Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 6 [8] July 2017: 58-63

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD
Global Impact Factor 0.533
Universal Impact Factor 0.9804

ORIGINAL ARTICLE **OPEN ACCESS**

Eco-genotoxic effects of certain Pesticides mixtures to freshwater fish, *Oreochromis mossambicus* Peters

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ABSTRACT

Eco-genotoxicity of three commercially available pesticides mixtures, viz., Fipronil 40% + Imidacloprid 40% WG, Indoxacarb 14.5 % + Acetamiprid 7.7 % SC and Thiamethoxam 12.6 % + Lambdacyhalothrin 9.5 % ZC was evaluated on Oreochromis mossambicus Peters. *Initially LC50 for each pesticides mixture was determined by conducting acute toxicity studies. Then the fish were exposed to 1/20th and 1/10th of LC50 values of each pesticides mixture for 21 days. At the end of the exposure period, the gills were isolated from the fish and processed for comet assay. Nucleoids were visually scored and categorized into various degrees of damages. Significant increase ($p < 0.05$) in the percentage and distribution of damaged nucleoids was recorded in fish exposed to the mixtures. Scores of DNA damage (AU) augmented with exposure concentrations. From the results it is concluded that the pesticides mixtures evaluated in the study could be potentially genotoxic to fish O. mossambicus.*

Keywords: *Eco-genotoxic, pesticides mixtures, acute toxicity, semi-static bioassay, genotoxic, comet, DNA damage.*

Received 20.05.2017 Revised 11.06.2017 Accepted 24.06.2017

INTRODUCTION

The current risk assessment, especially the regulatory limits for the use of pesticides mixtures are based on separate toxicological tests of the individual pesticides in the mixture and not on the basis of combined toxic effects of the pesticides in the mixtures. Most of the toxicity studies carried out with individual pesticides or mixtures of pesticides on aquatic organisms are aimed to understand mortality, behavioral changes and sometimes hematological and biochemical changes. Though studies are also conducted on reproduction in fishes with individual pesticides or mixtures of pesticides, reports on genotoxicity induced by the pesticides on fish are scarce. Fish are sensitive to pesticide residues and other toxic pollutants because they are able to uptake and accumulate the dissolved pollutants in water via active or passive processes and also respond to toxicants in a similar way to higher vertebrates [1, 3]. Carcinogenesis and the formation of some tumors in different tissues of fish exposed to insecticides may also be caused by genotoxic properties of these xenobiotic. The eco-genotoxic effects not only reduce the fitness in wild fish production, but also pose high risk to human health via food chain (10). Some insecticides that behave as endocrine disruptors can change the expression of vital genes resulting in unusual concentrations of plasma steroid hormones and reproductive dysfunction or immunosuppression [9]. The pesticides that demonstrate non genotoxic using existing battery of tests may or may not be non genotoxic for ecologically important organisms. Thus the analysis of DNA alterations in aquatic organisms has shown to be an adequate technique to evaluate the genotoxic contamination of the environments [6] [8], with the benefit of distinguishing and enumerating the genotoxic damages deprived of the complete knowledge of the physical and chemical properties of the pollutants existing in the environment. Biomarkers frequently used to assess genotoxic effects of pesticides include chromosomal aberration, micronuclei formation, sister chromatid exchange and comet assay [17]. The past few years, single cell gel electrophoresis (SCGE) or comet assay has been used as a subtle, visual, consistent, swift and inexpensive technique for measuring and analyzing DNA single and double-strand breaks, alkali-labile sites, DNA cross- linking and delayed repair-site detection in eukaryotic individual cells [16] [15]. In the present study eco-genotoxic effects of three pesticides mixtures were evaluated in the freshwater fish, *O. mossambicus*.

