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Research Article

Determination of genotoxic effects of aluminum on *Cirrhinus mrigala* and *Ctenopharyngodon idella* using comet assay and micronucleus test

Kousar S.[1*]; Javed M[2]; Ambreen F[1]; Abbas S.[3]

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Abstract

Metals are the major pollutants in aquatic environment. A lot of research literature is available globally about accumulation of heavy metals in aquatic ecosystems as well as in different body organs of aquatic animals, especially fish. Heavy metals mainly generate reactive oxygen species (ROS) in aquatic animals that lead to oxidative stress production. ROS cause oxidative damage to different animal tissues and cellular components, like protein and DNA. Aluminum exists in aquatic environments of Pakistan in concentration higher than permissible limits. So, the present study is conducted to determine toxic effects of aluminum on DNA in peripheral blood erythrocytes of fish viz., *Cirrhinus mrigala* and *Ctenopharyngodon idella*. For this purpose, fingerlings of each species were exposed to four sublethal concentrations of 17, 25, 33 and 50%. The effects of aluminum on 96-h LC_{50} concentrations were evaluated for a period of 30 days under controlled laboratory conditions. Results revealed significant toxic effect of this metal on genetic material of fish. Among the four treatments, 50% sublethal concentration 96-h LC_{50} of aluminum caused significantly higher percentage of DNA damage, genetic damage index and cumulative tail length of comets. Highest frequency of micronuclei was observed in 50% treatments of 96-hr LC_{50} exposure of aluminum which was not statistically (p<0.05) similar to micronuclei frequency caused by 33% of 96-hr LC_{50} of aluminum in fish. The findings of this experiment showed that aluminum exposure caused genetic damage in fish. Moreover, comet assay and micronucleus test appeared to be reliable indicators of metallic ion toxicity in aquatic animals.

Keywords: Aluminum, Fish, Comet assay, Micronucleus test, Blood, DNA damage

1-Department of Zoology, GC Women University Faisalabad, Pakistan
2-University of Agriculture, Faisalabad, Pakistan
3-Department of Zoology, University of Jhang, Pakistan
*Corresponding author's Email: dr.safinakousar@gcwuf.edu.pk

Introduction

Metals are present as dissolved ions, suspended and collide ions and solids in aquatic habitats. Metallic toxicity is strongly dependent upon biological processes, redox potential, ionic strength, pH, activities of organic and inorganic chelators and scavenging processes (Larocque and Rasmussen, 1998). Metal pollution gained attention because of toxicity, accumulation (Javed, 2004) and harmful effects of metals in aquatic ecosystems, fish and human health (Javed, 2006; Mudgal *et al*., 2010). Metals can induce DNA damage in different ways. Elevated Aluminum (Al) concentration in water bodies represents a significant environmental threat due to its toxic effects on fish (Jabeen *et al*., 2012). It turns into more soluble form in acidic waters and becomes more toxic, resulting in freshwater fish kill (Gensemer and Playel, 1999). Aluminum acts as pro-oxidant agent endorsing biological oxidation both in vitro and in vivo (Exley, 2004).

Due to growing number of agricultural, commercial and industrial chemicals, the rate of genetic disorders, diseases and mortality of exposed organisms in the natural habitats is increased significantly (Livingstone, 2001; Nehls and Segner, 2001). This stresses the need to study the impacts of these chemicals on the integrity and function of cellular DNA in organisms (Bombail *et al*., 2001; Kousar *et al*., 2018). Genotoxic compounds represent major ecological challenge because they may lead to unusual disorders transmitted to the progeny (Haldsrud and Krøkje, 2009). In general, metallic ion genotoxicity appears to be associated with formation of reactive oxygen species (Soto-Reyes *et al*., 2005). Oxidative stress is ascertained whenever the rate of reactive oxygen species formation surpasses its decomposition by an antioxidant defense system that would cause oxidation of fatty acids, proteins and DNA (Sies, 1993). As a result of oxidative DNA damage, DNA strand breaks, which is characterize as a major class of DNA damage under oxidative stress and a number of modifications occurs in DNA bases (Cadet *et al*., 1997).

Monitoring xenobiotics in freshwater ecosystems by means of resident species can help promote environmental quality as well as human health. Determination and localization of the toxicant's concentration ensure the safety of animal species that are important for human health (Nicareta, 2004). Fish can act as a pragmatic indicator to understand mechanism of stressors' toxic effects in aquatic ecosystems (Vutukuru *et al*., 2006; Birungi *et al*., 2007; Kousar and Javed, 2014a; Ambreen *et al*., 2019).

Several tests are developed to detect the genotoxic effect of metals and other existing genotoxicants in aquatic ecosystems (Fenech *et al*., 2003; Kousar and Javed, 2016). Both micronuclei and comet assay are more popular over other assays in aquatic toxicity research due to their sensitivity in detecting DNA damage and the short

required time to complete the study (Bopp *et al*., 2008; Kousar *et al*., 2018). Micronuclei (MN) are produced from chromosome fragments or whole chromosomes that lag at cell division due to lack of centromere, damage in centromere or a defect in cytokinesis. In tissues with actively dividing cells, micronucleus records reflect clastogenic or aneugenic compounds (Heddle *et al*., 1991). Different approaches are used for evaluation of DNA strand breakage. Comet assay is used as one of the best approaches to study the genotoxic effects of pollutants on fish (Ambreen *et al*., 2018; Ilyas *et al*., 2018). It is used to estimate DNA damage in single cell to evaluate the genetic risk associated with xenobiotic exposures. It is considered a reliable, responsive and fast technique (Tice *et al*., 2000) for the detecting DNA single/double-strand breakage and alkali labile sites, induced in individual eukaryotic cells by physical and chemical agents (Kim *et al*., 2001). This assay is effectively applied to peripheral erythrocytes of various fish species exposed to diverse genotoxicants (Matsumoto *et al*., 2006). It allows investigators to access DNA damage at individual cell level by measuring migration of broken DNA fragments in gel-embedded cells (Bombail *et al*., 2001). Micronuclei test is also used as one of the measures to assess clastogenic effects of a toxicant on erythrocytes of fish. It is a standard method to detect structural chromosomal aberrations derived from initial damage in S-phase (Obe *et al*., 2002).

Pakistan faces acute freshwater pollution problems where only 1% of industrial water is treated before discharge into rivers and streams (Khan *et al*., 2012). Thus, heavy discharge of untreated water into rivers of Pakistan adversely affected the freshwater fisheries (Jabeen *et al*., 2012) and indigenous fish species were on the verge of extension in rivers of Pakistan (Rauf *et al*., 2009). So, comparative toxicity studies are required to identify the effects of metal pollution on fish and devise some proper strategies for sustainability and conservation of fish in their natural habitats. Thus, it is imperative to study selected fish species' acute and chronic responses to toxicity of metallic ions that could affect fish growth and induce genotoxicity in their bodies. This work will help sustainable conservation of freshwater fisheries in Pakistan.

Materials and methods

Experimental conditions

The experiments were conducted to determine the extent of DNA damage in the peripheral erythrocytes of two freshwater fish species (*Cirrhinus mrigala* and *Ctenopharyngodon idella*) caused by aluminum. For this purpose, 150-day old fish (n=08) were acclimatized to laboratory conditions in cemented tanks before the experiment. For this experiment, 96-h LC_{50} of aluminum determined by Kousar and Javed (2016) was used to prepare four sublethal concentrations (17, 25, 33 and

50%) of aluminum for determination of their genotoxic effects. Each fish species was exposed to these four sublethal concentrations, separately, in the aquarium containing 60 liters of water for 30 days. Each test was conducted with three replications. Analytical grade aluminum was selected to test its effect on selected fish species. For preparation of stock solutions, appropriate quantity of aluminum in the form of nitrate (Al_2NO_3. $9H_2O$: Merck) was poured into 1000 ml flask and the flask was filled up to 1000 ml with deionized water. The stock solutions were diluted to make the required concentrations of metallic ions for use in this experiment. Temperature, pH and hardness of each test medium were maintained at 30°C, 7.5 and 300 mgL^{-1}, respectively. During the exposure period, the fish were fed twice daily (34% digestible protein and 3.00 kcal/g digestible energy). Each species of fish was exposed to the following four sublethal concentrations of aluminum (Table 1):

Table 1: Sub-lethal exposure concentration of aluminum metal for *Cirrhinus mrigala* and *Ctenopharyngodon idella*

Metals	Concentrations (mgL^{-1})	Fish Species	
		Cirrhinus mrigala	***Ctenopharyngodon idella***
		Exposure concentrations (mgL^{-1})	
Aluminum	17% of LC_{50}	15.65	13.81
	25% of LC_{50}	23.02	20.31
	33% of LC_{50}	30.39	26.81
	50% of LC_{50}	46.04	40.62

The control fish were kept in clean metal free water for comparison as negative control while Cyclophosphamide (Sigma) was used as positive control. The positive control fish were subjected to an intra-peritoneal injection of cyclophosphamide in a 4% saline solution with a concentration of 20 μgg^{-1}. Blood samples were collected after five days, and slides were prepared for analysis of DNA damage. The test media were replaced weekly and desired metallic ion concentrations were maintained throughout the test duration of 30 days. At the end of 30-day exposure to various aluminum concentrations, the DNA damage in peripheral erythrocytes of fish was evaluated using comet assay and micronucleus test.

