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Acute and Chronic Toxicity of Endocrine Disruptive Heavy Metals and Pesticides Exposed to Freshwater Fish *P. reticulata* and *P. Sphenops*

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Abstract

Environmental monitoring is a recent world-wide approach to save the future in line. The present study focuses on toxicity of heavy metals and pesticides on the freshwater aquarium fish (*Poecilia reticulata* and *Poecilia sphenops*). The study intense on drawing the acute and chronic safe levels of metals and pesticide exposed to 96 h and 28 day respectively. On exposure, the fish were observed to be under stress and showed excitability. It was observed that both species were highly sensitive to arsenic and cypermethrin, meanwhile resistant to cadmium and chlorpyrifos. The acute safe levels for As irrespective of species was comparatively lesser than that of Cd exposed fish and the range of safe values for pesticides exposure were between 0.018 and 0.059 ppb. As the water quality is influenced by many environmental factors, the present study helps the aquarist in monitoring the safe level of toxicants in the medium. This in turn increases the economic growth of the aquarist.

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Toxicity.

Introduction

India is rich in diversity of organisms and plays a prominent role in international market in ornamental fish due to its rusticity and color patterns. Despite its potential, India ranks low in the Asian market, asmost of the ornamental fish is of wild variety.[1] Among the total 156 species in Tamil Nadu, 16% of them were under threat due to problem in maintaining the stock, seed production, environmental factors including contamination of water etc. Hence research in aquaculture is indeed important to maintain the potential resource of income generation through entrepreneurship in the rural areas of Tamil Nadu.[2] Water quality is very important for survival and well-being of all the aquatic organisms. Toxicants from both point and non-point sources reach the aquatic ecosystem and pollute the environment through their receiving water bodies.[3] These toxicants cause

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adverse effects on the aquatic biota through food chains. Critical habitats are particularly vulnerable to pollution. The pollutants can directly or indirectly affect the metabolism, growth and development, and affect endocrine functions, reproduction, translation etc.,

The Endocrine Disrupting Chemicals (EDCs) affects the hormone receptors by competing with the natural hormones.[4] EDCs are widely distributed in various products such as plastic materials, pesticides, fungicides, metals, personal care products and even heavy metals.[5] When these chemicals are released into the water bodies it affects the metabolism of aquatic life. The mechanism of action is more complex which include direct actions on the receptors (mainly hormonal and/or neurotransmitter receptors), enzymes and hormone.[6,7] The interference in the hormones can alter the behavioural dimorphisms particularly when exposure occurs during critical developmental periods.[8,9] They are widespread throughout the aquatic ecosystems such as water and sediment disturbing the aquatic life through various routes, as runoff water from the agricultural fields, sewage/effluents of industries reaches the river and ocean.[10,11]

Among the aquatic life, fishes are mainly vulnerable to toxicants and express behavioural, structural, chemical and molecular changes.[12] Therefore, fish are considered as indicator organisms to assess the EDCs[13] which imitate the effects of natural oestrogens (xenoestrogens).[14,15,16,17] Mostly heavy metals, pesticides and insecticides acts as EDCs individually or synergistically and become toxic to the organisms. This in turn affects survival, growth and reproduction of organisms.[18] When the exposure level to these chemicals exceed, it can induce cancer, damage the nuclear materials and ultimately lead to lethality.[7] The lethality depends on species and concentration of toxicants, which may lead to aquatic loss and disrupts the food chain.[19,20] Apart from lethality the toxicants may also affect the economy of the aquarist by affecting the behaviour, structure and health of the ornamental fishes.

Hence, regular monitoring helps in identifying the ill effects of the toxicants in organisms. Therefore the present study was aimed to derive safe levels on acute and chronic concentrations of EDCs such as heavy metals (Cadmium and Arsenic) and pesticides (Chlorpyrifos and Cypermethrin) in the fresh water fish.

