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A Study on the Effect of Chlorpyrifos (20% EC) on Thyroid Hormones in Freshwater Fish, *Heteropneustes fossilis* (Bloch.) by using EIA Technique

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ABSTRACT

Chlorpyrifos, an organophosphate, is very acutely and chronically toxic to aquatic organisms. It is genotoxic, immunotoxic, an endocrine disruptor, embryotoxic and a developmental neurotoxicant. Chlorpyrifos also induces alterations in thyroid and adrenal glands and differentially affects levels of thyroid stimulating hormones. As a result, these chemicals cause disease and are accumulated in the fatty tissue of organisms and thus the concentration increases as they move up through the food chain. Many studies show a direct relationship between concentrations of chemicals in fish tissue and depressed hormone concentrations. This study on Heteropneustes fossilis (Bloch.) showed that exposure of sublethal concentration of chlorpyrifos induce alternations in serum concentration of tri-iodothyronine (T3), thyroxine (T4) and thyroid stimulating hormone (TSH) in comparison to the normal (untreated) group. In particular, possible impairment of thyroid function was demonstrated by the significant decrease ($p < 0.01$) in serum T3, T4 and TSH levels in treated fishes with very low concentration of chlorpyrifos (0.284 ppm i.e., $1/10^{th}$ of LC_{50}).

Keywords: *Endocrine disruptor, chlorpyrifos, thyroid hormones, Heteropneustes fossilis (Bloch.), EIA technique.*

INTRODUCTION

Fisheries and aquatic resources (ponds, lakes, rivers, streams and oceans) are exceptionally valuable assets enjoyed by millions of people. Pesticide chemicals disturb the balance between the ecosystem and the species that belongs to that particular ecosystem. Unintentional pesticide related fish kills occurs throughout the world, linked to human use. Pesticides are used widely in agriculture to control pests and in public health to control diseases. Pesticides produce many physiological, biochemical and behavioral changes in freshwater particularly those of fishes, by influencing the activities of several enzymes and hormones. [Radhaiah *et al*., 1987].

Fishes are exposed to insecticides in 3(three) ways –(i) dermally, direct absorption through the skin,(ii) breathing, direct uptake through the gills,(iii) orally, by drinking pesticide contaminated water or by feeding pesticide-contaminated prey. Different concentrations of insecticides are present in many types of wastewater and numerous studies have found them to be toxic to aquatic organisms especially fish species. [Talebi 1998; Uner *et al.*, 2006; Banaee *et al.*, 2008]. Fish are being used as useful genetic models for evaluation of pollution in aquatic ecosystems. Fish as bio-indicators of pollutant effects are very sensitive to the changes in their environment and play significant roles in assessing potential risk associated with contaminations of new chemicals in aquatic environment. [Lakra *et al*., 2009] Moreover, pesticides have been noticed to interfere with fish health and reproduction.[Mani *et al*., 1988].

Chlorpyrifos (O, O – diethyl O-3, 5, 6 trichloro-2-pyridyl-phosphorothioate) is used as broad-spectrum chlorinated organophosphate insecticide. Organophosphates (abbreviated as OP) are a common name for phosphoric acid esters. Use of chlorpyrifos has greatly increased since its introduction in 1965(by Dow Chemical Company).

The EPA (Environment Protection Agency) classifies chlorpyrifos as Class II: moderately toxic to humans and chronic exposure has been directly linked to neurological complications, developmental disorders & autoimmune disorders. But this substance is highly toxic to aquaculture fish [NPIC] and bees. Using the Globally Harmonised System of Classification and Labelling, the EU has categorised chlorpyrifos as Aquatic Acute Tox 1, with the hazard phrase “H400 – very toxic to aquatic life”; and Aquatic Chronic Tox 1, with the hazard phrase “H410 – very toxic to aquatic life with long lasting effects”[Chernyak et al., 1996]. Scientific study have found that as the chemical breaks down naturally in the environment, it releases chlorpyrifos oxon, which has been found to be even more toxic than the original form of the chemical.[US EPA 2011][32]. Chlorpyrifos is normally supplied as a 23.5% or 50% liquid concentrate. The recommended concentration for direct spray pin point application is 0.5% and for wide area application is 0.03-0.12% mix is recommended (US) [30, 31].