Comet assay

Fish blood samples were processed for comet assay according to Singh *et al.* (1988) with minor modifications. After 30-day exposure of aluminum, blood samples were collected from caudal vein of fish (n=5) through sterilized syringe. Utmost care was exercised to avoid the mixing of water and mucus into the blood samples. Heparin sodium salt was used for stabilization of the blood samples. Each blood sample was centrifuged at 1000rpm for 2 minutes in order to separate erythrocytes from the whole blood. The diluted blood sample (60 µL) was mixed with 200 µL of

1.7% low-melting-point (LMP) agarose. Then normal melting point agarose (0.5%) was evenly applied on the glass slide. Mixture (110 µL) of blood and agarose was layered on the slides that were precoated with 0.5% normal melting point (NMP) agarose. The slides were covered with a third layer of 90 µL 0.8% low-melting-point agarose and covered with glass slips. After that slides were placed in lysis buffer at 4 C for 1 hour. Then slides were placed in comet assay tank (CSL-COM 20; Cleaver, UK; Fig. 1) side by side. The tank was filled with fresh electrophoresis solution to a level approximately 0.25 cm above the slides. The slides were left in the solution for 20 minutes to allow unwinding. Electrophoresis was performed using the same solution at 25V, 300 mA for a period of 25 minutes. At the end, slides were neutralized gently with tris buffer and DNA was stained with ethidium bromide.

Analyses of slides

One hundred and fifty cells (50 per replicate) were scored and examined randomly under Epi-Fluorescence microscope (N-400M, American Scope; UK) equipped with a 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. Cells with no DNA damage possess intact nuclei without a tail, whereas cells with DNA damage showed comet like appearance. The length of comet tail formed due to DNA fragments migration is an estimate of DNA damage (Grover *et al.*, 2003). The DNA damage was quantified by visual classification of cells into the following five categories of "comets" corresponding to the tail length, undamaged (Type 0), low-level damage (Type I), medium-level damage (Type II), high-level damage (Type III) and complete damage (Type IV). The extent of DNA damage was expressed as the mean percentage of cells with medium. High and complete damaged DNA was calculated as the sum of cells with damage Types II, III and IV. Genetic Damage Index (GDI) was calculated for each subject by using the following formula:

$$GDI = \frac{(\text{Type I}) + 2(\text{Type II}) + 3(\text{Type III}) + 4(\text{Type IV})}{\text{Type 0} + \text{Type I} + \text{Type II} + \text{Type III} + \text{Type IV}}$$

TriTek CometScore™ (Summerduck, USA) software was used to measure the comet tail length of damaged cells (Costa *et al*., 2011; Jose *et al*., 2011). Cumulative tail length (µm) was obtained by adding tail lengths of all examined cells (n=50 per replicate).

Micronucleus test

For micronucleus test, a blood drop taken from the caudal vein of fish was directly smeared on slides and air-dried. Smears were subsequently fixed in methanol for 10 minutes and stained with 10% Giemsa solution (Wright's stain powder 300mg+Giemsa stain powder 30mg+Absolute methyl alcohol 100 mL) for 8 minutes (Heddle, 1973). Slides were prepared and examined in duplicate. Nuclear abnormalities and frequency of micronuclei were scored under oil emersion (100 X) lens of

binocular microscope (LABOMED CX_{R3}).

Scoring cells with micronuclei and other nuclear abnormalities

Blind scoring of micronuclei and other nuclear abnormalities were performed on coded slides. A total of 2,000 erythrocytes (1000/slide) with intact cellular and nuclear membranes were examined for each fish specimen. The frequency of micronuclei and other nuclear abnormalities, including bi-nucleated, dumbbell shaped, blebbed, notched and de-shaped nuclei were evaluated (per 1,000 cells) by scoring them at a 1,000 X magnification using a binocular microscope (LABOMED CX_{R3}). Micronuclei frequency was calculated by using the following formula:

$$\text{Micronucleus Frequency (\%)} = \frac{\text{Number of cells with micronuclei}}{\text{Total number of cells counted}} \text{ x } 100$$

Statistical analyses of data

Data were statistically analyzed by using Factorial design (RCBD). Means were compared for statistical differences through Tukey's student Newnan-Keul test. To compare the frequency of micronuclei and other nuclear abnormalities between control and treated fish groups and DNA damage in fish, the non-parametric Mann-Whitney U-test was performed. The relationships among various parameters were evaluated using correlation and regression analyses using MSTAT C software (Steel *et al*., 1997).

Results

DNA Damage in Fish Induced due to Aluminum Exposure

Cirrhinus mrigala

The proportion of damaged nuclei varied significantly (p<0.05) in peripheral erythrocytes of *Cirrhinus mrigala* at different exposure concentrations of aluminum, positive and negative controls. Percentage of damaged cells (%) was significantly higher due to exposure of 50% LC_{50} of aluminum, followed by 33%, 25%, positive control, 17% LC_{50} and negative control. However, non-significant difference in the damaged cells (%) was observed between 50% and 33% LC_{50}; and 25% LC_{50} and positive control. *Cirrhinus mrigala* showed significantly higher Genetic Damage Index (GDI) as 2.01±0.06 at 50% of aluminum LC_{50} exposure, followed by that of 33% (1.85±0.03), positive control (1.70±0.02), 25% (1.62±0.07), 17% LC_{50} (1.11±0.07) and negative control (0.08±0.00). However, the value of GDI in erythrocytes of *Cirrhinus mrigala* did not differ significantly between 25% aluminum LC_{50} and positive control treatments. The cumulative tail length of comets varied significantly (p<0.05) due to aluminum exposure at different concentrations, positive and negative controls. The mean tail lengths of comets observed at

33 and 50% of LC_{50} were significantly higher than that of positive control fish (Table 2).

Table 2: Mean±standard deviation values of DNA damages induced in peripheral erythrocytes of fish due to aluminum exposure.

