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Assessment of eco-genotoxic effects of pesticide mixtures on freshwater fish, *Catla catla*

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Abstract

Eco-genotoxicity was evaluated in fresh water fish, *Catla catla* using two commercially available pesticide mixtures, viz., Deltamethrin 1% + Triazophos 35% EC and Profenofos 40% EC + Cypermethrin 4% EC. Acute toxicity studies were conducted primarily to determine LC_{50} of each pesticide mixture. The fish were exposed to each pesticide mixture for 21 days at a dose value of 1/ 20^{th} and $1/10^{th}$ of LC_{50}. The gills were isolated from the fish at the end of the exposure period, and processed for comet assay. Nucleoids were scored visually, observed significant increase of damage and categorized into various degrees of damages. Based on the results obtained from this study it is concluded that the pesticide mixtures evaluated could be potentially genotoxic to fresh water fish, *Catla catla*.

Key words: Eco-genotoxicity, acute toxicity, comet assay, DNA damage.

Introduction

The current scenario to assess risk, particularly the regulatory limits for the use of pesticides mixtures are based on selective toxicological tests of the individual pesticides in the mixture and not on the basis of combined toxic effects of the pesticides in the mixtures. The aim of the most of the toxicity studies being conducted usually with individual pesticides or mixtures of pesticides on aquatic organisms are to understand mortality, behavioral changes and sometimes hematological and biochemical changes. The reports on genotoxicity induced by the pesticides or in combination on fish are very limited. The reason to choose fish for assessment of eco genotoxicity is because of sensitive to pesticide residues and other toxic pollutants. Fish accumulates pollutants dissolved in water and respond to toxicants in a similar way to higher vertebrates (1, 2). Carcinogenic activities such as formation of tumors in different tissues of fish exposed to insecticide may be the cause of xenobiotic used. The eco-genotoxic activity of certain pesticides can reduce the fitness of wild fish production, and also pose high risk to human health via food chain (3). Endocrine disruptors being used in insecticides can change the expression of vital genes that are responsible for reproductive dysfunction or immunosuppression (4).

The pesticides that are proved to be non genotoxic using existing battery of tests may or may not be non genotoxic for ecologically important organisms. The analysis of DNA changes in aquatic organisms will be a useful tool to evaluate contaminants existed in the aquatic environments with genotoxic compounds (5,6). Chromosomal aberrations, micronuclei formation, sister chromatid exchange and comet assay are the frequently using biomarkers to assess genotoxic effects of pesticides (7). Single cell gel electrophoresis (SCGE) or comet assay are the inexpensive techniques have been used for the past few years for measuring and analyzing DNA single and double-strand breaks, DNA cross-

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linking and delayed repair-site detection in eukaryotic individual cells (8, 9). Based on the availability of limited literature on combination of pesticide mixtures in the present study eco-genotoxic effects of two pesticides mixtures were evaluated in the freshwater fish, *Catla catla.*

Materials and methods

Acute toxicity: Two pesticides mixtures viz., Deltamethrin 1% + Triazophos 35% EC (D+T(EC)) and Profenofos 40% EC + Cypermethrin 4% EC (P+C(EC)) were procured commercially. Fresh water fish (*Catla catla*) (6 – 7 cm in length and approximately 6g in weight) were procured from a commercial supplier. Fish were transported to the laboratory in aerated water and quarantined for 12 days. Prior to initiation of the experiment fish were, acclimatized for 7 days and feed was withdrawn for 24 h before conduct of the study. Fish were fed with commercially available fish feed during the acclimatization and quarantine period. The acute toxicity of pesticide mixtures in fish was determined based on OECD guideline 203 (10) and Guidance document on toxicology for registration of pesticides in India, 2017 (11).

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 (A 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 D+T (EC), viz., 0.1, 0.5, 1, 5, 10mg/L and P+ C (EC) viz., 0.05, 0.1, 0.5, 1.0, 5.0 mg/L. Fish were observed for mortality and morbidity 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 (12). LC_{50} values for different concentrations of pesticides mixture were calculated by AAT Bioquest® calculator (13).

