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Microelectrophoretic study of environmentally induced DNA damage in fish and its use for early toxicity screening of freshwater bodies

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Abstract This study investigates the potential of the comet and micronucleus assays of fish DNA as a means of screening the toxicity of aquatic environments. *Catla catla* and *Cirrhinus mrigala* collected from the River Chenab in Pakistan were used as a case study for the application of comet and micronucleus techniques. Comet and micronucleus assays were used to compare DNA damage in *C. catla* and *C. mrigala* collected from polluted areas of the River Chenab and farmed fish. Atomic absorption spectrophotometry showed an acute level of toxicity from Cd, Cu, Mn, Zn, Pb, Cr, Sn, and Hg in river water. Comet assay showed significant ($p < 0.05$) DNA damage in *C. catla* representing 17.33 ± 2.42, 11.53 ± 2.14, and 14.17% DNA in the comet tail, averaged from three sites of the polluted area of the river. Tail moment was observed as 10.06 ± 2.71, 3.11 ± 0.74, and 14.70 ± 1.89, while olive moment was 8.85 ± 1.84, 3.83 ± 0.76, and 7.11 ± 0.73, respectively. Highly significant ($p < 0.01$) damage was reported in *C. mrigala* as 37.29 ± 2.51, 34.96 ± 2.53, and 38.80 ± 2.42% DNA in comet tail, tail moment was 23.48 ± 3.90, 19.78 ± 4.26, and 14.30 ± 1.82, and olive moment was 16.22 ± 2.04, 13.83 ± 1.96, and10.99 ± 0.90. Significant ($p < 0.05$) differences were observed in genotoxicity between farmed and polluted area fish. Micronucleus assay showed a similar picture of significant difference in respect to single and double micronucleus induction: i.e., 23.20 ± 4.19 and 2.80 ± 1.07‰ in *C. catla* and 44.80 ± 3.73 and 06.20 ± 0.97‰, respectively, in *C. mrigala*. Nuclear abnormalities were found as 6.00 ± 0.84 and 09.60 ± 1.72/thousand cells, respectively, in both species. The results of this study suggest that these novel fish DNA damage assays can be used as an expedient toxicity screening for aquatic environments.

Keywords DNA fragmentation · Biomarker · Indian major carps · Genotoxicity

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Introduction

Contamination of freshwater bodies is one of the most worrying issues of mankind and the primary responsibility moves towards domestic and industrial effluents (Claxton et al. 1998; White et al. 1998). Fish playing an important role in the food chain are excellent subjects to be used as bioindicators of the aquatic ecosystems. Fish can bioaccumulate toxic substances directly and indirectly through ingestion of both compounds dissolved in water and previously contaminated organism (Cavas

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and Gozukara 2005 ; Biagini et al. 2009). Ecogenotoxicology is an approach that applies the principles and techniques of genetic toxicology to assess the potential effects of environmental pollution in the form of genotoxic agents on the health of the ecosystem (Shugart and Theodorakis 1998). Biochemical and physiological parameters have a wider role as indicators of water quality and to detect sublethal impacts of freshwater pollutants. Genotoxicants include certain chemical compounds like heavy metals (Matsumoto et al. 2005; Igwilo et al. 2006), microbial toxins (Smith 1996), and polycyclic aromatic hydrocarbons (Fernandez and L'Haridon 1992). A number of genotoxicants cause structural changes in cellular DNA that could eventually lead to alteration in the form of single strand breaks, as well as, secondary double-strand breaks (Ferri et al. 1994; Sarker et al. 1995; Spencer et al. 1996). Such kinds of strand breaks are not uncommon but are pre-mutagenic lesions (Kaufman et al. 2011) and may provide a meaningful indication of the degree of the oxidative damage of the DNA (Imlay et al. 1988). Biomarkers of DNA damage are useful tools to evaluate Chronic and the acute exposure of aquatic organisms to genotoxic agents.

The comet assay (single cell gel electrophoresis) is a method for the detection of single and double strand of DNA. Singh et al. (1988) develop the basis of the current method. This assay presents advantages in comparison to other cytogenotoxicity assays, in that it is fast and sensitive enough to detect low levels of DNA damage requiring only a small number of cells per sample (Tice et al. 2000). Within few hours of exposure, detection of minor genotoxic damage is also possible with the comet assay (Masuda et al. 2004). Since the development and description of the SCGE, it has been widely used to examine the DNA damage in blood cells, hemocytes, and cells of the liver, gill, and gut (Cotelle and F′erard. 1999; Lee and Steinert 2003). Another assay, micronucleus test (MT), detects general disruptions in the chromosomal distribution. Micronuclei are small cellular chromatin bodies, separated from the main nucleus (Dopp et al. 1996). MT has been widely applied to fishes (Russo et al. 2004; Rodriguez-Cea et al. 2003; Sanchez-Galan et al. 2001) to biomonitor freshwater bodies for toxic substances (Sturm et al. 1999; Sturm et al. 2000; Katsiadaki et al. 2002). Many studies reported elevated frequencies of micronuclei in erythrocytes of fish collected from an area of heavy metal pollution (Richards et al. 2000; Pietripiana et al. 2002) but no one has yet compared fish species according to their feeding niches as in this case. The aim of this study, therefore, was to adopt both of these assays to *Catla catla* (surface feeder) and *Cirrhinus mrigala* (bottom feeder) erythrocytes in order to analyze if these assays can be used in these species as a reliable indicator of DNA damage and water pollution load for early monitoring of Asian rivers. Our concern was to test the usefulness of a bottom feeder species specifically as a biomarker of freshwater pollution load and its early monitoring by using simple and reliable techniques, comet and micronucleus assays.

