NUCLEAR DAMAGE IN PERIPHERAL ERYTHROCYTES OF *CYPRINUS CARPIO* EXPOSED TO BINARY MIXTURE OF PESTICIDES

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A B S T R A C T

The present study was undertaken to examine the DNA damage in peripheral blood erythrocytes of *Cyprinus carpio* under the binary exposure of bifenthrin and chlorpyrifos by using single cell gel electrophoresis (SCGE). Limited efforts have been made to study the genotoxic effect for long duration period. Therefore, the present investigation was aimed to assess the genotoxicity of pesticide mixture to the freshwater carp, *Cyprinus carpio* at sub-lethal concentration exposure (33% LC_{50}). At first 96-hr LC_{50} value of pesticide, the mixture was determined for *Cyprinus carpio* in a static system and then sub-lethal concentration was calculated and fish was exposed to this sub-lethal concentration of the mixture in glass aquaria for 70 days (five fortnights) at constant laboratory conditions. Peripheral blood erythrocytes were taken on a fortnightly basis for the time-dependent DNA damage assessment in-terms of percentage of damaged cells, genetic damage index and a cumulative tail length of comets. Concentration-dependent increase in the percentage of DNA damaged cells were observed up to a 4th fortnight, followed by a slight decrease in the 5th fortnight. Similarly, statistically significant time-dependent DNA damage was observed in terms of percentage of damaged cells, genetic damage index and a cumulative tail length of comets in treated fish (at 33% of LC_{50}) as compared to control groups. The results supported the use of SCGE for evaluating the toxicity of pollutants which may be used as part of environmental monitoring programs.

Keywords: Pesticide mixture, *Cyprinus carpio*, DNA damage, single cell gel electrophoresis.

INTRODUCTION

Pesticides, in particular, have received increasing attention given their suggested important role in the global loss of freshwater biodiversity and ecosystem functioning (Malaj *et al*., 2014; Ramussen *et al*., 2015) because pesticides of various groups are used indiscriminately against a number of pests in the field to increase crop yield. Residues of these pesticides reach the aquatic environment, representing a risk for the non-target organisms (Ruiz-Suarez *et al*., 2015). Genotoxicity of pesticides for non-target organisms and their impact on ecosystem are of worldwide concern.

In the last decades, organophosphate pesticides are among the most widely used classes of pesticides. China, India and other countries have substantially increased the production of organophosphate pesticides even in recent years (Yen *et al*., 2011). Organophosphate pesticides are often implicated as toxic and alkylating agents and potential genotoxicants. As a well-known organophosphate pesticide, chlorpyrifos is extensively used for controlling agriculture and household pests all over the world (Yen *et al*., 2011). In 2007, chlorpyrifos was the fourteenth most commonly used conventional active ingredient in the agricultural pesticide market sector (Grube *et al*., 2011). Runoff events, erosion and leaching are the major routes of chlorpyrifos entry into surface waters (Jin *et al*., 2015). Another pesticide class, pyrethroid is recently extensively used nowadays and some evidence indicates that these chemicals are genotoxic in some systems including fish (Ambreen and Javed, 2015). Bifenthrin is active insecticide belongs to pyrethroid group of pesticide and is used in agriculture and public health control programs (control of mosquitoes) that act as a contact stomach poison. It

affects the central and peripheral nervous system and cause synaptic discharge, depolarization and ultimately cause death and it also acts as ATPase inhibitor in fish (Ponepal *et al.*, 2010).