ABBREVIATIONS

Fipronil 40% + Imidacloprid 40% WG	-	Fip+Imida
Indoxacarb 14.5 % + Acetamiprid 7.7 % SC	-	Indo+ Aceta
Thiamethoxam 12.6 % + Lambdacyhalothrin 9.5 % ZC	-	Thia+Lambda

MATERIALS AND METHODS

ACUTE TOXICITY (semi-static bioassay)

Three pesticides mixtures viz., Fip+Imida, Indo+Aceta and Thia+Lambda were procured commercially. Freshwater fish (*Oreochromis mossambicus*) (4 – 6 cm in length and approximately 2g in weight) was procured from a commercial supplier, (Tamil Nadu Fish Seed Farm, Poondi, Thiruvallur dist., Tamil Nadu, India). Fish were transported to the laboratory in aerated water. On arrival at the laboratory, the fish were quarantined for 12 days. They were, acclimatized for 7 days and feed was withdrawn for 24 h before conduct of the study. During the acclimatization and quarantine period, the fish were fed with commercially available fish feed. The acute toxicity study (semi-static bioassay) was conducted based on OECD guideline 203 and Guidance document on toxicology for registration of pesticides in India, 2014. The temperature of the test room and the test medium was maintained between 21 - 25°C and a photoperiod of 12 h light and 12 h darkness was maintained using a timer. Blended water (mixture of well water and reverse osmosis water in the ratio of 1:1) was used as the exposure medium. Ten fish each were exposed to 5 different test concentrations (Table 1) of each pesticides mixture and blended water as a control for 96 h. The concentrations of pesticides were prepared separately and transferred to glass aquaria containing 20 L of blended water. The exposure media were renewed at the end of every 24 h with the respective concentrations or blended water. An initial study was performed to assess the acute toxicity of the pesticides mixtures with various concentrations of Fip+Imida, viz., 0.1, 0.5, 1, 5, 10mg/L, Indo+ Aceta viz., 0.5,1,5, 10, 50 mg/L and Thia + Lambda viz., 0.01, 0.05, 0.1, 0.5 and 1 mg/L. Fish were observed for mortality and toxicity signs at 3 h and 6 h at the start of exposure and thereafter at the end of 24, 48, 72 and 96 h. Physico-chemical parameters such as pH, dissolved oxygen, temperature were analyzed daily in the exposure media (2). LC50 with 95% confidence limits were determined using ECOSTAT software version 9.3.

SUB-LETHAL TOXICITY

Ten fish each were exposed to sub-lethal concentrations (1/20th and 1/10th of LC50 values) of each pesticides mixtures for 21 days. The 1/20th and 1/10th of LC50 values were 0.01 and 0.02 mg/L, respectively for Fip+Imida, 0.26 and 0.51 mg/L, respectively for Indo + Aceta and 0.001 and 0.002 mg/L, respectively for Thia+Lambda. A concurrent control was also maintained. The concentrations of the pesticides mixtures were prepared as given above. Exposure medium was renewed daily. The fish were fed with commercially available fish feed once in two days. The fish were observed for toxicity signs and mortality if any, daily.

GENOTOXICITY (COMET ASSAY)