Materials and Methods

Experimental Fish

Freshwater fish *Poecilia reticulata* and *Poecilia sphenops* were used to study the acute and chronic effects of the chosen chemicals. Healthy fish (1.0 ± 0.1 g) were procured from hatchery in Madurai. They were transported in the plastic bag separately with freshwater filled with oxygen to the laboratory at Madurai Kamaraj University, (9.9404°N and 78.0105°E) in Madurai, Tamil Nadu, India. Before acclimation, fish were treated with KMnO4 solution (0.05%) for 2 min to protect from dermal infection. Healthy fish were transferred to tank in the ratio of 1 g of fish weight in 10 litre of chlorine free tape water and acclimated in the laboratory at ambient environmental conditions for 15 days. During acclimation period no mortality was observed. Local manufactured pellet fish feed was fed twice a day ad *libitum* and the excretory matters were frequently removed. The essential physico-chemical parameters such as temperature, pH, DO, total ammonia, phosphate, nitrate, and electrical conductivity were estimated during the test every 24 hrs, following the standard methods.[21] The photoperiod of 10 hrs light and 14 hrs dark was maintained.

Chemicals

Metals, i.e., Arsenic chloride ($AsCl_3$) and Cadmium chloride ($CdCl_2$) and commercially formulated pesticides, i.e.,Chlorpyrifos 20% EC and Cypermethrin 25% EC were used for assessing the acute and chronic toxic effects. One percent stock solutions were prepared separately, for each metal and pesticide, in 1000 ml volumetric flasks. Whose concentration was 1 mg mL-1.The pesticides were dissolved in 0.5% acetone and also used in solvent control group.

Experimental Design for Acute and Chronic toxicity studies

Acute toxicity study was conducted to analyse the median lethal concentration (LC_{50}) and safe level of the toxicants under static renewal bioassay method after 96 hrs of exposure. Before the definitive toxicity tests, the wide range of toxicant concentrations such as 0.0001, 0.001, 0.01, 0.1, 1.0, 10 & 100 mg L^{-1}

were used to find out the concentrations for definitive toxicity tests.[21,22,23]

For acute and chronic toxicity studies, ten acclimated fish were introduced per toxicants in a separate tank. For pesticide toxicity, solvent control was maintained simultaneously. Based on the median lethal concentrations derived through definitive toxicity studies, the sub-lethal concentrations of each toxicant were selected for chronic toxicity studies by calculating 1/10th of the LC_{50} (96h) value of each toxicant.[24] During the chronic exposure studies the NOEC (No Observed Effect Concentration) and LOES (Lowest observed effect Concentration) were derived.

The test media were renewed every day to maintain the concentration of the toxicants. During the study period, the dead fish were removed immediately from the medium. No food was provided for test animals during the acute toxicity test. For acute toxicity testing, the mortality was observed up to 96h at an interval of 24 h.

The LC_{50} values of the toxicants were analysed at respective hours of exposure using Probit Analysis computer programme.[25,26] Safe concentrations of the toxicants were also calculated following the application factor, 1/100th of the 96h LC_{50} values.[27,28] The dose (concentration) vs response (mortality) curves were drawn using Excel programme to find out the effect of toxicants.

To find out the chronic toxicity endpoints a 28 day exposure was conducted. The artificial pellet fish feed was given ad *libitum*, during chronic studies.[29] The live animals in each media were counted every day. NOEC and LOEC were derived based on percentage survival of fishes on 28th day of the sub-lethal tests using Dunnet analysis computer program.[26,30] The statistical analysis was done using SPSS Version 23 to study the significance of the data.

Result and Discussion

Environment plays a significant role in the survival of an organism. When the fish were introduced to an induced environment, they exhibit behavioral changes to sustain in the new environment. Irrespective to the toxicants exposed both the fish species exhibited behavioral changes at higher concentration. On exposure the fish were alert and still in position. The fish exposed to metals and pesticides were found to be unsteady and showed erratic movements a sign of hyper excitability. Respiratory distresses with excess mucous secretion were observed in the toxicants exposed medium.

Their pectoral and pelvic fins were found to be hard may be due to muscle fatigue on pesticide exposure. On increasing the concentration of toxicants fish remained in a vertical position, exposing the mouth on to the surface of water indicating its effort to swallow air. The observed behavioural activity in the present study is due to increase in the level of ammonia and decrease in $Na+$, $K+$, $Ca2+$ in plasma which indirectly affects muscular contraction and impulse transmission.[31]