Treatments	Exposure Concentration (mg L^{-1})	Un-damaged Nuclei (%)	Proportions of Damaged Nuclei (%)				Percentage of Damaged Cells (II+III+III)	Genetic Damage Index* (GDI)	Cumulative Tail Length (µm)
		Type 0	Type I	Type II	Type III	Type IV			
Cirrhinus mrigala									
Negative control	0.00	93.33± 1.15 a	5.33± 2.31 e	1.33± 1.15 f	0.00± 0.00 f	0.00± 0.00 e	1.33± 1.15 d	0.08± 0.00 e	3.84± 0.33 f
Positive control	CP (20 µgg^{-1})	20.00± 2.00 d	32.00± 2.00 b	18.67± 2.31 d	16.67± 1.15 d	12.67± 1.15 a	48.00± 2.00 b	1.70± 0.02 c	126.28± 0.28 c
17% of LC_{50}	15.65	31.33± 1.15 b	42.00± 2.00 a	13.33± 2.31 e	11.33± 1.15 e	2.00± 2.00 d	26.67± 2.31 c	1.11± 0.07 d	84.43± 0.20 e
25% of LC_{50}	23.02	22.67± 3.06 c	23.33± 4.16 c	29.33± 8.33 b	18.67± 3.06 c	6.00± 2.00 c	54.00± 4.00 b	1.62± 0.07 c	93.04± 0.21 d
33% of LC_{50}	30.39	19.33± 1.15 de	15.33± 2.31 d	32.00± 2.00 a	27.33± 1.15 b	6.00± 2.00 c	65.33± 1.15 a	1.85± 0.03 b	187.04± 1.00 b
50% of LC_{50}	46.04	18.67± 1.15 e	15.33± 2.31 d	22.00± 2.00 c	34.67± 3.06 a	9.33± 1.15 b	66.00± 2.00 a	2.01± 0.06 a	229.85± 1.97 a
Ctenopharyngodon idella									
Negative control	0.00	91.33± 2.31 a	8.00± 2.00 e	0.67± 1.15 e	0.00± 0.00 f	0.00± 0.00 f	0.67± 1.15 f	0.09± 0.03 f	3.78± 0.48 e
Positive control	CP (20 µgg^{-1})	19.33± 1.15 c	18.67± 4.16 d	29.33± 1.15 b	20.00± 5.29 c	12.67± 1.15 a	62.00± 5.29 c	1.88± 0.10 c	116.22± 0.23 c
17% of LC_{50}	13.81	32.00± 2.00 b	48.00± 2.00 a	12.67± 2.31 d	4.67± 1.15 e	2.67± 3.06 e	20.00± 2.00 e	0.98± 0.11 e	69.22± 0.27 d
25% of LC_{50}	20.31	10.00± 0.00 d	38.00± 2.00 b	40.00± 2.00 a	8.00± 0.00 d	4.00± 0.00 c	52.00± 2.00 d	1.58± 0.02 d	120.23± 0.51 c
33% of LC_{50}	26.81	4.00± 0.00 e	20.67± 1.15c	21.33± 1.15 c	50.67± 1.15 b	3.33± 1.15d	75.33± 1.15 b	2.29± 0.04 b	190.11± 1.08 b
50% of LC_{50}	40.62	5.33± 1.15 e	6.67± 1.15 e	22.67± 1.15 c	54.67± 1.15 a	10.67± 1.15b	88.00± 2.00 a	2.59± 0.06 a	213.87± 0.95 a

*GDI = {Type I + 2(Type II) + 3(Type III) + 4(Type IV) / Type 0 + Type I + Type II + Type III + Type IV}; CP=Cyclophosphamide. Means with different letters in a single column for each variable are significantly different at $p<0.05$.

Ctenopharyngodon idella

Peripheral blood erythrocytes of this fish showed significantly variable proportions of damaged nuclei that were affected due to different exposure concentrations of aluminum, negative and positive controls. The fish exhibited a significantly ($p<0.05$) higher percentage of damaged cells with the mean value of 88.00±2.00% due to exposure of 50% aluminum LC_{50} than that of the positive control (62.00±5.29%). Maximum and minimum GDI values were observed at 50% LC_{50} exposure of metal and negative control, respectively. The cumulative tail length of comets, induced in the peripheral erythrocytes of fish varied significantly ($p<0.05$) within the mean values of 213.87±0.95 and 3.78±0.48µm between 50% aluminum LC_{50} and negative controls, respectively. Exposure of 50% aluminum LC_{50} caused significantly

higher damaged cells, GDI and a cumulative tail length of comets, in the erythrocytes of *Ctenopharyngodon idella*, than those of the positive control (Table 1).

Nuclear abnormalities in fish induced due to aluminum exposure

Cirrhinus mrigala

Exposure of aluminum at 50% LC_{50} to *Cirrhinus mrigala* caused significantly higher induction of micronuclei with the mean frequency of 32.16±0.03%. The sum of other nuclear abnormalities, observed under various concentrations, varied significantly with the mean higher frequency of 24.13±4.35% observed due to 33% LC_{50} exposure that was significantly more than that observed in positive control fish (Table 3).

Table 3: Chronic exposure effects of aluminum on micronuclei frequency (mean ± standard deviation) and other nuclear abnormalities in peripheral blood erythrocytes of *Cirrhinus mrigala*.

	Negative control	Positive control	17% of LC_{50}	25% of LC_{50}	33% of LC_{50}	50% of LC_{50}
Total number of cells with micronuclei	21.00	241.00	198.00	270.00	501.00	658.00
Micronuclei frequency (%)	1.01±0.05 f	11.55±0.12 d	9.80±0.04 e	13.29±0.02 c	24.93±0.10 b	32.16±0.03 a
Other nuclear abnormalities (%)						
Binucleated cells	0.29±0.07 e	1.15±0.06 b	1.09±0.00 c	0.49±0.01 d	1.44±0.03 a	0.00±0.00 f
Cells with dumbbell shaped nucleus	0.14±0.05 f	3.79±0.10 d	1.53±0.02 e	5.02±0.03 b	4.13±0.06 c	5.47±0.11 a
Cells with blebbed nucleus	0.43±0.10 f	1.49±0.13 e	2.38±0.17 d	6.89±1.00 b	2.64±0.01 c	7.38±0.13 a
Cells with notched nucleus	0.34±0.04 f	6.85±0.15 a	0.45±0.02 e	2.26±0.11 d	3.53±0.05 b	2.64±0.10 c
De-shaped cells	0.67±0.10 f	5.27±0.02 b	1.24±0.18 e	1.77±0.19 d	12.39±0.02 a	2.10±0.09 c
Total frequency of other nuclear abnormality (%)	1.87±0.20 f	18.54±2.44 b	6.68±0.70 e	16.44±2.60 d	24.13±4.35 a	17.60±2.91 c
Total number of cells with other nuclear abnormality	39.00	387.00	135.00	334.00	485.00	360.00
Total number (±SD) of analyzed cells	2081.00±1.35	2087.00±1.22	2020.00±1.76	2032.00±2.66	2010.00±3.63	2046.00±2.00

Means with different letters in a single row are significantly different at $p<0.05$.

Ctenopharyngodon idella

In *Ctenopharyngodon idella*, aluminum exposure at 50% of 96h LC_{50} induced significantly higher mean micronuclei frequency of 38.07±0.29%, while it was the lowest as 2.41±0.05% in the negative control treatment. All other nuclear abnormalities, except binucleated nuclei, were significantly higher due to 50% LC_{50} exposure. Mean total frequency of other nuclear abnormalities was significantly higher

due to 50% LC_{50}, followed by 33%, positive control, 25%, 17% LC_{50} and negative control (Table 4).

Table 4: Chronic exposure effects of aluminum on micronuclei frequency (mean ± standard deviation) and other nuclear abnormalities in peripheral blood erythrocytes of *Ctenopharyngodon idella*.

	Negative control	Positive control	17% of LC_{50}	25% of LC_{50}	33% of LC_{50}	50% of LC_{50}
Total number of cells with micronuclei	50.00	372.00	180.00	410.00	612.00	764.00
Micronuclei frequency (%)	2.41±0.05 f	18.41± 1.18 d	9.00±0.88 e	20.05±0.28 c	29.78±0.11 b	38.07±0.29 a
Other nuclear abnormalities (%)						
Binucleated cells	0.10±0.01 f	3.46±0.21 a	1.00±0.03 e	1.37±0.42 d	1.56±0.18 c	2.29± 0.28 b
Cells with dumbbell shaped nucleus	0.19±0.02 f	5.05±0.04 b	3.00±0.10 e	3.23±0.21 d	4.18±0.21 c	5.98±0.03 a
Cells with blebbed nucleus	0.87±0.05 f	3.71±0.03 e	4.00±0.22 d	5.13±0.38 c	5.45±0.08 b	7.42±0.10 a
Cells with notched nucleus	0.39±0.02 f	5.39±0.03 b	3.00±0.10 e	4.25±0.05 d	4.57±0.39 c	6.88±0.22 a
De-shaped cells	0.53±0.01 e	3.86±0.21 d	4.00±0.04 d	5.67±0.18 c	6.42±0.10 b	10.16±0.10 a
Total frequency of other nuclear abnormality (%)	2.07±0.30 f	21.47±0.87 c	15.00±1.22 e	19.66±1.71 d	22.19±1.83 b	32.74±2.85 a
Total number of cells with other nuclear abnormality	43.00	434.00	300.00	402.00	456.00	657.00
Total number (±SD) of analyzed cells	2075±3.86	2021±2.92	2000±4.65	2045±3.37	2055±2.77	2007±3.51

Means with different letters in a single row are significantly different at $p<0.05$.

