecticides in the world replacing the persistent and problematic organochlorine compounds. Exposure of aquatic ecosystems to these insecticides is difficult to assess because of their short persistence in the water column due to low solubility and rapid degradation. However, monitoring of these insecticides is important, because they are highly toxic to aquatic organisms.

Fish are ideal sentinels for behavioural assays of various stressors and toxic chemical exposure due to their 1) constant, direct contact with the aquatic environment where chemical exposure occurs over the entire body surface, 2) ecological relevance in many natural systems (Little *et al*., 1993), 3) ease of culture, 4) ability to come into reproductive readiness (Henry and Atchison, 1986) and 5) long history of use in behavioural toxicology.

Behavior provides a unique perspective linking the physiology and ecology of an organism and its environment (Little and Brewer, 2001). Behavior is both a sequence of quantifiable actions, operating through the central and peripheral nervous systems (Keenleyside, 1979) and the cumulative manifestation of genetic, biochemical and physiologic processes essential to life such as feeding, reproduction and predator avoidance. Behavior allows an organism to adjust to external and internal stimuli in order to THE best meet the challenge of surviving in a changing environment. Conversely, behavior is also the result of adaptations to environmental variables. Thus, behavior is a selective response that is constantly adapting through direct interaction with physical, chemical, social and physiological aspects of the environment. Selective evolutionary processes have conserved stable behavioural patterns in concert with morphologic and physiologic adaptations. This stability provides the best opportunity for survival and reproductive success by enabling organisms to

efficiently exploit resources and define suitable habitats (Little and Brewer, 2001).

Since behavior is not a random process, but rather a highly structured and predictable sequence of activities designed to ensure maximal fitness and survival (i.e. success) of the individual (and species), behavioural endpoints serve as valuable tools to discern and evaluate effects of exposure to environmental stressors. Fish are able to uptake and retain different xenobiotics dissolved in water via active or passive processes. They can be used to detect and document pollutants released into their environment. Sublethal concentrations of pesticides in aquatic environments cause structural and functional changes in aquatic organisms and this is more common than mortality (Sancho *et al*., 2003). Behavioural modification is one of the most sensitive indicators of environmental stress and many affect survival (Olla *et al*., 1983; Byrne and O'Halloran, 2001). Alterations in fish behavior, particularly in non-migratory species, can also provide important indices for ecosystem assessment. Any change in the behavior of fish indicates the deterioration of water quality, as fish are the biological indicator and hence index of environmental suitability and the cost of survival.

Chlorpyrifos is a synthetic organophosphate (OP), non-systemic, broad-spectrum insecticide and acaricide, acting as a cholinesterase inhibitor, with contact, stomach and respiratory action. Commercial manufacture of chlorpyrifos started in 1969, since then chlorpyrifos has been used for many purposes. The major use of chlorpyrifos in farming is to protect corn, cotton and fruit trees against insects. Common carp is a prime cultured and very important staple freshwater fish generally found in rivers, ponds and reservoirs. Contamination of aquatic biota at sublethal levels by chlorpyrifos is common in our region affecting the non-target icthyofauna. Hence, the present study was undertaken to evaluate the aquatic toxicity of chlorpyrifos with special emphasis on behavioural responses of the fish, *C. carpio* exposed to sublethal concentrations of commercial grade chlorpyrifos.

Materials and Methods

Sample Collection, Maintenance and Acute Toxicity Test

Healthy and active *C. carpio* fingerlings (840 test species) were procured from the State Fisheries Department, Dharwad, India. Fish were brought to the laboratory in large aerated crates. Later they were acclimatized for 30 days in large cement tanks (22 x 12 x 5 feet) and fed with commercial dry feed pellets (Nova, Aquatic P. Feed).

The carp (2±0.22 g, 4±0.25 cm) were acclimatized to laboratory conditions for 20 d at 24±1ºC and are held in 100 L glass aquaria (120 x 45 x 80 cm) containing dechlorinated tap water of the quality used in the test, whose physico-chemical characteristics were analyzed following the methods mentioned in APHA (2005) and found as follows, temperature 24±2ºC, pH 7.1±0.2 at 24ºC, dissolved oxygen 9.6±0.8 mg/L, carbon dioxide 6.3±0.4 mg/L, total hardness 23.4±3.4 mg as $CaCO_3$/L, phosphate 0.39±0.002 µg/L, salinity, nil, specific gravity 1.001 and conductivity less than 10 µS/cm. Water was renewed every day and a 12-12 h photoperiod was maintained during acclimatization and test periods. The fish were fed regularly with commercial fish food pellets during acclimatization and test periods, but feeding was stopped two days prior to exposure to the test medium for acute toxicity test only.