A primary risk factor of genotoxic pesticides is that they can interact directly or indirectly with chromosomal DNA, causing chronic genotoxicity such as carcinogenic and reproductive toxicity (Xiang et al., 2013). It is evident that chemical, physical and biological agents could interact with the genetic material, resulting in mutations which are associated with genomic instability and cancer (Malling, 2004). Regulatory agencies like Food and Drug Administration (FDA) and European Medicines Agency (EMA) now begun to require that different genotoxicity tests (Hartmann, 2004). These tests include in vitro and in vivo assays to detect the potential of genotoxic agents to induce genetic mutations and/or chromosomal aberrations (Araldi *et al.*, 2015). Among different tests used for genotoxicity, SCGE is a very sensitive and rapid assay that can be applied to nucleated cells for the detection of DNA damage at a single cell level. Single cell gel electrophoresis assay allows us to detect DNA strand breaks, which can be visualized by the increased migration of free DNA segments, resulting in images similar to comets (Azqueta and Collins, 2013). The single cell gel electrophoresis or comet assay was introduced by Ostling and Johanson (1984). Since its development, several methodological modifications were proposed however, the Alkaline method, developed by Singh *et al.* (1988) that allows the DNA denaturation, double-strand breaks (DSBs), single strand breaks (SSBs), detection of alkali-labile sites, became the most used and recommended because of its broad spectrum for DNA damage detection. These DSBs and SSBs are associated with chromosomal aberrations and genomic instability (Pfeiffer *et al.*, 2000).

In natural environments, contaminants usually present as complex mixtures (Ambreen and Javed, 2015) because cocktails of compounds create a huge problem as the toxicity of a mixture is not easily linked to individual toxicities of components in the mixture. The common carp was selected as the test fish because it is one of the most economically important freshwater fishes of the world and because there is a scarcity of information regarding the genotoxicity of pesticide mixture on freshwater fishes.

MATERIALS AND METHODS

The fingerlings of freshwater fish, *Cyprinus carpio* (180-day age) were purchased from local fish seed hatchery and transported to Fisheries Research Farms, University of Agriculture, Faisalabad, Pakistan. Healthy fingerlings of similar weight were acclimatized under laboratory conditions in cemented tanks for about ten days and fed with a nutritious diet. Bifenthrin and chlorpyrifos were dissolved, separately, in 95% analytical grade methanol (J.T Baker) as a carrier solvent to prepare the stock-I solutions (1g/100 ml) while a binary mixture of these pesticides were prepared by its further dilutions in deionized water (stock-II). The acute toxicity bioassay to determine the 96-hr LC_{50} value of pesticide mixture (bifenthrin+chlorpyrifos) was conducted in a static system. The 96-hr LC_{50} was determined as 0.76 μgL^{-1} for *Cyprinus carpio* by using the Probit analyses method. Based on this 96-hr LC_{50} value, one test concentration i.e. 33% of LC_{50} (0.25 μgL^{-1}) was calculated and used for the assessment of sub-lethal genotoxicity experiment.

Single Cell Gel Electrophoresis (SCGE): The experiments were conducted in glass aquaria to determine the extent of DNA damage in the peripheral blood erythrocytes of *Cyprinus carpio*. For this purpose, the thirty-six fingerlings of *Cyprinus carpio* were divided into three groups. The first group were exposed to 33% LC_{50} of pesticide mixture (test concentration), the second group of fish were maintained in tap water considered as "Negative Control" (unstressed group), while in third group cyclophosphamide (20 μgg^{-1}) was used as "Positive Control". During the whole experimental period, fish were fed daily with a small quantity of food. Water temperature (30 ºC), pH (7.75) and total hardness (225 mgL^{-1}) were kept constant throughout the experimental duration. Exposure was continued for 70 days and blood erythrocytes slides were prepared on a fortnightly basis from all experimental groups and subjected to alkaline single cell gel electrophoresis (Comet assay). The experiment was conducted with three replications. Alkaline single cell gel electrophoresis was performed as three layer procedure by following the methods of Singh *et al.* (1988). Blood samples were collected from the caudal vein of fish transferred in Eppendorf and treated with anticoagulants and diluted with 1 ml of phosphate buffer saline (PBS). Blood (60 µl) was mixed with 110 µl of 1.7% low melting point (LMP) agarose. Sample mixture then layered on the glass slides pre-coated with 0.5% normal melting point (NMP) agarose and immediately covered with a cover slip and kept for 10 minutes in a refrigerator to solidify. After gently removing the