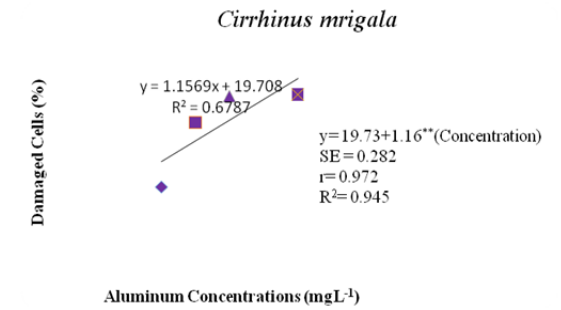
Discussion

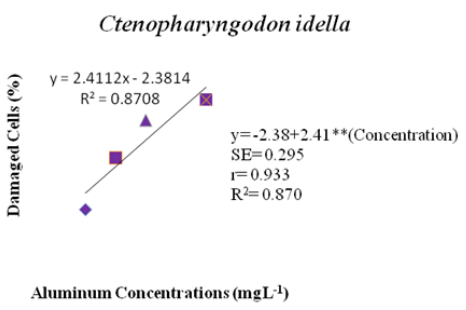
Due to industrial advancement, a variety of toxic chemicals, including metals, are released into aquatic environments of Pakistan (Jabeen *et al*., 2012; Atique *et al*., 2020). Toxic pollutants not only disturb the physico-chemical properties of water bodies (Khanom *et al.*, 2020; Saeed *et al.*, 2020) but also influence the aquatic food chain to cause physiological and cytogenetic alterations in the aquatic animals (Barbosa *et al*., 2010; Kousar *et al*., 2018; Zaib-Un-Nisa *et al*., 2021).

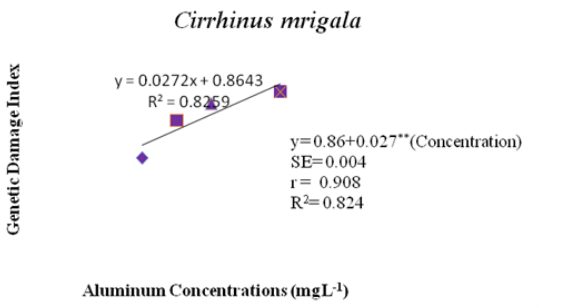
The responses of the two fish species to induce GDI in their erythrocytes were statistically non-significant under the exposures of aluminum. ROS generation and inhibition of DNA repair would lead to genomic instability and oxidative stress in fish (Rossman, 2003). Metals can also act through the redox cycle to induce ROS, which possibly causes DNA strand breakage (Ventura-Lima *et al*., 2009).

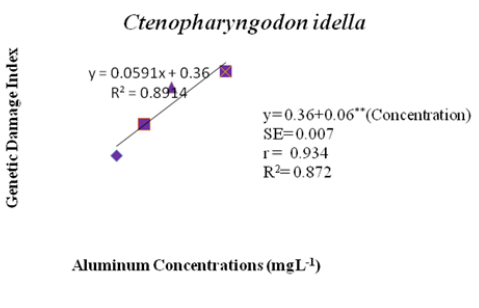
Pereira *et al*. (2013) observed genotoxic effects of cadmium and aluminum on embryonic cells of zebrafish using comet assay that showed significantly higher double-strand breakages due to aluminum than cadmium exposure. DNA damage in aluminum exposed organisms may lead to cytotoxic, mutagenic or carcinogenic gene modifications in their cells (Kumar *et al*., 2010). This higher genotoxic potential of aluminum may be attributed to its ability to oxidize purines and pyrimidines (Niu *et al*., 2005; Jackson and Bartek, 2009).

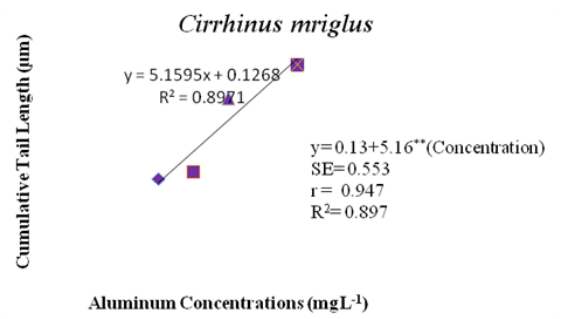
The percentage of damaged cells, GDI and cumulative tail length of comets increased significantly with concomitant exposure concentration, based on their 96-h LC_{50}. In general, the impacts of various treatments / metals to cause damage in cell nuclei,

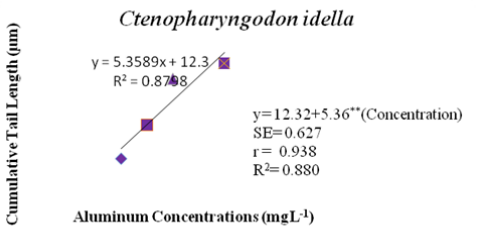


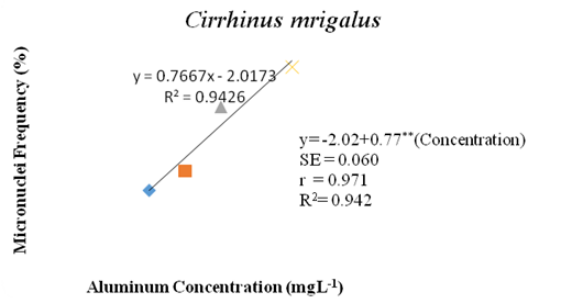


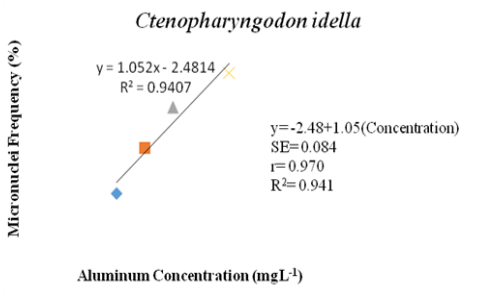












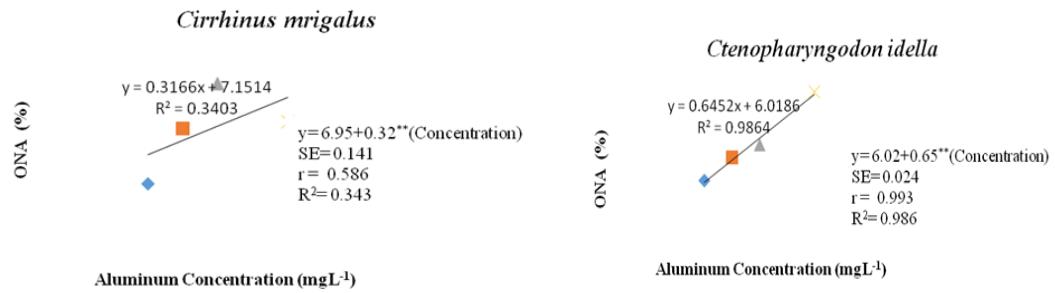


Figure 1: Relationships between exposure concentrations of aluminum and different genetic damages induced in peripheral erythrocytes of fish.

GDI and cumulative tail length of comets varied significantly, that followed the order: 50%> 33%> positive control> 25%> 17% LC_{50}> negative control treatment. Comet assay gained popularity in ecotoxicological studies and successfully detected DNA damage in aquatic organisms (Frenzilli *et al*., 2009), especially in fish (Kosmehl *et al*., 2008; Pereira *et al*., 2010). Comet assay allows examination of whole-genome at any step of cell cycle, rather than just during mitosis (Sunjog *et al*., 2012; Bae *et al*., 2020). Metals produce ROS either through redox cycling or by impairing antioxidant defense system of the animals (Stohs and Bagchi, 1995). In addition to that Fenton reactions are important for generating ROS (Valko *et al*., 2005). Single strand breakage occurred either due to the direct attack of ROS on the deoxyribose or its indirect effects on intermediate steps of the excision repair pathway leading to disruption of the repair process of oxidized DNA bases. This all ultimately ends up in double-strand breakage during the process of replication (Hedge *et al*., 2008). Coogan *et al*. (1991) detected DNA damage in catfish, *Heteropneustes fossilis*, due to both single and double-strand breakage of DNA, DNA-protein and DNA-DNA cross links as a result of chromium interaction with DNA (Fairbairn *et al*., 1995) or due to significant interaction of metal with ROS or this may be the resultant of excision repair enzyme blockage (Speit and Hartman, 1995). Generally, the DNA damage resulting from metal toxicity is broadly used as biomarker of biological effects of toxicants (Van-Der Oost *et al*., 2003; Moon *et al*., 2020). With increasing DNA damage in the cell nucleus, more broken DNA fragments migrate towards the tail region of the comet resulting in an increased amount of fluorescence in the tail region, as well as enlargement of tail length (Mitchelmore and Chipman, 1998) as observed during present investigation. Comet tail length is appeared as a basic parameter in quantifying DNA damage in peripheral fish erythrocytes. García-Medina *et al*. (2010) reported aluminum concentration-dependent increase in DNA damage in lymphocytes of *Cyprinus carpio*. Higher DNA damage in erythrocytes of mosquito fish, *Gambusia holbrooki*, is also observed

due to aluminum toxicity by Ternjej *et al*. (2010).