Materials and methods

Water analyses

Drain water samples were taken from selected sections of the Chakbandi Main Drain while river "water samples were collected from the points of fish harvest to analyze for selected heavy metals and water quality parameters defined by the Environmental protection Agency of Pakistan (2015). In order to maintain calculation stan- dards, seven water samples of about 1.5 L each were collected in polypropylene bottles from each site. Each water sample was analyzed by protocol adopted by Boyd (1981). Metals analyzed were tin (Sn), chromium (Cr), lead (Pb), zinc (Zn), manganese (Mn), copper (Cu), cadmium (Cd), and mercury (Hg). The concentra- tion of each metal was determined using metal kits (Spectroquant® Analysis System, Merck) and using nitrous oxide/acetylene flame atomic absorption spec- trophotometry (2000 series, High-Technologies Corpo- ration, Chiyoda, Tokyo, Japan) with Zeeman back- ground correction. All chemicals and reagents used were of analytical grade (Merck, Darmstadt, Germany)".

Procurement of fish

Two fish species, *C. catla* and *C. mrigala*, were harvested from the River Chenab through its 190 km of length upstream of Trimu Head. "This is an area of high pollu- tion due to sewage and industrial waste disposal into the river through Chakbandi Drain at this point (latitude 31.570°, longitude 72.534°). Three sites, Wara Thatta Muhammad Shah (R1), Bela Reta (R2), and Bandimahni Beg (R3), were selected along the length of the River Chenab after receiving

Chakbandi Drain (Fig. 1). Drag nets and gill nets were used to harvest seven fishes of each species from three different sites (R1, R2, and R3) of the river, upstream of this area, with farmed fish being used as a control. Farmed fish were used as positive control supplied with toxic water, and toxicant-free farmed fish was designated as –ve control". The weight of the collected fish ranged from 800 to 1150 g.

Preparation of fish for experiment

"All the fish specimens were freshened out in running de- chlorinated tap water. Venous blood was collected from the caudal vein of each fish in heparin-coated tubes. Water samples were taken from the River Chenab at each point at which fish were harvested and analyzed for water quality parameters and selected heavy metals".

Comet assay

"Immediately after blood sampling, a small amount of blood (40 μL) was diluted with phosphate buffer saline and stored in ice. The comet assay was performed on erythrocytes following the technique of Singh et al. (1988) with slight modifications (Cavalcante et al. 2008)".

Single-cell suspensions were prepared and embedded in low melting point agarose on frosted microscope slides. "These slides were placed in a lysing buffer for 1 h at 4 °C, to facilitate unwind- ing of DNA. It was subsequently followed by electrophoresis (20 min, 300 mA, 25 V) and neu- tralization (three washes for 5 min each in buffer). Slides were stained with ethidium bromide and analyzed under a fluorescent microscope. DNA damage was measured by the length of DNA mi- gration, which was visually determined in 250 randomly selected cells as 50 per slide for each fish through Comet Score V5 and classified into five classes based on the comet tail length and DNA damage".

Micronucleus assay

"Fresh blood was smeared on the slides. Slides were air- dried and fixed in cold Corney fixative for 5 min and stained in aqueous 10% Giemsa for 30 min. For each fish species, five fish were analyzed from each site of fish harvest for a total of 25,000 erythrocytes/fish sam- ple. The frequencies of micronuclei induction in eryth- rocytes were detected under binocular microscope at T1200x magnification. Erythrocytes with intact cellular and nuclear membranes were scored according to Cavas and Gozukara (2005) and classified as in Carrasco et al. (1990)".