coverslips, the slides were coated with a third layer of 75 µl low-melting point agarose and covered with glass slip again. After solidification of gel, the slides were immersed in the cold lysing solution and refrigerated at 4 °C, followed by electrophoresis (25 V, 300 mA for 25 minutes) and staining with ethidium bromide. Two slides were prepared and one hundred and fifty cells per slide were scored randomly and analyzed by using an image analysis system attached to Epi-Fluorescence microscope (N-400M, American Scope; UK) equipped with light source of mercury short arc reflector lamp filters for ethidium bromide at 400 X magnification and low lux (MD-800, American Scope; UK) camera.
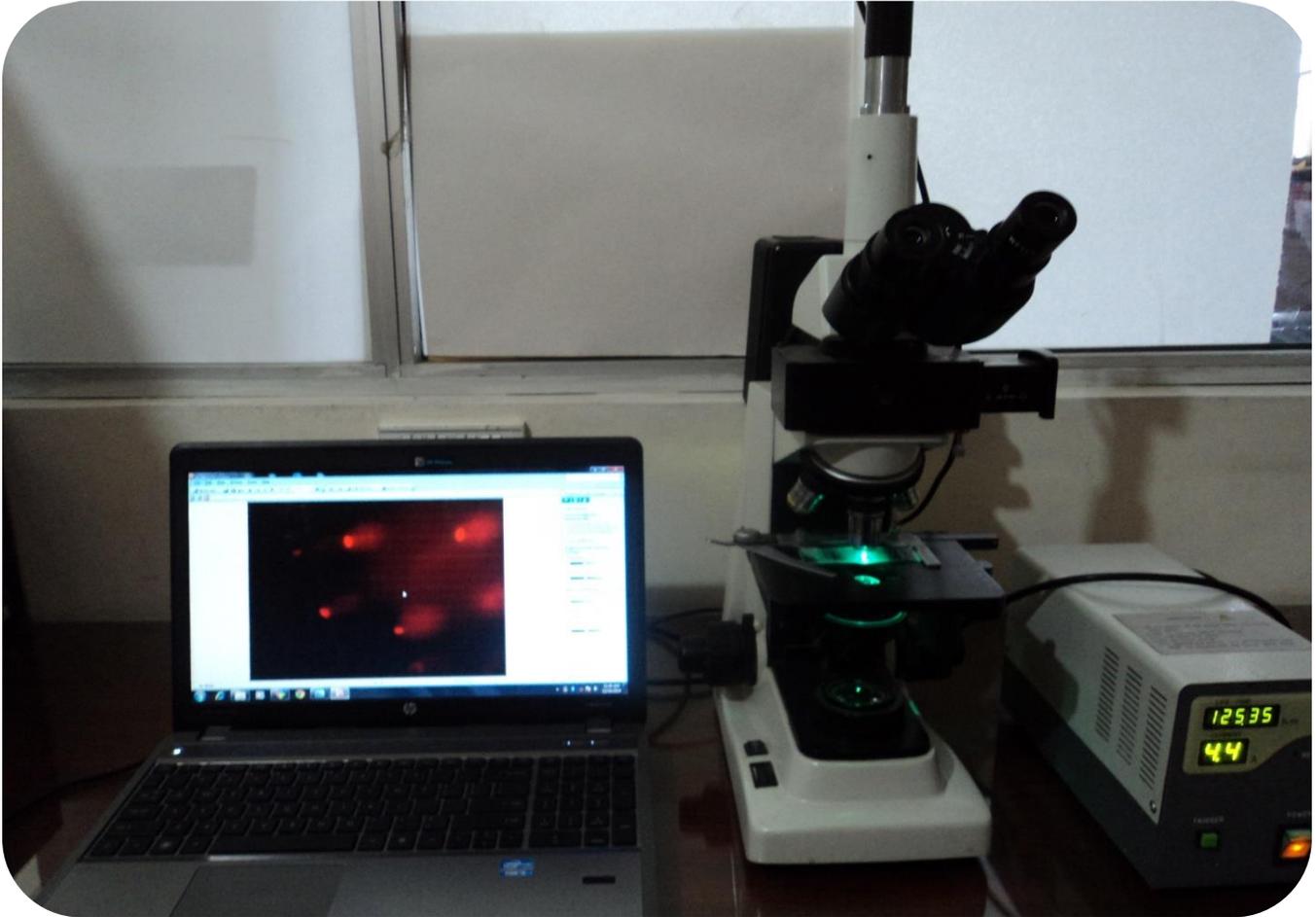