Chlorpyrifos (20% EC:emulsifiable concentrate) was procured from the local market of Dharwad, Karnataka, India, under the trade name Hyban, supplied by Hyderabad Chemical Supplies Limited, Hyderabad, India. The expiry date of the test substance checked prior to initiation of the treatment was found suitable for the exposure. Required quantity of chlorpyrifos was drawn directly from this 20% EC using micropipette.

In range finding test, fish were exposed in batches of ten (in 20 L of test medium) to varying concentrations (0.120 to 0.200 mg/L) of chlorpyrifos with six replicates for each test concentration along with the control sets. Water medium was replaced every 24 h followed by an addition of desired concentration of the test compound. Concentrations of the test compound used in short term definitive tests were between the highest concentration at which there was 0% mortality and the lowest concentration at which there was 100% mortality (Table 1).

Mortality was recorded every 24 h and the dead fish were removed when observed, every time noting the number of fish death at each concentration upto 96 h. Duncan's multiple range test (Duncan, 1955) was employed for comparing mean mortality values after estimating the residual variance by repeated measures ANOVA (Winner, 1971) for arc sine transformed mortality data (dead individuals/initial number of individuals). Time of exposure was the repeated measure factor while treatment (concentration and control) was the second factor. In addition, LC_{50} were compared by the method of APHA (2005). The LC_{50} with 95% confidence limits for chlorpyrifos were determined/estimated for 96 h by probit analysis (Finney, 1971).

Study Periods and Toxicant Concentrations

Sublethal concentrations of one-seventh (0.0224 mg/L) and one-fourteenth (0.0112 mg/L) of the 96 h LC_{50} (0.160 mg/L) were selected for subacute studies. Fish were exposed to both the sublethal concentrations of chlorpyrifos for 1, 7 and 14 days and allowed to recover in toxicant free medium for seven days only after 14^{th} day of exposure. The

Table 1. Mortality of *C. carpio* fingerlings in different concentrations of chlorpyrifos (20% EC) at 96 h exposure periods

Conc. of chlorpyrifos (mg/L)	Log conc.	No. of fish alive out of ten	% Corrected mortality	Probit kill
0.120	-0.920	10	0	--
0.130	-0.886	9	10	3.72
0.140	-0.853	8	20	4.16
0.150	-0.823	7	30	4.48
0.156	-0.806	6	40	4.75
0.160	-0.795	5	50	5.00
0.165	-0.782	4	60	5.25
0.170	-0.769	3	70	5.52
0.178	-0.749	2	80	5.84
0.188	-0.725	1	90	6.28
0.200	-0.698	0	100	--

control (exclusively toxicant free medium) and chlorpyrifos exposed fish were kept under continuous observation for study of behavioural responses and morphological deformities.

Analysis of Chlorpyrifos by HPLC

Concentrations of chlorpyrifos in the test medium were confirmed by High Performance Liquid Chromatography (HPLC) analysis by the method described by Rao *et al.* (2003). Test medium (100 ml) was extracted thrice with 50 ml of pet ether. After separation of layers, the pet ether extract was filtered through anhydrous sodium sulfate column. The extracts were passed through an activated Florisil column for cleanup of the sample. The resultant extract was evaporated to dryness under reduced pressure in a rotary evaporator at 40ºC. Dry extract was dissolved in 1 ml of acetonitrile for HPLC analysis. Briefly, the HPLC program (Shimadzu, Japan, Tokyo) was operated by using a UV detector with a mobile phase consisting of acetonitrile (65%) and water (35%) in 0.1% of acetic acid through a C_{18} (ODS) column (250 millimeter length and 4.6 millimeter internal diameter) with a flow rate of 1.5 ml/min. The obtained peak areas of chlorpyrifos in individual samples (µg/L of the sample) were analyzed using standards.

Statistical Analysis

Data correspond to the average of six replicates. The data obtained were analyzed statistically by following Duncan's multiple range test (Duncan, 1955).

Results and Discussion

Acute toxicity (96 h LC_{50}) of chlorpyrifos for the freshwater fish, *C. carpio* was found to be 0.160 mg/L. The upper and lower 95% confidence limits are presented in Table 2. Thus, chlorpyrifos can be rated as highly toxic to fish. No significant mortality was observed during the experimental periods in both the sublethal concentrations.