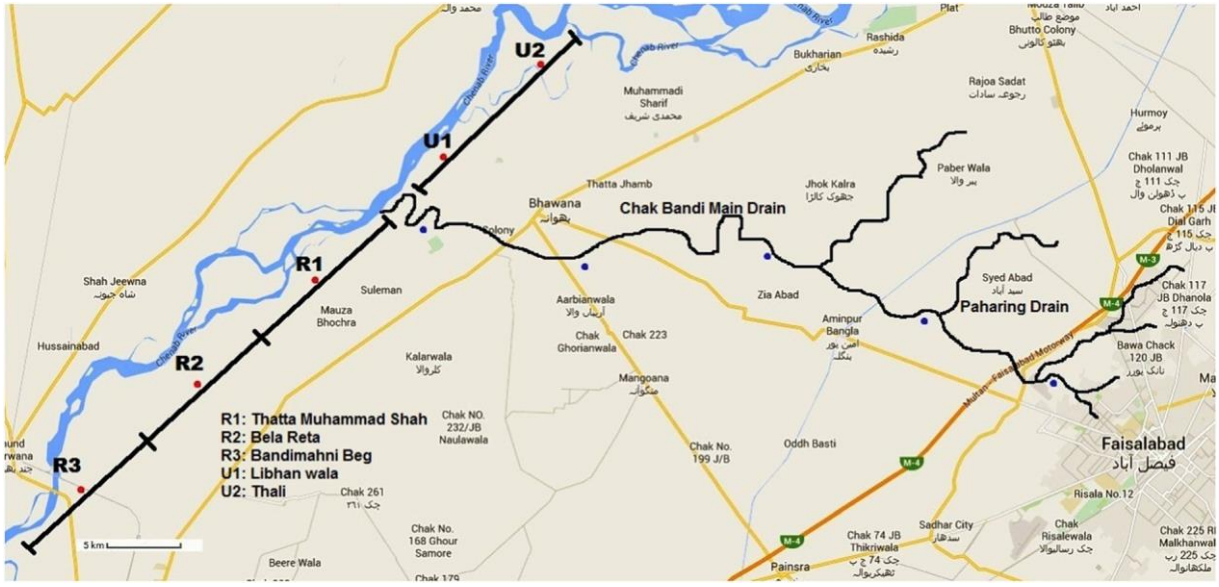

Fig. 1 Site map indicating the sites *R1*, *R2*, and *R3* along the polluted area of the River Chenab and upstream sites *U1* and *U2* before the entrance of the Chakbandi Main Drain. Source: www.google.com/maps/@31.522282,72.7814512,10z

Statistical analysis

The mean, standard error, and analysis of variance (ANOVA) were worked out using SPSS 9 for PC. The means were compared by using Duncan's multiple range test. Probability values of $p < 0.05$ were considered significant. TriTek Comet Score™ Freeware 1.6.1.13 software for image analysis, by Tritek Corporation (2010), was used for DNA damage analyses.

Results and discussion

Two fish species, *C. catla* and *C. mrigala*, from the polluted areas of the River Chenab were analyzed for DNA damage. All water quality parameters and selected heavy metals were found to be far higher than the permissible limits defined by the WHO (Table 1). Significant ($p < 0.05$) DNA damage was observed in *C. mrigala* collected from the polluted areas compared to the fish from the non-polluted area (upstream to polluted area) and farmed fish and also in comparison with *C. catla* (Fig. 2). In the case of the head diameter in the comet assay for *C. catla* for sites R1, R2, and R3, significant differences were reported between polluted and upstream samples, indicating significant DNA migration from the comet head. In respect to comet tail length for site R1 and R3, significant differences were observed between farmed, polluted, and upstream fishes. "The proportion of DNA in the tail for site R1 showed significant differences in respect to farmed and polluted fish (4.423 and 17.330%, respectively) but no differ- ences were observed for farmed and upstream fish (Fig. 3). For site R2, significant differences were obtain- ed among all four types. For site R3, significant differ- ences were obtained in respect to polluted and upstream fish (35.991 and 13.026%, respectively) (Table 2). Sig- nificant differences were also reported in respect to comet tail moment among polluted and upstream fish species".

"The head diameter revealed in the comet assay of *C. mrigala* blood for sites R1, R2, and R3 showed significant ($p < 0.05$) differences between farmed, polluted, and upstream fish (Fig. 4). Similar findings were observed in the case of the comet tail in the comet assay (Table 2). The percentage of DNA in the tail showed significant ($p < 0.05$) differences across all selected sites between farmed and polluted area fish and between polluted and upstream fish. The maximum DNA damage was observed in the fish collected from site R3, followed by site R1 and site R2, respectively". Similar findings were reported in respect to tail moment and

Table 1 Comparison of means of water quality parameters of the River Chenab (mean ± SE)