Sub-lethal toxicity: Ten fish each were exposed to sub-lethal concentrations (1/20th and 1/10th of LC_{50} values) of each pesticide's mixtures for 21 days. The 1/20th and 1/10th of LC_{50} values were 0.05 and 0.10 mg/L, respectively for D+T (EC), 0.02 and 0.05 mg/L, respectively for P + C (EC). 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 morbidity 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 using a mincing solution comprised of (HBSS, 20 mM EDTA, DMSO and Sterile water) with a micropestle and allowed to settle. The supernatant containing single cells extracted from the gills tissue was used for comet assay (9, 14). For the basal layer, 1% normal melting agarose in phosphate buffered saline (PBS) was prepared. To this about 25 µl of the cell suspension from each sample was mixed with 75 µl of low melting agarose (0.5% in PBS) covered with cover glass and allowed to solidify. After removal of the cover glass, the slides were immersed in 50 ml of cold lysing solution and maintained in dark condition at 4°C for 1 h. Slides were then transferred to a tank containing electrophoresis buffer (300 mM NaOH, 1 mM Na2 EDTA, pH >13), for 20 min to make the DNA unwinding. After electrophoresis, the 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 (2 µg/mL) and screened for comets using a fluorescence microscope at 400X magnification. The cells were scored by their tail intensities and the scores were categorized as 0 (undamaged), 1 (mild), 2 (moderate), 3 (severe) and 4 (extensive) based on (15). The total amount

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of DNA strand breakage was expressed in total arbitrary units (AUT) defined as:

AUT = N0 x 0+N1 x
1+N2 x2+N3 x3+ N4 x 4,

where Ni is the number of nuclei scored in each category (16).

Statistics : Statistical evaluation was performed using SPSS 16.0 version.

Results and discussion

Acute toxicity: It's a continuous process that aquatic organisms are being exposed to various pollutants in the environment. A group of organisms such as plants, animals, fish or wildlife under controlled conditions when exposed to toxic pollutants can be easily evaluated the toxicity. Pesticides can produce adverse effects in a biological system, seriously damaging its structure and function of living system finally leads to death of organism. Those adverse responses may be defined in terms of a measurement as acute toxicity. Pesticides are entering into aquatic ecosystem by agriculture runoff from land, impairing the quality of the water and making it unfavorable for aquatic life (17).

Treatment with Pesticide mixture D+T (EC): No mortality was observed in control and fish treated with D+T (EC) at a concentration of 0.1 and 0.5mg/L, whereas 30% mortality was

Table 1. Mortality in *Catla catla* exposed to various concentrations of D+T (EC) at 3, 6, 24, 48, 72 and 96 hours (h).

Concentration (mg/L water)	No of Fish tested	Mortality at						Mortality (%) upto 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	0	0	0	0	0
1	10	0	0	0	1	2	0	30
5	10	0	0	4	6	0	0	100
10	10	0	0	10	0	0	0	100

Table 2. Mortality in *Catla catla* exposed to various concentrations of P+C (EC) at 3, 6, 24, 48, 72 and 96 hours (h).

Concentration (mg/L water)	No of Fish tested	Mortality at						Mortality (%) upto 96 h
		3h	6h	24h	48h	72h	96h	
Control	10	0	0	0	0	0	0	0
0.05	10	0	0	0	0	0	0	0
0.1	10	0	0	0	0	0	0	0
0.5	10	0	0	0	1	3	0	40
1	10	0	0	2	6	0	0	80
5	10	0	0	5	5	0	0	100

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observed in fish exposed to the concentration of 1 mg/L after 96 h. Fish exposed to the concentration of D+T (EC) at 5 mg/L exhibited 100% mortality at the end of 48 h, while fish exposed to 10 mg/L exhibited 100% mortality at the end of 24 hrs (Table 1). Fish exposed to the concentrations of 1, 5 and 10 mg/L exhibited clinical signs such as pigmentation, loss of equilibrium, rapid opercular movement and lateral lying at the bottom of the aquaria. Acute toxicity study conducted by (18) revealed that Triazophos exposed to fish individually at lower concentrations indicates no mortality but when exposed to higher concentrations showed death after 2-3 hours of exposure period.

Treatment with Pesticides mixture P+C (EC): Control and fish treated with P+C (EC) at a concentration of 0.05 and 0.1 mg/L showed no mortality until 96 h, whereas 40% mortality was observed in fish exposed to the concentration of 0.5 mg/L at 72 h. Fish exposed to the concentration of 1 mgL exhibited 80% mortality at the end of 48 hrs, respectively (Table 2). Similarly, 100% mortality was observed when fish treated with 5mg/L at 48hrs. Fish exposed to the concentrations of 0.5, 1 and 5 mg/L exhibited toxicity signs such as pigmentation and loss of equilibrium. A dose dependent increase and time dependent decrease were observed in *Catla catla* when exposed to Profenofos individually and the mortality rate at the exposure time increased from 24 to 96 hours (18).