At the end of 21 days sub-lethal toxicity study, the exposed fish gills were isolated and minced [14]. Cells isolated from the gills were taken for comet analysis using the method of Singh *et al*. (1988), with slight modifications. For the basal layer, 1% normal melting agarose in phosphate buffered saline (PBS) was prepared; slides were dipped in the agarose solution and air dried. About 20 µl of the cell suspension was mixed with 80µl low melting agarose (0.7% in PBS) and added to the basal agarose layer, covered with cover glass and allowed to solidify. This was followed by the addition of the third layer of agarose (100 µl 0.5% low melting agarose) cover slipped and allowed to solidify. After removal of cover slips, the slides were immersed in 50 mL cold lysing solution and slides were kept in dark at 4ºC for 1 h in refrigerator. To avoid any additional DNA damage, the procedure was performed under dim light. The slides were removed from the lysing solution and placed on a horizontal electrophoresis tank for DNA unwinding for 20 min in the electrophoresis buffer (300 mM NaOH, 1 mM Na2 EDTA, pH >13). The cells were exposed to alkali for DNA unwinding. After electrophoresis, the gel slides were washed in neutralizing buffer (0.4 M Tris-HCl, pH 7.5) for 15 min and then stained with 75 µl of ethidium bromide (2µg/mL) and screened for comets using a Carl Zeiss Axiostar fluorescence microscope (Carl Zeiss, Germany) at 400X magnification. The migrated cells resemble the comets with a head region containing undamaged DNA and a tail containing broken DNA. The amount of DNA able to migrate and the distance of migration indicate the number of strand breaks present in that cell and the extent of DNA migration indicates the level of DNA breakage in the cell. Cells with increased DNA damage display an increased migration of chromosomal DNA from the nucleus towards the anode. The cells were scored visually based on their tail intensities and the scores were categorized as 0 (undamaged), 1 (mild), 2 (moderate), 3 (severe) and 4 (extensive) [11]. About 100 comet images were visually scored at random for each fish covering a total of 1000 cells per group. The percentage of damage was calculated and statistically analyzed among the control and

treated groups. Arbitrary Unit was used to express the extent of DNA damage and calculated as follows (Equation 1):

$$\text{Arbitrary unit} = \sum_{i=0}^{4} Ni \times i$$

Where *Ni* =Number of cells in *i* degree; *i* = degree of damage (0, 1, 2, 3, 4).

Statistics

ECOSTAT 9.3 Version was used for statistical evaluation. Statistical comparison of DNA damage in the controls and treatment group was performed using the Kruskal-Wallis test.

RESULTS

ACUTE TOXICITY

Fip+Imida

At the end of 96 h no mortality was observed in control and 0.1 mg/L concentration of Fip+Imida, whereas 90% mortality was observed in fish exposed to the concentration of 0.5 mg/L. Fish exposed to the concentrations of 5 and 10 mg/L Fip+Imida exhibited 100% mortality at the end of 24 h, while fish exposed to 1 mg/L exhibited 100% mortality at the end of 48 h (Table 1). Fish exposed to the concentrations of 0.5, 1, 5 and 10 mg/L exhibited toxicity signs such as pigmentation, loss of equilibrium, rapid opercular movement and lateral lying at the bottom of the aquaria.

Table 1. Mortality in *O. mossambica* exposed to various concentrations of Fip+Imida at 3, 6, 24, 48, 72 and 96 hours (h).

Concentration of Fip+Imida (mg/L water)	**No. of fish tested**	**Mortality at**						**Mortality (%) up to 96 h**
		3h	6h	24h	48h	72h	96h	
Control	10	0	0	0	0	0	0	0
0.1	10	0	0	0	0	0	0	0
0.5	10	0	0	1	0	8	0	90
1	10	0	0	3	7	-	-	100
5	10	0	0	10	-	-	-	100
10	10	0	0	10	-	-	-	100

Indo+Aceta

At the end of 96 h, no mortality was observed in control, 0.5 and 1 mg/L, whereas 20% mortality was observed in fish exposed to the concentration of 5 mg/L. Fish exposed to the concentrations of 10 and 50 mg/L exhibited 100% mortality at the end of 48 and 24 h, respectively (Table 2). Fish exposed to the concentrations of 5, 10 and 50 mg/L exhibited toxicity signs such as pigmentation and loss of equilibrium.

Table 2. Mortality in *O. mossambica* exposed to various concentrations of Indo+ Aceta at 3, 6, 24, 48, 72 and 96 hours (h).