Sites	Physicochemical parameters of water from the River Chenab			
	Cadmium mg L^{-1}	Copper mg L^{-1}	Manganese mg L^{-1}	Zinc mg L^{-1}
R1	0.141 ± 0.01^{C}	0.907 ± 0.21^{E}	1.59 ± 0.15^{C}	0.215 ± 0.04^{E}
R2	0.135 ± 0.01^{C}	0.863 ± 0.13^{EF}	1.53 ± 0.148^{C}	0.207 ± 0.03^{F}
R3	0.130 ± 0.02^{CD}	0.826 ± 0.20^{F}	1.36 ± 0.14^{D}	0.206 ± 0.04^{F}
	Lead mg L^{-1}	Chromium mg L^{-1}	Tin mg L^{-1}	Mercury mg L^{-1}
R1	1.602 ± 0.16^{C}	0.349 ± 0.05^{D}	0.304 ± 0.04^{D}	0.996 ± 0.03^{BC}
R2	1.348 ± 0.12^{D}	0.289 ± 0.04^{E}	0.273 ± 0.03^{DE}	1.013 ± 0.02^{BC}
R3	1.298 ± 0.12^{D}	0.246 ± 0.03^{F}	0.261 ± 0.03^{E}	0.893 ± 0.01^{CD}
	Phenols mg L^{-1}	Sulfates mg L^{-1}	BOD mg L^{-1}	COD mg L^{-1}
R1	1.67 ± 0.15^{E}	264.79 ± 28.23^{D}	70.64 ± 2.33^{F}	146.43 ± 13.6^{F}
R2	1.48 ± 0.10^{F}	250.36 ± 47.27^{E}	61.70 ± 1.88^{G}	135.00 ± 13.4^{G}
R3	1.32 ± 0.13^{G}	246.07 ± 45.68^{E}	50.88 ± 1.44^{H}	124.07 ± 13.9^{G}
	pH	TDS mg L^{-1}	Salinity mg L^{-1}	Conductivity mS/m
R1	10.37 ± 0.05^{CD}	1597.64 ± 221.95^{E}	1392.86 ± 153.16^{E}	2.25 ± 0.26^{E}
R2	10.28 ± 0.02^{D}	1475.43 ± 280.16^{F}	1250.00 ± 145.19^{F}	2.11 ± 0.27^{F}
R3	10.06 ± 0.04^{E}	1214.43 ± 237.61^{G}	921.43 ± 87.15^{G}	1.70 ± 0.31^{G}

"Means sharing a similar letter in a row or in a column are statistically non-significant ($P > 0.05$)

R1–R3 polluted experimental sites in the River, *BOD* biochemical oxygen demand, *COD* chemical oxygen demand"

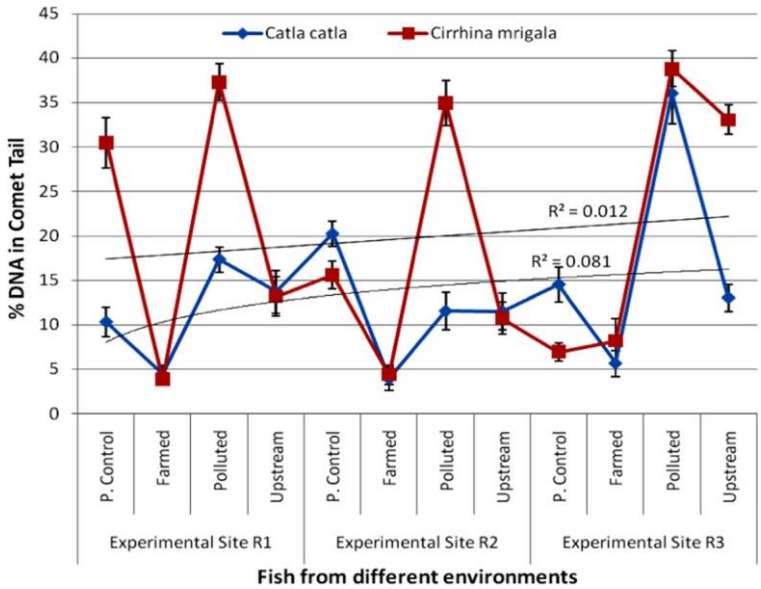

Fig. 2 % DNA damage in *Catla catla* and *Cirrhinus mrigala*, type, and site interactions. *R1–R3*: polluted experimental sites along the River Chenab

olive moment (Fig. 5). *C. mrigala* tail length also showed a highly significant correlation to DNA in tail and head diameter (Table 3), signifying the magnitude and precision of single cell gel electrophoresis (comet assay).

