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Changes in Behavior and Brain Acetylcholinesterase Activity in Mosquito Fish, *Gambusia affinis* in Response to the Sub-Lethal Exposure to Chlorpyrifos

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Abstract: Sub-lethal studies of chlorpyrifos, O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl) phosphorothioate on mosquito fish, *Gambusia affinis* were carried out *in vivo*, for 20 days to assess the locomotor behavior in relation to bioaccumulation and interaction with a targeted enzyme, acetylcholinesterase (AChE, EC: 3.1.1.7). Fish exposed to sub-lethal concentration of 60 µg/L (1/5 of LC_{50}) were under stress, and reduced their locomotor behavior like distance travelled per unit time (m/min) and swimming speed (cm/sec) with respect to the length of exposure. The alteration in locomotor behavior of fish may be due to an accumulation of acetylcholine (ACh), a neurotransmitter at synaptic junctions, due to the inhibition of AChE enzyme activity (40 to 55%) in brain and also bioaccumulation of the toxicant in different parts of fish. The bioaccumulation values indicated that the accumulation of chlorpyrifos was maximum in viscera followed by head and body. The average bio-concentration values are 0.109, 0.009 and 0.004 µg/g for viscera, head and body with depuration rates of 2.24, 1.69 and 0.39 ng/h respectively. It is evident from the results that the sub-lethal concentration [1/5 of LC_{50}; equivalent to Lowest Observed Effect Concentration (LOEC)] of chlorpyrifos can able to alter the locomotor behavior of *G. affinis* in relation to the length of exposure. The findings revealed that the locomotor activity of test organism could be considered as a suitable marker to evaluate the affect of toxicant even at LOEC levels.

Keywords: *Gambusia affinis*, chlorpyrifos, bioaccumulation, behavior, acetylcholinesterase

Introduction

Pesticides are widely used in agriculture and there is a need for tools to monitor the impact of these pesticides on fish population. There is a growing concern worldwide over the indiscriminate use of such chemicals, resulting in environmental pollution and toxicity risk to non-targeted organisms [1-2]. Responses of aquatic organisms are broad-ranged depending on the toxic compound, exposure time, water quality and the species [3-5]. Fish are continuously exposed to comparatively low concentrations of pesticides affecting their behavioral responses. Mortality is obviously not the only endpoint to consider and there is a growing interest in the development of behavioral markers to assess the sub-lethal affects of toxicant. Behavior is considered a promising tool in ecotoxicology [6-8] and these studies are becoming prominent in toxicity assessments in unicellular organisms [9], insects [10, 11], fish [12] and even rodents [13]. Locomotory behavior is commonly affected by contaminants [14] and the pattern of fish swimming is a highly organized species-specific response. Various methods were employed to investigate the altered locomotor behavior of stressed organisms. The advanced time-lapse video techniques have been successfully used to facilitate the documentation of behaviors in insects, fish and rats of normal and stressed organisms [15-17]. With the recent development of computer-assisted electronics, video-camera tracking systems have been greatly improved (Ethovision, Noldus, The Netherlands) and used extensively in quantification of locomotor behavior with a high degree of precision [10, 18].

In the present study, we have made an attempt to evaluate the sub lethal effects (60 µg/L; 1/5 of LC_{50}) of chlorpyrifos, O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl) phosphorothioate on the behavior of fish, *Gambusia affinis*, chosen as an experimental model because of its

wide availability and suitability to evaluate the toxicity of xenobiotics [19-21]. Swimming behavior of fish is frequently assessed as a response in toxicity investigations because altered locomotor activity can indicate effects to the nervous system. The work focused mainly to study the locomotor behavior (distance travelled per unit time in m/min and swimming speed in cm/sec) with special emphasis on target enzyme (AChE) interaction and bioaccumulation of chlorpyrifos 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. The fish species, *Gambusia affinis* (Order: Cyprinodontiformes, Family: Poeciliidae), were obtained from Andhra Pradesh Fisheries Department, Medchal (Hyderabad) and were transported from the farm in oxygenated polythene bags to the laboratory and immediately transferred into glass aquaria of 100 L capacity containing well-aerated unchlorinated ground water. Fish weighing 125±5mg were transferred to a 40 L glass aquarium (60 x 30 x 30 cm) for seven days and fed commercial dry feed pellets (Hello Fish Dry Pellets; CVM Products, Beijing, China) for conditioning. The water in the aquarium was renewed daily and was aerated with a Jumbo-Jet aquarium air pump (Super-8300, made in India). The natural photoperiod of 13:11 h (L:D) was maintained. The conditions for acclimatization and tests were maintained as temperature 26±2°C, pH 7.10±0.05 and dissolved oxygen 8.15±554 mg/L [22].