Concentration of Indo+ Aceta (mg/L water)	No. of fish tested	Mortality at						Mortality (%) up to 96 h
		3h	6h	24h	48h	72h	96h	
Control	10	0	0	0	0	0	0	0
0.5	10	0	0	0	0	0	0	0
1	10	0	0	0	0	0	0	0
5	10	0	0	0	1	1	0	20
10	10	0	0	1	9	-	-	100
50	10	0	0	10	-	-	-	100

Thia+Lambda

No mortality was observed in control and 0.01 mg/L at the end of 96 h whereas 100% mortality was observed in fish exposed to the concentrations of 0.05 and 0.1 mg/L at the end of 24 h. Fish exposed to the concentrations of 0.5 and 1 mg/L exhibited 100% mortality at the end of 6 h(Table 3). Fish exposed to the concentration of 0.01 mg/L exhibited loss of equilibrium.

Table 3. Mortality in *O. mossambica* exposed to various concentrations of Thia+Lambda at 3, 6, 24, 48, 72 and 96 hours (h).

Concentration of Thia+Lambda (mg/L water)	No. of fish tested	Mortality at						Mortality (%) up to 96 h
		3h	6h	24h	48h	72h	96h	
Control	10	0	0	0	0	0	0	0
0.01	10	0	0	0	0	0	0	0
0.05	10	0	0	10	-	-	-	100
0.1	10	0	0	10	-	-	-	100
0.5	10	0	10	-	-	-	-	100
1	10	0	10	-	-	-	-	100

DETERMINATION OF LC50

Based on the above mortality data, the LC50 with 95% confidence limits were determined as follows:

Table 4. LC50 with 95% confidence limits of pesticides formulations for *O. mossambica*

Pesticides mixture	**LC50 (mg/L)**	**95% confidence limits (mg/L)**
Fip+Imida	0.2	0.1 to 0.4
Indo+Aceta	5.1	2.8 to 8.6
Thia+Lambda	0.02	0.01 to 0.05

PHYSICO-CHEMICAL PARAMETERS

The physico-chemical parameters such as pH, dissolved oxygen, temperature, conductivity and hardness of the exposure media determined during the experiment were, pH- 7.72 – 8.12; temperature - 22.7 – 24.7°C; dissolved oxygen - 71 – 112%; hardness - 206 – 208 mg/L and conductivity - 758 – 790 µs/cm, and were within the acceptable range [2].

SUB-LETHAL TOXICITY

No mortality and toxicity signs were observed in control and in fish exposed to the sub- lethal concentrations of the pesticides mixtures for 21 days.

COMET ASSAY (GENOTOXICITY IN FISH)

On analysis it was observed that the pesticide mixtures, Fip+Imida (Table 5), Indo+Aceta (Table 6) and Thia+Lambda (Table 7) induced strand breaks, when exposed to the concentrations of 0.01 and 0.02 mg/L,, 0.26and 0.51 and 0.001 and 0.002 mg/L, respectively.

Table 5. Analysis of DNA damage as measured by comet assay in gills tissue of *O. mossambicus*.

Concentration	**Proportion of Damaged nuclei [a]**					**%DNA damage (1+2+3+4)[b]**	**DNA Damage score (AU)[c]**
	0	**1**	**2**	**3**	**4**		
Control (water)	76.8 ± 5.18	22.1 ± 5.36	0.9 ± 0.74	0.2 ± 0.42	0 ± 0	23.2 ± 5.18	24.5 ± 5.36
0.01mg/L	45.2 ± 4.54	27.8 ± 5.81	17.2 ± 4.85	7.5 ± 2.72	2.3 ± 0.95	54.6* ± 4.72	93.9 ± 9.77
0.02 mg/L	25.9 ± 2.56	26.3 ± 2.06	28.3 ± 2.75	13.2 ± 2.57	6.3 ± 1.57	74.1* ± 2.56	147.7 ± 6.78

* Significantly different from control ($p < 0.05$ - Kruskal-Wallis test).
a: 0-4 – indicates grade of DNA damage
b: percentage of damaged cells = (1+2+3+4)
c: AU - Arbitrary units of DNA Damage score

Table 6. Analysis of DNA damage as measured by comet assay in gills tissue of *O. mossambicus*