Pesticide such as chlorpyrifos (organophosphate) can cause adverse effects by interfering the body's hormones (or chemical messengers). These substances i.e. pesticides are therefore called hormone disruptors or endocrine disruptors. Fish species are sensitive to enzymatic & hormone disruptors. Doses of pesticides that are not high enough to kill fish are associated with subtle changes in behavior & physiology that impair both survival and reproduction [Kegley *et al.,* 1999]. There is increasing evidence that a wide range of chemicals can interfere with thyroid and adrenal functions [De Angelis *et al*., 2007; Maranghi *et al*., 2003] and that the developmental life stages are critically vulnerable to endocrine disrupting chemicals (EDC) [Mantovani, 2006].

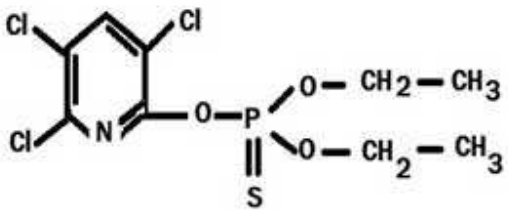
Thyroid hormones regulate a number of biological processes essential for growth, metabolism as well as brain maturation [Bernal *et al*., 2003]. Thyroid hormone disruption can result in negative impacts on foetal brain development [Ghisari & J.Bonefeld, 2005]. Specifically, a few studies have suggested that chlorpyrifos may target thyroid and adrenal homeostasis both in human and animal models. [Jacobsen *et al.,* 2004; Jeong *et al*., 2006].Hence pollutants such as insecticides may significantly damage certain physiological and biochemical processes when they enter into the organs of fishes [John, 2007; Banaee et al., 2011]. Since fishes are important sources of protein and lipids for humans and domestic animals, so health of fishes is very important for human beings. Therefore, the aim of the present study was to examine the effect of chlorpyrifos (20% EC) on thyroid hormones such as triiodothyronine (T3), thyroxine (T4) and thyroid stimulating hormone (TSH) in *Heteropneustes fossilis* (Bloch.).

MATERIALS AND METHODS

Normal healthy freshwater fish, *Heteropneustes fossilis* weighing 10 ± 0.5g and average length of 11cm were collected from local ponds of Dhubri District, Assam. The experimental fishes were acclimatized in aquariums under necessary laboratory conditions for 4-5 days and then LC_{50} was determined. During the whole process of treatment no food was supplied.

Sub-lethal dose of chlorpyrifos (0.284 ppm i.e.,$1/10^{th}$ of LC_{50}) were administered in the experimental species *Heteropneustes fossilis* for a period of days intervals as 5days,10days,15days,20days,25days and 30 days. Immediately after the treatment of the above mentioned day intervals, blood samples were collected in sterilized vials from the caudal fins of the normal control group and the treated group (chlorpyrifos treated). The collected blood is handled carefully to prevent haemolysis. It is allowed to clot for 10 minutes and is then centrifuged for the collection of serum, which is later used for the estimation of triiodothyronine (T3), thyroxine (T4) and thyroid stimulating hormone (TSH) concentrations.

Chlorpyrifos is an organophosphate pesticide. The molecular formula is $C_9H_{11}Cl_3NO_3PS$ and the chemical structure is



Measurement of thyroid hormone levels:

Serum concentration of T3, T4 and TSH were assessed in *Heteropneustes fossilis* by EIA (enzyme immune assay) technique using an UVvisual spectrophotometer. Serum concentration of these hormones was selected as appropriate parameters to assess thyroid toxicants in fish.