Table 1: Water quality analysis

Parameters	Control	Metal Exposure	Pesticide Exposure
Odour	-	-	-
Phosphate (mg/L)	1	0.9	1.2
Hardness	Nil	Nil	Nil
Nitrate	1.5	0.9	1.2
Total Ammonia (mg /L)	2.00	2.42	2.54
Dissolved Oxygen (mg /L)	5.79	5.06	4.84
pH	7.2	7.4	7.4
Temperature (ºC)	32 -33	31 – 32	33-35
Electrical conductivity ($mScm^{-1}$)	3.43	3.2	3.26

Acute Toxicity

The physicochemical parameters maintained throughout the experiments were listed in Table - 1. The toxicity endpoints are the primary criteria for the protection of fauna in the aquatic medium. Therefore, the present investigation aimed to elicit the acute and chronic endpoints of the chosen Endocrine Disrupting Chemicals such as heavy metals (Arsenic and Cadmium) and pesticides (Chlorpyrifos and Cyperimethrin) in freshwater fish viz., *P. reticulata* and *P. sphenops*.

The dose dependent increase in the mortality of metals exposed fish were shown in Fig.1. There was no mortality during the experimental period in the control and solvent control (acetone 0.5%) groups.

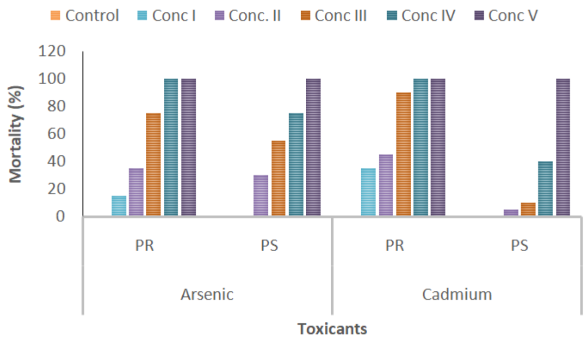

Fig. 1: Percentage of mortality on exposure to Arsenic and Cadmium metals. PR – *P. reticulata* and PS - *P. sphenops*

Table 2: Acute lethal toxicity test under static bioassay

Toxicants used	Exposure (h)	LC_{50}	*P. reticulata* 95% Confidence Interval	Safe Conc. (µg L^{-1})	Chi-square calculated*	LC_{50}	*P. sphenops* 95% Confidence Interval	Safe Conc. (µg L^{-1})	Chi-square calculated*
Heavy metals									
Arsenic	24	158.02	82.404 - 3410.558		0.527	93.886	62.692-212.881		0.525
(mg L^{-1})	48	35.593	26.789 - 50.624		3.128	62.171	36.344-204.698		0.298
	72	27.747	20.284 -39.970		0.304	24.822	14.591-41.115		0.857
	96	**11.387**	8.838 -14.378	**113.87**	1.703	**10.755**	7.589-13.933	**107.55**	3.303
Cadmium	24	360.812	NC		0.914	104.15	NC		0.386
(mg L^{-1})	48	106.25	68.708-280.560		0.47	89.058	NC		0.136
	72	42.799	32.095 - 60.349		1.717	37.233	28.581-56.827		0.838
	96	**24.572**	19.019 - 31.651	**245.72**	3.746	**20.036**	16.081-25.204	**200.36**	3.635
Pesticides									
Chlorpyrifos	24	42.981	26.398 -142.738		0.455	6.911	3.486-819.585		1.079
(µg L^{-1})	48	26.988	17.834- 58.561		1.613	5.724	2.883-71.577		0.608
	72	11.168	7.907- 16.281		1.634	2.334	1.606-4.587		0.892
	96	**5.886**	3.967- 8.087	**0.059**	4.441	**1.046**	0.645-1.993	**0.011**	1.355
Cypermethrin	24	5.893	3.967-417.830		0.086	5.984	4.018-19.105		0.216
(µg L^{-1})	48	3.924	2.872-8.042		0.34	4.409	3.205-8.326		0.828
	72	2.572	2.001-3.657		0.292	2.547	1.844-4.035		1.642
	96	**1.826**	1.422-2.448	**0.018**	0.224	**1.362**	1.008-1.867	**0.01**	5.012