Figure 1. Caption is missing

The DNA damage was quantified by visual classification of cells into four classes of "comets" (Figure 1) corresponding to the tail length (measured through TriTek CometScore™) as undamaged (Class 0); low level damage (Class I); medium level damage (Class II); high level damage: (Class III) and complete damage (Class IV). All steps were conducted in dim light to avoid any non-specific additional breakage of DNA. The extent of DNA damage was examined as the mean percentage of cells with medium, high and complete damaged DNA, which was calculated as the sum of cells with class II+III+IV. Statistical analyses were performed by using MSTATC computer software. Results were expressed as Means±SD. A p-value of less than 0.05 were considered statistically significant. Means of data were compared for the statistical differences by using Duncan Range Multiple tests (Steel et al., 1996).
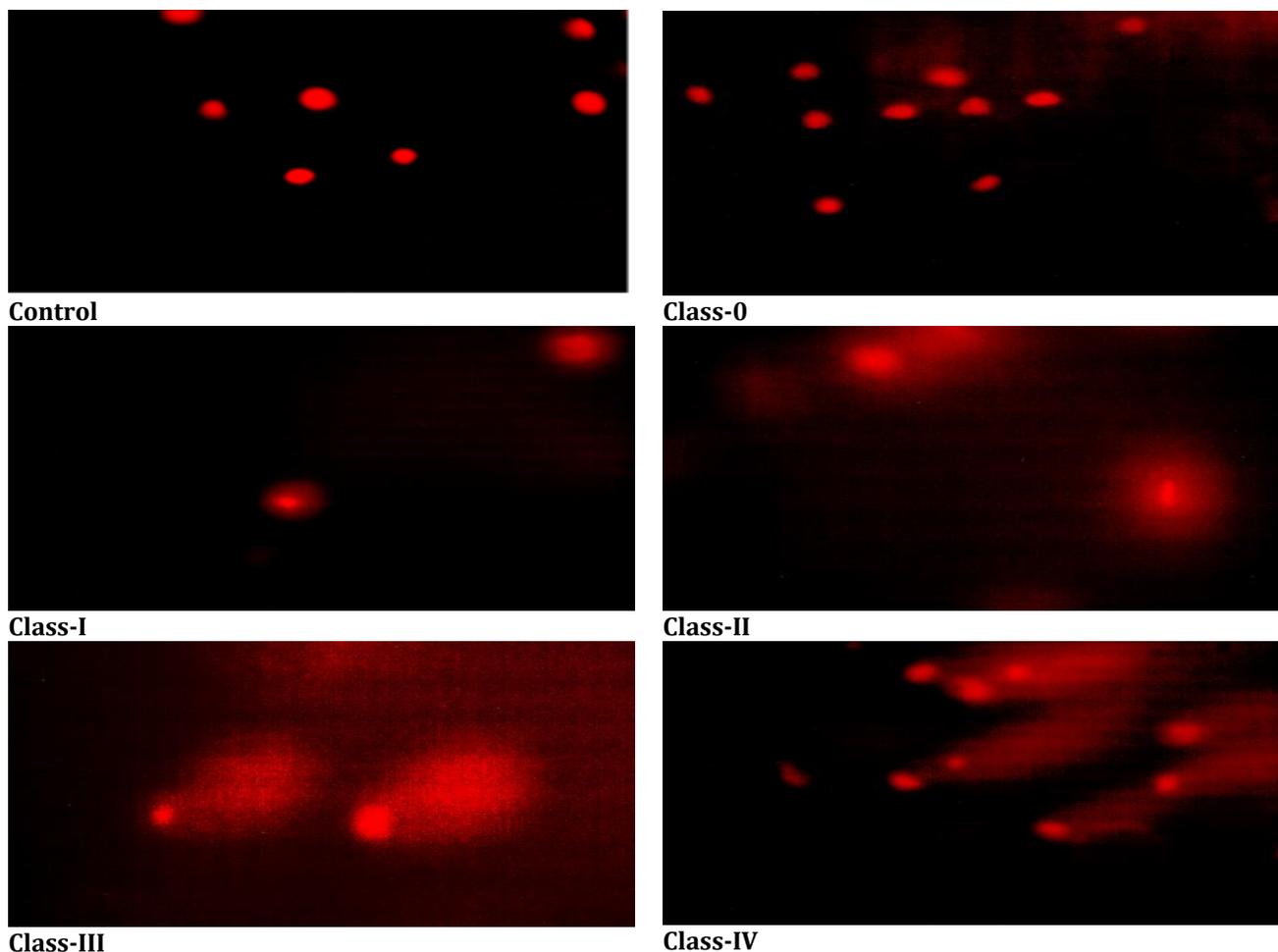
Figure 1. Comet class categories used for the estimation of the percentage of damaged cells.