During the present investigation, mean sensitivity of both fish species in terms of percentage of damaged cells, GDI and cumulative tail length of comets varied significantly due to exposure to different concentrations of metal. *Cirrhinus mrigala* was significantly more susceptible to metal's toxicity among the two fish species. They had significantly higher percentage of DNA damaged cells, GDI and a cumulative tail length of comets. Different fish species showed significantly variable responses towards metal's toxicity, due to differences in their genetic susceptibilities, ability of DNA repair (Wojewódzka *et al*., 1998), the number of alkali-labile sites, concentration of antioxidant enzymes and fish metabolic rate (Buschini *et al*., 2004). Nagpure *et al*. (2008) observed species specific variations for comet tail lengths in erythrocytes of *Labeo rohita* and *Puntius puntio*. Significant higher tail DNA percentages in *Cirrhinus mrigala* indicate its higher susceptibility to metal under study, than the other fish species. Kopjar *et al.* (2008) reported that DNA damage in comet tail length, induced in peripheral erythrocytes of Balkan loach (*Cobitis elongata*), increased significantly due to toxicity of industrial effluents containing arsenic, copper, mercury, chromium, manganese and strontium.

The variability in DNA damage due to various metals in different fish species may be attributed to different metallothionein levels which is a molecular biomarker used extensively in aquatic organisms (Andrews, 2000). It works as metal-chelating agent that plays an important role in detoxifying metals in aquatic organisms, through oxygen-free radical scavenging actions and metal binding (Kalpaxis *et al*., 2004). Higher aluminum accumulation in *Ctenopharyngodon idella* stimulated the production of reactive oxygen species that ultimately leads to oxidative stress in fish (Frenández-Dávila *et al*., 2012) as observed during the present investigation.

Micronucleus test is commonly used to estimate biological impacts of water pollutants on genotoxic damage in fish (Ergene *et al*., 2007). Micronuclei are cytoplasmic chromatin containing bodies developed from broken chromosome sections or from the chromosomes that could not be incorporated into daughter nuclei (Fagr *et al*., 2008). Various metallic ions act as valuable genotoxins at particular concentrations just because of their ability to bind to thiol groups and induce instability in the spindle formation in cells (Patra *et al*., 2004).
During the present investigation, the extant of micronuclei and other nuclear abnormalities in their peripheral erythrocytes of both fish species varied significantly due to aluminum exposure. The 50% LC_{50} exposure of aluminum-induced significantly higher micronuclei in fish erythrocytes than positive control. It is also reported to cause oxidative damage to the proteins and enzymes due to its high affinity for their sulfhydryl groups (Bhattacharya

and Bhattacharya, 2007). Yadav and Trivedi (2009) assessed mutagenic potential of mercuric chloride, arsenic trioxide and copper sulphate in vivo using spotted snakehead fish, *Channa punctata*. A previous study indicated a relationship between genotoxicity response variability and metabolic and pharmacokinetic factors in animals (Al-Sabti and Metcalfe, 1995). In general, such nuclear abnormalities can be considered good indicators of genotoxic damage, and therefore, they may complement micronuclei scoring in routine genotoxicity surveys. Ahmed *et al*. (2011) observed concentration dependent increase in micronuclei frequency in the erythrocytes of *Oreochromis mossambicus*. In *Channa punctatus*, the frequency of micronuclei was found significantly higher due to arsenic exposure while it was lower in control treatment fish (Patowary *et al*., 2012).

Cirrhinus mrigala appeared significantly more sensitive and induced a higher frequency of micronuclei in its peripheral erythrocytes than *Ctenopharyngodon idella*. So, the induction of other nuclear abnormalities in the erythrocytes of fish species followed the order: *Cirrhinus mrigala*> *Ctenopharyngodon idella*. Micronucleus test showed inter species variability in micronuclei frequency as reported by Buschini *et al*. (2004), Kim and Hyun (2006), Kumar *et al*. (2008) and Barbosa *et al*. (2010). These variations may occur due to difference in metabolic indices of different fish species, DNA repair ability, as well as cell proliferation in tissues. Genotoxic damage mainly depends on the type of pollutant involved and fish species exposed to that pollutant (Ali *et al*., 2008). Ambreen and Javed (2016) reported concentration and time-dependent genotoxic damage in peripheral blood erythrocytes of fish, *Cyprinus carpio*, exposed to metals mixture. Similarly, Kousar and Javed (2014b) reported that arsenic exposure could cause concentration dependent DNA damage in peripheral erythrocytes of *L. rohita, C. mrigala* and *Gibelion catla*. Galindo *et al*. (2010) reported that aluminum exposure caused significant increase in frequency of other nuclear abnormalities, while micronuclei frequency did not vary significantly in the erythrocytes of streaked prochilod of South America, *Prochilodus lineatus*. The DNA damage, determined in terms of percentage of damaged cells, GDI, cumulative tail length (µm), micronuclei frequency (%), and frequency of other nuclear abnormalities (%) showed significant direct relationships with the metallic ion concentrations. Summak *et al*. (2010) reported a significant positive correlation (r=0.980) between metal concentrations and frequency of nuclear abnormalities in *Oreochromis niloticus*. Results exhibited variable genotoxic damage in peripheral blood erythrocytes of both fish species. Moreover, it is concluded that micronucleus and comet assay can be

used as reliable tools to determine genotoxic effects of pollutants in fish.

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References

Ahmed, M.K., Al-Mamun, M.H., Hossain, M.A., Arif, M., Parvin, E., Akter, M.S., Khan, M.S. and Islam, M.M., 2011. Assessing the genotoxic potentials of arsenic in tilapia (*Oreochromis mossambicus*) using alkaline comet assay and micronucleus test. *Chemosphere*, 84(**1**), 143–149. https://doi.org/10.1016/j.chemosphere.2011.02.025.

Ali, F.K., El-Shehawi, A.M. and Seehy, M.A., 2008. Micronucleus test in fish genome: A sensitive monitor for aquatic pollution. *African Journal of Biotechnology*, 7(**5**), 606–612.

Al-Sabti, K. and Metcalfe, C.D., 1995. Fish micronuclei for assessing genotoxicity in water. *Mutation Research*, 343(**2–3**), 121–135. https://doi.org/10.1016/0165-1218(95)90078-0.

Ambreen, F. and Javed, M., 2016. Effect of 30 day sub-lethal exposure of cadmium and lead mixture on DNA damage in fish. *The Journal of Animal and Plant Sciences*, 26(**3**), 674–679.

Ambreen, F., Javed, M., Abbas, S., Kousar, S., Ilyas, R. and Batool, M., 2018. DNA damage in peripheral erythrocytes of *Ctenopharyngodon idella* during chronic exposure to pesticide mixture. *Pakistan Journal of Zoology*. *Supplementary Series*, 13, 19–25.

Ambreen, F., Javed, M., Kousar, S., Abbas, S. and Ilyas, R., 2019. Genotoxic potential of pesticide mixture against common carp. *Proceeding of Pakistan Congress of Zoology*, 39, 7–12.

Andrews, G.K., 2000. Regulation of metallothionein gene expression by oxidative stress and metal ions. *Biochemical Pharmacology*, 59(**1**), 95–104. https://doi.org/10.1016/s0006-2952(99)00301-9.