Determination of LC_{50}: Based on the above mortality data, the LC_{50} were determined by AAT Bioquest® calculator (13) and results were incorporated in Table 3.

Table 4. Analysis of DNA damage as measured by comet assay in gills tissue of *Catla catla*.

Concentration	Proportion of damaged nuclei					% DNA Damage (1+2+3+4)	DNA Damage score (AU)
	0	1	2	3	4		
Control	71.00	23.00	0.90	0.10	0.00	24	25.1
	74	29	1	0.4	0.00	30.4	32.2
	79	24	0.7	0.3	0.00	25	26.3
Average	**74.67**	**25.33**	**0.87**	**0.27**	**0.00**	**26.47**	**27.87**
SD	**4.04**	**3.21**	**0.15**	**0.15**	**0.00**	**3.44**	**3.80**
0.05mg/L	46	26	14	7	2	49	83
	43	29	20	11	2	63	110
	48	28	17	5	3	53	89
Average	**45.67**	**27.67**	**17.00**	**7.67**	**2.33**	**55.00**	**94.00**
SD	**2.52**	**1.53**	**3.00**	**3.06**	**0.58**	**7.21**	**14.18**
0.10mg/L	29	30	31	14	6	81	158
	23	26	30	17	6	79	161
	25	25	27	10	7	69	137
Average	**25.67**	**27.00**	**29.33**	**13.67**	**6.33**	**76.33**	**152.00**
SD	**3.06**	**2.65**	**2.08**	**3.51**	**0.58**	**6.43**	**13.08**

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Table 3. LC_{50} of pesticides mixtures for *Catla catla*

Pesticides mixtures	LC_{50} (mg/L)
D+T (EC)	1.09
P+C (EC)	0.51

Physico-chemical parameters: The physico-chemical parameters in the exposure media determined during the experiments were, pH- 7.2 – 8.0; temperature - 21.2 – 23.5°C; dissolved oxygen - 75 – 108%; hardness - 201 – 209 mg/L and conductivity - 741 – 783 µs/cm, and were within the acceptable range (12).

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

Concentration	**Proportion of damaged nuclei**					**% DNA Damage (1+2+3+4)**	**DNA Damage score (AU)**
	0	1	2	3	4		
Control	78.00	19.00	0.60	0.40	0.00	20	21.4
	79	18	0.5	0.6	0.00	19.1	20.8
	81	25	0.8	0.2	0.00	26	27.2
Average	**79.33**	**20.67**	**0.63**	**0.40**	**0.00**	**21.70**	**23.13**
SD	**1.53**	**3.79**	**0.15**	**0.20**	**0.00**	**3.75**	**3.53**
0.02	39	23	15	13	7	58	120
	41	27	19	12	8	66	133
	42	23	10	12	8	53	111
Average	**40.67**	**24.33**	**14.67**	**12.33**	**7.67**	**59.00**	**121.33**
SD	**1.53**	**2.31**	**4.51**	**0.58**	**0.58**	**6.56**	**11.06**
0.05	19	20	18	17	15	70	167
	22	21	18	21	17	77	188
	25	21	19	25	18	83	206
Average	**22.00**	**20.67**	**18.33**	**21.00**	**16.67**	**76.67**	**187.00**
SD	**3.00**	**0.58**	**0.58**	**4.00**	**1.53**	**6.51**	**19.52**

Sub-lethal toxicity: Mortality and any toxicity signs were not observed in control and in fish exposed to the sub- lethal concentrations of the pesticides mixtures for 21 days.

Comet assay (genotoxicity in fish): DNA damage if any by the pesticide mixtures was analyzed and observed that the pesticide mixtures, D+T (EC) (Table 4) and P+C (EC) (Table 5) induced strand breaks, when exposed to the concentrations of 0.05, 0.10 mg/L and 0.02, 0.05mg/L, respectively. 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 (19, 20).

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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 (21).

Conclusion

On analysis it was concluded that 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. 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.