RESULTS

DNA damaging effect of bifenthrin and chlorpyrifos mixture was investigated by using the comet assay. Table 1 shows the significantly variable proportion of undamaged (normal cells) and damaged class nuclei in the peripheral erythrocytes of *Cyprinus carpio* underexposure of negative control, positive control and 33% LC$_{50}$ during five fortnights. It was observed that the proportion of damaged cells changed with the duration of exposure to pesticide mixture (bifenthrin + chlorpyrifos). Results showed that fish exposed to 33% LC$_{50}$ exhibited significantly higher DNA damage in their peripheral blood erythrocytes as compared to control groups. Among all treatments, viz. negative control, positive control and 33% LC$_{50}$, the proportion of class-0 (normal cells) were observed significantly higher in the negative control group during all fortnights while the proportion of normal cells were minimum under the exposure of 33% LC$_{50}$. However, the proportion of comet class-I damaged cells were maximum during the 1st, 2nd and a 5th fortnight at 33% LC$_{50}$ exposure while during 3rd and 4th fortnight same were observed higher due to the positive control treatment. Proportions of comet class-II damaged nuclei were also varied significantly during the various fortnights of this study period. During the first two fortnights, proportions of comet class-II cells were observed higher due to the positive control treatment. However, during 3rd, 4th and 5th fortnights, 33% LC$_{50}$ gave a significantly higher proportion of class-II cells. Among various treatments, viz. negative control, positive control and 33% LC$_{50}$, the frequency of cells with high-level damage (class-III) and complete damage (class-IV) were observed significantly higher due to 33% LC$_{50}$ exposure of pesticides mixture. The proportion of class-III damaged cells were ranged from 24.67±1.15 – 33.33±1.15, exhibited a concomitant increase in damage from 1st to a 3rd fortnight. The proportion of all comet classes exhibited time-dependent increase or decrease in DNA damage from 1st to a 5th fortnight.

Table 1 DNA damage in peripheral blood erythrocytes of Cyprinus carpio exposed to a binary mixture of bifenthrin and chlorpyrifos.