"Fish collected from the polluted area of the River Chenab showed the highest frequency of micronucleus (MN) induction and nuclear abnormalities (Table 4). *C. mrigala* showed significantly higher frequencies of micronucleus induction compared to *C. catla* (Figs. 6 and 7). *Cirrhinus* showed significant micronucleus induction, even from the area of lower pollution intensity (upstream of Chakbandi drain), possibly because *C. mrigala* is a bottom feeder" and therefore is more

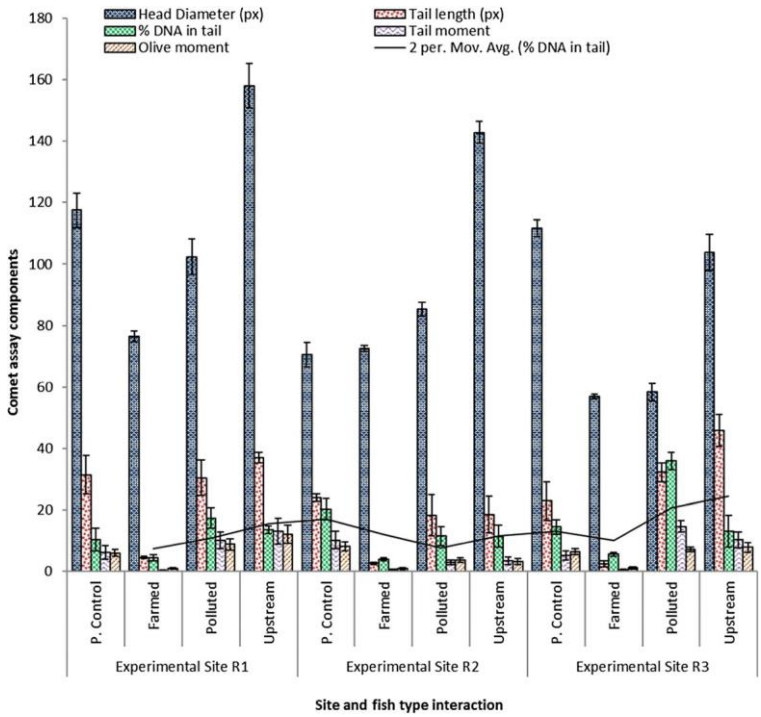

Fig. 3 Comparative analyses of comet assays of blood from *Catla catla* collected from different environments. *Line* on the plot indicates moving average for % DNA in tail. *R1–R3*: polluted experimental sites along the River Chenab

Table 2 Comet assay results for *Catla catla* and interaction (fish, site, and type interactions)

Sites	Comet assay components				
	Head diameter (px)	Tail length (px)	DNA in tail %	Tail moment	Olive moment
Catla catla species and site interaction (means ±SE)					
Site 1	114.69 ± 3.52	26.77 ± 2.85	11.43 ± 1.04	7.44 ± 1.38	6.96 ± 0.95
Site 2	92.82 ± 2.99	15.87 ± 1.53	11.79 ± 1.51	4.34 ± 0.81	4.03 ± 0.53
Site 3	82.71 ± 2.84	25.86 ± 2.09	17.28 ± 1.42	7.72 ± 0.96	5.68 ± 0.55
Mean	97.38 ± 1.91^{A}	23.42 ± 1.30^{B}	13.50 ± 0.78^{B}	6.50 ± 0.62^{B}	5.56 ± 0.41^{B}
Catla catla species and type interaction (mean ± SE)					
Control $^{+ve}$	98.76 ± 3.32^{bc}	25.07 ± 2.55^{bc}	15.00 ± 1.46^{cd}	7.25 ± 1.28^{bc}	6.90 ± 0.71^{bc}
Farmed	68.70 ± 2.08^{fg}	3.27 ± 0.71^{e}	4.66 ± 0.73^{e}	0.50 ± 0.14^{d}	1.02 ± 0.18^{d}
Polluted	82.08 ± 3.415^{de}	25.95 ± 2.41^{bc}	21.62 ± 1.78^{b}	9.29 ± 1.19^{bc}	6.59 ± 0.72^{bc}
Upstream	135.88 ± 3.91^{a}	33.78 ± 3.43^{ab}	12.72 ± 1.72^{d}	8.98 ± 1.69^{bc}	7.71 ± 1.19^{bc}
Cirrhinus mrigala species and site interaction (means ±SE)					
Site 1	89.79 ± 4.16	31.37 ± 2.87	21.19 ± 1.45	12.16 ± 1.53	08.83 ± 0.88
Site 2	77.18 ± 3.59	22.30 ± 2.57	16.40 ± 1.31	07.65 ± 1.33	06.09 ± 0.71
Site 3	90.92 ± 3.07	30.96 ± 2.53	21.75 ± 1.51	10.45 ± 1.10	08.52 ± 0.68
Mean	86.86 ± 2.04^{B}	29.32 ± 1.44^{A}	19.78 ± 0.83^{A}	10.08 ± 0.77^{A}	07.82 ± 0.44^{A}
Cirrhinus mrigala species and type interaction (mean ± SE)					
Control $^{+ve}$	109.77 ± 5.70^{b}	36.47 ± 361^{ab}	17.66 ± 1.53^{bc}	09.72 ± 1.46^{b}	07.92 ± 0.87^{bc}
Farmed	52.31 ± 1.07^{h}	02.20 ± 0.38^{e}	05.45 ± 0.93^{e}	00.57 ± 0.20^{d}	01.22 ± 0.31^{d}
Polluted	89.22 ± 3.15^{cd}	42.49 ± 3.22^{a}	37.01 ± 1.43^{a}	19.19 ± 2.03^{a}	13.68 ± 1.00^{a}
Upstream	95.39 ± 4.11^{cd}	34.88 ± 3.12^{ab}	19.00 ± 1.52^{bc}	10.86 ± 1.45^{b}	08.44 ± 0.87^{b}