Johnson and Finley (1980) and Clark *et al.* (1985) reported 96 h LC_{50} of chlorpyrifos to channel catfish, *Ictalurus punctatus* and sheepshead minnow, *Cyprinodon variegatus* as 0.280 mg/L and 0.136 mg/L, respectively. Chlorpyrifos toxicity reported by Rao *et al.* (2003 and 2005) to euryhaline and mosquito fish, *Oreochromis mossambicus* (Tilapia) and *Gambusia affinis* by semi-static method is 0.0259 mg/L and 0.297 mg/L, respectively.

We can infer from our results that chlorpyrifos is highly toxic to freshwater fish, *C. carpio* and comparison of the different LC_{50} values clearly indicates that the acute toxicity of chlorpyrifos varies with the fish species.

Behaviour of the Control and Exposed Fish

In the present study, the control fish were active for feeding and alert to slightest of the disturbance with their well-synchronized movements. The behavior did not significantly vary between the control groups; therefore, these results were taken as standards for the entire experimentation.

Carp exposed to chlorpyrifos exhibited disrupted school behavior, localization to the bottom of test chamber and independency (spread out) in swimming. This followed loss of co-ordination and occupancy of twice the area to that of control group were the early responses of the carp following exposure to chlorpyrifos in both the sublethal concentrations. Subsequently, fish moved to the corners of the test chambers, which can be viewed as an avoidance behavior of the fish to chlorpyrifos. Further, carp exhibited irregular, erratic and darting swimming movements and loss of equilibrium followed by hanging vertically in water. The above symptoms may be due to inhibition of acetylcholinesterase (AChE) activity leading to accumulation of acetylcholine (ACh) in cholinergic synapses ensuing hyperstimulation. Since, inhibition of AChE activity is a typical characteristic of organophosphate compounds (Holmstedt, 1963; Habig and DiGiulio, 1991; Padilla *et al*., 1996; Timchalk *et al*., 2002). Our

Table 2. Acute toxicity (96 h LC_{50}), slope and 95% confidence limits of chlorpyrifos to the fingerlings of common carp

Pesticide	Slope	96 h LC_{50} (mg/L)	95% Confidence limits	
			Upper limit	Lower limit
Chlorpyrifos	1.009	0.160 ± 0.007	0.168	0.151

findings corroborate with the observations made by Hülya *et al.* (2006) in the sentinel freshwater fish, *Oreochromis niloticus* following sublethal exposure to diazinon.

The primary molecular mechanism of action of the OP pesticides is inhibition of AChE activity, a widely distributed serine esterase (Ecobichon, 1996). AChE occurs throughout the central and peripheral nervous system of vertebrates and its normal physiological action is to hydrolyze the neurotransmitter ACh, so that activation of cholinergic receptors is transient. AChE hydrolyses ACh into choline and acetic acid and is responsible for the removal of the neurotransmitter ACh from the synaptic cleft through hydrolysis (Habig and DiGiulio, 1991). ACh is the primary neurotransmitter in the sensory and neuromuscular systems in most species. Activity of AChE system is vital to normal behavior and muscular function and represents a prime target on which some toxicants can exert a detrimental effect. Once bound, organophosphorus compounds are considered irreversible inhibitors, as recovery usually depends on new enzyme synthesis (Habig and DiGiulio, 1991).

Fish slowly became lethargic, restless and secreted excess mucus all over the body. Intermittently some of the carp were hyper excited resulting in erratic movements. These behavioural alterations persisted even during the recovery periods. An excess secretion of mucus in fish forms a non-specific response against toxicants, thereby probably reducing the toxicant contact. Mucus also forms a barrier between the body and the toxic medium, to minimize its irritating effect, or to scavenge it through epidermal mucus. Rao (2006) made similar observations following RPR-V (a novel phosphorothionate insecticide, 2-butenoic acid-3-[diethoxy phosphinothionyl] ethyl ester) exposure to euryhaline fish, *Oreochromis mossambicus*.

Disrupted shoaling behavior, easy predation, gulping air and swimming at the water surface (surfacing phenomenon) were observed on the day of exposure to sublethal concentrations of chlorpyrifos. This situation further continued intensely throughout the test periods, which is in accordance with the observations made by Ural and Simsek (2006). Gulping of air may help to avoid contact of toxic medium and to ease respiratory stress. Surfacing phenomenon i.e., significant preference of upper layers in exposed groups may be due to elevated demand for oxygen during the exposure periods (Katja *et al.,* 2005). Surfacing phenomenon and easy predation continued even under recovery periods of seven days in both the test concentrations. This reflects the catastrophic impact posed by the toxicant. Of all, phenomenon of easy predation is one of the most serious damage caused by a pollutant on sensitive species like fish, which ultimately decide the survival of a species in a given ecosystem.