RESULTS

Median lethal concentration (LC_{50}) is determined by Probit Analysis (Finney, 1952) in the acclimatized *Heteropneustes fossilis* (Bloch) for chlorpyrifos. The value of LC_{50} -36 hrs of chlorpyrifos was determined as 2.84 ppm for *H.fossilis* in the laboratory condition. On the basis of LC_{50} value a sub-lethal concentration viz., 0.284 (i.e., $1/10^{th}$ of LC_{50}) ppm was determined. (LC_{50} means Lethal Concentration-50, i.e., 50% death/ alive specimen out of the total studied population and Sub-lethal concentration is the concentration lower than that of LC_{50}, hence $1/10^{th}$ of LC_{50} is 0.284 ppm. The fishes were exposed to this sub-lethal concentration (0.284 ppm) of chlorpyrifos for different day intervals. The results as obtained are shown in the Table (1-3). The analysis revealed decreased levels of mean serum T3, T4 and TSH in chlorpyrifos treated group of *Heteropneustes fossilis* during the experimental period of 1 month as compared to normal control group.

Table 1: Presenting the Mean Values of Serum Level of Tri-Iodothyronine, T3 (ng/ml) and Significance of differences between normal and treated group in *Heteropneustes fossilis* (Bloch.).

PARAMETERS	*H.fossilis*		DAYS OF TREATMENT					
			5 DAYS	10 DAYS	15 DAYS	20 DAYS	25 DAYS	30 DAYS
T3 (in ng/ml)	NORMAL GROUP	MEAN	0.129	0.148	0.129	0.155	0.128	0.127
		± SD	0.018	0.077	0.018	0.077	0.016	0.018
		SEM	0.006	0.024	0.006	0.024	0.005	0.006
	TREATED GROUP	MEAN	0.066	0.140	0.107	0.130	0.091	0.106
		± SD	0.011	0.003	0.012	0.003	0.011	0.009
		SEM	0.003	0.001	0.004	0.001	0.004	0.003
	SIGNIFICANCE OF DIFFERENCE	t	9.656	0.333	3.290	1.020	5.962	3.302
		p	< 0.01	> 0.01	< 0.01	> 0.01	< 0.01	< 0.01
		df	18	18	18	18	18	18

Table 2: Presenting the mean values of serum level of thyroxine,t4 (nmol/l) and significance of differences between normal and treated group in *Heteropneustes fossilis*(Bloch.).

PARAMETERS	*H.fossilis*		DAYS OF TREATMENT					
			5 DAYS	10 DAYS	15 DAYS	20 DAYS	25 DAYS	30 DAYS
T4 (in nmol/l)	NORMAL GROUP	MEAN	0.409	0.403	0.409	0.408	0.403	0.423
		± SD	0.035	0.038	0.034	0.035	0.043	0.017
		SEM	0.011	0.012	0.011	0.011	0.014	0.005
	TREATED GROUP	MEAN	0.137	0.399	0.221	0.359	0.220	0.424
		± SD	0.011	0.016	0.011	0.005	0.018	0.013
		SEM	0.004	0.005	0.003	0.002	0.006	0.004
	SIGNIFICANCE OF DIFFERENCE	t	23.622	0.311	16.800	4.413	12.372	-0.257
		p	< 0.01	> 0.01	< 0.01	< 0.01	< 0.01	> 0.01
		df	18	18	18	18	18	18

Table 3: Presenting the mean values of serum level of thyroid stimulating hormone, TSH (µ IU/ml) and significance of differences between normal and treated group in *Heteropneustes Fossilis*(Bloch.).