Determination of Median Lethal Concentration (LC_{50})

The acute LC_{50} value of chlorpyrifos was determined in the laboratory using semi-static method [23]. The test concentrations were chosen based on the initial experiments to determine the lethal concentration (LC_{50}) for 96 h. The required concentrations (200, 250, 300, 400 and 500 µg/L) were maintained in 40 L of water by adding the toxicant dissolved in 2 ml of acetone and renewed daily with out aeration. Fish starved for two days were released into each aquarium (10 fish of both sexes) and were exposed to five different concentrations with two replicates. The control experiments were also performed simultaneously with an addition of carrier solvent alone. The mortality record of the fish was maintained (during 96 h of exposure and further seven days of observation) in each concentration of the toxicant and the data was used to estimate the median lethal concentration (LC_{50}) by using a computerized programme developed by the method of Finney [24].

Further, the sub lethal test concentration, 60 µg/L (1/5 of LC_{50}) was maintained in 40 L of water (5 replicates) and 50 numbers of two-day starved fish of both sexes were released into each aquarium. The toxicant was dissolved in 2 ml of acetone to maintain the desired concentration and renewed daily (till the exposure tenure of 20 days), with out aeration. The exposed fish had free access to food pellets. The aquarium receiving only carrier solvent, acetone was the control. During the exposure tenure, the altered locomotor behavior of fish was monitored at regular intervals of day-4, 8, 12, 16 and day-20 by computer-assisted electronics, video-camera tracking system (EthoVision®, Noldus Information Technology, Wageningen, The Netherlands). Briefly: Before recording of the behavior, fish from control and treated lots were acclimatized individually for ten minutes in a recording glass aquarium (15x15x15 cm), containing 2.5 L of 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 visual disturbances.

The behavior of fish was recorded for five minutes in a fixed monitoring arena (internal diameter of aquarium and height of the water, 14.5x14.5x11 cm) with a high-resolution CCD camera, SONY CCD-IRIS (Model No: SSC-M370CE) mounted 20 cm away from the left over plain side of the recording aquarium. The behavioural pattern of video sequence was digitized using 'EthoVision' software interfaced with a personal computer. A minimum of 10 fish from each test interval was used to evaluate individually for determining their locomotor behavior (distance travelled per unit time in m/min and swimming speed in cm/sec).

Immediately after behavior recording of each lot, five numbers of fish were dissected out and their viscera, head and remaining body (hence forth called as body) were frozen in liquid nitrogen to estimate the bioaccumulation of chlorpyrifos. The remaining five fish were sacrificed and their brains were homogenized individually (10% w/v) in 0.1 M phosphate buffer (pH-7.5) using Potter-Elvehjam homogenizer fitted with a Teflon pestle for estimating AChE activity. The homogenates were centrifuged at 5,000 g for 10 minutes and the supernatant was further centrifuged at 5,000 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*., [25]. The AChE activity was estimated at different intervals of day-4, 8, 12, 16 and day-20 in brain tissues by the method described by Ellman *et al*., [26].

The deep frozen tissues for bioaccumulation were ground and homogenized in petroleum ether and the extract was filtered through anhydrous sodium sulphate. 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 Chromatography (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 (µg/g wet tissue) were analysed with standard peaks.

Statistical Analysis

Data on locomotor behavior are expressed as mean ± SE of ten fish from each interval was evaluated

individually. Statistical significance was determined by student's *t* test, and $P < 0.05$ was considered significant as compared to control.

Results and Discussions

Chlorpyrifos was commercially used for more than a decade particularly to control foliar insects effecting on different agricultural crops and also to control subterranean termites [11]. It is an extensively used Organophosphorous (OP) insecticide and second largest selling in India that resulting an increasing load in the environment, causing adverse effects on non-targeted fish [27]. In the present paper, acute toxicity of chlorpyrifos on fish, *Gambusia affinis* was carried out by semi static method and its LC_{50} value for 96 h is presented in table 1. It is evident from the results that chlorpyrifos can be rated as highly toxic to *G. affinis* with an LC_{50} value of 297.63±21.72 µg/L (ppb). Based on our previous findings, it can be concluded that *G. affinis* is several fold resistant than a euryhaline fish, *Oreochromis mossambicus* (Tilapia) with a median lethal concentration of 25.97±0.01 µg/L [28].