Caudal bending was noticed in both the toxicant concentrations with time and persisted even under recovery periods, which greatly retarded the normal swimming pattern. The extent of caudal bending was pronounced in the highest toxicant concentration ($1/7^{th}$ of 96 h LC_{50}). Caudal bending may be a sort of paralysis, which might be due to the inhibition of muscular AChE activity resulting in blockage of neural transmissions. This produces rapid twitching of voluntary muscles followed by paralysis (Ware, 1989; Habig and DiGiulio, 1991). Bending of caudal base is owing to the fact that caudal portion is the thinnest structure and hence can be conferred any sort of orientation due to paralysis of caudal musculature because of AChE activity inhibition. Thus, chlorpyrifos reduced instinctive behavioural responses and affected morphological features.

Hyper extension of fins, dullness in body colour and fish body became lean towards abdomen and carp under stress were observed with time and concentration in experimental periods. Intermittently, some of the fish sank to the bottom with their least opercular movements, failing to fight chlorpyrifos stress in both the sublethal exposures and in recovery periods. In later period's there was slight swelling in the abdominal region, which persisted even under recovery periods in both the test concentrations. In general, fish poisoned with anticholinesterase insecticides show signs of muscle paralysis, especially of the fins and respiratory apparatus, hyperactivity and loss of balance (Sancho *et al.,* 1998). Leaning of fish indicates reduced feeding behavior and diversion of fish metabolism towards adaptability to the toxic media. Feeding preferences were affected and consumption of food in fish was impaired and reduced drastically. This was more pronounced in one-seventh of sublethal exposure periods and continued even under recovery periods. For these animals, it might be profitable to decrease their food uptake under toxic environmental conditions to lower the energetic costs of digestion. Depression in appetite is a common response of fish to stress and intermittence of feeding for longer periods can have a

clear impact on growth and reproduction (Rice, 1990). A substantial growth reduction caused by toxicant stress has important implications for survival in the natural situations. Dembele *et al.* (2000) indicated that the abnormalities in fish behavior observed in exposure to OP insecticides (chlorfenvinphos, chlorpyrifos and diazinon) could be related to failure of energy production or the release of stored metabolic energy, which may cause severe stress, leading to the death of the fish. Fish in the lowest ($1/14^{th}$ of 96 h of LC_{50}) sublethal concentration of chlorpyrifos were alert and fed actively.

Behavioural anomalies were evidenced right from the day of exposure to sublethal concentrations of chlorpyrifos and are due to inhibition of brain AChE. Inhibition of AChE activity results in the accumulation of ACh and signs of cholinergic toxicity. Chawanrat *et al.* (2007) reported that inhibition of brain AChE activity is an early process of sublethal exposure to chlorpyrifos in hybrid catfish and hence support the above observed behavioural changes in the exposed fish. Overall impairments in fish behavioural responses and morphological deformities even under recovery 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 (CYP) (Amitai *et al.*, 1998; Poet *et al.*, 2003), dearylation reaction utilizing the same enzymes and A-esterase (Poet *et al.*, 2003). Furthermore, physiological reactions, such as activation of biotransformation enzyme systems in the presence of xenobiotic substances enable the organisms to survive in subacute exposures. This may be the reason for insignificant mortality observed during this study.

Conclusion

The current study evidenced that chlorpyrifos is highly toxic and had a detrimental impact on the behavioural responses of *C. carpio* at sublethal concentrations. It reduced/decreased the animals' ability to adapt to its environment by 1) increasing the time required to learn to escape or to avoid external noxious stimuli, 2) decrease the animal sensitivity to subtle changes in the environment, or 3) interfere with the animals' ability to retain previously learned behavior. Thus, chlorpyrifos reduced instinctive behavioural response and affected morphological features. Impairments in behavioural responses even under recovery periods may be due to inhibition of brain AChE activity by chlorpyrifos-oxon via biotransformation of bioaccumulated chlorpyrifos in the tissues into their active oxygen analog (chlorpyrifos-oxon). These behavioural responses can be used as a tool in biomonitoring programme to monitor ecotoxicity risk of chlorpyrifos to the test species.

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