References

1. Al-Sabti, K. and Metcalfe, C.D. (1995). Fish Micronuclei for Assessing Genotoxicity in Water. Mutation Research, 343: 121-135.
2. Bailey, E., Crespo, S., Nonnotte, C., Colino, A., Leray, L. and Aubree, A. (1992). Morphological and functional alterations induced in trout intestine by dietary cadmium and lead. Fish Biology, 28: 69-80.
3. Kapour, D. and Nagpure, N.S. (2005). Training on genotoxic assays in fishes. National Bureau of Fish Genetic Resources, Dilknsha, Telibagh, India, 68.
4. Jin, Y.X., Chen, R.J., Liu, W.P. and Fu, Z.W. (2010). Effect of endocrine disrupting chemicals on the transcription of genes related to the innate immune system in the early developmental stage of zebrafish (Daniorerio). Fish Shellfish Immunology, 28: 854-61.
5. Frenzilli, G., Nigro, M. and Lyons, B.P. (2009). The Comet assay for the evaluation of genotoxic impact in aquatic environments. Mutation Research, 681: 80-92.
6. Jha, A.N. (2008). Ecotoxicological applications and significance of the comet assay. Mutagenesis, 23: 207-221.
7. Zeljezicm, D. and Garaj-Vrhovac, V. (2001). Chromosomal aberration and single cell gel electrophoresis (Comet) assay in the longitudinal risk assessment of occupational exposure to pesticides. Mutagenesis, 16: 359-363.
8. Rojas, E., Lopez, M.C. and Valverde, M. (1999). Single cell gel electrophoresis assay: Methodology and applications. Journal of Chromato Biomedical Science and Application, 54: 722-225.
9. Singh, N.P., McCoy, M.T., Tice, R.R. and Schneider, E.L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. Experimental Cell Research, 175: 184-191.
10. OECD (2019). Organization for Economic Cooperation and Development. Guideline for Testing of Chemicals, No. 203. Fish Acute Toxicity Test. Paris, France.
11. Guidance document on toxicology for registration of pesticides in India, 2017.
12. American Public Health Authority, (1975). Standard Methods for the Examination of Water and Wastewater, 14th Edition, American Public Health Association, Washington D.C.
13. AAT Bioquest Inc. 2018. Quest Graph, LC50 Calculator. https://www.aatbio.com/tools/lc50-calculator.
14. Rajaguru, P., Suba, S., Palanivel, M. and Kalaiselvi, K. (2003). Genotoxicity of a polluted river system measured using the

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DOI: 10.5530/ctbp.2021.1.5

alkaline comet assay on fish and earthworm tissues. Environmental Molecular Mutagen, 41: 85-91.

15. Collins, A.R., Dusinska, A., Franklin, M., Somorovska, M., Petrovska, H., Duthie, S., Fillion, L., Panayoitidis, M., Raslova, K. and Vaughan, N. (1997). Comet assay in human biomonitoring studies: reliability, validation and applications. Environmental Molecular Mutagenesis, 30:139-146.

16. Collins, A.R. (2002). The comet assay, principles, applications and limitations. In: Didenko VV (ed.) In Situ Detection of DNA Damage. Methods and Protocols, v. 203. Humana Press Inc., Totowa, 163-177.

17. Anitha Smruthi, C.H., Lalitha., Hari babu, G. and Venkata Rathnamma, V. (2018). Toxicityy Evaluation and Behavioural Studies of Catla Catla induced Fipronil 5%SC. International Journal of Recent Scientific Research, 9(2): 23843-23847.

18. Ghazala, S., Mahboob, K.A., Al-Ghanim, S., Sultana, H.F., Alkahem Al- Balawi, T., Sultana, F., Al-Misned. and Ahmed, Z. (2014). Acute toxicity II: effect of organophosphates and carbmates to Catla catla fingerlings. The Journal of Animal & Plant Sciences, 24(6):1795-1801.

19. Fairbairn, D.W., Olive, P.L. and O'Neil, K.L. (1995). The comet assay: A comprehensive review. Mutation Research Review/ Genetic Toxicology, 339: 37-59.

20. Mitchelmore, C.L. and Chipman, J.K. (1998). DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring. Mutation Research/Fundamental Molecular Mechanisms Mutagen, 399: 135-147.

21. Maria Aparecida Marin-Morales., Franco Dani Campos-Pereira., Fernanda Flores Navarro. and Jaqueline Aparecida de Oliveira. (2016). Eco-Genotoxicity in Aquatic Systems. Hudson Polonini and Roberta Brayner, editors, OMICS Group, USA, 1-19.