Atique, U., Iqbal, S., Khan, N., Qazi, B., Javeed, A., Anjum, K.M., Haider, M.S., Khan, T.A., Mahmood, S. and Sherzada, S., 2020. Multivariate assessment of water chemistry and metals in a river impacted by tanning industry. *Fresenius Environmental Bulletin*, 29, 3013–3025.

Bae, D.Y., Atique, U., Yoon, J.H., Lim, B.J. and An, K.G., 2020. Ecological risk assessment of urban streams using fish biomarkers of DNA damage and physiological responses. *Polish Journal of Environmental Studies.* 29(**2**), 1077–1086. https://doi.org/10.15244/pjoes/104660.

Barbosa, J.S., Cabral, T.M., Ferreira, D.N., Agnez-Lima, L.F. and de-Medeiros, S.R.B., 2010. Genotoxicity assessment in aquatic environment impacted by the presence of heavy metals. *Ecotoxicology and Environmental Safety*, 73(**3**), 320–325. https://doi.org/10.1016/j.ecoenv.2009.10.008.

Bhattacharya, A. and Bhattacharya, S., 2007. Induction of oxidative stress by arsenic in *Clarias batrachus*: Involvement of peroxisomes. *Ecotoxicology and Environmental Safety*, 66(**2**), 178–187. https://doi.org/10.1016/j.ecoenv.2005.11.002.

Birungi, Z., Masola, B., Zaranyika, M.F., Naigaga, I. and Marshall, B., 2007. Active biomonitoring of trace heavy metals using fish (*Oreochromis niloticus*) as bioindicator species. The case of Nakivubo wetland along Lake Victoria. *Physics and Chemistry of the Earth, Parts A/B/C*, 32(**15–18**), 1350–1358. https://doi.org/10.1016/j.pce.2007.07.034.

Bombail, V., Gordon, D.A.E. and Batty, J., 2001. Application of the comet and micronucleus assays to butterfish (*Pholis gunnellus*) erythrocytes from the Firth of Forth, Scotland. *Chemosphere*, 44(**3**), 383-392. https://doi.org/10.1016/s0045-6535(00)00300-3.

Bopp, S.K., Abicht, H.K. and Knauer, K., 2008. Copper-induced oxidative stress in rainbow trout gill cells. *Aquatic Toxicology*, 86(**2**), 197–204. https://doi.org/10.1016/j.aquatox.2007.10.014.

Buschini, A., Martino, A., Gustavino, B., Monfrinotti, M., Poli, P., Rossi, C., Santoro, M., Dorr, A.J.M. and Rizzoni, M., 2004. Comet assay and micronucleus test in circulating erythrocytes of *Cyprinus carpio* specimens exposed in situ to lake waters treated with disinfectants for potabilization. *Mutation Research*, 557(**2**), 119–129. https://doi.org/10.1016/j.mrgentox.2003.10.008.

Cadet, J., Berger, M., Douki, T. and Ravanat, J.L, 1997. Oxidative damage to DNA: Formation, measurement and biological significance. *Reviews of Physiology, Biochemistry and Pharmacology*, 131, 1–87. https://doi.org/10.1007/3-540-61992-5_5.

Coogan, T.P., Mots, J. Snyder, C.A., Squibb, K.S. and Costa, M. 1991. Differential DNA-protein crosslinking in lymphocytes and liver following chronic drinking water exposure of rat to potassium chromate. *Toxicology and Applied Pharmacology*, 109(**1**), 60–72. https://doi.org/10.1016/0041-008x(91)90191-g.

Costa, P.M., Neuparth, T.S., Caeiro, S., Lobo, J., Martins, M., Ferreira, A.M., Caetano, M., Vale, C., DelValls, T.Á. and Costa, M.H., 2011. Assessment of the genotoxic potential of contaminated estuarine

sediments in fish peripheral blood: Laboratory versus in situ studies. *Environmental Research*, 111(**1**), 25–36. https://doi.org/10.1016/j.envres.2010.09.011.

Ergene, S., Cavaş, T., Celik, A., Köleli, N., Kaya, F. and Karahan, A., 2007. Monitoring of nuclear abnormalities in peripheral erythrocytes of three fish species from the Goksu Delta (Turkey): genotoxic damage in relation to water pollution. *Ecotoxicology*, 16(**4**), 385–391. https://doi.org/10.1007/s10646-007-0142-4.

Exley, C., 2004. The pro-oxidant activity of aluminum. *Free Radical Biology and Medicine*, 36(**3**), 380–387. https://doi.org/10.1016/j.freeradbiomed.2003.11.017.

Fairbairn, D.W., Olive, P.L. and O'Neill, K.L., 1995. The comet assay: a comprehensive review. *Mutation Research*, 339(**1**), 37–59. https://doi.org/10.1016/0165-1110(94)00013-3.

Fagr, A., El-shehawi, A.M. and Seehy, M.A. 2008. Micronucleus test in fish genome: A sensitive monitor for aquatic pollution. *African Journal of Biotechnology*, 7(5): 606-612.

Fenech, M., Chang, W.P., Kirsch-Volders, M., Holland, N., Bonassi, S. and Zeiger, E., 2003. HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte culture. *Mutation Research*, 534(**1–2**), 65–75. https://doi.org/10.1016/s1383-5718(02)00249-8.

Frenández-Dávila, M.L., Razo-Estrada, A.C., Garcia-Medina, S., Gomez-Oliván, L.M., Piñón-López, M.J. and Ibarra, R.G., 2012. Aluminum-induced oxidative stress and neurotoxicity in grass carp (Cyprinidae – *Ctenopharyngodon idella*). *Ecotoxicology and Environmental Safety*, 76(**2**), 87–92. https://doi.org/10.1016/j.ecoenv.2011.09.012.

Frenzilli, G., Nigro, M. and Lyons, B.P., 2009. The comet assay for the evaluation of genotoxic impact in aquatic environments. *Mutation Research / Reviews in Mutation Research*, 681(**1**), 80–92. https://doi.org/10.1016/j.mrrev.2008.03.001.

Galindo, B.A., Troilo, G., Cólus, I.M.S., Martinez, C.B.R. and Sofia, S.H., 2010. Genotoxic effects of aluminum on the neotropical fish, *Prochilodus lineatus*. *Water, Air and Soil Pollution*, 212, 419–428. https://doi.org/10.1007/s11270-010-0357-5.

García-Medina, S., Razo-Estrada, A.C., Gómez-Oliván, L.M., Amaya-Chávez, A.A., Madrigal-Bujaidar, E. and Galar-Martínez, M., 2010. Aluminum-induced oxidative stress in lymphocytes of common carp (*Cyprinus carpio*). *Fish Physiology and Biochemistry*, 36(**4**), 875–882.

https://doi.org/10.1007/s10695-009-9363-1.

Gensemer, R.W. and Playle, R.C., 1999. The bioavailability and toxicity of aluminum in aquatic environments. *Critical Reviews in Environmental Science and Technology*, 29(**4**), 315–450. https://doi.org/10.1080/10643389991259245.

Grover, P., Danadevi, K., Mahboob, M., Rozati, R., Saleha Banu, B. and Rahman, M.F., 2003. Evaluation of genetic damage in workers employed in pesticide production utilizing the comet assay. *Mutagenesis*, 18(2), 201–205. https://doi.org/10.1093/mutage/18.2.201.

Haldsrud, R. and Krøkje, A., 2009. Induction of DNA double-strand breaks in the H4IIE cell line exposed to environmentally relevant concentrations of copper, cadmium, and zinc, singly and in combinations. *Journal of Toxicology and Environmental Health, Part A.*, 72(**3–4**), 155–163. https://doi.org/10.1080/15287390802538964.

Heddle, J.A., 1973. A rapid in vivo test for chromosomal damage. *Mutation Research*, 18(2), 187–190. https://doi.org/10.1016/0027-5107(73)90035-3.

Heddle, J.A., Cimino, M.C., Hayashi, M., Romagna, F., Shelby, M.D., Tucker, J.D., Vanparys, P. and MacGregor, J.T., 1991. Micronuclei as an index of cytogenitic damage: past, present and future. *Environmental and Molecular Mutagenesis*, 18(**4**), 277–291. https://doi.org/10.1002/em.2850180414.

Hedge, M.L., Hazra, T.K. and Mitra, S., 2008. Early steps in the DNA base excision/single-strand interruption repair pathway in mammalian cells. *Cell Research*, 18(**1**), 27–47. https://doi.org/10.1038/cr.2008.8.