Concentration	Proportion of Damaged nuclei [a]					%DNA damage (1+2+3+4)[b]	DNA Damage score (AU)[c]
	0	1	2	3	4		
Control (water)	78.40 ± 8.19	20.60 ± 8.82	0.60 ± 0.70	0.40 ± 0.52	0.00 ± 0.00	21.60 ± 8.19	23.00 ± 7.32
0.26 mg/L	40.1 ± 6.47	23.7 ± 3.92	15.3 ± 2.87	13.5 ± 4.77	7.4 ± 3.37	59.2* ± 6.71	124.4 ± 24.19
0.51 mg/L	21.3 ± 3.62	20.4 ± 2.84	18.5 ± 3.47	22 ± 4.14	17.8 ± 4.52	78.7* ± 3.62	194.6 ± 17.06

* Significantly different from control ($p < 0.05$ - Kruskal-Wallis test).
a: 0-4 – indicates grade of DNA damage
b: percentage of damaged cells = (1+2+3+4)
c: AU - Arbitrary units of DNA Damage score

Table 7. Analysis of DNA damage as measured by comet assay in gills tissue of *O. mossambicus*

Concentration	Proportion of Damaged nuclei [a]					%DNA damage (1+2+3+4)[b]	DNA Damage score (AU)[c]
	0	1	2	3	4		
Control (water)	77.90 ± 5.59	21.00 ± 5.16	0.50 ± 0.71	0.60 ± 0.70	0.00 ± 0.00	22.10 ± 5.59	23.80 ± 6.39
0.001 mg/L	44.60 ± 4.55	32.40 ± 3.78	13.40 ± 2.63	6.10 ± 1.91	2.50 ± 1.08	54.00* ± 4.78	87.50 ± 8.64
0.002 mg/L	26.30 ± 4.62	23.60 ± 2.32	22.90 ± 4.31	12.30 ± 2.63	14.90 ± 2.69	73.70* ± 4.62	165.90 ± 13.09

* Significantly different from control ($p < 0.05$ - Kruskal-Wallis test).
a: 0-4 – indicates grade of DNA damage
b: percentage of damaged cells = (1+2+3+4)
c: AU - Arbitrary units of DNA Damage score

DISCUSSION

The current genetic toxicology studies of pesticides are more concerned about the potential effects towards humans. Though eco-genotoxicology studies are considered to evaluate the direct or indirect effects of the pollutants on the genetic materials of the organisms in the ecosystem [7], not many studies were carried out on the eco-genotoxicity of pesticides. At lower concentrations, pesticides may not be toxic to aquatic organisms directly/immediately but may alter the genomic function of the organisms as revealed in the present study where the sub-lethal concentration of the pesticides mixtures did not show any signs of toxicity, but caused genotoxicity. In the present study, the pesticides mixtures exhibited a significant increase ($p < 0.05$) in the % DNA damage compared to the control. The DNA damage observed could possibly be initiated from DNA single or double strand breaks or through the formation of DNA adducts and/or DNA cross links, which might have resulted due to the interaction of DNA with the pesticides mixtures [5] [13]. Studies of this nature are useful to monitor the ecosystem health and, consequently, for the well-being of all the organisms exposed to it, including man [12]. However, from the comet assay one can point out only the general damages to the DNA and cannot specify the region, affected, which may impair the growth, reproduction and population dynamics of the organisms in the long term exposure which in turn gradually leads to the extinction of these species, further molecular studies are essential to understand about the mode of action of these chemicals on genome of the beneficial organisms, on its DNA repair mechanisms and the genome area, affected.

ACKNOWLEDGEMENT

The authors are grateful to the Management of IIBAT and the supporting staffs for the encouragement and providing facilities for conducting the study.

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CITATION OF THIS ARTICLE

P. Dhandapani, R. A. Gopi, T. N. Sathya and K. Sadasivan Pillai. Eco-genotoxic effects of certain Pesticides mixtures to freshwater fish, *Oreochromis mossambicus* Peters. Bull. Env. Pharmacol. Life Sci., Vol 6 [8] July 2017: 58-63