Table 1: Acute toxicity of chlorpyrifos on *Gambusia affinis*

Compound	*Regression Equation* $Y=(\bar{y}-b\bar{x})+bX$	*Acute Toxicity Range 98% Confidence Limit*		*Median LC_{50} (µg/L)*
		Upper (µg/L)	*Lower (µg/L)*	
Chlorpyrifos	-13.86 + 4.21X	340.21	255.06	297.63 ± 21.72

The fish, *G. affinis* exposed to the higher concentrations (400 and 500 µg/L) of chlorpyrifos, used for determining the lethal concentration, exhibited abnormal behaviors like erratic swimming with jerky movements, loss of equilibrium and secreted copious amount of mucous from whole body. Fish were lethargic and at the time of death they exhibited transient hyperactivity before collapsing. Similar behavioral changes were reported previously in the fish exposed to OP pesticides [16, 28-30]. However, no significant difference was observed in the rate of opercular movements of treated fish (60 µg/L) when compared to control.

The video tracking (computer algorithm) method for automated behavioral monitoring implemented in this present paper has the potential for generating results that may be utilized for evaluating risks related to contamination by or discharge of pesticides into stream ecosystems. Hence, a sub lethal concentration of 60 µg/L was used for behavioral monitoring of fish at laboratory conditions. It is evident from the figure 1 that the locomotor behavior of fish was greatly influenced during the exposure tenure. It is apparent from the data that the mobility (distance travelled) of fish was gradually decreased significantly ($P < 0.05$) by the action of toxicant from day-4 to day-20 in comparison to control value.

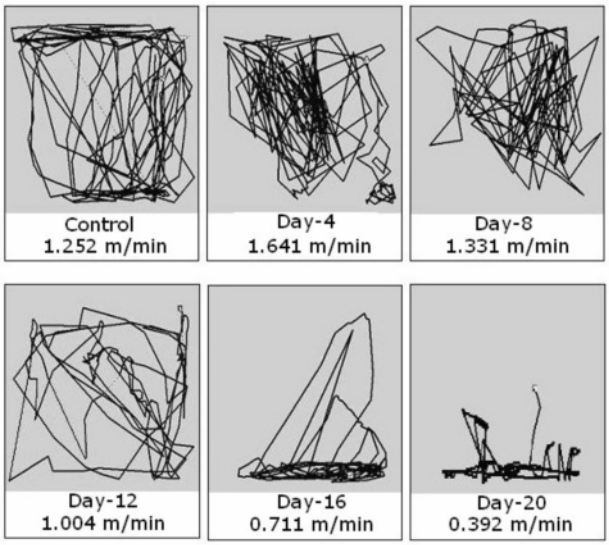

Figure 1: *In-vivo* effect of chlorpyrifos on locomotor behavior (distance travelled per min) of *Gambusia affinis* during sub-lethal exposure (60 µg/L) tenure of 20 days.

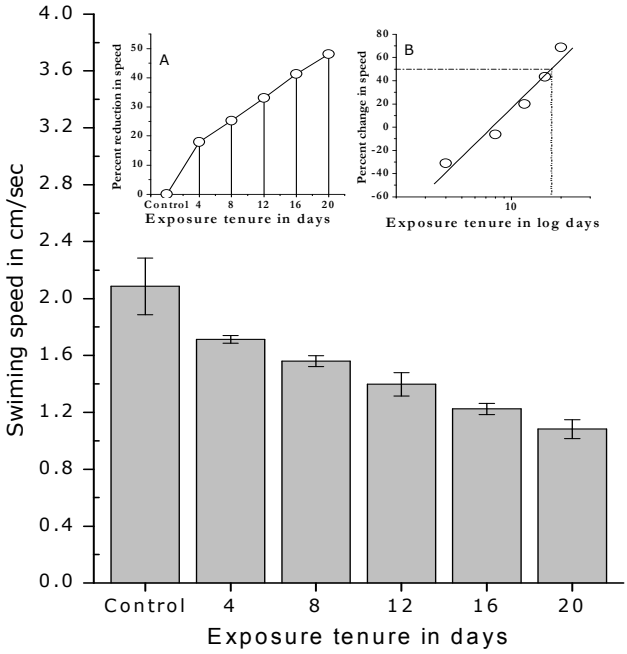

Figure 2: Average swimming speed of locomotor activity (swimming speed in cm/sec) of mosquito fish, *Gambusia affinis* at regular intervals of 20 days exposure to the sub-lethal concentration (60 µg/L; 1/5 of LC_{50}) of chlorpyrifos. Inset A: Percent change in their swimming speed. Inset B: Determination of time required to reduce 50% of their mobility.