Fortnights	Treatments	Undamaged Nuclei (%) Class-0	Comet Classes (Damaged Nuclei (%))				*Percentage of Damaged Cells	**GDI	***CTL (µm)
			Class-I	Class-II	Class-III	Class-IV			
1st	Negative Control	98.00±0.00 a	2.00±0.00 c	0.00±0.00 c	0.00±0.00 c	0.00±0.00 c	0.00±0.00 c	0.02±0.00 c	3.23±0.03 c
	Positive Control	34.00±0.00 b	14.67±1.15 b	18.00±2.00 a	10.67±1.15 b	22.67±1.15 a	51.33±1.15 b	1.73±0.01 b	128.15±0.05 b
	33% of LC50	21.33±1.15 c	25.33±1.15 a	10.67±1.15 b	24.67±1.15 a	18.00±2.00 b	53.33±2.31 ab	1.93±0.08 a	572.37±0.06 a
2nd	Negative Control	98.00±0.00 a	2.00±0.00 c	0.00±0.00 c	0.00±0.00 c	0.00±0.00 b	0.00±0.00 c	0.02±0.00 c	3.19±0.08 c
	Positive Control	32.00±2.00 b	14.00±2.00 b	19.33±1.15 ab	14.67±1.15 b	20.00±2.00 a	54.00±0.00 b	1.77±0.04 b	130.56±0.05 b
	33% of LC50	14.67±1.15 c	21.33±1.15 a	17.33±3.06 bc	26.67±1.15 a	20.00±2.00 a	64.00±0.00 a	2.16±0.04 a	678.20±0.05 a
3rd	Negative Control	98.00±0.00 a	2.00±0.00 c	0.00±0.00 c	0.00±0.00 c	0.00±0.00 c	0.00±0.00 c	0.02±0.00 c	3.22±0.04 c
	Positive Control	38.00±2.00 b	15.33±1.15 ab	14.67±1.15 b	10.67±1.15 b	21.33±1.15 b	46.67±2.31 b	1.62±0.07 b	128.70±0.10 b
	33% of LC50	10.67±1.15 c	12.00±2.00 b	20.00±2.00 a	33.33±1.15 a	24.00±2.00 a	77.33±1.15 a	2.48±0.04 a	694.51±0.14 a
4th	Negative Control	98.00±0.00 a	2.00±0.00 c	0.00±0.00 c	0.00±0.00 c	0.00±0.00 c	0.00±0.00 c	0.02±0.00 c	3.42±0.08 c
	Positive Control	30.67±1.15 b	12.67±2.31 ab	20.00±2.00 b	16.67±1.15 b	20.00±2.00 ab	56.67±1.15 b	1.83±0.04 b	134.58±0.07 b
	33% of LC50	9.33±2.31 c	11.33±1.15 b	34.00±3.46 a	27.33±1.15 a	18.00±2.00 bc	79.33±1.15 a	2.33±0.01 a	681.35±0.05 a
5th	Negative Control	97.33±1.15 a	2.67±1.15 c	0.00±0.00 c	0.00±0.00 c	0.00±0.00 b	0.00±0.00 c	0.03±0.01 c	3.45±0.05 c
	Positive Control	30.00±2.00 b	14.67±1.15 b	16.67±1.15 b	18.00±2.00 b	20.67±1.15 a	55.33±1.15 b	1.85±0.06 b	139.54±0.04 b
	33% of LC50	8.67±1.15 c	18.00±2.00 ab	21.33±1.15 a	31.33±3.06 a	20.67±1.15 a	73.33±3.06 a	2.37±0.09 a	713.70±0.06 a

The means with similar letters in a single column for each variable are statistically non-significant at p<0.05.

*Percentage of Damaged Cells = Class-II + Class-III + Class-IV;

**GDI (Genetic Damage Index) = {(Class-I) +2 (Class-II) +3 (Class-III) +4 (Class-IV) / Class-0 + Class-I + Class-II + Class-III + Class-IV};

***CTL = Cumulative Tail Length of Comets.

Table 2. Time and dose-related DNA damage in *Cyprinus crpio*.

		Percentage of Damaged Cells	*GDI (Genetic Damage Index)	**CTL (µm) (Cumulative Tail Length)
Dose-Dependent Genotoxicity				
Treatments	Negative Control	0.00±0.00 c	0.02±0.00 c	3.30±0.12 c
	Positive Control	52.80±3.95 b	1.76±0.09 b	132.31±4.77 b
	33% of LC$_{50}$	69.46±10.77 a	2.25±0.21 a	668.03±55.27 a
Time-Dependent Genotoxicity				
Fortnights	1st	34.89±30.23 e	1.23±1.05 e	234.58±299.13 e
	2nd	39.33±34.43 d	1.32±1.14 d	270.65±358.65 d
	3rd	41.33±38.94 bc	1.37±1.25 c	275.48±368.28 b
	4th	45.33±40.86 a	1.39±1.22 b	273.12±359.57 c
	5th	42.89±38.22 b	1.42±1.23 a	285.56±376.97 a

The means with similar letters in a single column for each variable are statistically non-significant at $p < 0.05$.