PARAMETERS	*H.fossilis*		DAYS OF TREATMENT					
			5 DAYS	10 DAYS	15 DAYS	20 DAYS	25 DAYS	30 DAYS
TSH (in µ IU/ml)	NORMAL GROUP	MEAN	0.082	0.083	0.082	0.081	0.080	0.082
		± SD	0.016	0.016	0.016	0.015	0.024	0.016
		SEM	0.005	0.005	0.005	0.005	0.008	0.005
	TREATED GROUP	MEAN	0.036	0.035	0.018	0.028	0.029	0.055
		± SD	0.002	0.004	0.008	0.002	0.004	0.008
		SEM	0.001	0.001	0.003	0.001	0.001	0.003
	SIGNIFICANCE OF DIFFERENCE	t	8.842	9.500	10.988	11.166	6.817	4.777
		p	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
		df	18	18	18	18	18	18

± SD = standard deviation, SEM = standard error of mean, t = student's t-test, t value,
p = probability value, df = degree of freedom
If $t < 2.55$ then $p > 0.01$ which is not significant.
If $t > 2.55$ then $p < 0.01$ which is highly significant.

Statistical Analysis:

The results obtained were statistically analyzed for t-test, probability value and others following Croxton [4].

DISCUSSION

Exposure of sublethal concentrations of chlorpyrifos has caused the following effects in species of freshwater and marine fauna: delayed maturation, growth and reproductive impairment, change in hormone level, deformities and depressed populations [Marshall and Roberts, 1978; Jarvinen *et al*.,1983; Odenkirchen *et al*.,1988]. The present study on *H.fossilis* showed that exposure of sublethal concentration of chlorpyrifos induce alternations in serum concentration of tri-iodothyronine(T3), thyroxine(T4) and thyroid stimulating hormone (TSH) in comparison to the normal control group. In particular, possible impairment of thyroid function was demonstrated by the significant decrease ($p < 0.01$) in serum T3, T4 and TSH levels in treated fishes [Table-1, 2, 3] with very low concentration of chlorpyrifos (0.284 ppm).Similar results have been obtained in other studies conducted in experimental models, although at higher dose level than those used in the present study. Specifically, in ewes a significant decrease in serum T4 was observed following 36 days of treatment with two weekly doses of 12.5 mg/kg of chlorpyrifos orally [Rawlings *et al*, 1998]. In a study involving male Wistar albino rats subjected to acute organophosphate (methamidophos; dimethyl phosphoramidothioate) exposure, a decrease in serum T4, T3, and TSH levels was observed and resulted in secondary hypothyroidism and sick euthyroidism [Satar *et al.*,2005]. Haviland *et al*., (2010) found increased thyroid hormone levels and altered learning behavior in female mice exposed to 1 and 5 mg/kg chlorpyrifos on gestation days 17-20. A decrease in serum T4 levels was also observed in CD1 mice (both in dams and F1) after developmental exposure to chlorpyrifos at doses low enough to not elicit inhibition of brain acetylcholinesterase (AchE). Both sexes of F1 CD1 mice showed reduced serum T4 levels, a more significant effect was observed in males compared to females [De Angelis *et al*., 2009]. Jeong *et al.*, (2006) reported that chlorpyrifos-methyl induces hypothyroidism (decreased serumT4 and increased serum TSH) and altered thyroid and pituitary gland weights through sexual maturation and adulthood in rats after long-term *in utero* and postnatal exposure. In an occupational study of 136 male floriculture workers that examined the association between thyroid hormones (T3, T4, and TSH) and OP exposure, urinary dialkyl phosphate (DAP) concentrations were associated with increased levels of TSH and total T4[Lacasana *et al*.,2010]. Finally, the thyroid disrupting potential of chlorpyrifos was also demonstrated *in vitro* by a study of rat pituitary GH3 cells, where chlorpyrifos exposure altered T3-induced cell growth [Ghisari *et al*., 2006]. Several animal studies investigating thyroid effects related to chlorpyrifos or other OPs may support our results suggesting that chlorpyrifos administration at very low concentration will cause thyroid alterations possibly leading to hypofunction in test species.

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