The average swimming speed (cm/sec) of fish at different length of exposures revealed that the swimming speed was also considerably reduced with an effect of the toxicant (figure 2). The percent reduction in swimming speed at regular intervals is calculated based on the control values and presented in the inset-A of figure 2. The gradual reduction in the swimming speed was observed with a prolonged exposure and reached up to 62.5% on day-20. The estimated time required for 50% reduction in the mobility (moving speed) was

calculated by converting the time period into log values, thus taken on X axis and plotted linear regression against percent reduction on Y axis (inset-B of figure 2). The derived time value from the linear regression, for inhibiting 50% of locomotor activity in the exposed fish is 14.7 days, (which is equals to 14 days 16 h 48 minutes). Earlier findings indicated that the food deprivation, anoxic conditions and inhibition of AChE enzyme were responsible for the reduction in locomotor activity of rats [31, 32]. In the present experiments the exposed fish were not facing such conditions like the deficiency of food. Therefore, the altered locomotor activity (swimming speed) of fish may be due to the toxicant induced stress, which is an indication of neurotoxicity. It is evident from the figure 3 that the brain AChE activity was inhibited maximum on day-4 (55%) and slightly recovered and exhibited 40% inhibition on day-20. The inhibition of AChE enzyme leads to the accumulation of ACh at synaptic junctions. Hence, the altered locomotor behavior of fish could be due to the accumulation of ACh, which interrupted coordination between the nervous and muscular junctions. Similar, reduction in locomotor activity was observed in adult Carabid, *Pterostichus cupreus* after copper treatment [33] and exposure to an OP pesticide on locomotor activity of laboratory mice and five species of wild rodents [34].

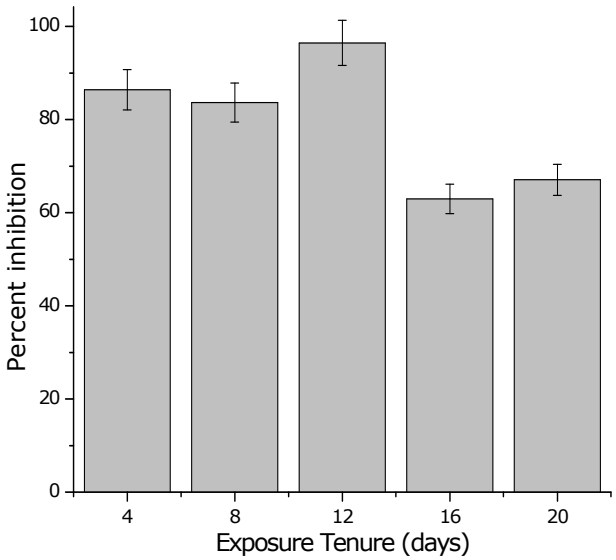

Figure 3: *In vivo* effect of chlorpyrifos on brain acetylcholinesterase activity in *Gambusia affinis* during sub-lethal exposure of 60 µg/L (1/5 of LC_{50}) for a period of 20 days.

Chlorpyrifos is known to bio-accumulate in the tissues of test organisms and the estimates of tissue concentrations may be more valuable for the assessment of situations in the natural environment [35]. Hence, the accumulation of the toxicant in different parts of the test organism was estimated by HPLC method and is presented in figure 4.

The maximum amount of bioaccumulation in all the three regions of head, body and viscera was observed on day-4 than other intervals. It is clear from the results that the accumulation of chlorpyrifos is more in viscera followed by head and body. The average bio-concentration factor (µg/g) values are 0.109, 0.009, and 0.004 for viscera; head and body respectively (table 2).