Table 2 shows dose and time-based variations in the percentage of damaged cells, genetic damage index and a cumulative tail length of comets (µm) in the peripheral blood erythrocytes of *Cyprinus carpio*. In the present study, DNA damage showed time and dose-dependent relationship. All comet parameters viz. percentage of damaged cells, genetic damage index and a cumulative tail length of comets (µm) were observed higher at 33% LC$_{50}$, followed by that of positive and negative control exhibiting dose-dependent DNA damage with statistically significant differences among them. Damaged cells (%) increased gradually and reached at maximum during 4th fortnight (45.33±40.86 %) while during 5th fortnight (42.89±38.22 %) slight decrease was observed exhibiting that DNA damage increased significantly with duration of exposure. However, genetic damage indices were maximum (1.42±1.23) and minimum (1.23±1.05) during 5th and 1st fortnight, respectively. The cumulative tail length of comets (µm) gradually increased from 1st to a 3rd fortnight (234.58±299.13 to 275.48±368.28 µm) however, at 4th-fortnight slight decrease in length of comets were observed as evident from their mean value of 273.12±359.57 µm but during 5th fortnight it again increased linearly up to 285.56±376.97 µm.

DISCUSSION

Pesticide effects on organisms were often investigated by using single toxicants under laboratory conditions. However, under environmental conditions a mixture of pesticides or pesticide metabolites were present which may cause interactive effects. Pesticides may cause DNA damage directly due to the action of a parental compound or their metabolites and indirectly by the generation of reactive oxygen species (Oliveira *et al.*, 2009). During present study time and dose-dependent DNA damage was observed underexposure of bifenthrin + chlorpyrifos mixture. Similarly, a significant increase in DNA damage was observed by Polard *et al.* (2011) under the exposure of pesticide mixture, chlorotouron + atrazine + isoproturon + metachlor + deethylatrazine as compared to the control group. Dose and time-dependent genotoxicity in fish associated with pesticide exposure using the single cell gel electrophoresis assay in fish erythrocytes is well documented (Nwani *et al.*, 2010 and 2013; Pandey *et al.*, 2011; Yong *et al.*, 2011; Rani and Kumaraguru, 2013). Pesticide mixture, chlorpyrifos + endosulfan + thiraam has also been reported to cause

significant DNA damage (Tope and Rogers, 2009). Arcaute *et al.* (2014) observed that even acute exposure of herbicide (dicamba) can increase the frequency of primary DNA lesions in circulating blood cells of tropical fish estimated by alkaline single cell gel electrophoresis. All previously discussed genotoxicity assessment was based on evaluation of acute exposure effects. Such an approach fails to provide relevant information regarding the long-term effects of pesticide burden on the genome. Therefore, the main idea of the present study was to characterize DNA damage induced by prolonged exposure to bifenthrin + chlorpyrifos. The binding of chlorpyrifos with DNA to produce DNA adducts leads to an increasing concern about the genotoxic risk of chlorpyrifos in fish. Li *et al.* (2015) observed that the sub-lethal concentrations of chlorpyrifos induced significant concentration-dependent single strand DNA breaks in the treated cells as compared to control group and conclude that chlorpyrifos is a strongly genotoxic agent that can induce DNA damage and cell apoptosis. DNA strand breaks have been mainly ascribed to the excessive accumulation of reactive oxygen species therefore, it was possible that DNA adducts, and reactive oxygen species contribute to the bifenthrin + chlorpyrifos mediated DNA damage. The decrease in the percentage of DNA damage after 4th fortnight day of exposure as observed in the fish exposed to pesticide mixture might indicate the repair of damaged DNA, loss of heavily damaged cells or both. The tail length determines how far the DNA has migrated out of the cell. Smaller DNA fragments move the farthest; therefore, the tail length is predominantly dictated by the size of the DNA fragments generated during the alkaline unwinding step of the single cell gel electrophoresis (Kumaravel and Jha, 2006).

CONCLUSION

Knowledge of the sub-lethal effects of pesticides on the genetic level is very crucial for delineating fish health status and for understanding the future ecological impacts. The current study of DNA damage caused by pesticide mixture may become a key indicator in assessing the general health of freshwater fish. This would also identify the importance of genotoxicity as a monitoring factor for predicting the impacts of pollution on the fish.

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