*Chi - Square tabular value at 0.05 level = 7.815

The LC_{50}, 95% Confidence Interval and safe values of *P. reticulata* and *P. sphenops* calculated under acute static renewal test method were presented in Table 2. The LC_{50} values were higher during 24 hr exposure and declined on further increase in duration. The LC_{50} values of *P. reticulata* and *P. sphenops* exposure to metals and pesticides showed negative correlation with time. The calculated 96 h LC_{50} values for *P. reticulata* and *P. sphenops* exposed to As were 11.387 and 10.755 mg L^{-1} respectively and the LC_{50} values of respective fish exposed to Cd were 24.572 and 20.036 mg L^{-1} respectively (Table 2). The result showed that *P. sphenops* was more susceptible to heavy metals compared with *P. reticulata* irrespective of the metals. The results concluded that there was a significant effect of heavy metals in *P. sphenops* (R^2 = 0.9717 and 0.9476 for As and Cd respectively) hence, there exists a strong correlation between the dose and mortality compared with *P. reticulata*. Overall study inferred that the highest toxicity was reported in As exposed *P. sphenops* and the lowest toxicity in Cd exposed *P. reticulata*. On acute exposure, both fish were resistant to cadmium and sensitive to arsenic metal.

Table 3: Chronic toxicity assay

Test Species	Toxicants	NOEC (ppb)	LOEC (ppb)	Chronic value (ppb)
Heavy Metal				
P. reticulata	Arsenic	0.070	0.140	99.000
	Cadmium	0.150	0.310	216.000
P. sphenops	Arsenic	0.070	0.140	99.000
	Cadmium	0.130	0.250	180.000
Pesticide				
P. reticulata	Chlorpyrifos	0.038	0.075	0.053
	Cypermethrin	0.011	0.023	0.016
P. sphenops	Chlorpyrifos	0.007	0.014	0.010
	Cypermethrin	0.009	0.018	0.013

Similarly, in the acute toxicity test, *P. reticulata* and *P. sphenops* were shown to be sensitivity to Cypermethrin and resistant to Chlorpyrifos. The 96 h LC_{50} values for *P. reticulata* and *P. sphenops* exposed to Chlorpyrifos were 5.886 and 1.046 ppb and to Cypermethrin were 1.826 and 1.362 ppb respectively (Table-3; Fig 2). It was apparent that *P. sphenops* was highly sensitive to Chlorpyrifos under acute exposure. The present study on acute toxicity concluded that the pesticides were highly toxic to both fish species than heavy metals.

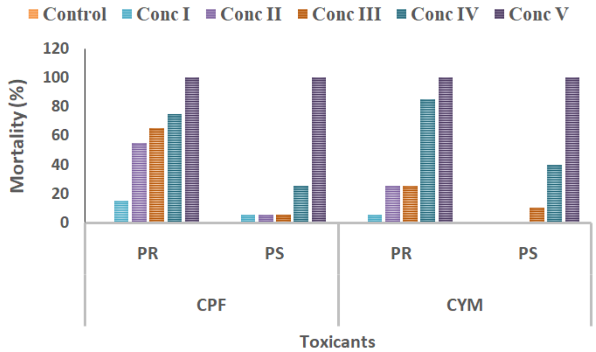

Fig. 2: Percentage of mortality on exposure to CPF – Chlorpyrifos; CYM – Cypermethrin; PR – *P. reticulata* and *PS - P. sphenops*

The mean survival of control animals and environmental factors was substantial to EPA/ COE.[31] Thus confirmed the holding facilities of test animals and other environmental factors are under acceptable levels of the toxicity testing. Under acute toxicity studies, the mortality of test fish was positively correlated with increasing acute concentrations and exposure time, coincides with earlier reports for marine organisms.[32]

Among the chosen metals *P. reticulata* was more susceptible to cadmium and *P. sphenops* to arsenic. Hence, the study showed a species dependent response on exposure to heavy metals and also shown significant result to the previous investigation in freshwater fish exposed to heavy metal and pesticide under static bioassay method. The lethality depended on the test methodology, Shuhaimi *et al.*,[33] reported less lethal concentration under static non-renewal test and Yilmaz[34] reported higher lethal level for the same metals under static renewal test. The study confirmed that the test methods have influence on the toxic effect, due to continuous exposure to toxicants and bioaccumulation. In the present study the estimated LC_{50} values for As and Cd to test fish were higher than that of the previous studies[35] invariable of test methods.