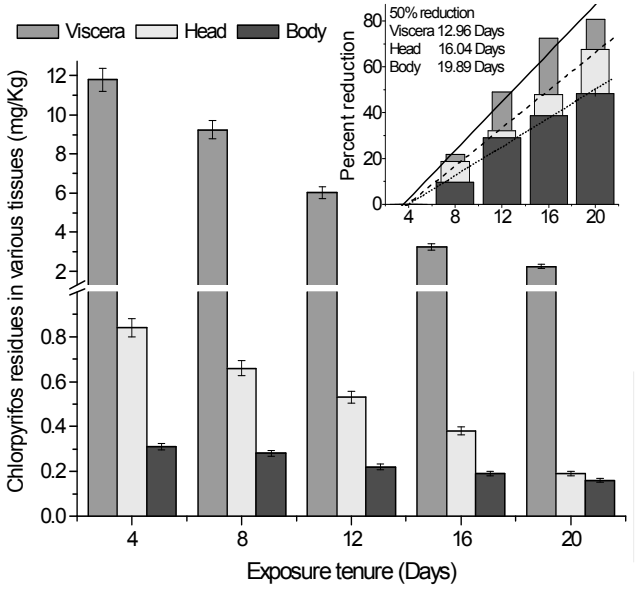

Figure 4: Bioaccumulation of chlorpyrifos in viscera, head and body of *Gambusia affinis*, during sub-lethal exposure of 60 µg/L (1/5 of LC_{50}) for a period of 20 days. Inset: Regression line for time required to determine 50% depuration in tissues.

Table 2: Bio-concentration factors of chlorpyrifos in viscera, head and body of fish *Gambusia affinis* during sub-lethal exposure to 60 µg/L for a period of 20 days. The values in parenthesis indicate percent reduction, based on the day-4 value as an initial accumulation. The data are derived from the mean values of figure 4. The values are significant at $P<0.05$

Exposure Period (Days)	Time After Initial Value (Hours)	Bio-Concentration Factors* in Different Parts of Fish		
		Viscera	Head	Body
4	0	0.197 (0.00)	0.014 (0.00)	0.005 (0.00)
8	96	0.154 (21.83)	0.011 (21.43)	0.005 (0.00)
12	192	0.100 (49.24)	0.009 (35.71)	0.004 (20.00)
16	288	0.054 (72.59)	0.006 (57.14)	0.003 (40.00)
20	384	0.038 (80.71)	0.003 (78.57)	0.002 (60.00)
Average bio concentration values ± SE		0.109 ±0.029	0.009 ±0.002	0.004 ±0.001

*Bio-concentration factor = accumulated concentration in tissue (µg/g)/concentration in media (µg/L).

The factors of bioaccumulation in different parts were calculated based on the available concentration in the medium (water) and noticed gradual decrease in the length of exposure. It may be due to induction in the detoxifying enzymes in liver and they might have played a critical role in the depuration of the toxicant. Based on the observations from day-4 to day-20 in the tissues of viscera, head and body, the depuration rates of chlorpyrifos are 2.235, 1.687 and 0.385 ng/h respectively (figure 4). The order of time required for 50% depuration in tissues is viscera> head > body (12.96, 16.04 and 19.89 days, respectively). Previously, the depuration rate of profenofos in *Brachydanio rerio* at 100th and 500th concentration of LC_{50} was reported as 0.09 and 0.10 µg/h [36].

Identical observations like high accumulation and depuration rates in visceral organs than the muscle and head region were noticed, when fish, *Labeo rohita* and *Saccobrachus fossilis* exposed to metasysntox; *Mugil cephalus* and *Mystus gulio* to endosulfan and *Clarias batrachus* to sub-lethal concentrations of dimethoate respectively [37-39]. The organs in the visceral region (liver, heart, intestine, kidneys) carry out the primary activities related to absorption, distribution and elimination. The rate of depuration is more in visceral part due to the enzymes induced or enhanced by the toxicant stress, which decrease the lipid solubility of organic contaminant facilitating assimilation and excretion of the contaminant. The enzymes involved mainly are cytochromes P-450s, glutathione-S-transferases, rhodanese, sulfotransferase, etc., and other enzymes mainly belonging to mono-oxygenase system [40-42].

Conclusion

The study showed that the effects of chlorpyrifos at sub lethal concentration for a prolonged period of 20 days include not only direct effect but also on the locomotor behavior of fish, *Gambusia affinis.* The prime focus of this study was to use the locomotor behavior of test organism as a promising tool in ecotoxicology, to assess the adverse effect of the toxicant. The reasons for altering the behavior were further analyzed by estimating the activity of target enzyme AChE and the bioaccumulation of the toxicant. The inhibition of AChE enzyme activity, and the presence of residues (bioaccumulation) in different parts of the fish might have been altered the locomotor behavior of exposed fish. Currently, the individual secondary metabolites were not considered at this point of time. Further research is necessary to study the effect of other pesticides and their combinations on the behavior of fish in the laboratory as well as field conditions before implementing such markers in the current scenario.

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