Ilyas, R., Javed, M., Kousar, S., Ambreen, F., Batool, M. and Azmat, H., 2018. Genotoxicity of dietary bifenthrin in peripheral blood erythrocytes of fresh water fish, *Cirrhina mrigala*. Pakistan Journal of Zoology, Supplementary Series, 13, 26–31.

Jabeen, G., Javed, M. and Azmat, H., 2012. Assessment of heavy metals in the fish collected from the river Ravi, Pakistan. *Pakistan Veterinary Journal*, 32(**1**), 107–111.

Jackson, S.P. and Bartek, J., 2009. The DNA-damage response in human biology and disease. *Nature*, 461, 1071–1078. https://doi.org/10.1038/nature08467.

Javed, M., 2004. Comparison of selected heavy metals toxicity in the planktonic biota of the river Ravi. *Indus Journal of Biological Sciences*, 1(**2**), 59–62.

Javed, M., 2006. Studies on growth responses of fish during chronic exposures of nickel and manganese. *Pakistan Journal of Biological Sciences*, 9(**2**), 318–322.

https://doi.org/10.3923/pjbs.2006.318.322.

Jose, S., Jayesh, P., Mohandas, A., Philip, R. and Singh, I.S.B., 2011. Application of primary haemocyte culture of *Penaeus monodon* in the assessment of cytotoxicity and genotoxicity of heavy metals and pesticides. *Marine Environmental Research*, 71(**3**), 169–177. https://doi.org/10.1016/j.marenvres.2010.12.008.

Kalpaxis, D.L., Theos, C., Xaplanteri, M.A., Dinos, G.P., Catsiki, A.V. and Leotsinidis, M., 2004. Biomonitoring of Gulf of Patras, *N. Peloponnesus*, Greece. Application of a biomarker suite including evaluation of translation efficiency in *Mytilus galloprovincialis* cells. *Environmental Research*, 94(**2**), 211–220. https://doi.org/10.1016/s0013-9351(03)00048-3.

Khanom, D.A., Nesa, A., Jewel, M.A.S., Haque, M.A., Paul, A.K., Iqbal, S., Atique, U. and Alam, L., 2020. Muscular tissue bioaccumulation and health risk assessment of heavy metals in two edible fish species (*Gudusia chapra* and *Eutropiichthys vacha*) in Padma River, Bangladesh. *Punjab University Journal of Zoology*, 35(**1**), 81–89. https://doi.org/10.17582/journal.pujz/2020.35.1.81.89.

Khan, B., Khan, H., Muhammad, S. and Khan, T., 2012. Heavy metals concentration trends in three fish species from Shah Alam River (Khyber Pakhtunkhwa Province, Pakistan). *Journal of Natural and Environmental Science*, 3(**1**), 1–8.

Kim, G.W., Noshita, N., Sugawara, T. and Chan. P.H., 2001. Early decrease in DNA repairs proteins, Ku70 and Ku86, and subsequent DNA fragmentation after transient focal cerebral ischemia in mice. *Stroke*, 32(**6**), 1401–1407. https://doi.org/10.1161/01.str.32.6.1401.

Kim, I.Y. and Hyun, C.K., 2006. Comparative evaluation of the alkaline comet assay with the micronucleus test for genotoxicity monitoring using aquatic organisms. *Ecotoxicology and Environmental Safety*, 64(**3**), 288–297. https://doi.org/10.1016/j.ecoenv.2005.05.019.

Kopjar, N., Mustafic, P., Zanella, D., Buj, I., Caleta, M., Marcic, Z., Milic, M., Dolenec, Z. and Mrakovcic, M., 2008. Assessment of DNA integrity in erythrocytes of *Cobitis elongata* affected by water pollution: the alkaline comet assay study. *Folia Zoologica*, 57(**1**), 120–130.

Kosmehl, T., Hallare, A.V., Braunbeck, T. and Hollert, H., 2008. DNA damage induced by genotoxicants in zebrafish (*Danio rerio*) embryos after contact exposure to freeze-dried sediment and sediment extracts from Laguna Lake (The Philippines) as measured by the comet assay. *Mutation Research Genetic Toxicology and Environmental Mutagenesis*, 650(**1**),

1–14. https://doi.org/10.1016/j.mrgentox.2007.09.009.

Kousar, S. and Javed, M., 2014a. Assessment of DNA damage in peripheral blood erythrocytes of fish exposed to arsenic under laboratory conditions. *International Journal of Current Microbiology and Applied Sciences*, 3(11), 877–888.

Kousar, S. and Javed, M., 2014b. Heavy metals toxicity and bioaccumulation patterns in the body organs of four freshwater fish species. *Pakistan Veterinary Journal*, 34(**2**), 161–164.

Kousar, S. and Javed, M., 2016. Determination of arsenic induced nuclear abnormalities in peripheral blood erythrocytes of carps using micronucleus test. *The Journal of Animal and Plant Sciences*, 26(**5**), 1501–1506.

Kousar, S., Javed, M., Ambreen, F., Ashraf, A., Ilyas, R., Batool, M. and Azmat, H., 2018. Assessment of copper induced genotoxicity in peripheral erythrocytes of *Cirrhina mrigala* by using comet assay and micronucleus test. *Pakistan Journal of Zoology, Supplementary Series*, 13, 65–73.

Kumar, V.J.R., Achuthan, C., Manju, N.J., Philip, R. and Singh, I.S.B., 2008. Activated packed bed bioreactors (PBBR) for the rapid nitrification in brackish water hatchery systems. *Journal of Industrial Microbiology and Biotechnology*, 36(**3**), 355–365. https://doi.org/10.1007/s10295-008-0504-9.

Kumar, R., Nagpure, N.S., Kushwaha, B., Srivastava, S.K. and Lakra, W.S., 2010. Investigation of the genotoxicity of malathion to freshwater teleost fish *Channa punctatus* (Bloch) using the micronucleus test and comet assay. *Archives of Environmental Contamination and Toxicology*, 58(**1**), 123–130. https://doi.org/10.1007/s00244-009-9354-3.

Larocque, A.C.L. and Rasmussen, P.E., 1998. An overview of trace metals in the environment, from mobilization to remediation. *Environmental Geology*, 33, 85–91. https://doi.org/10.1007/s002540050227.

Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. *Marine Pollution Bulletin*, 42(**8**), 656–666. https://doi.org/10.1016/s0025-326x(01)00060-1.

Matsumoto, S.T., Mantovani, M.S., Malaguttii, M.I.A., Dias, A.L., Fonseca, I.C. and Marin-Morales, M.A., 2006. Genotoxicity and mutagenicity of water contaminated with tannery effluents, as evaluated by the micronucleus test and comet assay using the fish *Oreochromis niloticus* and chromosome aberrations in onion root-tips. *Genetics and Molecular Biology*, 29(**1**), 148–158.

https://doi.org/10.1590/s1415-47572006000100028.

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*, 399(**2**), 135–147. https://doi.org/10.1016/s0027-5107(97)00252-2.

Moon, W.K., Atique, U. and An, K.G., 2020. Ecological risk assessments and eco-toxicity analyses using chemical, biological, physiological responses, DNA damages and gene-level biomarkers in Zebrafish (*Danio rerio*) in an urban stream. *Chemosphere*, 239, 124754. https://doi.org/10.1016/j.chemosphere.2019.124754.

Mudgal, V., Madaan, N., Mudgal, A., Singh, R.B. and Mishra, S. 2010. Effect of toxic metals on human health. *The Bentham Open Nutraceuticals Journal*, 3, 94–99.

Nagpure, N.S., Sharma, S., Pandey, S., Kumar, R., Srivatava, S.K., Verma, M.S. and Kapoor, D., 2008. Use of comet assay for genotoxicity assessment in fishes from Gomti River. *Indian Journal of Fisheries*, 55(**3**), 285–288.

Nehls, S. and Segner, H., 2001. Detection of DNA damage in two cell lines from rainbow trout, RTG-2 and RTL-W1, using the comet assay. *Environmental Toxicology*, 16(**4**), 321–329. https://doi.org/10.1002/tox.1039.

Nicareta, L., 2004. Biomarkers for the detection of sublethal effects caused by deltamethrin in *Ancistrus multispinnis*. Master's Dissertation, University of Parana, Brazil.