"Means sharing similar letter in a row or in a column are statistically non-significant ($P > 0.05$). Small letters represent comparison among interaction means and capital letters are used for overall mean. R1–R3, polluted experimental sites in the river; fish types (F, farmed; P, polluted; U, upstream; +ve, positive control)"

exposed to the polluted sediments. Farmed (control) fish showed a negligible amount of this kind of DNA damage.

"The urban and industrial discharges are known to be responsible for high concentrations of toxic contami- nants in aquatic environments (Richards et al. 2000), most of the current literature has sought to assess the effects of this contamination, mainly through the use of a holistic approach that takes into account the combined effects of heavy metals on fish through histopathologi- cal and physiological studies. In the literature, informa- tion about the potential genotoxic effects of pollution on these fish species is still scarce, therefore (Galindo et al. 2010)". Many contaminants present in these environ- ments induce genetic alterations leading to amber mu- tations (Russo et al. 2004). This project sought to esti- mate such effects on *C. catla* and *C. mrigala*'s genetic makeup and to use fish DNA fragmentation as a bio- marker of freshwater pollution. This will allow early detection of freshwater pollution having genotoxic ef- fects to fish. This project corroborates the study of Van-Der-Oost et al. (2003) with respect to the use of fish biomarkers as indices of the effect of pollution. Another study reported elevated frequencies of micronuclei in erythrocytes of fish collected from an area of heavy metal pollution (Richards et al. 2000; Pietripiana et al. 2002) but no one has as yet compared fish species according to their feeding niches as in this case.

"In this study, we used a simple and more comprehen- sive comet assay technique for the direct measurement of DNA damage in fish caught from highly contaminat- ed areas of the River Chenab. The results showed in- creased levels of genotoxic damage compared to the farmed fish devoid of any pollution also described by Baršienė et al. (2013)". These results from comet assay data for *C. catla* and *C. mrigala* are in concordance with another study in the context of environmental biomon- itoring and genotoxicity in fish (Pavlica et al. 2011).

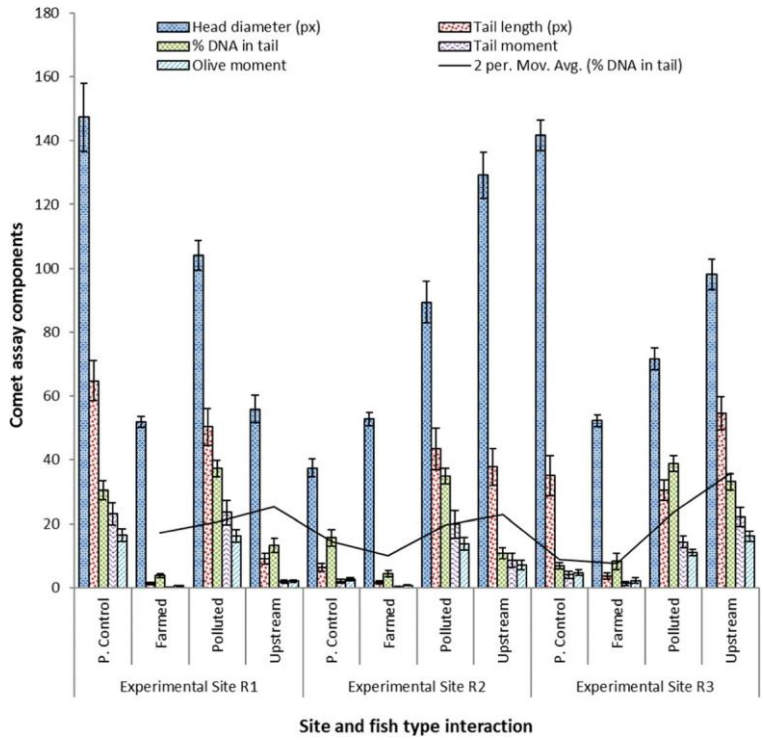

Fig. 4 Comparative analyses of comet assays of blood from *Cirrhinus mrigala* collected from different environments

Significant relationships were observed in the comet assay results for micronucleus frequencies, nuclear abnormalities, and DNA damage. The present results are in agreement with earlier studies that had detected elevated micronucleus frequencies in fish inhabiting contaminated environments (Bombail et al. 2001; Andrade