The calculated 96 LC_{50} values in the present study reveals that both *P. reticulata* and *P. sphenops* were more sensitive to As, compared to *Heteropneustes fossilis* (35.10 mg L^{-1}; Kermiu *et al.*,[36]) and slightly resistant than *Catla catla*[37] (10.16 mg L^{-1}); and on exposure to Cd the test fish was resistance than reported in *Ophiocephalus striatus* (0.63 mg L^{-1}) and sensitive than *Gambusia holbrooki*[38] (37.3 mg L^{-1}). Similarly for cadmium metal toxicity, present study reveals that the *P. reticulata* and *P. shenops* were sensitive than *Heteropneustes fossilis*[39] (50.41 mg L^{-1}) and resistant than Rasbora sumatrana[33] (0.10 mg L^{-1}).

The lethal concentration observed in the present study on exposure to Chlorpyrifos and Cypermethrin concentrations were less when compared to the earlier reported LC_{50} value for various organisms including *P. reticulata* under different experimental setup[40,41,42,43]; Danio rerio[44] and rainbow trout.[45] The present study also reveals that *P. shenops* was more sensitive to Chlorpyrifos and the LC_{50} values observed in the study is less compared to earlier reports.

P. reticulata is also more sensitive to chlorpyrifos than other organic chemicals including pyrethroid.[46] Similarly, sensitive than other freshwater species i.e., *Oreochromis niloticus*[47] (12.6 µg L-1); *Heterobranchus bidorsalis*[48] (36 mg L-1) and *Cyprinus carpio*[49] (3.31 µg L-1). Hence the present study concluded that there exists a species dependent response to the toxicant.

Chronic Toxicity

In the present study the NOEC, LOEC and chronic value for Arsenic exposed *P. reticulata* and *P. sphenops* were similar i.e., 0.07, 0.140 and 99.0 µg L^{-1} respectively. Nevertheless, there was variation in the value for Cadmium exposed *P. reticulata* (0.15, 0.31 and 216.0 µg L^{-1}) and *P. sphenops* (0.13, 0.25 and 180.0 µg L^{-1}) (Table 3). The pesticide exposed fish showed very less chronic endpoints (NOEC and LOEC) than that of metal exposed fish. The NOEC and LOEC value of *P. reticulata* and *P. sphenops* exposed to Chlorpyrifos were 0.038 and 0.007 ppb respectively and the Chronic value ranged between 0.53 and 0.010 ppb. At the same time the chronic value for cypermethrin was noted to be lower for *P. reticulata*.

Similar to acute exposure, the fish reared in control media (freshwater control and solvent control) showed 100% survival during the period of chronic studies. The survival rate was observed to be 85% for *P. reticulata* on exposure to higher sub-lethal concentration of cadmium and chlorpyrifos. In the lowest sub-lethal concentrations of metals and pesticides, the fish survival was between 97.5% and 100%, hence these concentrations were found to be suitable for survival test.[50,51,52]

Acute and Chronic Safe Concentrations

Analogous to the acute safe level, the chronic safe values were also derived using the NOEC and LOEC values obtained through survival studies. The chronic values for Arsenic to *P. reticulata* and *P. sphenops* were same i.e., 99 µg L^{-1} while the chronic values of Cd for respective fish were 216 and 180 µg L^{-1}. The chronic value for Cd exposed *P. reticulata* was slightly higher. Likewise, the chronic values of chlorpyrifos to respective fish were 0.053

and 0.010 µg L^{-1} and for cypermethrin were 0.016 and 0.010 µg L^{-1}.

No mortality in the control and solvent control groups were noticed in the present study ie., 100% survival rate observed in chronic test. Only 32.5% mortality was observed that too in the higher concentration of pesticide on the third week of exposure.

Conclusion

Maintenance of water quality is essential for survival, growth and physiology of aquatic organisms. The release of various pollutants including endocrine disruptive chemicals affects the quality of aquatic medium and leads to impairment of life in the aquatic environment. Therefore, the strict acute and chronic safe level criteria should be adopted to maintain water quality for the protection of most natural bio resources. Thus the present study on acute and chronic values of the chosen EDCs reveals that it was in good agreement with the criteria developed by USEPA[26] under safe levels to protect the freshwater bodies. The data obtained in the present study can be used to assess and monitor the organic or inorganic contaminates in the aquarium fish culture. This in turn helps to improve the economy of the aquarist and growth of entrepreneurship in the ornamental fish trade of the world market.

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Conflict of Interest

The authors declare no conflict of interest.

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