Niu, P.Y., Niu, Q., Zhang, Q.L., Wang, L.P., He, S.E., Wu, T.C., Conti, P., Gioacchino, M.D. and Boscolo, P., 2005. Aluminum impairs rat neural cell mitochondria in vitro. *International Journal of Immunopathology and Pharmacology*, 18(**4**), 683–689. https://doi.org/10.1177/039463200501800410.

Obe, G., Pfeiffer, P., Savage, J.R.K., Johannes, C., Goedecke, W., Jeppesen, P., Natarajan, A.T., Martínez-López, W., Folle, G.A. and Drets, M.E., 2002. Chromosomal aberrations: formation, identification and distribution. *Mutation Research*, 504(**1–2**), 17–36. https://doi.org/10.1016/s0027-5107(02)00076-3.

Patowary, K., Hazarika, N.S. and Goswami, M., 2012. Studies on the toxic impact of arsenic on some enzymes and chromosomes of *Channa punctatus*. *The Clarion*, 1(**2**), 148–153.

Patra, M., Bhowmik, N., Bandopadhyay, B. and Sharma, A., 2004. Comparison of mercury, lead and arsenic with respect to genotoxic effects on plant systems and the development of genetic tolerance. *Environmental and Experimental Botany*, 52(**3**), 199–223.

https://doi.org/10.1016/j.envexpbot.2004.02.009.

Pereira, C.S.A., Guilherme, S.I.A.G., Barroso, C.M.M., Verschaeve, L., Pacheco, M.G.G. and Mendo, S.A.L.V., 2010. Evaluation of DNA damage induced by environmental exposure to mercury in *Liza aurata* using the comet assay. *Archives of Environmental Contamination and Toxicology*, 58(**1**), 112–122. https://doi.org/10.1007/s00244-009-9330-y.

Pereira, S., Cavalie, I., Camilleri, V., Gilbin, R. and Adam-Guillermin, C., 2013. Comparative genotoxicity of aluminum and cadmium in embryonic zebrafish cells. *Mutation Research*, 750(**1–2**), 19–26. https://doi.org/10.1016/j.mrgentox.2012.07.007.

Rauf, A., Javed, M., Ubaidullah, M. and Abdullah, S., 2009. Assessment of heavy metals in sediments of river Ravi, Pakistan. *International Journal of Agriculture and Biology*, 11(**2**), 197–200.

Rossman, T.G., 2003. Mechanism of arsenic carcinogenesis: an integrated approach. *Mutation Research*, 533(**1–2**), 37–65. https://doi.org/10.1016/j.mrfmmm.2003.07.009.

Saeed, F., Iqbal, K.J., Atique, U., Javid, A., Khan, N., Iqbal, S., Majeed, H., Azmat, H., Khan, B.Y.A., Baboo, I., Shahid, M.T. and Afzal, G., 2020. Toxic trace metals assessment in selected organs of edible fish species, sediment and water in Head Punjnad, Punjab, Pakistan. *Punjab University Journal of Zoology*, 35(**1**), 43–50. https://doi.org/10.17582/journal.pujz/2020.35.1.43.50.

Sies, H., 1993. Strategies of antioxidant defense. *European Journal of Biochemistry*, 215(**2**), 213–219. https://doi.org/10.1111/j.1432-1033.1993.tb18025.x.

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(**1**), 184–191. https://doi.org/10.1016/0014-4827(88)90265-0.

Soto-Reyes, E., Razo, L.M.D., Valverde, M. and Rojas, E., 2005. Role of the alkali-labile sites, reactive oxygen species and antioxidants in DNA damage induced by methylated trivalent metabolites of inorganic arsenic. *BioMetals*, 18(**5**), 493–506. https://doi.org/10.1007/s10534-005-0858-7.

Speit, G. and Hartmann, A., 1995. The contributions of excision repair to the DNA effects seen in the alkaline single cell gel test (comet assay). *Journal of Mutagenesis*, 10(**6**), 555–559. https://doi.org/10.1093/mutage/10.6.555.

Steel, R.G.D., Torrie, J.H. and Dickey, D.A., 1997. Principles and procedures of statistics. A biometrical approach, 3rd edition. McGraw-Hill Book Company, New York, USA.

Stohs, S.J. and Bagchi, D., 1995. Oxidative mechanisms in the toxicity of metals ions. *Free Radical Biology and Medicine*, 18(**2**), 321–336. https://doi.org/10.1016/0891-5849(94)00159-h.

Summak, S., Aydemir, N.C., Vatan, O., Yilmaz, D., Zorlu, T. and Bilaloğlu, R., 2010. Evaluation of genotoxicity from Nilufer Stream (Bursa/Turkey) water using piscine micronucleus test. *Food and Chemical Toxicology*, 48(**8-9**), 2443–2447. https://doi.org/10.1016/j.fct.2010.06.007.

Sunjog, K., Gačić, Z., Kolarević, S., Višnjić-Jeftić, Ž., Jarić, I., Knežević-Vukčević, J., Vuković-Gačić, B. and Lenhardt, M., 2012. Heavy metal accumulation and the genotoxicity in barbel (*Barbus barbus*) as indicators of the Danube River pollution. *Scientific World Journal*, 2012, 351074. https://doi.org/10.1100/2012/351074

Ternjej, I., Mihaljević, Z., Stanković, I., Kerovec, M., Sipos, L., Zeljezić, D. and Kopjar, N., 2010. Estimation of DNA integrity in blood cells of eastern mosquitofish (*Gambusia holbrooki*) inhabiting an aluminum-polluted water environment: an alkaline comet assay study. *Archives of Environmental Contamination and Toxicology*, 59(**2**), 182–193. https://doi.org/10.1007/s00244-010-9469-6.

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C. and Sasaki, Y.F., 2000. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environmental and Molecular Mutagensis*, 35(**3**), 206–221. https://doi.org/10.1002/(sici)1098-2280(2000)35:3<206::aid-em8>3.0.co;2-j.

Valko, M., Morris, H. and Cronin, M.T.D., 2005. Metals, toxicity and oxidative stress. *Current Medical Chemistry*, 12(**10**), 1161–1208. https://doi.org/10.2174/0929867053764635.

Van der Oost, R., Beyer, J. and Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology*, 13(**2**), 57–149. https://doi.org/10.1016/s1382(02)00126-6.

Ventura-lima, J., de Castro, M.R., Acosta, D., Fattorini, D., Regoli, F., de Carvalho, L.M., Bohrer, D., Geracitano, L.A., Barros, D.M., Marins, L.F.F., da Silva, R.S., Bonan, C.D., Bogo, M.R. and Monserrat, J.M., 2009. Effects of arsenic (As) exposure on the antioxidant status of gills of the zebrafish *Danio rerio* (Cyprinidae). *Comparative Biochemistry and Physiology - Part C - Toxicology and Pharmacology*, 149(**4**), 538–543. https://doi.org/10.1016/j.cbpc.2008.12.003.

Vutukuru, S.S., Chintada, S., Madhavi, K.R., Rao, J.V. and Anjaneyulu, Y., 2006. Acute effects of copper on superoxide dismutase, catalase and lipid peroxidation in the freshwater teleost fish, *Esomus danricus. Fish Physiology and Biochemistry*, 32, 221–229. https://doi.org/10.1007/s10695-006-9004-x.

Wojewódzka, M., Kruszewski, M., Iwaneñko, T., Collin, A.R. and Szumiel, I., 1998. Application of comet assay for monitoring DNA damage in workers exposed to chronic low-dose irradiation. I. Strand breakage. *Mutation Research*, 416(**1-2**), 21–35. https://doi.org/10.1016/s1383-5718(98)00073-4.

Yadav, K.K. and Trivedi, S.P., 2009. Sublethal exposure of heavy metals induces micronuclei in fish, *Channa punctata*. *Chemosphere*, 77(**11**), 1495–1500. https://doi.org/10.1016/j.chemosphere.2009.10.022.

Zaib-Un-Nisa, Sultana, S., Al-Ghanim, K.A., Ghazla, Khan, F.Q., Al-Misned, F., Atique, U., Ahmed, Z. and Mahboob, S., 2021. Comparative assessment of heavy metal bioaccumulation in skeletal muscles of softshell and hard-shell freshwater turtles. *Journal of King Saud University – Science*, 33(**5**), 101463. https://doi.org/10.1016/j.jksus.2021.101463.