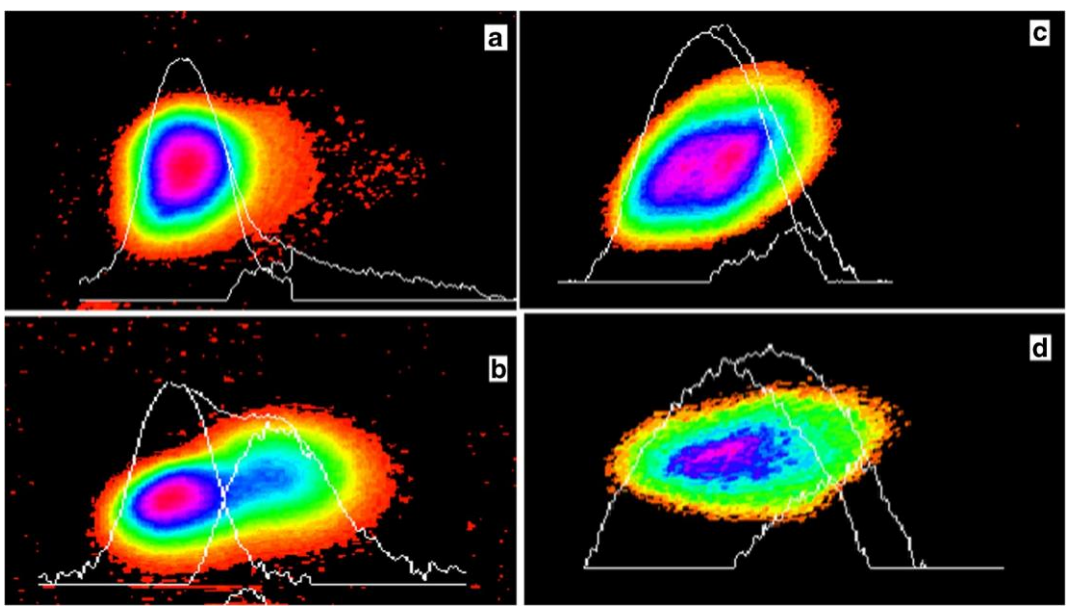

Fig. 5 Photomicrographs of comet assay. a, b *Cirrhinus mrigala*. c, d *Catla catla*. TriTek Comet Score™ Freeware 1.6.1.13

Table 3 Correlation matrix for comet assay results of blood from *Cirrhinus mrigala* and *Catla catla*

	Catla catla					*Cirrhinus mrigala*				
	T.DNA.	Type	H.dia	T.length	T.mome	T.DNA.	Type	H.dia	T.length	T.mome
Type	0.139					0.299				
	0.672					0.345				
H.dia	−0.135	0.428				0.283	−0.022			
	0.683	0.166				0.373	0.945			
T.length	0.571*	0.392	0.480			0.733**	0.175	0.835**		
	0.050	0.208	0.114			0.007	0.587	0.001		
T.mome	0.822**	0.322	0.232	0.861**		0.906**	0.264	0.574*	0.922**	
	0.001	0.307	0.468	0.000		0.000	0.408	0.050	0.000	
O.mome	0.579*	0.262	0.489	0.871**	0.904**	0.903**	0.253	0.602*	0.937**	0.997**
	0.050	0.410	0.107	0.000	0.000	0.000	0.428	0.038	0.000	0.000

"Upper values indicated Pearson's correlation coefficient. Lower values indicated level of significance at 5% probability
T.DNA % DNA in tail, *H.dia* head diameter, *T.length* tail length, *T.mome* tail moment, *O.mome* olive moment"
*Significant ($P < 0.05$); **Highly significant ($P < 0.01$)

et al. 2004; Kumar et al. 2010). Previous laboratory investigations showed that fish exposed to cyclophosphamide, cypermethrin, and textile mill effluents produced higher micronucleus frequencies in gill cells than in erythrocytes, and "Cavas and Gozukara (2003) reported that the gill cells of *Oreochromis mossambicus* are more sensitive to genotoxic exposure than cells from other tissues such as the kidney or liver. Use of the kidney, liver, or other such tissues for genotoxicity biomonitoring has many limitations, since there is a low mitotic index for cells from such tissues. The use of connective tissues such as blood erythrocytes has therefore been shown to be advantageous for such stud- ies (Cavas and Gozukara 2005)".

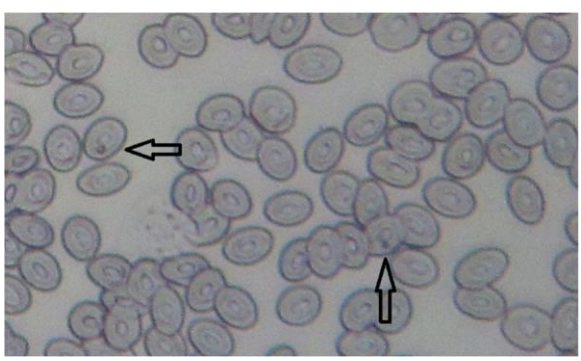

Fig. 6 Micronucleus assay of fish (*Catla catla*) blood harvested from the polluted area of the River Chenab indicating nuclear abnormalities and micronucleus induction

In recent decades, morphological nuclear abnormalities (NAs) "together with micronucleus induction have received considerable attention, although the mecha- nisms responsible for the induction of NAs are not yet fully understood. Several studies specify that NAs are induced in response to exposure to genotoxic agents (Serrano-Garcia and Montero-Montoya 2001; Bombail et al. 2001). Significant DNA damage was reported in this study in *C. mrigala* collected from the polluted area of the River Chenab and even from less polluted areas of the river upstream to the entrance of the Chakbandi Main Drain into the river. Water quality parameters that were even in less polluted area were beyond the permis- sible limits indicating higher intensities of the pollution. Pulkrabová et al. (2007) and Viarengoa et al. (2007)".

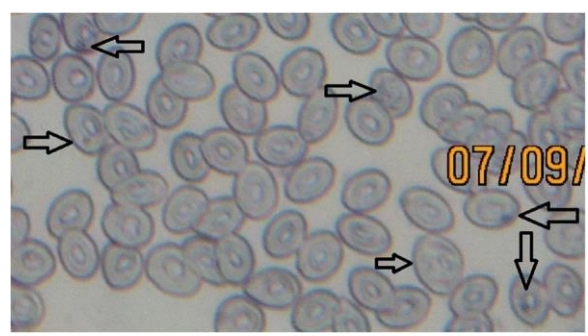

Fig. 7 Micronucleus assay of fish (*Cirrhinus mrigala*) blood harvested from the polluted area of the River Chenab indicating

Table 4 Analysis of variance for micronucleus test of *Catla catla* and *Cirrhinus mrigala* blood (mean ± SE)

Source of variation	Degrees of freedom	*F* value for MNs	*F* value for MNd	*F* value for NAs
Species	2	11.90**	3.70*	4.69*
Type	3	60.70**	14.10**	22.26**
Species × type	6	3.05*	2.94*	2.62*
Fish type	Micronucleus assay (*Catla catla*)			
	Single micronucleus	Double micronucleus	Nuclear abnormalities	
Polluted	23.20 ± 4.19^{bc}	02.80 ± 1.07^{b}	06.00 ± 0.84^{cd}	
Upstream	08.00 ± 1.05^{cd}	01.40 ± 0.75^{b}	03.20 ± 0.37^{d}	
Control (farmed)	02.20 ± 0.58^{d}	00.00 ± 0.00^{b}	01.00 ± 0.32^{d}	
+ve. control	43.60 ± 5.35^{a}	08.60 ± 3.67^{ab}	17.80 ± 2.92^{a}	
Mean	19.25 ± 4.00^{B}	03.20 ± 1.17^{B}	07.00 ± 1.65^{B}	
Fish type	Micronucleus assay (*Cirrhinus mrigala*)			
	Single micronucleus	Double micronucleus	Nuclear abnormalities	
Polluted	44.80 ± 3.73^{a}	06.20 ± 0.97^{ab}	$09.60 \pm 1.72^{a–d}$	
Upstream	20.60 ± 4.02^{bcd}	05.20 ± 1.53^{b}	$10.00 \pm 1.05^{a–d}$	
Control (farmed)	08.20 ± 2.20^{cd}	00.80 ± 0.37^{b}	06.20 ± 1.85^{bcd}	
+ve. control	37.40 ± 3.92^{ab}	08.40 ± 2.80^{ab}	15.20 ± 2.06^{ab}	
Mean	27.75 ± 3.66^{A}	05.15 ± 1.00^{AB}	10.25 ± 1.08^{A}	

"Means sharing similar letter in a row or in a column are statistically non-significant ($P > 0.05$). Small letters represent comparison among interaction means and capital letters are used for overall mean. Frequency calculated per thousand cells"

MNs micronucleus single, *MNd* micronucleus double, *NAs* nuclear abnormalities

*Significant ($P < 0.05$); **Highly significant ($P < 0.01$)

described that benthic species are more affected by the freshwater pollution. In this study, more DNA damage was observed in the bottom dweller fish *C. mrigala*, compare to surface feeder *C. catla*. These findings verify the genotoxicity of water in the River Chenab to a bottom feeder fish *C. mrigala* along with its sensitive response to the pollution when compared to the surface feeder fish *C. catla*. Early monitoring of DNA damage in *C. mrigala* using simple and reliable techniques such as comet and micronucleus assay could act as a biomarker of freshwater pollution load.

Conclusion

Water of the River Chenab was found to be highly polluted by genotoxicants. This study showed that bottom dwelling species could be used as bioindicators for a particular aquatic environment, through assessment of DNA fragmentation as a biomarker of pollution for early warning and monitoring of the freshwater bodies.

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